UNIVERSIDAD DE OVIEDO

Departamento de Ingeniería Química y Tecnología del Medio Ambiente

Hidrólisis, separación y aprovechamiento de proteína de yema de huevo

TESIS DOCTORAL

Por

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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RESUMEN (en español)

El huevo es un alimento completo, rico en proteínas de alto valor biológico y en lípidos, que es altamente consumido por sí mismo debido a sus cualidades nutricionales, pero que además, gracias a sus propiedades funcionales, puede ser utilizado en multitud de productos con el objetivo de modificar la textura y añadir palatabilidad. El huevo está compuesto por la clara, rica en agua y con un 10% de proteína, y la yema, en la cual los lípidos que alimentarán al embrión se encuentran ensamblados en forma de lipoproteínas. La clara y la yema pueden ser fácilmente separadas. Además, la yema puede ser a su vez fraccionada en dos partes, una en la que se concentran los lípidos, denominada plasma, y otra en la que se concentran las proteínas que recibe el nombre de gránulos. El estudio de cada una de las fracciones que constituyen al huevo y su aprovechamiento tecnológico repercutirá en la revalorización del huevo en su conjunto. Durante esta tesis se ha procedido al estudio de los gránulos de la yema de huevo con el objetivo de aumentar el rango de aplicaciones en las cuales pueden ser aprovechados.

En primer lugar, debido a que en la fracción granular se concentran una parte importante de las proteínas de la yema de huevo, se ha estudiado la hidrólisis enzimática y con agua en condiciones subcríticas de la proteína granular. La hidrólisis con tripsina de los gránulos disueltos en agua salada resultó en la producción de un hidrolizado que mantenía las propiedades emulsionantes de los gránulos completos, y con el cual se podía elaborar una mayonesa con unas propiedades reológicas similares a las de una mayonesa comercial tomada como referencia. En el caso de utilizar gránulos delipidados y muy insolubles, la recuperación de péptidos solubles mediante la hidrólisis con tripsina resultó disminuida, obteniéndose un 50% de la proteína original en forma de péptidos tras 6 horas de operación. Cuando se utilizó agua en condiciones subcríticas y una corriente de nitrógeno las recuperación de los péptidos alcanzó un 95% en 4 horas de operación, tiempo que se vió disminuido cuando se empleó una corriente oxidante (oxígeno) en lugar de nitrógeno, también en condiciones subcríticas. Las propiedades funcionales y antioxidantes evaluadas en estos hidrolizados resultaron similares cuando se comparaba el tratamiento con tripsina con el tratamiento con agua en condiciones

subcríticas con corriente de nitrógeno, sin embargo, el uso de oxígeno resultó en hidrolizados con algunas propiedades funcionales y antioxidantes disminuidas, probablemente debido a variaciones en la estructura primaria de los péptidos.

Además, se abordó la separación de la fosvitina del resto de componentes granulares. La fosvitina resulta interesante ya que es la proteína más fosforilada encontrada en la naturaleza, presentando una fuerte capacidad quelante de iones de hierro y calcio. En este estudio se propone un método de separación basado en la precipitación selectiva de las otras proteínas granulares mediante el ajuste de la fuerza iónica del medio y el pH, quedando en disolución la fosvitina. El desalado (NaCl) de la fosvitina soluble se desarrolló por diálisis y por ultrafiltración.

Finalmente, debido a que los gránulos de yema de huevo comparten muchas características funcionales con la yema entera, se ha procedido a la sustitución de la yema por cantidades equivalentes de gránulos en un producto alimentario complejo. Para ello se ha utilizado como modelo un producto de horneado ampliamente consumido como la madalena. Este modelo permite conocer el comportamiento de los gránulos en crudo, mediante la evaluación de las propiedades físicas de la masa, así como la influencia de los gránulos en el producto horneado, una vez que el calor desestabiliza su estructura. Para ello se ha recurrido a técnicas reológicas y de texturometría, además de análisis de imagen. Los resultados revelaron que sustituciones mayores a un 50% producían cambios acentuados en ciertas propiedades del producto horneado y crudo, sin embargo, son posibles sustituciones completas pero compensando la fórmula con emulsificantes.

RESUMEN (en Inglés)

Egg is a complete food, with a high content in high biological value proteins and lipids. The egg is mainly consumed due to its nutritional properties, but furthermore, thanks to its functional properties, the egg can be used in a lot of products with the aim of changing their texture and adding flavour and palatability. Egg is composed by the albumen, which is rich in water, with a 10% protein content, and the yolk, which contains the lipids that will feed the embryo and that are assembled in form of lipoproteins. Albumen and yolk can be separated easily. Furthermore, the yolk can be separated in two other fractions, one of them called plasma and with high lipid content, and another, the granular one, with high protein content. The study of each fraction and their technological use will have repercussions in the revaluation of the whole egg yolk. In this Thesis the egg yolk granules fraction has been studied in order to

increase the range of applications in which they can be used.

Firstly, and because in the granular fraction the proteins are concentrated, the enzymatic hydrolysis and the sub-critical water hydrolysis of this fraction have been studied. The solubilized granules treated with trypsin resulted in the production of a hydrolysate which maintains the emulsifying properties of the unhydrolysed granules. Using this hydrolysite it was possible the elaboration of a mayonnaise with similar rheological properties to those found in a commercial mayonnaise used as reference. The pre-treat of the granular fraction with ethanol resulted in a lipid-free protein precipitate. Fifty percent of this protein was recovered in form of soluble peptides through its hydrolysis with trypsin after 6 hours of reaction. However, when the insoluble protein was hydrolysed using sub-critical water the 95% of the protein was recovered in form of soluble peptides after 4 hours of operation. These 4 hours were decreased to 2 hours when an oxygen stream was used during the hydrolysis instead of a nitrogen stream. The functional and antioxidant properties of the hydrolysed obtained using sub-critical water under a nitrogen stream resulted similar to those obtained by means of the enzymatic hydrolysis with htrypsin. However, the oxidant stream in the sub-critical water hydrolysis resulted in peptides with some functional and antioxidant properties decreased, likely due to variations in the primary structure of the peptides.

Furthermore, the separation of the phosvitin of the other granular molecules using a novel organic solvent-free method was developed too. The phosvitin is the most phosphorylated protein found in the nature, with high calcium and ferrous chelating properties. In this study, the phosvitin remains soluble and the other granular proteins are precipitated due to changes in the medium pH and ionic force. The enriched phosvitin solution was desalted (NaCl) by means of dialysis and ultrafiltration.

Finally, owing to the fact that egg yolk granules maintain functional properties similar to those of the egg yolk, a bakery product (muffins) was developed substituting the whole egg yolk by its low-cholesterol granular fraction. This bakery product allows knowing how the granules affect the characteristics of the raw food (the batter) and the cooked one (the muffin). In the case of the elaboration of gluten-free muffins, all the egg yolk was substituted by the granular fraction, and some changes in several rheological and textural parameters were observed. To study these changes more precisely, other muffin recipes, with different plasma/granules ratios were developed. The results obtained reveal that substitutions higher than 50% result in greater changes in some properties of the raw batter and the cooked muffin, however, complete substitution is possible, but the recipe must be balanced with the use of emulsifiers.

SR. DIRECTOR DE DEPARTAMENTO DE Ingeniería Química y Tecnología del Medio Ambiente/
SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN Biotecnología alimentaria.

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ÍNDICE

RESUMEN

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En primer lugar, debido a que en la fracción granular se concentran una parte importante de las proteínas de la yema de huevo, se ha estudiado la hidrólisis enzimática y con agua en condiciones subcríticas de la proteína granular. La hidrólisis con tripsina de los gránulos disueltos en agua salada resultó en la producción de un hidrolizado que mantenía las propiedades emulsionantes de los gránulos completos, y con el cual se podía elaborar una mayonesa con unas propiedades reológicas similares a las de una mayonesa comercial tomada como referencia. En el caso de utilizar gránulos delipidados y muy insolubles, la recuperación de péptidos solubles mediante la hidrólisis con tripsina resultó disminuida, obteniéndose un 50% de la proteína original en forma de péptidos tras 6 horas de operación. Cuando se utilizó agua en condiciones subcríticas y una corriente de nitrógeno las recuperación de los péptidos alcanzó un 95% en 4 horas de operación, tiempo que se vió disminuido cuando se empleó una corriente oxidante (oxígeno) en lugar de nitrógeno, también en condiciones subcríticas. Las propiedades funcionales y antioxidantes evaluadas en estos hidrolizados resultaron similares cuando se comparaba el tratamiento con tripsina con el tratamiento con agua en condiciones subcríticas con corriente de nitrógeno, sin embargo, el uso de oxígeno resultó en hidrolizados con algunas propiedades funcionales y antioxidantes disminuidas, probablemente debido a variaciones en la estructura primaria de los péptidos.

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una vez que el calor desestabiliza su estructura. Para ello se ha recurrido a técnicas reológicas y de texturometría, además de análisis de imagen. Los resultados revelaron que sustituciones mayores a un 50% producían cambios acentuados en ciertas propiedades del producto horneado y crudo, sin embargo, son posibles sustituciones completas pero compensando la fórmula con emulsificantes.

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Firstly, and because in the granular fraction the proteins are concentrated, the enzymatic hydrolysis and the sub-critical water hydrolysis of this fraction have been studied. The solubilized granules treated with trypsin resulted in the production of a hydrolysate which maintains the emulsifying properties of the unhydrolysed granules. Using this hydrolysite it was possible the elaboration of a mayonnaise with similar rheological properties to those found in a commercial mayonnaise used as reference. The pre-treat of the granular fraction with ethanol resulted in a lipid-free protein precipitate. Fifty percent of this protein was recovered in form of soluble peptides through its hydrolysis with trypsin after 6 hours of reaction. However, when the insoluble protein was hydrolysed using sub-critical water the 95% of the protein was recovered in form of soluble peptides after 4 hours of operation. These 4 hours were decreased to 2 hours when an oxygen stream was used during the hydrolysis instead of a nitrogen stream. The functional and antioxidant properties of the hydrolysed obtained using sub-critical water under a nitrogen stream resulted similar to those obtained by means of the enzymatic hydrolysis with htrypsin. However, the oxidant stream in the sub-critical water hydrolysis resulted in peptides with some functional and antioxidant properties decreased, likely due to variations in the primary structure of the peptides.

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1. INTRODUCCIÓN A LA TESIS

INTRODUCCIÓN

El huevo de gallina se ha revelado en los últimos años como un alimento completo, rico en proteínas de alto valor biológico y lípidos, que además de nutrir puede conferir a los alimentos propiedades texturales y organolépticas únicas. Entre las propiedades funcionales que distinguen al huevo podemos destacar sus cualidades emulsionantes, espumantes o gelificantes, que han sido aprovechadas por la industria alimentaria de tal forma que actualmente el 30% del huevo consumido es a través de procesados que contienen este ingrediente en su fórmula. Así por ejemplo, el huevo se introduce en productos cárnicos como las albóndigas, las croquetas y algunas salchichas; en postres como pudines, natillas, helados, bombones rellenos de crema, pasteles o galletas; en la industria de los condimentos, con una gran variedad de salsas, e incluso en pastas alimenticias a base de harina como los espaguetis o similares.

El huevo está constituido además de por la cáscara, de escasa utilidad en la industria alimentaria, por la clara y la yema. Una ventaja tecnológica importante es lo fácilmente separable que son estas dos fracciones del huevo entero, además, la clara posee una composición completamente diferente a la de la yema de huevo. Mientras que la clara está constituida en su mayoría por agua y tiene alrededor de un 10% de proteínas, la yema de huevo presenta alrededor de un 34% de lípidos y de un 15% de proteínas. Esta diferencia en composición también conlleva diferencias nutricionales y funcionales bien marcadas que pueden ser aprovechadas por separado, comercializándose incluso la clara pasteurizada separada de la yema entera. De este modo, se proporciona algo de valor añadido al huevo obteniéndose dos productos que pueden ser explotados por separado, pero sin embargo, esta revalorización podría considerarse escasa y de bajo valor tecnológico.

En este sentido, atendiendo al número de estudios bibliografiados, el interés por la yema ha resultado menos intenso que el de la clara de huevo, entre otras cosas debido a que es un sistema más complejo, con la presencia de lípidos que dificultan el estudio de las proteínas y viceversa. Sin embargo, tal y como la clara puede ser separada fácilmente de la yema, dando dos productos distinguibles, la yema puede dividirse a su vez también con gran sencillez resultando en una fracción plasmática y una fracción granular. Cada una de estas dos subfracciones se diferencia de la otra no sólo por la cantidad de lípidos y proteínas, sino también por el tipo de lípidos y proteínas, lo cual permite el estudio y revalorización de cada parte por separado.

Desde la década de los 70 aparecieron diferentes estudios que se centraban en la separación de la yema de huevo en sus dos fracciones. Teniendo en cuenta que la yema de huevo está constituida por un conjunto de partículas que flotan en un líquido viscoso, la metodología para su fraccionamiento se puede resumir en disminuir su apelmazamiento, diluyendo con agua de baja fuerza iónica, y centrifugar para obtener las partículas precipitadas (gránulos) y el líquido constituyente (plasma). Ya que el plasma es la fracción rica en lípidos en general y fosfolípidos y colesterol en particular, ha resultado tener importantes cualidades emulsionantes que pueden ser aprovechadas en la elaboración de cremas cosméticas. Además, su contenido en inmunoglobuina Y la hace interesante para la industria farmacéutica. En cuanto a los gránulos de yema de huevo su contenido en proteínas es mayor en comparación con el plasma, y su

contenido en colesterol también se encuentra muy reducido. Esta particular composición hace que los gránulos sean interesantes desde el punto de vista nutricional, por otro lado también mantiene cualidades emulsionantes que pueden ser interesantes en el diseño de alimentos.

Por tanto, una estrategia para revalorizar al huevo en su conjunto podría ser abordar el estudio pormenorizado de cada una de las fracciones resultantes. Los gránulos de yema de huevo presentan una serie de ventajas que los hacen particularmente interesantes en este sentido: debido a su composición (baja en grasa y colesterol) pueden ser investigados como sustitutos de la yema de huevo en productos que lo demanden. Además, su contenido en proteínas permite el estudio de la hidrólisis de las mismas, y cómo esta hidrólisis proporciona péptidos con capacidades antioxidantes. Finalmente podría ser interesante la separación de las proteínas constituyentes, con el fin de facilitar la aplicación de las mismas.

OBJETIVOS

La investigación tiene como objetivo el estudio de los gránulos de yema de huevo desde diferentes puntos de vista con el fin de aumentar las aplicaciones, y por tanto el valor añadido, del huevo en su totalidad. Para ello se han desarrollado los siguientes objetivos:

- Estudio de la hidrólisis de las proteínas de los gránulos de yema de huevo. Se realizarán diferentes aproximaciones, evaluando la hidrólisis enzimática de gránulos enteros de yema de huevo y de gránulos delipidados e insolubles. Además se procederá a la hidrólisis con agua en condiciones subcríticas. El análisis de los péptidos se desarrollará mediante electroforesis y cromatografías. Además, se evaluarán las propiedades antioxidantes y funcionales de los hidrolizados.

- Separación de fosvitina de los gránulos de yema de huevo con el objetivo de facilitar el aprovechamiento de esta proteína. La fosvitina resulta ser la proteína más fosforilada encontrada en la naturaleza y tiene una gran capacidad de acomplejar hierro y calcio. El rendimiento se comparará con el de otros métodos.

- Estudio de los gránulos como sustitutivo bajo en colesterol y rico en proteínas de la yema entera en un sistema alimentario complejo. Con este fin se elaboraron fórmulas de un producto de horneado, el cual permite evaluar el efecto de los gránulos tanto en crudo como después de haber aplicado calor. Para ello se eligió como modelo las madalenas. En este caso las cualidades de la masa y el efecto de la temperatura sobre su estructura serán evaluadas fundamentalmente con técnicas reológicas, mientras que la madalena horneada será analizada mediante texturometría.

ESTRUCTURA DE LA MEMORIA

Esta tesis se encuentra dividida en 8 capítulos a través de los cuales quedan recogidos los resultados obtenidos en cuanto a hidrólisis, separación y aprovechamiento de proteínas de yema de huevo se refiere.

En el capítulo 1 se presenta la introducción de la memoria, la cual a su vez se divide en un texto introductorio, los objetivos, y una explicación sobre la estructura de la memoria. En el capítulo 2 se recogen los fundamentos teóricos, dentro de los cuales se explican principios sobre la estructura y la composición del huevo en general y de la yema de huevo y sus fracciones en particular. Además, se resumen las características de la hidrólisis enzimática y de la hidrólisis con agua en condiciones subcríticas de proteínas, así como anotaciones sobre teoría de reología y texturometría. En el capítulo 3 se describen brevemente las técnicas y los materiales empleados durante el trabajo experimental.

En el capítulo 4 se recogen los trabajos experimentales sobre hidrólisis de proteína de yema de huevo. El trabajo experimental se ha centrado en la hidrólisis de la fracción granular, puesto que es en esta fracción donde se concentra una mayor cantidad de proteína. De esta manera, en la sección 4.1 se trata la hidrólisis de los gránulos de yema de huevo con tripsina. El hidrolizado resultante fue utilizado para la elaboración de mayonesas con el objetivo de ensayar sus propiedades emulsionantes en un producto alimentario. En la sección 4.2 se describe la hidrólisis de proteína de gránulos utilizando agua en condiciones subcríticas, empleando una corriente de gas de nitrógeno o de oxígeno, y comparando los resultados de rendimiento así como las propiedades funcionales de los hidrolizados con aquellos obtenidos mediante hidrólisis enzimática con tripsina. En la sección 4.3 se evalúan las propiedades antioxidantes de los hidrolizados obtenidos según las condiciones descritas en la anterior sección (4.2). En la sección 4.4 se expone una revisión sobre la obtención de hidrolizados de proteínas mediante la hidrólisis ácida y básica, así como con agua en condiciones subcríticas, y que son de interés para la industria alimentaria.

En el capítulo 5 se muestran los resultados obtenidos sobre la separación de la fosvitina del resto de componentes de la fracción granular.

El capítulo 6 se refiere al aprovechamiento directo de los gránulos de yema de huevo usándolos como sustitutivos de yema de huevo entera. Para ello, en la sección 6.1 se utilizaron los gránulos en un producto de horneado sin gluten, analizándose los cambios reológicos que se producían en la masa cruda y los cambios físicos en la madalena horneada cuando dicha sustitución era total. En la sección 6.2, ya en madalenas con gluten, se ensayaron diferentes grados de sustitución que equivalían a diferentes proporciones de plasma/gránulos para evaluar y modelizar el efecto de esta fracción rica en proteínas sobre la masa cruda y horneada.

El capítulo 7 recoge las conclusiones obtenidas. Finalmente, en el capítulo 8 se detallan los apéndices, en los cuales se recogen ensayos que no pueden ser explicitados en el cuerpo de la memoria y que lo complementan. Además se explican las abreviaturas y se muestra la lista de tablas y figuras, la bibliografía conjunta de todo el documento, propuestas de trabajo futuro y la referencia al estado de difusión de este trabajo.

2. FUNDAMENTOS TEÓRICOS

1. EL HUEVO

1.1. CARACTERÍSTICAS GENERALES

Los huevos de gallina tienen tres partes distinguibles y fácilmente separables entre sí: la cáscara, la yema y la clara o albumen. La cáscara se compone principalmente de carbonato cálcico, y representa el 10% p/p del total del huevo. Es una estructura porosa con una membrana interna que rodea a la clara. La porosidad de la cáscara es necesaria para el intercambio de gases y agua[1].

El albumen o clara representa entre el 60-63% del peso total del huevo. El principal componente del albumen es el agua, constituyendo entre el 84% y el 89% del total, además presenta un 10% de proteínas y un 0.03% de lípidos, su proteína más abundante es la albúmina, la cual representa más del 54% de toda la proteína de clara de huevo [2]. Por otro lado la yema puede considerarse un sistema más complejo, con partículas suspendidas en una solución de proteínas y rodeada por la membrana vitelina. Representa un total de entre el 28% y 29% del peso del huevo entero, su contenido en proteínas es de entre el 15.7% al 16.6% y su composición en lípidos varía entre el 31.8%-35.5% [3].

1.2. ESTRUCTURA DE LA YEMA DE HUEVO

Los lípidos en la yema de huevo se encuentran empaquetados junto a proteínas formando lipoproteínas. Las proteínas están presentes como proteínas libres o apoproteínas asociadas a los lípidos. Las lipoproteínas constituyen el principal componente de la yema de huevo, así, la yema de huevo está formada en un 68% por lipoproteínas de baja densidad (LDL), en un 16% por lipoproteínas de alta densidad (HDL), 19% por proteínas globulares (livetinas) y un 4% por fosvitina.

La yema de huevo es un sistema complejo de partículas en suspensión en un fluido amarillento (plasma) que contiene proteínas. Estas partículas en suspensión pueden ser fácilmente separadas del plasma diluyendo la yema de huevo con agua y centrifugándola a 10000 g durante 30 minutos [4]. El agua disminuye la viscosidad de la yema de huevo y facilita la migración de las partículas, de esta forma se obtiene un sobrenadante de color anaranjado, el plasma, y un sedimento formado por la fracción granular. Los constituyentes de la yema de huevo quedan repartidos en el plasma y en los gránulos tal y como se indica en la Tabla 1.

Tabla 1. Distribución de los constituyentes de la yema de huevo. Adaptado de Powrie y Nakay [3].

	Yolk dry matter (%)	Yolk lipids (%)	Yolk proteins (%)	Lipids (%)	Proteins (%)
Yolk	100	100	100	64	32
Plasma	78	93	53	73	25
LDL	66	61	22	88	10
Livetins	10	-	30	-	96
Others	2	-	1	-	90
Granules	22	7	47	31	64
HDL	16	6	35	25	75
Phosvitin	4	-	11	-	95
LDLg	2	1	1	88	10

1.2.1. FRACCIÓN PLASMÁTICA

El plasma comprende el 77-81% de la materia seca de la yema de huevo, y su componente principal son las LDL (85%) y las livetinas (15%) [5]. En el plasma se concentran prácticamente todos los carotenoides. El 5% del total de los lípidos presente en el plasma es colesterol [6].

1.2.2. FRACCIÓN GRANULAR

Los gránulos representan entre el 19-23% de la materia seca de la yema de huevo, reuniendo aproximadamente el 50% del total de la proteína de la yema y el 7% de los lípidos [6]. La estructura de los gránulos fue dilucidada por Mineki y Kobayashi [7] mediante microscopía electrónica de barrido (SEM), apareciendo como formas poliédricas con una geometría y tamaño variable desde las capas internas hasta las externas de la yema de huevo. Estas esferas contienen los componentes granulares.

La matería seca de los gránulos es del 44%, y contiene un 64% de proteínas y un 31% de lípidos [8]. Los gránulos están en un 70% consitituidos por HDL y en un 16% de fosvitina, las lipoproteínas y proteínas que forman parte de los gránulos aparecen asociadas por puentes de fosfocalcio [5], además, en los gránulos se incluyen LDLg (8%) que son lipoproteínas de baja densidad muy similares a las encontradas en el plasma.

La estructura granular se mantiene siempre y cuando la fuerza iónica del medio sea baja, lo cual hace a las proteínas y lípidos constituyentes poco accesibles a la desnaturalización por calor y a la acción de enzimas hidrolíticas. Cuando la fuerza iónica del medio es igual a 0.3 M NaCl los puentes de fosfocalcio se rompen debido a que el sodio reemplaza al calcio divalente, consiguiéndose solubilizar el 80% del contenido granular [8]. La solubilización completa de los gránulos se produce cuando la fuerza iónica alcanza el 1.71 M NaCl.

Las proteínas granulares derivan de un precursor de 480 kDa denominado vitelogenina [9], sintentizado en el hígado y secretado al torrente sanguíneo hasta alcanzar el oocito, tranportándose al interior de este por endocitosis. Durante este transporte la vitelogenina sufre una ruptura proteolítica dando lugar a las apoproteinas que forman parte de las HDL, la fosvitina y el péptido YGP40 [10]. Las HDL o lipovitelinas son proteínas diméricas de 400 kDa constituidas en un 75-80% por proteínas y en un 20-25% de lípidos. Estas lipoproteínas presentan un aspecto globular, con una pequeña oquedad formada por dos láminas-beta ricas en residuos hidrofóbicos, en la cual se pueden alojar 35 moléculas de fosfolípidos [11]. Los triglicéridos quedarían encerrados en esta cavidad hidrofóbica gracias al entorno formado por los fosfolípidos (Figura 1). Cada monómero de HDL está compuesto por 5 apoproteinas principales, con pesos moleculares que varían desde los 35 hasta los 110 kDa [8]. Finalmente, las HDL se agrupan en submicelas que interactúan mediante puentes de fosfocalcio con la fosvitina. En este entramado, cómo interactúan las LDLg con el resto de componentes granulares permanece actualmente en estudio, pero parece ser importante el grado de compactación de las HDL en función del pH del medio para una mayor incorporación de LDLg al interior del gránulo [12]. La estructura esquemática de un gránulo a pH 6.6 se presenta en la Figura 2.

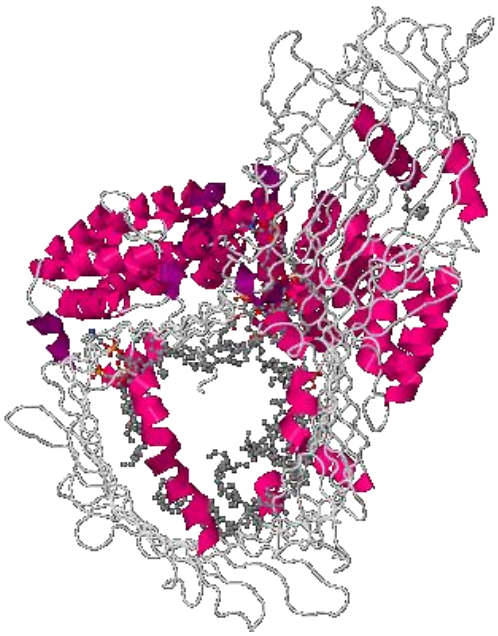

Figura 1. Modelo 3D de la lipovitelina obtenido del RCSB protein data bank (1LSH), de acuerdo con los estudios de Thompson y Banaszak [13].

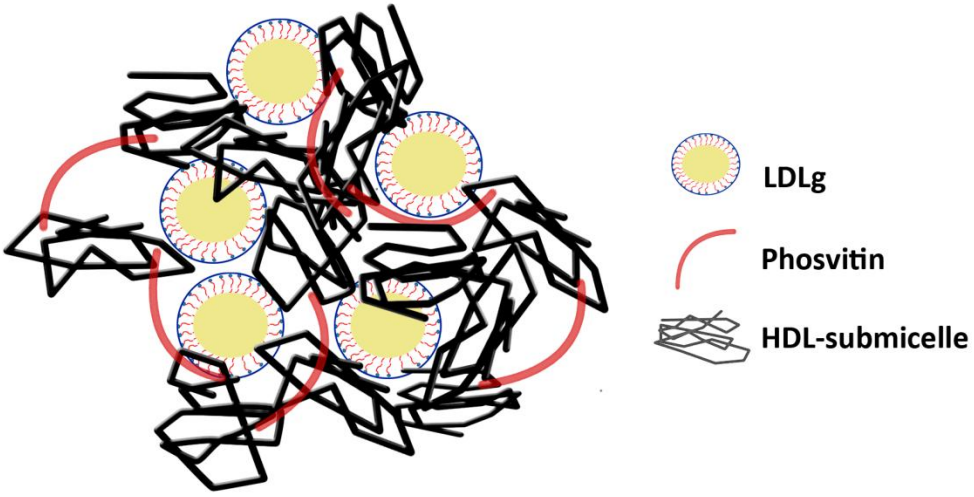

Figura 2. Modelo esquemático de un gránulo de yema de huevo a pH6, con las submicelas de HDL unidas mediante puentes de fosfocalcio a la fosvitina, y con las vesículas de LDL embebidas en el conjunto [12].

2. HIDRÓLISIS DE PROTEÍNAS

2.1. HIDRÓLISIS ENZIMÁTICA

2.1.1. CONSIDERACIONES GENERALES

Las enzimas son moléculas de origen biológico que tienen la facultad de catalizar diferentes reacciones químicas. Aunque no es estrictamente cierto, se tiene en cuenta que en general, las enzimas son proteínas. En la acción enzimática se produce una disminución de la energía libre de activación de Gibbs (ΔG^{*}) lo cual supone un aumento exponencial de las velocidades de reacción del orden de 10^{8} a 10^{12} veces. En cuanto a la variabilidad de enzimas y su nomenclatura estas se pueden subdividir sucesivamente según la acción, la subclase, su substrato o cosubstrato y su número de serie dentro de las diferentes subclases. Teniendo en cuenta las diferentes acciones enzimáticas, la que es de interés en cuanto a la tecnología de proteínas alimentarias se refiere, es la de acelerar la hidrólisis del enlace peptídico.

La hidrólisis de proteínas por acción enzimática ha sido ampliamente estudiada por Adler-Nissen en los años 80, y sus trabajos siguen vigentes hoy día. Este tipo de hidrólisis es el más utilizada actualmente para hidrolizar proteínas en tecnología alimentaria, siendo muy populares en bibliografía enzimas como la tripsina, quimotripsina, alcalasa o la pepsina por citar las más relevantes. La hidrólisis enzimática de proteínas resulta especialmente atractiva para los investigadores principalmente por el hecho de que es predecible si se sabe la secuencia de aminoácidos del substrato, por tanto definiendo las condiciones de hidrólisis se originarán siempre los mismos péptidos. Esta hidrólisis dirigida permite la obtención “a la carta” de péptidos con bioactividades conocidas e interesantes, centrándose la labor investigadora en la selección de la proteína a tratar y de las enzimas que van a participar en el proceso. Sin embargo, a la hora de describir la hidrólisis enzimática también se deben de tener en cuenta sus desventajas y limitaciones: las enzimas pueden considerarse un producto caro

en comparación con las alternativas de hidrólisis no enzimáticas, el sustrato debe de tener las dianas accesibles al medio, además, las enzimas deben de ser inactivadas irreversiblemente para asegurar la total parada de la reacción catalizada, finalmente la acción enzimática requiere de un fuerte control de los parámetros de la reacción:

- Temperatura: En una reacción sin catalizador las moléculas al colisionar lo tienen que hacer en la orientación y con la suficiente energía para que esta se lleve a término. Por tanto, cuanto mayor sea la energía cinética media del conjunto de moléculas más colisiones eficaces se producirán. Según Arrhenius, el logaritmo neperiano de la constante de velocidad es función de la energía de activación necesaria para que se dé la reacción y de la temperatura. A partir de sus observaciones experimentales se llegó a la siguiente ecuación:

$$\ln k = \ln A - \frac{Ea}{R}\frac{1}{T} \qquad \text{Ecuación 1.}$$

En una reacción catalizada por enzimas la dependencia de la velocidad de la reacción con la temperatura se produce de una manera similar, sin embargo hay que tener en cuenta la complejidad de este tipo de biopolímeros. En general las proteínas tienen un rango óptimo de actividad frente a la temperatura de entre los 25 y los 40 ^{o}C *in vitro.* Se estima una temperatura óptima para cada enzima por encima de la cual los procesos de desnaturalización merman su capacidad catalítica, con un impacto sobre la V_{max} cada vez más acentuado. La dependencia de la V_{max} con la temperatura se puede esquematizar según la Figura 3.

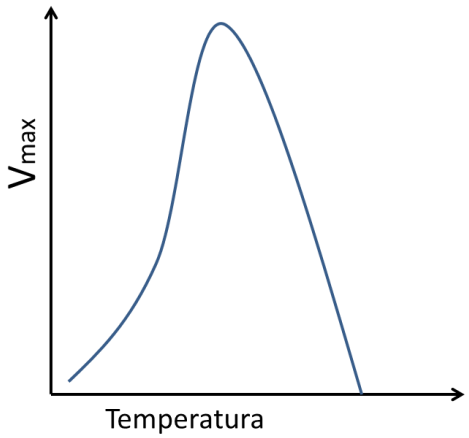

Figura 3. Relación de la velocidad máxima de la reacción enzimática con la temperatura.

-Fuerza iónica del medio: Variaciones en la fuerza iónica del medio pueden llevar a cambios en los pK_a de los aminoácidos del centro activo y por tanto a la pérdida de actividad. Cambios más abruptos pueden incluso comprometer la solubilidad de las proteínas, produciéndose su precipitación por *salting out*.

-Efecto del pH: El grado de protonación o desprotonación de los aminoácidos que forman parte del centro activo afecta críticamente a la actividad de las enzimas. De este modo podemos dividirlas en enzimás ácidas, neutras o alcalinas (Figura 4) en

función de su grado de actividad a diferentes pHs. La relación de la V_{max} con el pH del medio en cada tipo de enzima se presenta en la Figura 4.

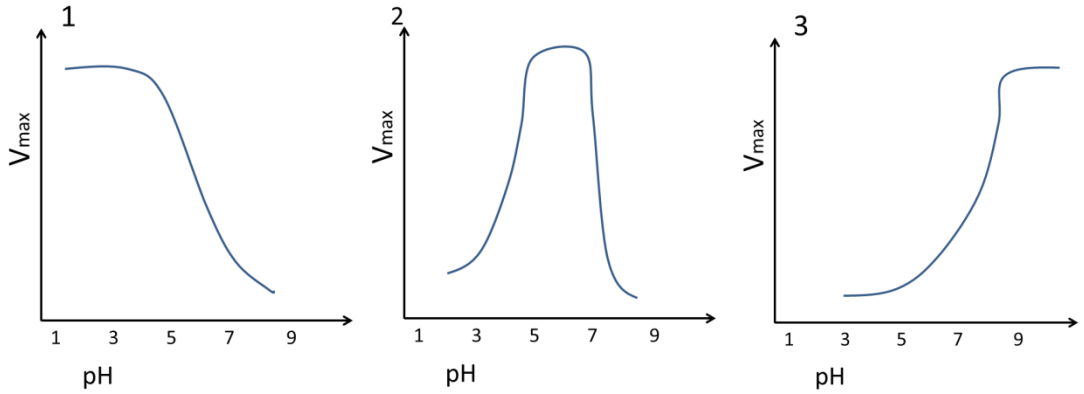

Figura 4. Relación de la velocidad máxima de la reacción enzimática con el pH. Comportamiento típico de enzimas ácidas (1), neutras (2) y básicas (3).

2.1.2. OBTENCIÓN DE PÉPTIDOS CON NUEVAS CAPACIDADES ANTIOXIDANTES Y FUNCIONALES

Las proteínas han sido utilizadas ampliamente en tecnología alimentaria como ingrediente con cualidades gelificantes, emulsionantes o espumantes. Sin embargo, todas estas propiedades funcionales derivan de la capacidad de las proteínas de ser solubles en el medio en el que se encuentran. Por tanto todas aquellas proteínas que no se solubilizan, ya sea porque se han extraído utilizado condiciones agresivas que producen su precipitación o porque son formuladas en un medio en el cual el pH coincide con el pI de la proteína, tienen una utilidad limitada en tecnología alimentaria. A este respecto mediante la hidrólisis de proteínas insolubles se puede originar péptidos solubles con nuevas cualidades funcionales. En la bibliografía aparecen diferentes ejemplos de estudios desarrollados en este sentido usando enzimas y un extracto insoluble de proteína de soja [14], proteína de garbanzo [15], germen de trigo [16], pescado [17, 18], proteína de colza [19] o proteína de suero de leche [20] entre otros.

2.2. HIDRÓLISIS DE PROTEÍNAS CON AGUA EN CONDICIONES SUBCRÍTICAS

2.2.1. CONSIDERACIONES GENERALES

Se considera el agua en condiciones subcriticas cuando su temperatura varía entre los 100°C y los 374°C, manteniéndose líquida gracias a la aplicación de altas presiones [21]. En este estado las propiedades del agua cambian de manera acentuada, produciéndose una disminución de su constante dieléctrica desde los 78 F m^{-1} a 25°C y 0.1 MPa hasta los 14.07 F m^{-1} a 350°C y 20 MPa [22]. Esta variación permite que las moléculas de agua puedan interaccionar con substancias no polares, disminuyendo sus fuerzas de unión y disolviéndolas. Además se produce un aumento considerable de la K_w pudiendo pasar de

10^{-14} a temperatura ambiente a 10^{-12} en condiciones sub-criticas. Este aumento de H^+ y OH^- aumenta la reactividad del agua y su capacidad de catalizar reacciones tipo ácido/base.

La hidrólisis de proteínas con agua en condiciones sub criticas conlleva primero la ruptura del enlace peptídico y la liberación de péptidos y aminoácidos al medio. Cuando la reacción progresa lo suficiente en el tiempo hay que considerar la ruptura de toda la proteína en sus aminoácidos constituyentes. Finalmente los aminoácidos pueden terminar descomponiéndose debido a la alta temperatura, dando a lugar ácidos orgánicos como el ácido propiónico, láctico, acético, fórmico, succínico y málico , además de gases como el CO_2 o CO entre otros [23].

Habitualmente se considera que la hidrólisis de proteínas y la posterior degradación de sus aminoácidos puede ser descrita mediante una reacción irreversible de primer orden, según la cual se podrían calcular dos constantes cinéticas (Figura 5).

$$\text{Proteína} \xrightarrow{k_1} \text{Aminoácidos} \xrightarrow{k_2} \text{Productos de degradación}$$

Figura 5. Reacción irreversible de primer orden que describe la hidrólisis de proteínas en condiciones subcríticas. k_1 y k_2 son las constantes cinéticas de la ruptura de la proteína y la degradación de los aminoácidos respectivamente.

Por tanto, a la hora de elegir las condiciones de presión, temperatura y tiempo de residencia hay que tener en cuenta la velocidad a la que se dan los fenómenos de ruptura de proteína y degradación de aminoácidos. Por ejemplo, Rogalinsky et al. [24], investigando con BSA, determinó que a 310°C y 330°C todos los aminoácidos se descomponían después de 140 segundos de reacción, resultando 230°C la mejor temperatura para preservar los aminoácidos en el medio.

2.2.2. HIDRÓLISIS CON AGUA EN CONDICIONES SUBCRÍTICAS EN LA INDUSTRIA ALIMENTARIA

Una de las aplicaciones que aparece con frecuencia en la bibliografía es el uso del agua en condiciones sub críticas para recuperar moléculas de interés a partir de deshechos de la industria alimentaria. Así, es recurrente el uso de esta tecnología para obtener péptidos y aminoácidos de residuos orgánicos, como por ejemplo a partir de entrañas de pescado [25], vísceras de vieiras [26], pelo de cerdo [27] o residuos de la industria avícola [28]. Utilizando desechos de habas se ha podido llegar a recuperar el 52.9% en forma de aminoácidos del contenido en proteína total, con salvado de arroz el 75% en forma de péptidos y el 5% en forma de aminoácidos, y usando cáscara de camarón 70mg de aminoácidos por cada gramo de producto procesado.

2.3. HIDRÓLISIS DE PROTEÍNAS DE HUEVO

La hidrólisis enzimática de proteínas de clara de huevo ha sido ampliamente estudiada. Se ha demostrado que la clara de huevo tratada controladamente con pepsina da lugar a péptidos con capacidades antioxidantes y con efecto anti hipertensivo [29]. Otros autores, también trabajando con clara de huevo cruda, encuentran los mismos efectos inhibitorios sobre la enzima agiotensin convertasa, principalmente cuando el tratamiento es también con pepsina, identificándose en este caso 14 peptidos provenientes principalmente de la ovoalbúmina con esta capacidad [30]. Otro estudio [31] exploró las cualidades antimicrobianas de los péptidos resultantes de la hidrólisis con pepsina y tripsina de la lisozima de clara de huevo. En este caso se determinaron 20 pequeños péptidos de menos de 1000 Da que mostraron actividad bacteriostática contra bacterias gram positivas y gran negativas.

En cuanto a la hidrólisis enzimática de yema de huevo, habitualmente se requiere su delipidación previa mediante el tratamiento con etanol a 40°C. Este pretratamiento produce la desnaturalización y precipitación de la proteína, que posteriormente se recupera en forma de péptidos solubles con diferentes cualidades. Sakanaka et al. [32] encontraron cualidades antioxidantes con respecto al ácido linoleico en los péptidos obtenidos mediante hidrolisis con proteasas de Bacillus sp. Los mismos autores con las mismas enzimas estudiaron también las propiedades antioxidantes de los péptidos obtenidos en sistemas alimentarios complejos como la carne de vaca y de atún [33]. También Wang and Wang [34] estudiaron la hidrólisis de la proteína de yema de huevo utilizando varias endoproteasas bacterianas,obteniendo nuevos péptidos con propiedades emulsionantes y espumantes. Goulas et al. [35]estudió el producto de la hidrólisis de la fosvitina, una proteína de los gránulos de yema de huevo, con tripsina, pepsina y chimotripsina, resultando que la proteína era de difícil digestión. Por otro lado, Orcajo et al. [36] hidrolizaron gránulos de yema de huevo previamente disueltos en agua con NaCl 0.55 M con tripsina alcanzando un grado de hidrólisis del 12% en 90 minutos. Los hidrolizados fueron caracterizados mediante electroforesis y cromatografía con columna de exclusión por tamaño. En este caso se elaboraron posteriormente mayonesas con los hidrolizados. Finalmente, Marcet et al. [37] hidrolizaron los gránulos de yema de huevo delipidados con etanol, pero utilizando agua en condiciones sub-críticas, obteniéndose una mezcla de péptidos con propiedades funcionales. En este último caso se aumentó el rendimiento en comparación con la hidrólisis de la misma proteína de yema de huevo con enzimas (tripsina).

3. REOLOGÍA

3.1. CONSIDERACIONES GENERALES

La reología describe la deformación de un líquido, sólido o gas bajo la influencia del estrés. Cómo la materia se deforma da información sobre su estructura y permite predecir su comportamiento en determinadas condiciones. La reología se ha beneficiado de un gran desarrollo científico gracias al estudio de los polímeros industriales y sus solventes, por tanto, es una disciplina que se ha adaptado recientemente para el estudio estructural de los alimentos. En muchas circunstancias, podríamos considerar un alimento como una serie de

polímeros, en este caso macromoléculas de origen biológico, esencialmente proteínas, hidratos de carbono y lípidos, suspendidas en un medio acuoso. Todas estas macromoléculas pueden estar interaccionando entre sí y con el solvente en el que se encuentran pudiéndose deducir su estructura conociendo su comportamiento reológico.

Los alimentos se pueden presentar de diferentes maneras: en estado sólido, líquido, como un gel, como una suspensión de sólidos en un líquido y en forma de emulsión. En los alimentos, la variación de la fórmula añadiendo o quitando una pequeña cantidad de un polímero puede afectar drásticamente al comportamiento reológico del mismo. Por tanto los estudios sobre sus propiedades reológicas son útiles e importantes para aplicaciones que incluyen el procesamiento, manipulación, el control de calidad y el análisis de sus cualidades sensoriales [38].

3.2. ESFUERZO CORTANTE Y VELOCIDAD DE DEFORMACIÓN

Teniendo en cuenta la medida del flujo de una muestra entre dos placas paralelas, el esfuerzo cortante es la fuerza aplicada tangencialmente sobre la muestra en un área dada (Figura 6, ecuación 2). La máxima velocidad de flujo se encontraría en la placa superior (Vmax), e iría bajando a medida que se desciende hacia la superficie fija. Esta caída de velocidad a lo largo del espacio en el que se encuentra alojada la muestra se denomina velocidad de deformación ($\dot{\gamma}$, ecuación 3). De acuerdo a estos parámetros, Newton expresó la Ley de la viscosidad, la cual describe el comportamiento de flujo de un líquido ideal, relacionando el esfuerzo cortante y la velocidad de deformación (ecuación 4).

$$\tau = \frac{Fuerza\ (Newton)}{Área\ (m^2)} = Pa \qquad \text{Ecuación 2.}$$

$$\dot{\gamma} = \frac{dv}{dy} = \frac{m/_s}{m} = \frac{1}{s} \qquad \text{Ecuación 3.}$$

$$\tau = \eta \cdot \dot{\gamma} \qquad \text{Ecuación 4.}$$

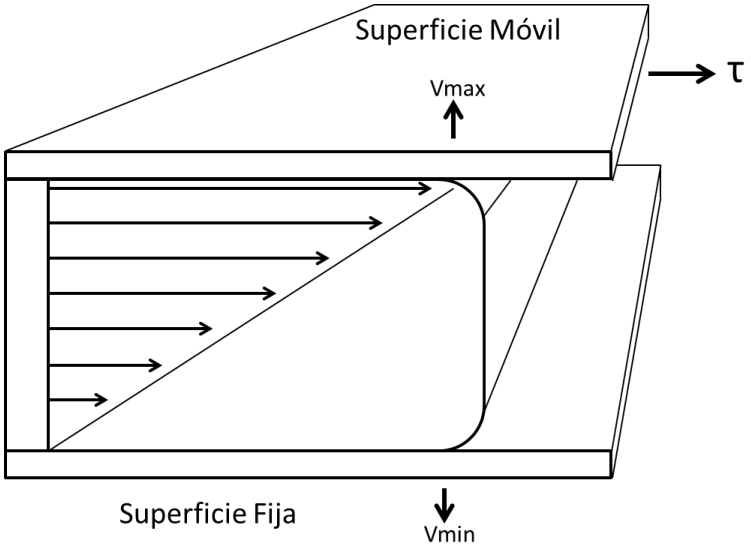

Figura 6. Flujo entre dos platos paralelos.

3.3. TIPOS DE FLUIDOS

3.3.1. FLUIDOS NEWTONIANOS

De acuerdo a la Ley de Newton, la representación del flujo de un fluido ideal describiría una línea recta con inicio en el origen (Figura 7.1), y puesto que la ecuación 4 puede reordenarse en la ecuación 5, para cualquier par esfuerzo cortante-velocidad de deformación la viscosidad mantendría el mismo valor. Este hecho puede apreciarse en la curva de viscosidad (Figura 7.2). Como ejemplos de fluidos Newtonianos tenemos el agua, la miel, bebidas carbonatadas o la leche.

$$\eta = \frac{\tau}{\dot{\gamma}}$$ Ecuación 5.

3.3.2. FLUIDOS NO NEWTONIANO

Todos los demás líquidos que no exhiben el comportamiento descrito anteriormente son denominados “no Newtonianos”. Podrían clasificarse en tres grandes grupos.

1. Comportamiento independiente del tiempo.
2. Comportamiento dependiente del tiempo.
3. Viscoelásticos.

3.3.2.1 COMPORTAMIENTO INDEPENDIENTE DEL TIEMPO

En este caso podemos encontrarnos con los fluidos pseudoplásticos, viscoplásticos y dilatantes. El comportamiento de estas sustancias en las curvas de flujo y viscosidad se detalla en la Figura 7.

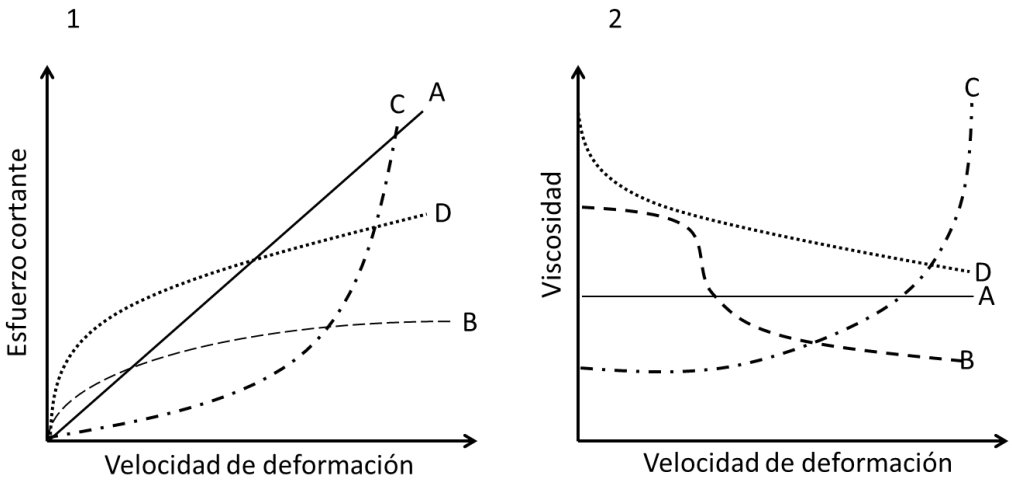

Figura 7. Curvas de flujo (1) y de viscosidad (2) de fluidos Newtonianos (A), pseudoplásticos (B), dilantantes (C) y viscoplásticos (D).

Tanto en los fluidos pseudoplásticos como dilatantes se produce una variación de la viscosidad en función de la velocidad de deformación. En el caso de los pseudoplásticos, la viscosidad disminuye conforme aumenta la velocidad de deformación, mientras que en el caso de los fluidos dilantantes se da el fenómeno inverso, aumentando la viscosidad conforme aumenta la velocidad de deformación. Estos fluidos pueden ser descritos mediante la ecuación de Ostwald o ley de la potencia (ecuación 6). De esta ecuación se deduce la consistencia (k) y el índice del comportamiento de flujo (n). Según el valor de ***n*** puede caracterizarse un fluido como newtoniano (n = 1), pseudoplástico (n < 1) o dilatante (n > 1). Muchos fluidos alimentarios tienen un comportamiento pseudoplástico, sin embargo, los fluidos dilatantes son más difíciles de encontrar.

$$\tau = k \cdot \dot{\gamma}^{n} \qquad \text{Ecuación 6.}$$

Otro tipo de fluido independiente del tiempo es el fluido viscoplástico. Estas sustancias exhiben un comportamiento sólido mientras el esfuerto de corte no supere un valor de

referencia, a partir del cual se comporta como un fluido Newtoniano (plástico de Bingham) o como un fluido pseudoplástico.

3.3.2.2. COMPORTAMIENTO DEPENDIENTE DEL TIEMPO

Los fluidos que exhiben comportamiento pseudoplástico habitualmente presentan tixotropía. Los fluidos tixotrópicos suelen ser sistemas heterogéneos con una fase dispersa muy fina. Estas partículas aparecen interaccionando entre sí cuando el sistema se encuentra en reposo, sin embargo, cuando las fuerzas de cizallamiento son suficientemente altas, estas interacciones se deshacen y la resistencia a fluir se ve disminuida con el tiempo [39], este comportamiento se describe según la Figura 8. En este tipo de fluidos, cuando se enfrenta el esfuerzo cortante con la velocidad de deformación, a velocidadades de deformación crecientes y decrecientes consecutivas, las dos curvas no coincidirán en valores. La tixotropía puede encontrarse en soluciones muy concentradas, como por ejemplo en quesos blandos o en salsas para ensalada.

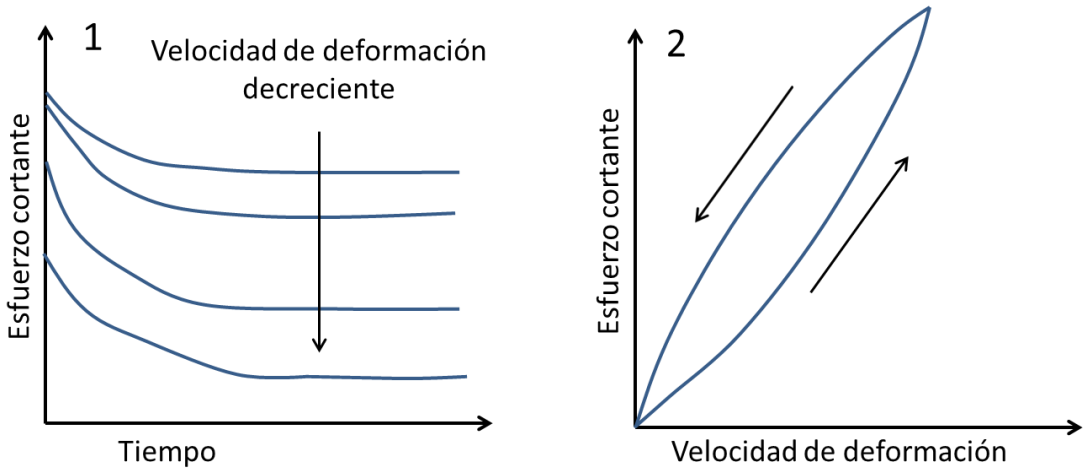

Figura 8. Comportamiento de fluidos tixotrópicos a velocidades de deformación constantes (1) y a velocidades de deformación crecientes-decrecientes (2).

3.3.2.3. VISCOELÁSTICOS

Los fluidos viscoelásticos se deforman después de aplicar una cierta energía al sistema, sin embargo, una vez cesado el esfuerzo cortante sólo son capaces de recuperar un porcentaje de la forma original, liberándose parte de la energía aplicada en forma de calor. Estos fluidos pueden estudiarse según el modelo de Maxwell, en el cual se entiende que en la sustancia aparecen a la vez estructuras tipo resorte capaces de recuperar la forma tras haberse aplicado la fuerza, y tipo pistón que se deforman sin posibilidad de recuperación (Figura 9). Si la deformación es lo suficientemente alta la estructura interna se colapsa produciéndose el flujo del material. Las pastas y emulsiones concentradas se comportan de este modo.

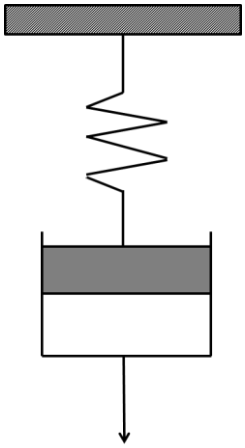

Figura 9. Modelo Maxwell simple, resorte-pistón.

3.4. MEDIDAS REOLÓGICAS

Las medidas reológicas se desarrollan con un reómetro o viscosímetro, presentando el instrumento diferentes sondas de medida (cono-plato, cilindro concéntrico, platos en paralelo…) para adecuarse a las necesidades de la muestra a tratar. En la Figura 10 se muestran los diferentes tipos de ensayos reológicos que pueden utilizarse en la caracterización de productos alimentarios.

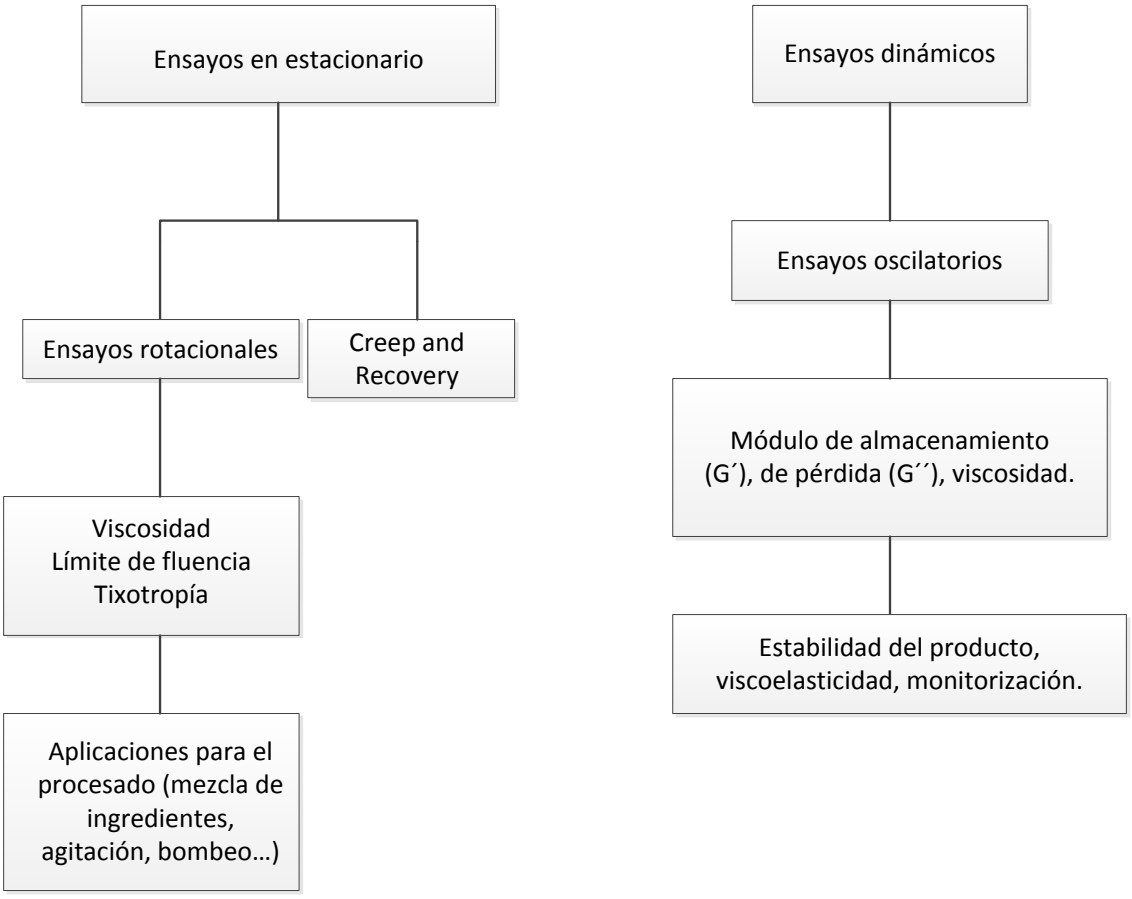

Figura 10. Ensayos reológicos utilizados en la caracterización de productos alimentarios. Adaptado de Tabilo-Munizaga y Barbosa-Cánovas [40].

4. TEXTUROMETRÍA

4.1. CONSIDERACIONES GENERALES

La textura es un atributo clave a la hora de determinar la calidad y aceptabilidad de cualquier producto alimentario, ya sea fresco o procesado. Especialmente durante el diseño de los alimentos, la formulación de los mismos se asocia con cambios en la textura que pueden variar su aceptabilidad. Por ejemplo, el consumo regular de inulina se considera beneficioso para la salud, sin embargo, a ciertas dosis la adición de inulina al pan produce un aumento de la dureza en el producto horneado y un empeoramiento en general de la calidad [41]. De igual manera, cuando se intenta ajustar la cantidad de grasa de un producto alimentario, el impacto sobre la textura debe de ser convenientemente evaluado. En este sentido Sudha et al. [42] estudiaron la variación en la textura de una galleta conforme se producía una disminución en la cantidad de aceite añadido.

La evaluación de la textura de un alimento puede analizarse de diferentes maneras: utilizando un panel compuesto por un variable número de individuos, muy frecuentemente 10 panelistas, que han sido entrenados previamente [43-45]. El grado de entrenamiento puede ser regulado en función de las habilidades de los valoradores, así por ejemplo, existen estudios en los que se determina la textura de un alimento con un panel altamente experimentado que apenas recibe formación adicional en este sentido [46]. Habitualmente, a la hora de describir la textura del alimento los investigadores hacen uso de escalas sensoriales que forman parte del entrenamiento de los panelistas.

Por otro lado se puede optar por la utilización de instrumentos para desarrollar medidas objetivas sobre la textura alimentaria. En este caso, el test de punción y compresión es el más común a la hora de caracterizar las propiedades texturales de los alimentos. Dentro de los tests de compresión, el análisis del perfil de textura (TPA) imita el proceso de masticación, con dos ciclos de compresión y se encuentra ampliamente desarrollado en la bibliografía. En un TPA el alimento debe de ser previamente acondicionado, disponiéndolo en forma cilíndrica para que las irregularidades no alteren la comparación de las diferentes muestras. Por ejemplo, en el caso del análisis textural de setas se usaron cilindros de 20 mm de longitud y diámetro [47]. En el caso de madalenas hay autores que usan cortes cilíndricos de 2.5 cm de altura [48], en el caso de pasteles horneados, Gómez et al. [49] dispusieron la miga en un tamaño de 40x40x20 mm y Marcet et al. [50], también con madalenas, hicieron cortes de 2 cm de altura de la miga.

Una vez preparada la muestra, la sonda, que está constituida por un plato cilíndrico y plano, desciende a una velocidad constante comprimiendo el alimento a analizar, resultando un perfil en el que se enfrenta la fuerza aplicada frente al tiempo (Figura 11).

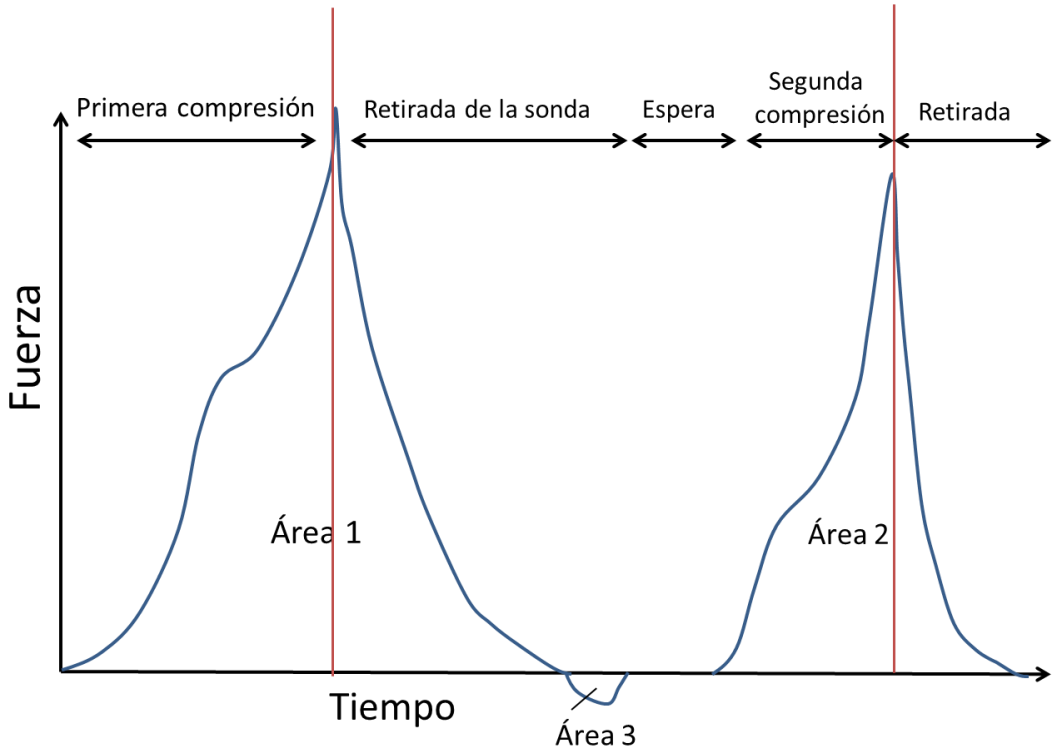

Figura 11. Curva típica obtenida de un ensayo de doble compresión (TPA).

Mediante un TPA se pueden caracterizar diferentes propiedades texturales que son deducidas a través del cálculo de las áreas y las alturas de los picos resultantes:

1. Dureza: El punto de fuerza máximo durante la primera compresión.
2. Fracturabilidad: No siempre aparece, se aprecia en el perfil como un primer pico en el primer ciclo de compresión muy anterior a la retirada de la sonda.
3. Elasticidad: El grado de recuperación de la muestra después de la primera compresión.
4. Cohesividad: Cómo la muestra soporta la segunda deformación en relación a como se comportó durante la primera compresión. Se calcula dividiendo el área 2 entre el área 1.
5. Masticabilidad: Sólo para productos sólidos y se relaciona con el esfuerzo que se requiere para masticarlo. Se calcula como el producto de la dureza por la cohesividad por la elasticidad.
6. Adhesividad: La resistencia del producto a despegarse de la sonda durante la primera retirada, se calcula como el área 3.
7. Gomosidad: Aparece en productos semisólidos, gomosidad y masticabilidad son mutuamente excluyentes. Se calcula como el producto de la dureza por la cohesividad.
8. Resiliencia: Se puede considerar como una primera elasticidad o la capacidad que tiene una muestra para volver a su posición inicial en un primer momento. Se calcula como el área durante la primera retirada de la sonda dividida entre el área 1.

4.2. REOLOGÍA Y TEXTUROMETRÍA DE PRODUCTOS DE HORNEADO.

En el pan, variaciones en la formulación y en el tratamiento de la masa que impliquen a parámetros como la cantidad de sal, temperatura de almacenamiento, tiempo de fermentación, presencia de aire, etc, afectan de diferente manera a la estructura interna del producto crudo y por tanto a la calidad del pan horneado. Así por ejemplo, el contenido en agua de la masa tiene un efecto negativo sobre G´ y G´´, y estos dos parámetros se ven reducidos conforme aumenta el contenido en agua [51, 52]. La adición de tensioactivos, como ésteres de sacarosa disminuyen la resistencia a la deformación y la extensibilidad de la masa [53], otros emulsionantes, como los mono y digliceridos y la lecitina mejoran la absorción de agua, el desarrollo de la masa y su estabilidad [54]. La adición de fibra de chocolate como un sustituto de parte del aceite en madalenas produce variaciones en la consistencia (k) y en el índice de comportamiento de flujo (n) de la masa cruda, aumentando el primero y disminuyendo el segundo con respecto a la madalena control. Estas variaciones dan lugar a una miga con mayor cohesividad y de más difícil masticación [48]. La adición de otras fibras insolubles como fibra de avena, y de fibras solubles como inulina y goma guar en pasteles, dieron como resultado un aumento en la viscosidad de la masa (excepto en el caso de la adición con inulina) y un aumento en la dureza del producto cocinado esto ya se dijo con anterioridad [55]. Por otro lado, la presencia de almidón modificado también ha sido estudiada desde el punto de vista reológico y en relación con la mejora del producto horneado. Sanz et al. [56] investigaron cuatro tipos de almidones resistentes en una fórmula de madalena, observándose las propiedades de flujo de la masa cruda, así como sus características viscoelásticas mediante el análisis de barridos de temperatura y el barrido de frecuencia a 80°C. Asimismo, el contenido y tipo de proteína también afecta a las propiedades reológicas ya que se producen variaciones en la red proteíca formada antes y después del calentamiento de la masa. Pernell et al. [57] estudiaron el efecto de cierta cantidad de proteína de clara de huevo o de suero de leche en un pastel de ángel, observándose como la reología entre ambas formulaciones era sensiblemente diferente y la textura del producto horneado final también.

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3. MATERIALES Y MÉTODOS

1. OBTENCIÓN DE GRÁNULOS DE YEMA DE HUEVO EN CONDICIONES NATIVAS Y DELIPIDADOS

Los gránulos fueron extraídos siguiendo el procedimiento desarrollado por Laca et al. [1]. La yema de huevo fue separada de la clara y las chalazas retiradas con pinzas, a continuación la membrana vitelina fue secada con papel de laboratorio y rota por un extremo. La yema se vertió en un vaso de precipitados y diluida 1:1.5 con agua destilada. El pH de la solución se ajustó a 7 con NaOH 1N y se mantuvo toda la noche a 4°C. Finalmente se procedió a centrifugar a 10000 g durante 45 minutos, quedando los gránulos en el sedimento y el plasma en el sobrenadante. El plasma fue descartado por decantación.

En caso de que los gránulos fuesen delipidados, primero fueron liofilizados y mezclados con etanol (96%, 1:12.5, p/v) a 40°C durante 2 h con agitación suave. A continuación los gránulos fueron recuperados con ayuda de una bomba de vacío filtrándolos con papel Whatman N° 1. Los gránulos delipidados fueron lavados con 200 mL de etanol fresco y secados durante toda la noche en un calentador a 50°C, obteniéndose un polvo deshidratado y libre de etanol.

2. ELABORACIÓN DE PRODUCTOS ALIMENTARIOS

2.1. PREPARACIÓN DE MADALENAS SIN GLUTEN

La preparación de madalenas fue elaborada de acuerdo a la receta de un manufacturero nacional de productos sin gluten. La formulación básica incluye 17 g de pectina de manzana, 102.12 g de harina de maíz, 65.36 gramos de clara pasteurizada, 36.76 gramos de yema de huevo, 93.6 g de aceite de girasol y 85.08 gramos de azúcar.

La yema de huevo y la clara fueron batidas por 3 minutos empleado una batidora de mano de 180 W (HB01, Morphy Richards, UK) a máxima velocidad. El azúcar y la gelatina fueron añadidos y mezclados por 2 minutos. El aceite de girasol fue añadido y batido por 1.5 minutos. Finalmente, la harina fue incorporada y mezclada por 2 minutos con una mezcladora de 200 W (SM3827, Severin Eletrogeräte, Germany) con la velocidad ajustada al nivel 3. Ya que los gránulos de yema de huevo tienen una cantidad de colesterol reducida, se evaluó el impacto de usar esta fracción de la yema de huevo como sustitutivo de la yema entera. En este caso se elaboraron cuatro diferentes tipos de madalenas: una madalena con la receta original y usando yema de huevo; un segundo tipo sin yema de huevo y con su peso equivalente en gránulos de yema de huevo; un tercer tipo sin yema de huevo y con un 60% del peso original de gránulos de yema de huevo, con el objetico de disminuir aún más el contenido en colesterol, y una cuarta madalena sin yema de huevo y con su peso equivalente en forma de gránulos liofilizados y reconstituidos con agua, con el fin de evaluar el impacto de un posible método de conservación en las cualidades físicas y organolépticas de la receta.

2.2. PREPARACIÓN DE MADALENAS CON GLUTEN

Las madalenas con gluten fueron elaboradas según una receta tradicional. La formulación incluye 100 g de harina de trigo, 3 g de levadura química (difosfato sódico (E-450i), Bicarbonato sódico (E-550ii), carbonato sódico (E-500i) y sulfato cálcico (E-516), 65 g de clara de huevo pasteurizada, 35 g de yema de huevo, 100 g de aceite de girasol y 100 g de azúcar. La yema de huevo y la clara fueron batidas durante 3 minutos empleado una batidora de mano de 180 W (HB01, Morphy Richards, UK) a máxima velocidad. El azúcar y el aceite fueron añadidos y mezclados durante 2.5 minutos con una mezcladora de 200 W (SM3827, Severin Eletrogeräte, Germany) con la velocidad ajustada al nivel 3. Finalmente, la harina y la levadura química fueron añadidas y mezcladas por 2 minutos. 6 recetas diferentes fueron desarrolladas, como se muestra en la Tabla 1. En cada receta la yema de huevo entera fue sustituida progresivamente por la fracción granular, en la sexta receta se sustituyó toda la yema de huevo por gránulos y se añadió un emulsificante ampliamente utilizado en la industria alimentaria (E-471).

Tabla 1. Porcentaje de yema de huevo, gránulos y emulsificante en cada receta.

	A	**B**	**C**	**D**	**E**	**F**
Yema de huevo % (p/p)	100	75	50	25	X	X
Gránnulos % (w/w)	X	25	50	75	100	100
E-471 (g)	X	X	X	X	X	2.5

2.3. PREPARACIÓN DE MAYONESAS

La formulación de la mayonesa fue desarrollada de acuerdo con Laca et al. [2] con ligeras modificaciones. En esta fórmula se incluyen 9 mL de vinagre blanco (6% de acidez), 0.94 g de sal fina, 1.3 g de azúcar y 70 mL de aceite de girasol. Como emulsionante se utilizaron gránulos de yema de huevo liofilizados y gránulos de yema de huevo hidrolizados liofilizados hasta obtener una concentración de proteína de 27 mg/g de mayonesa. Para la elaboración de la mayonesa primero se mezclaron la sal, el azúcar y el vinagre a 8000 rpm usando un homogeneizador SilentCrusher M (Heidoloph, Alemania) para a continuación ir añadiendo el agente emulsionante. Durante el mezclado, 20 mL de aceite de girasol fueron añadiéndose gota a gota, el resto del aceite fue añadido en cantidades de 10 mL. El tiempo total de mezclado fue de 15 minutos.

3. PROPIEDADES FÍSICAS Y SENSORIALES DE LOS ALIMENTOS

3.1. REOLOGÍA

Los ensayos reológicos fueron llevados a cabo con un reómetro rotacional Haake MARS II (Thermo Scientific, USA) capaz de realizar ensayos en estacionario y dinámicos. El reómetro presenta una unidad Peltier para controlar la temperatura en función del ensayo a realizar. La unidad de medida seleccionada fue plato/plato (pp60), con una apertura de 1 mm. Para evitar la deshidratación de la muestra se empleó aceite de silicona y una cúpula de vidrio. Los parámetros reológicos y los modelos obtenidos se calcularon según el programa informático Haake Rheowin.

3.1.1. ENSAYOS DINÁMICOS

3.1.1.1. BARRIDOS DE ESFUERZO

Las curvas de esfuerzo fueron desarrolladas con un esfuerzo cortante desde 0.01 hasta los 100 Pa y a una frecuencia 1Hz. Antes de desarrollar cualquier otro ensayo dinámico se realizaron barridos de esfuerzo a diferentes temperaturas para seleccionar esfuerzos cortantes que estuvieran dentro del rango viscoelástico lineal de la muestra a tratar.

3.1.1.2. BARRIDOS DE FRECUENCIA

Los barridos de frecuencia fueron llevados a cabo entre 10.0 Hz y los 0.01 Hz. Los datos experimentales fueron ajustados a la siguiente ecuación de acuerdo con Gabriele et al. [3].

$$G^* = A.\omega^{1/z} \qquad \text{Ecuación 1.}$$

Donde G^*es el modulo complejo en Pa, ω la frecuencia en Hz, Z el número de coordinación y A el coeficiente proporcional.

3.1.1.3. BARRIDOS DE TEMPERATURA

Fueron llevados a cabo entre 4°C-20°C en el caso de trabajar con mayonesas o entre los 25°C-100°C en el caso de las madalenas. La frecuencia se mantenía en 1Hz y la tasa de calentamiento en 1°C/min.

3.1.2. ENSAYOS ESTACIONARIOS

3.1.2.1. CREEP AND RECOVERY

La fase de deformación fue desarrollada teniendo en cuenta un esfuerzo cortante constante (2 Pa), que se encuentra dentro del rango viscoelástico lineal de la muestra a tratar, durante 500 segundos. El tiempo de recuperación se extendió durante 1500 segundos. Los valores de porcentaje de deformación fueron recolectados en función del tiempo. Los datos experimentales fueron ajustados al modelo de Burger [4] (Figura 1). La ecuación 2 se corresponde con la ecuación utilizada para modelar la fase *creep* [5]:

$$\gamma(t) = \frac{\sigma_0}{G_0} + \frac{\sigma_0}{\eta_0} t + \frac{\sigma_0}{G_1\left(1 - exp^{-t/\lambda ret}\right)}$$ Ecuación 2.

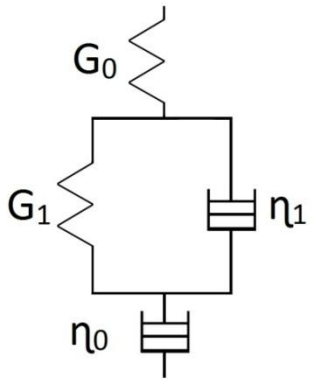

Figura 1. G_0 y η_0 representan el módulo elástico y la viscosidad del modelo de Maxwell. G_1 y η_1 son el módulo elástico y la viscosidad interna del módelo de Kelvin-Voigt.

3.1.2.2. CURVAS DE FLUJO

La viscosidad aparente fue obtenida en función de la velocidad de deformación desde los 0.01 1/s hasta los 100.0 1/s. En cada ensayo se anotaron 100 puntos con una distribución logarítmica. Los datos obtenidos se ajustaron al modelo de Ostwald de Waele [6].

$\eta = K\ \dot{\gamma}^{n-1}$ Ecuación 3.

Donde η es la viscosidad aparente, $\dot{\gamma}$ es la velocidad de deformación, K (Pa.s^n) es el índice de consistencia, y n es el índice de comportamiento de flujo.

3.2. GRAVEDAD ESPECÍFICA Y ANÁLISIS DE IMAGEN DE MASA DE MADALENA CRUDA

La gravedad específica de cada muestra fue calculada llenando un contenedor estándar con masa cruda y dividiendo su peso por el del mismo contenedor lleno de agua destilada. En cuanto al recuento de burbujas en la masa cruda, se utilizó un método similar al de Gómez et al. [7], utilizando microscopía confocal (BX50, Olympus, Japón). El número de burbujas fue calculado en una superficie de 4 mm^2 de masa cruda usando el programa informático ImageJ.

3.3. ANÁLISIS DEL PERFIL DE TEXTURA DE MADALENAS HORNEADAS (TPA)

Para el análisis de textura se empleó un TA.XT.plus Texture analyser (Stable Microsystems, UK). Para ello, 2 cm de la mitad inferior de cada madalena fueron evaluados mientras que la mitad superior fue descartada. Se utilizó una sonda cilíndrica de base plana (P/75) con una velocidad de descenso de 1.0 mm/s, y una doble compresión del 50% de la altura original de la muestra a estudiar (Figura 2). Los parámetros de TPA calculados fueron dureza, elasticidad, cohesividad, masticabilidad, y resiliencia.

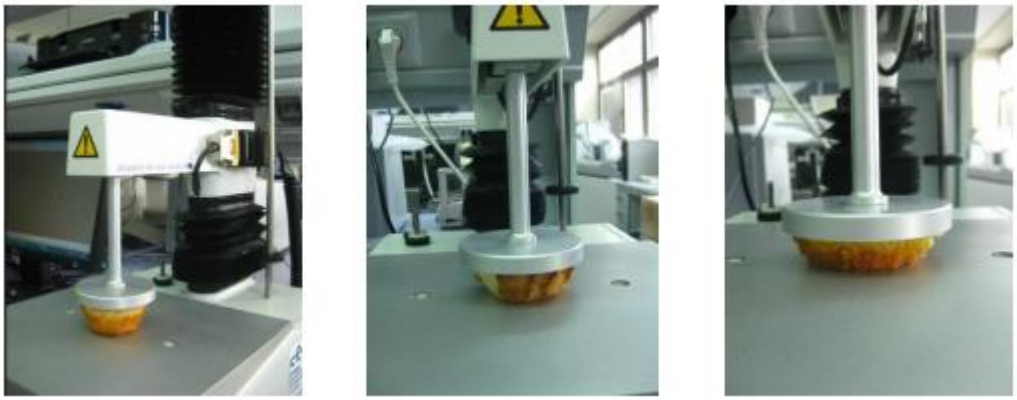

Figura 2. Análisis de la textura de una madalena.

3.4. DETERMINACIÓN DEL NÚMERO DE BURBUJAS EN LA MADALENA HORNEADA

Las madalenas fueron cortadas a la altura del molde, la mitad superior fue retirada y se obtuvo una imagen de la miga usando un scanner (HP PSC 1610, Hewlett Packard, USA). El número de burbujas fue calculado por análisis de imágenes empleando el programa informático ImageJ.

Figura 3. Izquierda: Madalena sin gluten con gránulos sustituyendo a la yema de huevo. Derecha: Madalena sin gluten con yema de huevo.

3.5. COLORIMETRÍA

El color de las madalenas fue medido en un sistema de medida L*, a*, b* (Ultrascan VIS spectrofotómetro, HunerLab, USA). Diferentes puntos de la corteza fueron analizados y un valor medio fue obtenido por cada muestra. Para el color de la miga la corteza fue descartada y la miga homogeneizada empleado un homogenerizador eléctrico de 180 W (AR100G, Moulinex, Francia). Los análisis fueron desarrollados en el modo de exclusión especular. Las diferencias de color entre las madalenas elaboradas con yema de huevo y gránulos fueron examinadas y el ΔE (cambio total de color con respecto a una referencia) calculado según la siguiente ecuación:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{Ecuación 4.}$$

3.6. EVALUACIÓN SENSORIAL

Para obtener información sobre la aceptabilidad de las madalenas, un test hedónico preliminar fue desarrollado usando 30 consumidores habituales de productos de horneado, de edades comprendidas entre los 24 y 58 años, aproximadamente la mitad hombres y mujeres. Estos consumidores recibieron 4 muestras que fueron puntuadas del 1 al 7, siendo el 1 un disgusto extremo y 7 una máxima aprobación. Las muestras fueron dispuestas en platos blancos e identificadas con un número aleatorio de 3 dígitos.

En cuanto a la textura, se llevó a cabo un análisis sensorial descriptivo para contrastar los datos obtenidos del TPA. 6 consumidores entrenados con experiencia en análisis sensoriales fueron seleccionados para desarrollar una evaluación de dureza en una escala de 5 puntos (1= muy blandas, 5= muy duras). La dureza fue definida a los panelistas como la fuerza necesaria para comprimir la muestra entre los molares. Como referencia de textura muy blanda se mostró a los panelistas miga de pan recién horneado, en el caso de la referencia de textura muy dura se

utilizó miga de pan de dos días. El ensayo fue repetido un segundo día con muestras recién preparadas.

4. HIDRÓLISIS DE GRÁNULOS

4.1. HIDRÓLISIS ENZIMÁTICA

La hidrólisis fue desarrollada en un biorreactor con un sistema de ajuste de pH automático (pH-Burette 2S, Crison, España). Los gránulos de yema de huevo liofilizados fueron disueltos en una solución 0.55 M de NaCl a una concentración de 0.4% (p/v). La temperatura en el biorreactor se mantuvo a 37°C y el pH se ajustó a 7.5 usando una solución 0.1 M de NaOH. Se utilizó tripsina de páncreas de cerdo (E.C. 3.4.21.4, T7409, Sigma-Aldrich) en proporción 1.5:80 enzima-sustrato. La inactivación de la enzima fue llevada a cabo por acidificación a pH 3 con HCl 1 M después de 90 minutos de reacción. La solución hidrolizada fue mantenida a 4°C durante la noche y centrifugada a 10000 g por 45 minutos, el sobrenadante fue descartado.

4.2. HIDRÓLISIS ENZIMÁTICA DE GRÁNULOS DELIPIDADOS

El sistema de hidrólisis fue el mismo que en el caso de la hidrólisis enzimática con los gránulos nativos con ligeras modificaciones: en este caso los gránulos de yema de huevo delipidados se mezclaron con agua destilada (1:20 p/v) y fueron precalentados a 90°C durante 7 minutos para incrementar su solubilidad y facilitar su dispersión. Se emplearon dos proporciones enzima-sustrato, 1:50 y 1:25, usando también tripsina de páncreas de cerdo. Los gránulos dispersos en agua fueron calentados a 37°C y el pH fue ajustado a 7.5. Finalmente la enzima fue añadida al reactor y después de 6 h la reacción enzimática fue detenida mediante un baño de agua a 90°C.

4.3. DETERMINACIÓN DEL GRADO DE HIDRÓLISIS

El método pH-STAT fue usado para determinar el grado de hidrólisis (DH) de acuerdo con Adler-Nissen [8]. Según este método, la cantidad de base consumida durante la reacción expresada en moles es proporcional a la cantidad de grupos amino liberados. El grado de hidrólisis fue calculado de acuerdo a la ecuación propuesta por Adler-Nissen:

$$DH\% = \frac{100 \times V_B \times N_B}{\alpha \times M_p \times h_{tot}} \qquad \text{Ecuación 5.}$$

Donde α es el grado de disociación de los grupos amino. Este parámetro se calculó según la ecuación 6. M_p es la masa de la proteína en gramos, h_{tot} es el número total de enlaces péptidos en la proteína (meq g^{-1} proteína) y V_B y N_B son el volumen (mL) y la concentración (normalidad) de la base añadida.

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \qquad \text{Ecuación 6.}$$

El valor de h_{tot} para la proteína de yema de huevo es actualmente desconocido y se asume que es 8 de acuerdo a una sugerencia bibliográfica [9]. El valor de pK usado para la hidrólisis es calculado mediante la ecuación de Guadix et al. [10]:

$$pK = 3.80 + 0.45pH \qquad \text{Ecuación 7.}$$

Se tomaron alícuotas a diferentes tiempos. El grado de hidrólisis se estimó para cada alícuota.

4.4. HIDRÓLISIS EN CONDICIONES SUBCRÍTICAS

Los gránulos delipidados fueron dispersos en agua (1:20) hasta obtener un volumen final de 400 mL. Estos 400 mL fueron dispuestos en el reactor mostrado en la Figura 4. A cada temperatura ensayada se alcanzó una presión de 40 bares usando un gas no reactivo (nitrógeno) o uno con capacidad oxidante (oxígeno) a un flujo de entrada de 1L/min. El gas fue previamente saturado con agua para evitar la evaporación de la muestra y calentado para mantener la temperatura del reactor constante.

Figura 4. Reactor utilizado en la hidrólisis de proteínas en condiciones subcríticas.

5. ANÁLISIS DE PROTEÍNAS

5.1. CUANTIFICACIÓN DE PROTEÍNAS

La cantidad de proteína fue determinada usando el método de combustión Dumas [11]. Para ese propósito se utilizó un CNHS/O Elementar Vario EL Analyzer (Elementar, Germany). El factor de conversión empleado fue 6.25 mg de proteína/mg de nitrógeno.

5.2. CUANTIFICACIÓN DE AMINOÁCIDOS

La cantidad de aminoácidos liberados durante las hidrólisis enzimáticas fue cuantificada mediante el método de Rosen [12]. Este método se basa en la interacción de la ninhidrina con los grupos amino, resultando en un compuesto coloreado.

5.3. ELECTROFORESIS EN GELES DE POLIACRILAMIDA

5.3.1. SDS-PAGE EN CONDICIONES DESNATURALIZANTES (SDS-PAGE)

Las electroforesis en condiciones desnaturalizantes se realizaron usando un gel de concentración del 3% y un gel de separación con un 12% de acrilamida en un bufer Tris-Glicina 5M con SDS al 0.1% (p/v). El desarrollo de la electroforesis fue según el método de Laemmli [13]. Las proteínas fueron teñidas con dos soluciones diferentes en el caso de la tinción de fosvitina. Primero se utilizó una solución específica para la tinción de fosfoproteínas: azul de Coomassie 0.05% (p/v), ácido acético 10% (v/v), triton 1% (v/v), etanol 25% (v/v) y nitrato de aluminio 0.1M, 3.75% (p/v). La segunda solución fue una estándar de azul de Coomassie (azul de Coomassie al 0.1% (p/V), metanol al 50% (v/v), ácido acético al 10% (v/v)). Las proteínas usadas como estándar presentan un peso molecular de amplio rango (Bio-Rad).

5.3.2. ELECTROFORESIS EN CONDICIONES NATIVAS

En este caso se utilizó un gel de separación con una concentración del 10% (p/v) de acrilamida. No se empleó SDS en ninguna solución. La solución de teñido usada fue la *stains all* (230960, Sigma-Aldrich) y empleada conforme a las instrucciones suministradas.

5.4. CROMATOGRAFÍAS

Los análisis de cromatografía fueron desarrollados en un FPLC ÄKTA (Amersham Biosciences, UK).

5.4.1. CROMATOGRAFÍA DE INTERCAMBIO IÓNICO

La columna empleado presenta un volumen de 5mL y una resina aniónica (HyperD® Q, Life Biosciences, USA), la tasa de flujo que se seleccionó fue de 1mL/min. La muestra fue filtrada por 0.45 µm antes de ser inyectada, además la columna fue equilibrada con 5 volúmenes de una solución 0.1M Trizma pH 8.0 (tampón A). El buffer de elución (tampón B) estaba compuesto por una solución de 0.1M Trizma pH 8.0 con una concentración 1M de NaCl. El gradiente desarrollado fue lineal, con una variación del tampón B desde el 0% hasta el 60% en 60 minutos. Los picos obtenidos fueron monitorizados a 280 nm.

5.4.2. CROMATOGRAFÍA DE EXCLUSIÓN POR TAMAÑO

Se utilizó un columna de exclusión por tamaño con posibilidad de separar péptidos desde los 100 Da hasta los 7000 Da (Superdex™ Peptide 10/300 GL). El buffer usado estaba compuesto por 100 mL de TrizMa pH 7.6, 1M, y 900 mL de agua destilada. Al igual que en el caso de la cromatografía de intercambio iónico las muestras fueron filtradas por filtros de 0.45 µm de tamaño de poro antes de ser inyectadas. El flujo de elución se ajustó a 1mL/min y el detector empleado emitía luz ultravioleta a 214 nm. La columna fue calibrada con marcadores de tamaño suministrados por Sigma-Aldrich. En consecuencia, el área de los cromatogramas obtenidos fue dividida en rangos de tamaños moleculares, de acuerdo con la calibración y usando el software suministrado con el FPLC (Unicorn 5.1), obteniéndose la concentración de proteína en cada caso.

6. PROPIEDADES FUNCIONALES Y ANTIOXIDANTES DE LOS HIDROLIZADOS

6.1. SOLUBILIDAD

La solubilidad de los péptidos resultantes de las operaciones de hidrólisis fue calculada según el método de Wang et al. [9] con ligeras modificaciones: los hidrolizados fueron diluidos en agua hasta obtener una concentración de proteína de 1 mg/mL. El pH de estas soluciones se ajustó con 1 N HCl o 1N NaOH y equilibrados a temperatura ambiente durante 60 minutos. Los valores de PH fueron reajustados a la media hora y después de los 60 minutos para corregir posibles variaciones. Finalmente las muestras diluidas fueron centrifugadas a 10000 g durante 30 minutos. El sedimento fue descartado en cada caso. El contenido en proteína de las muestras fue determinado según Markwell et al. [14] antes y después de la centrifugación. Una curva de calibrado fue llevada a cabo usando albúmina de suero bovino. La solubilidad de los hidrolizados fue calculada usando la siguiente ecuación:

$$PS(\%) = \frac{Proteína\ en\ el\ sobrenadante\ (mg)}{Proteína\ en\ la\ solución\ inicial\ (mg)} \times 100$$ Ecuación 8.

6.2. CAPACIDAD ESPUMANTE Y ESTABILIDAD DE LA ESPUMA

La capacidad espumante y la estabilidad de la espuma formada fue determinada según Deng et al. [15] con modificaciones: se dispusieron soluciones de péptidos en agua a una concentración de proteína de 5mg/mL y el pH fue ajustado a 7. Después de 30 minutos de equilibrado a temperatura ambiente, 30 mL de las soluciones fueron tomadas y agitadas a

15000 rpm usando un homogeneizador SilentCrusher M (Heidolph, Alemania) durante 1 minuto. El volumen de espuma formado fue inmediatamente calculado usando una probeta y la estabilidad de la espuma fue determinada en función de la variación del volumen durante 30 minutos de reposo. En cada caso, para calcular la capacidad espumante (FC) y la estabilidad de la espuma (FS) se utilizaron las ecuaciones siguientes:

$$FC(\%) = \frac{Volumen\ de\ espuma\ después\ de\ agitación\ (mL)}{Volumen\ de\ líquido\ inicial(mL)} \times 100$$ Ecuación 9.

$$FS(\%) = \frac{Volumen\ de\ espuma\ después\ de\ 30\ min\ (mL)}{Volumen\ de\ espuma\ inicial\ \ (mL)} \times 100$$ Ecuación 10.

6.3. PROPIEDADES EMULSIONANTES

Las propiedades emulsionantes fueron evaluadas de acuerdo con Pinciroli et al. [16] con algunas modificaciones: los péptidos fueron diluidos en una solución de NaCl (0.075% p/v) para obtener una concentración de proteína final de 10 mg/mL. El pH de estas soluciones fue ajustado a pHh 7 con HCl o NaOH 1N y equilibraod por 30 minutos con agitación continua a temperatura ambiente. Un total de 15 mL de estas soluciones fueron mezclados con 10 mL de aceite de semilla de algodón a 15000 rpm con un homogeneizador SilentCrusher M (Heidolph, Alemania). Inmediatamente estas emulsiones fueron dispuestas en un recipiente de medida y la estabilidad contra el *creaming* fue medida usando un Turbiscan (Formulaction, Francia). La luz retrodispersa fue monitorizada cada 2 minutos durante 1 h en función de la altura de la muestra. La clarificación fue medida en la base de la célula de medida desde los 0 hasta los 25 mm de altura (B%0-25) y el creaming fue determinado en lo alto de la célula (B%25-40 mm). Además, la retrodispersión inicial en la zona media ($B\%_{ini}$), el parámetro de estabilidad ($t_{0.1}$) y la constante cinética ($K_{0.1}$) fueron determinadas también. El parámetro de estabildiad $t_{0.1}$ fue calculado como el tiempo en el cual la retrodispersión en la base de la célula de medida disminuía un 10% con respecto a su valor inicial. La constante cinética $K_{0.1}$fue calculada de acuerdo a la siguiente ecuación:

$$K_{0.1}(h^{-1}) = (B\%_{ini,0-25mm} \times t_{0.1})^{-1}$$ Ecuación 11.

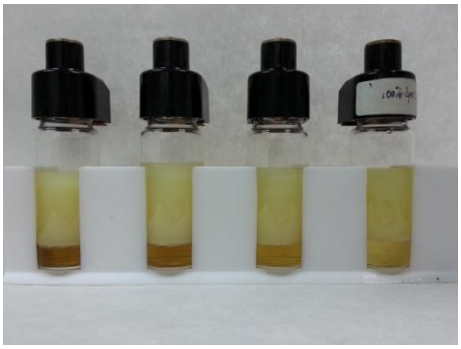

Figura 5. Diferentes muestras dispuestas en una célula de medida durante el ensayo de las propiedades emulsionantes de los hidrolizados.

6.4. ENSAYO DE ELIMINACIÓN DE RADICAL $ABTS^{\cdot+}$

La capacidad de eliminar del medio el radical ABTS (2,2´-azinobis-(ácido-3-etilbenzotiazolina- 6-sulfónico) por parte de los hidrolizados fue calculado en cada caso de acuerdo con Watchararuji et al. [17] con modificaciones. Se preparó una solución inicial 7mM de $ABTS^{\cdot+}$ y 2.45 mM de persulfato de potasio. Esta solución *stock* se guardó en oscuridad a temperatura de laboratorio durante 16 horas antes de su uso. A continuación, la solución fue diluida con agua destilada hasta alcanzar una absorbancia de 0.70±0.04 a 734 nm en una cubeta de medida de 1 cm de paso óptico. Las muestras liofilizadas fueron diluidas a diferentes concentraciones de proteína (de 0.5% a 0.015%(p/v)) y alícuotas de 166 mL fueron mezcladas con 4830 mL de ABTS e incubadas en oscuridad durante 20 minutos. Finalmente, la absorbancia de las muestras fue medida a 734 nm. Se usó GSH y agua como controles positivos y negativos respectivamente. La capacidad de eliminar el radical $ABTS^{\cdot+}$ fue expresada en porcentaje a cada concentración (SC%, ecuación 12). Además, se calculó la concentración de proteína necesaria para producir la eliminación del 50% del radical (IC_{50}).

$$SC\% = [1 - (A_t/A_r)] \times 100 \qquad \text{Ecuación 12.}$$

Donde A_t es la absorbancia de la muestra y A_r es la absorbancia del control negativo.

6.5. ELIMINACIÓN DEL RADICAL DPPH

La capacidad de los hidrolizados de eliminar el radical DPPH fue determinado por el método de Tanzadehpanah et al. [18] con ligeras modificaciones: se dispusieron 0.6 mL de una solución 1.0 mM DPPH, además, otros 0.6 mL de la solución que contiene los péptidos a diferentes concentraciones (0.5%-2%(p/v)). Finalmente a la mezcla anterior se le añadieron 4 mL de etanol (95%). La solución control estaba compuesta por 0.6 mL de agua destilada, 4 mL de etanol y 0.6 mL de 1.0 mM DPPH. En todos los casos la mezcla de ensayo se agitó vigorosamente y se incubó durante 30 minutos en la oscuridad a temperatura ambiente. A

continuación, la absorbancia fue medida a 517 nm. Los resultados fueron calculados como el porcentaje de inhibición de acuerdo con la siguiente fórmula:

$$\%\, inhibición = [(A_{control} - A_{sample})/ A_{control}] \times 100$$ Ecuación 13.

6.6. PODER REDUCTOR DE LOS PÉPTIDOS

los péptidos liofilizados fueron disueltos en tampón fosfato (pH 6.6, 0.2 M) a diferentes concentraciones de proteína. 4 mL de estos péptidos fueron mezclados con 2 mL al 1% (p/v) de ferricianuro de potasio. La mezcla fue mantenida a 50°C durante 20 minutos y enfriados rápidamente. Después de esto, 2 mL de ácido tricloroacético (10% p/v) fueron añadidos y la solución fue centrifugada a 10000 g por 10 minutos. El sedimento fue descartado y 2 mL del sobrenadante fueron mezclados con 2 mL de agua y 2 mL de cloruro férrico (0.1% p/v). Después de 10 minutos la medida de la absorbancia fue desarrollada a 700 nm. Una alta absorbancia es indicativa de una fuerte capacidad reductora.

6.7. CAPACIDAD QUELANTE DE ION FERROSO

La capacidad de unir ion ferroso por parte de los péptidos fue ensayado de acuerdo con el me´todo de Decker y Welch [19]. En resumen, 5 mL de soluciones elaboradas con los péptidos fueron mezcladas con 0.1 mL de cloruro ferroso 2 mM y con 0.2 mL de ferrozine 5 mM. Después de mantener las mezclas a temperatura ambiente por 10 minutos, la abosrbancia fue medida a 562 nm. El complejo Fe2+/ferrozine presenta una alta absorbancia a esta longitud de onda, así que una capacidad quelante alta es mostrada como una baja absorbancia. La capacidad quelante en porentaje fue calculada con la siguiente ecuación:

$$Habilidad\ quelante\ de\ hierro\ (\%) = (A_{blanco} - A_1/A_1) \times 100$$ Ecuación 14.

Donde A_1es la absorbancia de la muestra a ensayar.

6.8. EFECTO ANTIOXIDANTE DE LOS PÉPTIDOS EN HOMOGENEIZADOS DE CARNE

Se dispusieron soluciones de carne al 20% (p/v) en una solución tampón 50 mM HEPES pH 7 usando un homogeneizador (Grindomix GM300, Retsch, Alemania). Los hidrolizados fueron ensayados a diferentes concentraciones de péptidos. Las soluciones de ensayo contienen 0.8 mL de homogeneizado de carne y 0.2 mL de agua destilada o de péptidos. Estas soluciones fueron incubadas a 37°C por 60 minutos para luego ser ensayadas en la formación de sustancias reactivas al ácido tiobarbitúrico (TBARS). La oxidación se paró añadiendo hidroxitolueno butilado (20 µL, 0.2%).

A continuación se preparó una solución 0.25 M de HCl que contiene ácido tricloroacético al 15% (p/v) y ácido 2-tiobarbitúrico (TBA) al 0.375% (p/v). 2 mL de esta solución fueron añadidos a 1 mL de la mezcla incubada, siendo calentados durante 10 minutos en un baño de agua hirviendo. Finalmente las muestras se dejaron enfriar a temperatura ambiente y fueron centrifugadas por 20 minutos a 5000 g. El color rosado desarrollado fue medido a 532 nm. La formación de TBARS fue calculado de una curva estándar de malonaldehido (MDA), un producto de ruptura del tetraetoxipropano (TEP).

6.9. TEST ANTIMICROBIANO

Dos especies bacterianas diferentes, L. innocua (gram positiva) y E. coli (gram negativa) fueron seleccionadas para ensayar el efecto antimicrobiano de los péptidos. Estas bacterias crecieron en un medio líquido PBS a 37°C por 24 h. La concentración bacteriana final fue diluida hasta obtener una población de $1x10^7$ UFC/mL, 200 µL de esta solución bacteriana fueron extendidos en la superficie de un medio agar de PBS. En este medio sólido se realizaron tres pocillos y se dispusieron 50 µL de la muestra a ensayar en cada uno. La concentración de péptidos a ensayar fue creciente de 2 a 40 mg/mL. Las placas de agar fueron incubadas por 24 horas a 37°C. Un control negativo fue desarrollado usando agua destilada.

7. PRUEBAS QUÍMICAS

7.1. PORCENTAJE DE FÓSFORO INORGÁNICO

El porcentaje de fósforo inorgánico fue determinado usando un método colorimétrico [20] usando ácido 1-amino-2-naftol-4-sulfónico y molibdato de amonio como reactivos.

7.2. DETECCIÓN Y CUANTIFICACIÓN DE SODIO

Las muestras liofilizadas fueron diluidas a una concentración 1mg/ml en agua ultra pura. A continuación se añadió 8 mL de ácido nítrico al 1% por mL de liofilizado diluido. Esta solución fue calentada en un sistema de digestión por microondas (Milestone, USA) a 800W y 160°C por 30 minutos. El resultado de la digestión fue diluido nuevamente con agua a un volumen final de 15 mL. Finalmente, 1 mL de esta última solución fue preparado añadiendo 9mL de ácido nítrico al 1%. El sodio contenido en la muestra fue determinado mediante un ICP masas (ICP-MS 7500ce, Agilent Technololgies, USA) utilizando 5 ppb de Rh como estándar interno en cada muestra.

8. PROCEDIMIENTOS DE SEPARACIÓN DE PROTEÍNAS

8.1. EXTRACCIÓN DE IgY

El aislamiento de IgY fue desarrollado de acuerdo con Polson et al. (1985). Para extraer la IgY se utilizaron 10 mL de yema de huevo, separándose de la clara y rompiéndose la membrana vitelina. A continuación se añadieron 20 mL de tampón Tris-HCl 0.1 M a pH 7.6 y la mezcla se agitó vigorosamente. A continuación, se disolvió un 3.5% (p/v) de polietilenglicol (PEG6000) en la solución anterior y se centrifugó a 4500 g por 20 minutos. El precipitado se desechó y el sobrenadante fue filtrado por lana de vidrio. A este sobrenadante se le añadió PEG nuevamente, hasta alcanzar una concentración del 12% en la solución. El PEG fue completamente disuelto y la mezcla centrifugada a 12000 g por 10 minutos. El sedimento fue resuspendido en 30 mL de tampón Tris-HCl y finalmente se añadió PEG hasta alcanzar nuevamente la concentración de un 12%. El PEG fue disuelto y la mezcla centrifugada a 12000 g por 10 minutos. El sedimento final se resuspendió en 2 mL de tampón Tris-HCl. A lo largo del proceso se tomaron alícuotas para hacer un seguimiento de la separación de IgY.

8.2. EXTRACCIÓN DE FOSVITINA

Los gránulos de yema de huevo fueron dispersos en una solución 0.16 M de agua con NaCl (1:1 p/p). Después de una hora la mezcla se centrifugó a 10000 g durante 45 minutos, descartándose el sobrenadante y recuperando el precipitado. Este precipitado son los gránulos de yema de huevo lavados. A continuación los gránulos fueron disueltos en diferentes concentraciones de NaCl y el pH se ajustó a valores ácidos desde 2.0 a 4.0 con soluciones de ácido cítrico 1M y de ácido clorhídrico 1N. Estas soluciones fueron centrifugadas a 10000 g duratne 45 minutos, obteniéndose un sedimento que se descartó y un sobrenadante rico en fosvitina.

9. ENSAYOS DE DIÁLISIS Y ULTRAFILTRACIÓN

9.1. DESALADO POR DIÁLISIS DE LOS SOBRENADANTES RICOS EN FOSVITINA

El pH de la solución a dializar fue ajustado a 3.0, 5.0, 7.0 y 8.5 utilizando ácido cítrico o ácido clorhídrico 1M o hidróxido sódico 1N. Las membranas de diálisis fueron adquiridas en Sigma Aldrich (D7884-10FT). Para la diálisis se empleó agua destilada que iba siendo retirada periódicamente, midiéndose la conductividad electrolítica con un conductivímetro (Modelo HI98129, Hanna Instruments, USA) hasta que se obtuvieron valores por debajo de 1 microsiemen.

9.2. DESALADO POR ULTRAFILTRACIÓN DE LOS SOBRENADANTES RICOS EN FOSVITINA

Se empleó una membrana de polietersulfona (PXB010A50, Millipore, USA) para este propósito. La presión transmembrana fue fijada a 1.4 atm y el pH fue ajustado a 8.0 usando hidróxido

sódico 1N de acuerdo a Chay Pak Ting et al. [21]. El volumen inicial a dializar fue de 150 mL, produciéndose la diafiltración con 6000 mL de agua destilada.

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4. HIDRÓLISIS DE LA FRACCIÓN GRANULAR

4.1. HIDROLIZADO DE GRÁNULOS DE YEMA DE HUEVO: CARACTERÍSTICAS, PROPIEDADES REOLÓGICAS Y APLICACIONES

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Egg yolk hydrolysed granules: Characteristics, rheological properties and applications

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A B S T R A C T

Given its low-cholesterol content feature, granules from egg yolk can be used as a substitute of the whole egg yolk. However, the functional properties of the granular fraction should be improved. In this sense, hydrolysis of proteins frequently produces improvements in some of its nutritional and technological properties. For that reason, in this work egg yolk granules were treated with a proteolytic enzyme, trypsin (E.C. 3.4.21.4) with the purpose of making a comparative characterization of the products.

Results showed that the enzymatic reaction produced a degree of hydrolysis of 12%, being the size of the different peptides obtained and quantified by chromatographic and electrophoretic techniques. Mayonnaises made with these hydrolysed granules resulted more stable to temperature changes between 4 and 20 °C than the one made with non-hydrolysed ones. In the rheological tests carried out, the mayonnaise elaborated with hydrolysed granules has the most similar rheological behaviour to that of a commercial one used as reference. In general, the results obtained suggest that the recipe elaborated with hydrolysed granules had better rheological characteristics than those prepared using non-hydrolysed granules, maintaining the low-cholesterol feature.

Keywords: Egg yolk; Granules; Hydrolysis; Emulsification; Mayonnaise; Rheological properties

1. Introduction

People's concern about the rise of cardiovascular diseases and the obesity has led the consumers to select the ingredients that form part of the food products, trying to avoid these health issues. In response to this question, the researchers have focused their attention on two particular nutritional facts, namely the lipids and cholesterol content of foods. In this sense, an ingredient with high levels of cholesterol and lipids, commonly used in the food industry, is the egg yolk.

Egg yolk is broadly recognized to contain many substances with biological functions beyond basic nutritional ones, and for that reason, its substitution is complicated and usually implies a quality loss in the final product.

However, egg yolk can be easily separated by centrifugation into two fractions: the plasma and the granular one. Plasma is mainly composed of 85% low density lipoproteins (LDL) and 15% livetins. This fraction contains about 73% of lipids and 3/4 of the whole egg yolk cholesterol. On the other hand, egg yolk granules are mainly composed of 70% high-density lipoproteins (HDLs), 16% phosvitin and 12% low density lipoproteins. This fraction is high in protein content and it has approximately 1/4 of the total cholesterol found in egg yolk (Laca et al., 2010a).

In a study about egg yolk fractionation, it has been shown that egg yolk granules keep, even after lyophilization treatment, good emulsifying, gelling and other properties, with the additional advantage of its lower cholesterol content (Laca et al., 2010a). However, the emulsifying properties of this granular fraction are lower than those found in the whole egg yolk or in the plasma fraction (Le Denmat et al., 2000).

Concerning proteins, their functional properties are those physicochemical properties that govern their performance and behaviour in food systems during their preparation, processing, storage and consumption. These properties can be enhanced through enzymatic modification of food proteins by controlled proteolysis over a wide pH range, and other processing conditions. Choosing the right proteolytic enzyme,

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environmental conditions and degree of hydrolysis (DH) is crucial for enhancing the functional properties of proteins. Owing to the complex nature of proteins it is very difficult to reach high DH values (Panyam and Kilara, 1996).

The emulsifying capacity of the egg yolk has been broadly investigated: the effect of a high-pressure treatment (Anton et al., 2001a), the stability and rheology when adding another agent (Kontogiorgos et al., 2004) or the egg yolk protein gels and emulsions (Kiosseoglou, 2003) are some examples. However, there is little information on the effect of controlled enzymatic hydrolysis of the egg yolk and its fractions on their foaming and emulsifying properties. When used as emulsifiers, the hydrolysis of the surfactant proteins, either before or after the formation of the emulsions, can affect the stability of the emulsion system by making the emulsion inherently unstable or by altering its sensitivity to external influences (e.g., calcium ions, reduced pH, or high temperature). Nevertheless, in some cases hydrolysis may even promote stability, as it has been observed in the increase in calcium stability of caseinate emulsions treated with a serine protease, trypsin. This enzyme cleaves peptide bonds at carboxyl terminals of arginine and lysine, except when linked to a proline residue (Olsen et al., 2004). Hence, if hydrolysed proteins are used as emulsifiers, there is a risk that the stabilizing effect of the protein will be lost affecting their potential applications, but, otherwise, it must be set also the possibility that the disruption of the protein structure may permit more efficient adsorption of some peptides (Singh and Dalgleish, 1998). Besides, whenever enzymatic hydrolysis of protein causes breakdown of protein molecules, the protein solubility increases, and it is well known that solubility is essential for most proteins to provide good functionalities such as foaming and emulsification.

In this work, since the granular egg yolk fraction is low in cholesterol content, and with the aim to test the possibility of enhancing the functional properties of the granular proteins, they were hydrolysed to a value close to 12% degree using trypsin (E.C. 3.4.21.4). The procedure was developed at the optimal temperature and pH for the activity of trypsin, 37 °C and pH over 7.0 (Chelulei Cheison et al., 2011). Then, the applicability of the product of hydrolysis was tested, in particular elaborating mayonnaises with non-hydrolysed and hydrolysed granules. Rheological properties were measured in order to evaluate the mayonnaises characteristics compared to those of a first quality mayonnaise acquired from a local market, which was used as a reference.

2. Materials and methods

2.1. Fractionation of egg yolk

The fractionation method was developed modifying the procedure by Laca et al. (2010a). The general scheme for egg yolk fractionation is shown in Fig. 1.

The granules fraction was frozen at −80 °C overnight and then lyophilized at −70 °C and 0.1 mBa in a Telstar Cryodos Lyophilizator for 24 h, in order to increase its shelf life.

2.2. Enzymatic hydrolysis

The hydrolysis reaction was carried out in a 5 l bioreactor with a pH-STAT automatic titration (pH-Burette 24 2S, Crison) connected to an iso-thermal shaker.

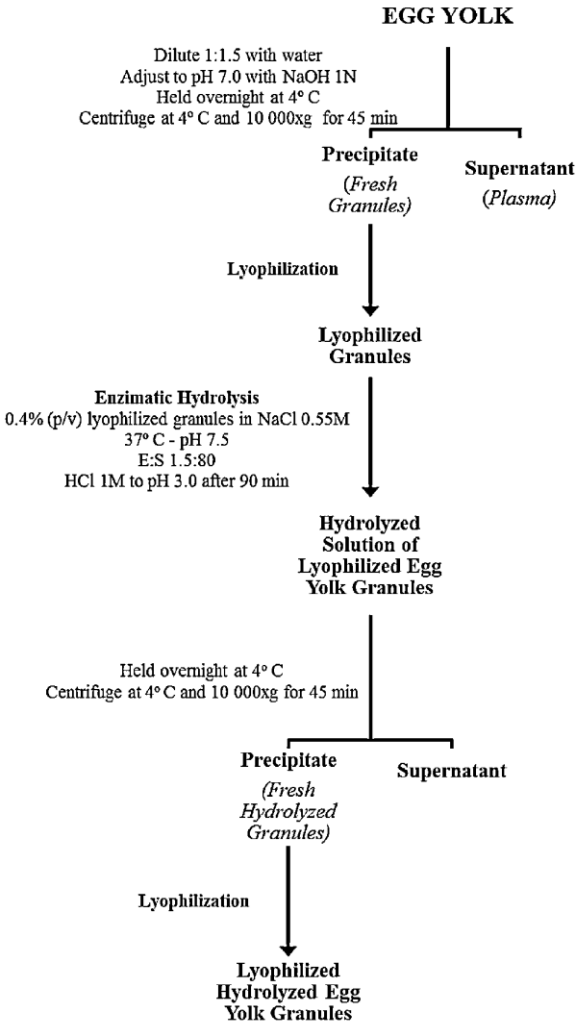

Fig. 1 – General scheme procedure to obtain lyophilized hydrolysed egg yolk granules.

Lyophilized egg yolk granules were dissolved at 0.4% (p/v) in 0.55 M sodium chloride solution for their total disruption (Antón et al., 2000) and stirred to fully disperse the protein. The protein content of this solution was calculated by Bradford method. Then, it was heated at 37 °C and pH adjusted to 7.5 by using 0.1 M sodium hydroxide solution. Trypsin from porcine pancreas E.C. 3.4.21.4 (T7409, Sigma–Aldrich) 1900 u/mg solid was added to the bioreactor (1.5:80 enzyme–substrate relation). Inactivation of the enzyme was done by acidifying with 1 M hydrochloric acid to pH 3.0 after 90 min of reaction.

The hydrolysed solution was kept at 4 °C overnight and then centrifuged (KUBOTA 6500 Centrifuge) at 10 000 × *g* and 4 °C for 45 min to separate into supernatant and the hydrolysed granule fraction (precipitate). The supernatant was separated from the hydrolysed granules by decantation.

The hydrolysed granules fraction was lyophilized by the same method as for the non-hydrolysed granules. The protein contents of both lyophilized hydrolysed and non-hydrolysed granules were calculated according to the Dumas combustion method using a CNHS/O Vario EL analyzer (Elementar).

2.3. Mayonnaises preparation

Two mayonnaise formulations were prepared using egg yolk granules and hydrolysed granules. A commercial mayonnaise acquired from a local market was used as reference.

The mayonnaise formulation was obtained from Laca et al. (2010b) with slight modifications, and included 9 ml white vinegar (6% acidity), 0.94 g fine sea salt, 1.3 g white sugar and 70 ml sunflower oil. As emulsifying agents were added lyophilized non-hydrolysed egg yolk granules (mayonnaise A) and lyophilized hydrolysed egg yolk granules (mayonnaise B) until obtaining a protein concentration of 27 mg/g of mayonnaise. Before mayonnaises preparation, both lyophilized non-hydrolysed and hydrolysed granules were rehydrated by adding water (1.38 g/g of lyophilized). Mayonnaise C is a commercial standard acquired from a local market and used as a reference, whose ingredients were water, wine vinegar, salt, sugar, sunflower oil, egg yolk, lemon extract, corn starch, modified corn starch, antioxidant and colouring (curcumin).

The amount of the emulsifying agent was adapted from the basic formulation described by Ghoush et al. (2008). Mustard and other spices were not included in the formulation since only egg yolk granules were to be evaluated as emulsifying agent.

The preparation process was as follows: salt, sugar and vinegar were mixed at 8000 rpm with a Heidolph Silentcrusher M (Typ 12 G/M) and emulsifying agent was added. During blending, 20 ml of sunflower oil was added drop by drop; the rest of the sunflower oil was added in amounts of 10 ml at each addition. The total time of mixing was 15 min.

2.4. Determination of the degree of hydrolysis (DH)

The pH-STAT method was used to determine the degree of hydrolysis (DH). According to this method that was established by Adler-Nissen (1986) the amount of base consumed expressed in moles is proportional to the amino groups liberated also in moles during the hydrolysis process. Degree of hydrolysis was calculated using Adler-Nissen's equation (1):

$$\mathrm{DH}\% = \frac{100 \times V_B \times N_B}{\alpha \times M_p \times h_{tot}} \quad (1)$$

where α is the degree of dissociation of α-amino group calculated by following Eq. (2), M_p is the mass of protein (g), h_{tot} is the total number of peptide bonds in the protein (meq g^{-1} protein), and V_B and N_B are the volume (ml) and the concentration (normality) of alkaline added.

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{pK}}}{1 + 10^{\mathrm{pH}-\mathrm{pK}}} \quad (2)$$

The value of h_{tot} for egg yolk protein is currently unknown and it was assumed to be 8 based on a reference suggestion (Wang and Wang, 2009).

The pK value used for the hydrolysis procedure is approximated calculated through the equation of Guadix et al. (2000) (3):

$$\mathrm{pK} = 3.80 + 0.45\mathrm{pH} \quad (3)$$

During three different hydrolysis reactions, aliquots were taken at different times (0, 1, 5, 10, 15, 20, 25, 30, 60 and 90 min). DH percentage was calculated at each aliquot.

2.5. Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify the egg yolk granules proteins hydrolysis. Laemmli method (Laemmli, 1970) was followed including a specific staining phase for phosphoproteins consisting on Coomassie blue 0.05% (p/v), ethanol 0.025% (v/v), acetic acid 10% (v/v), Triton X-100 1% (v/v), aluminium nitrate 3.75% (p/v) and distilled water 40% (v/v). SDS-PAGE Molecular Weight Standards Broad Range (Bio-Rad) were used as protein standards. Electrophoresis was run on polyacrylamide gels (stacking 3.5% and resolved 12%) with a migration buffer which consisted of a 0.02 M THAM, glycine 5 M and SDS (w/v) 0.1% solution in Power Pac 300 (Bio-Rad) operator. The gels were destained in a solution that contained acetic acid 10%, methanol 40% and distilled water 50%.

2.6. Size-exclusion chromatography

An ÄKTA FPLC (GE Healthcares) system was employed to determine the molecular weight distribution. A size exclusion column was used (Superdex^{TM} Peptide 10/300GL), which can separate peptides from 100 to 7000 Da. The buffer used was composed by 100 ml of TrizMa pH 7.6 1 M and 900 ml of distilled water. The samples were filtered by 0.45 μm before injecting them. Those samples were obtained at time 0, 1, 30, 60 and 90 min of hydrolysis. The elution flow was adjusted at 1 ml/min. The FPLC system used was dotted with an UV detector and the wavelength employed was 280 nm. Previously, the column was calibrated with standard weight markers supplied by Sigma–Aldrich. The molecular size distribution and the average molecular size obtained were analysed to determine the total soluble peptide concentration. Subsequently, the area of the chromatograms obtained was divided in ranges of molecular size, according to previous calibration. The area of each zone was measured employing the Unicorn 5.1 analysis software and transformed into protein concentration. The frequency of each molecular weight ranged was calculated with regard to the total amount of soluble peptides.

2.7. Rheological measurements of mayonnaises

The rheological tests were carried out with a Haake MARS II rotational rheometer using a Peltier unit to control the temperature. All the tests were carried out at 20 ± 0.1 °C (except for the temperature ramp) and before starting any measurement, the sample was allowed to rest for at least 15 min. Glass hood and silicone oil were employed to avoid sample desiccation during the analysis. The rheological measurements were performed on the mayonnaise samples after one-day storage.

In dynamic conditions, a plate/plate measuring system (PP60) was used, with a gap of 1 mm. The stress sweeps were performed from 0.01 to 100 Pa at a frequency of 1 Hz and 20 °C. The temperature sweeps were carried out from 4 to 20 °C at a heating rate of 1 °C/min, a frequency of 1 Hz and a constant shear stress adjusted to each mayonnaise in order to keep the measurements within the linear viscoelasticity regime.

In steady state, a serrated plate/plate measuring system (PP35) was used, with a gap of 1 mm. Flow properties were measured at 20 °C. Apparent viscosity was obtained as a function of shear rate from 0.01 1/s to 100.0 1/s. 100 points were collected with a logarithmic distribution in each case.

Triplicates presented differences lower than 10%. Data obtained were adjusted to the Ostwald de Waele model (4):

$$\eta = k\dot{\gamma}^{n-1} \quad (4)$$

where η is the apparent viscosity, $\dot{\gamma}$ is the shear rate, k (Pa s^n) is the consistency index, and n is the flow behaviour index.

Ostwald de Waele model parameters were calculated from the obtained curves using the Haake Rheowin Software.

2.8. Statistical analysis

Analysis of variance (ANOVA) was applied. Least significant differences (LSD) were calculated by Fisher's test to determine significant differences among the tested samples. These analyses were performed using a statistical software (statgraphics v.15.2.06). Experiments were carried out in triplicate, along with calculation of average and standard deviations.

3. Results and discussion

3.1. Hydrolysed granules characterization

To characterize the hydrolysed granules the degree of hydrolysis was calculated. Besides, a primary electrophoresis approach was made. It results difficult to separate and quantify the smallest peptides formed along the enzymatic reaction employing electrophoresis techniques. Hence, to complete this information, aliquots taken at different times of hydrolysis were analysed employing size exclusion chromatography.

3.1.1. Determination of the degree of hydrolysis (DH)

The change in the functional properties of a protein is a direct result of the hydrolysis effect. The DH (%) curve in Fig. 2 shows that protein degradation rate was high in the initial stage of the enzymatic incubation but the trend decreases after 30 min following the kinetic model of Michaelis–Menten (Seguel, 1993). The DH percentage reached was 10.00 ± 0.51 and 12.00 ± 0.5 after 60 and 90 min, respectively.

3.1.2. Gel electrophoresis

SDS-PAGE of egg yolk granules hydrolysis at different times is shown in Fig. 3. At t0, a band of approximately 80 kDa can be identified as α-livetin or as one HDL apoprotein (Anton, 2007; Le Denmat et al., 2000) below another two superimposed one each other corresponding to two apo-HDL (110 kDa and 105 kDa, respectively). The phosvitin, 39 kDa molecular

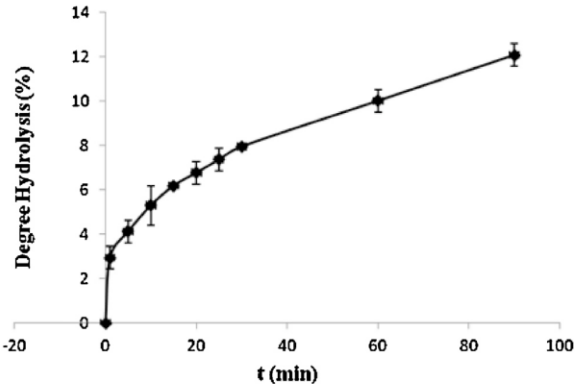

Fig. 2 – Degree of hydrolysis (DH) percentage of the egg yolk lyophilized granules.

weight, is found over another 32 kDa which feasibly belongs to the smallest HDL apoprotein. In the first minute the proteolytic enzyme hydrolyses high molecular weight proteins appearing peptides between 30 and 40 kDa, notably after 5 min. A faint band of 29 kDa can be detected from minute 10 to 90. According to bibliography, a large peptide fragment (Gln 49-Arg 212) and a smaller one (not detected in Fig. 3) are the product of the tryptic digestion of phosvitin (Goulas et al., 1996). Besides, not only a great amount of peptides of less than 30 kDa can be observed between 60 and 90 min lines, but also a band of about 74 kDa that cannot be seen at t0. This large fragment of 74 kDa suggests that, in the tested conditions, the biggest HDL apoproteins cannot be totally digested and a core of these apoproteins remained largely intact upon digestion with trypsin. A similar behaviour has been detected in the phosvitin.

These results reveal that trypsin is capable of partially hydrolysing egg yolk granules protein even at the high salt concentration of 0.55 M necessary to solve the proteins present in the egg yolk granules (Antón et al., 2000). However, smallest peptides cannot be detected by employing electrophoresis techniques and this information has been completed carrying out size exclusion chromatography.

3.1.3. Size-exclusion chromatography

An analysis to determine the quantity and weight of the smallest peptides obtained at different times of the hydrolysis was performed by size-exclusion chromatography (SEC). Employing this chromatography technique, peptides from 100

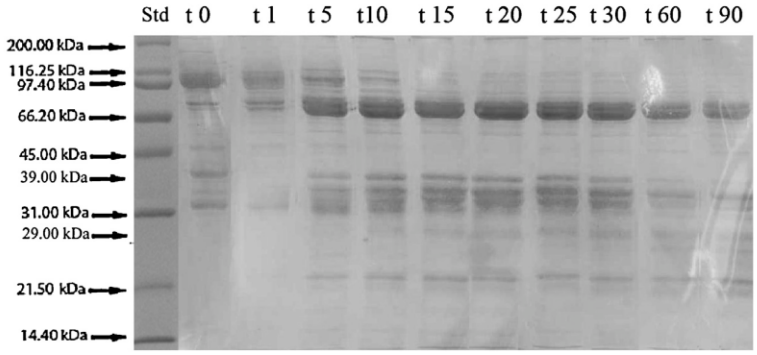

Fig. 3 – SDS-polyacrylamide gel electrophoresis of egg yolk lyophilized hydrolysed granules at different times (min) of the enzymatic hydrolysis.

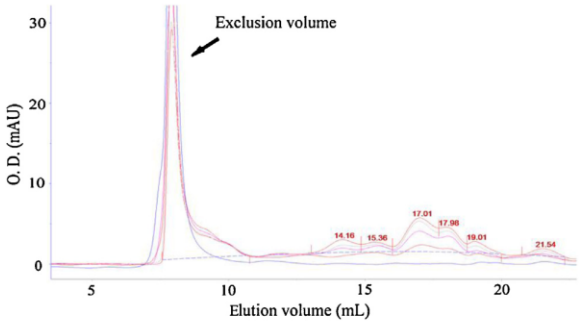

Fig. 4 – Size exclusion liquid chromatography of egg yolk lyophilized and hydrolysed granules at different times of the enzymatic reaction.

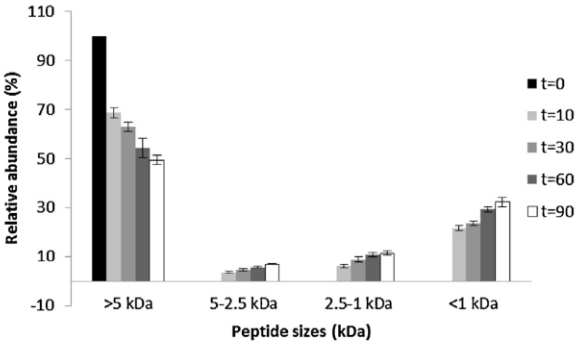

Fig. 5 – Relative amount of small peptides along the enzymatic reaction.

to 7000 Da can be separated and quantified. In Fig. 4, chromatograms at tested times are shown.

In these chromatograms farthest peaks correspond to higher times of hydrolysis excepting the exclusion volume where it is the opposite. There are proteins of more than 7000 Da in the exclusion volume that the column is not able to retain. These proteins were analysed in the gel electrophoresis in the previous section. As expected, several peaks were present growing progressively with time. Polypeptides from 960 to 4990 Da appear in the chromatogram at an elution volume between 14.16 and 17.01 ml before the tripeptides (562 Da) and the dipeptides (314 Da) which can be seen at 17.98 and 19.01 ml, respectively, as is described in Table 1. From a exclusion volume of 21 ml are considered amino acids of around 100 Da.

Comparing the granules proteolysis at different times, size-exclusion chromatogram reveals the increasing quantity of peptides obtained up to 1 h, but this trend decreases from 60 to 90 min. From the exclusion volumes indicated in Table 1 and peak areas from chromatograms of Fig. 4, the relative amount of peptides along the enzymatic reaction was calculated (Fig. 5). This figure shows that at t0, 100% of proteins were detected in the exclusion volume, with no peptides less than 7000 Da in solution. However, at every time the amount of protein in the exclusion volume decreased while small peptides were appearing. Even after 60 min, a high proportion of protein appeared in the exclusion volume, indicating the proteolytic resistance of the egg yolk granules protein. This agrees with the gel electrophoresis of the previous section, where peptides of more than 21 kDa persist in the medium at t90. Furthermore, peptides lower than 5 kDa were present always from 10 to 90 min of hydrolysis. The amount of these peptides constantly increased over time and it was more obvious as the molecular weight of peptides was lower; so relative abundance of smallest peptides (<1 kDa) were the highest compared to the other small peptides produced.

Table 1 – Molecular weight of the peaks from size-exclusion chromatography (Fig. 4).

Ve (ml)	MW (Da)
14.16	4990
15.36	2633
17.01	960
17.98	562
19.01	314
21.54	75

It was reported (Bautista et al., 2000; Clemente, 2000) that low molecular weight peptides, especially di- and tripeptides, with free amino acids have high nutritional and therapeutic values (Vijayalakshmi et al., 1986; Williams, 1995). On the other hand, large molecular weight peptides (more than 20 amino acid residues) are presumed to be associated with an improvement in the functional properties of hydrolysates (Gauthier et al., 1986).

3.2. *Mayonnaises*

3.2.1. *Rheological measurements*

Stress and temperature sweeps and viscosity curves were carried out for mayonnaises elaborated with: non-hydrolysed (A) and hydrolysed (B) egg yolk granules. Furthermore, a commercial mayonnaise was analysed too (C).

3.2.1.1. Stress sweeps. Results of stress sweeps at 20 °C are shown in Fig. 6. As it can be seen, the linear viscoelastic range varies from 0.1 to 30 Pa for both the hydrolysed recipe and the commercial mayonnaise. However, in the case of the non-hydrolysed recipe, the linearity is maintained through the whole stress range tested. Furthermore, the elastic module is higher in the non-hydrolysed recipe too, reaching values of around 3200 ± 60 Pa, meanwhile in the case of the commercial and the hydrolysed mayonnaise the G′ values are of 930 ± 20 and 330 ± 15 Pa, respectively. This behaviour denotes a more strong structure in the case of the non-hydrolysed mayonnaise, likely because the granular proteins can be involved in the formation of more interactions, since proteins are responsible for structure support. Furthermore, the

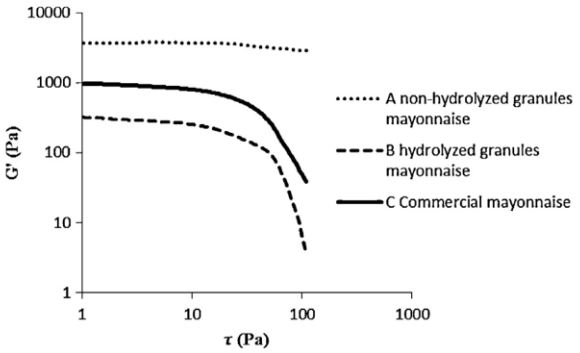

Fig. 6 – Storage modulus (G′) vs. stress sweeps of mayonnaises.

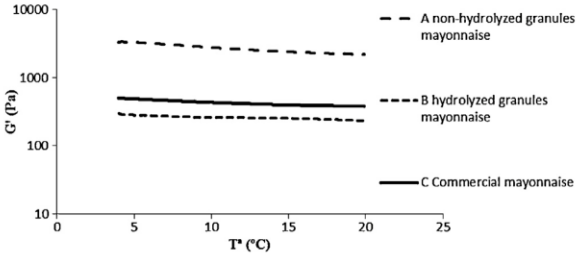

Fig. 7 – Storage modulus (G′) vs. temperature sweeps of mayonnaises.

granular fraction of the egg yolk has been described as an active filler (Anton et al., 2001b), which denotes the potential capacity of these proteins to interact between them in emulsions. However, according to the results obtained, this protein feature was reduced drastically after the hydrolysis treatment. On the other hand, commercial mayonnaise behaviour (*line* C) remains in the range between that of non-hydrolysed and hydrolysed granules mayonnaises.

3.2.1.2. *Temperature sweeps*. Shear stress was selected for each mayonnaise in order to develop their temperature sweeps in the linear range.

Between 4 and 20 °C (common temperature range for mayonnaises consumption) the linear trend of the mayonnaises does not mark important changes in the storage module as it can be seen in Fig. 7. However, the mayonnaise elaborated using non-hydrolysed granules, even with higher values of G′, looks to be a bit less stable to temperature changes at the range studied. It is remarkable that the mayonnaise made with hydrolysed granules has a behaviour quite close to that of the commercial used as a reference.

3.2.1.3. *Flow curve*. Flow curve was then studied for hydrolysed and non-hydrolysed granules mayonnaise comparing the results with those obtained from the commercial mayonnaise sample. Results are exposed in Fig. 8, responding the three samples to the Ostwald de Waele model.

In flow curves shown in Fig. 8, it can be observed that the apparent viscosity decreases with increments in the shear rate. This behaviour is a feature of shear thinning products. This fact is confirmed by the flow behaviour index value ($n < 1$). Furthermore, values of n close to 1 are a feature of

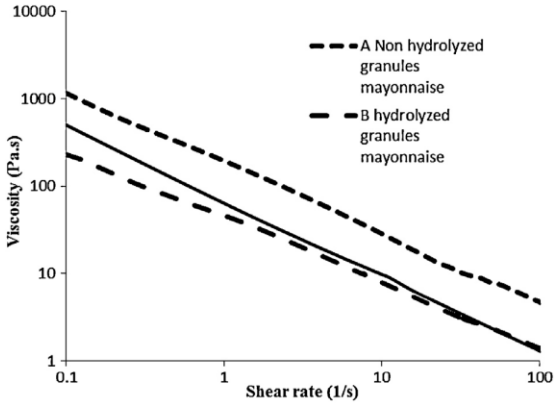

Fig. 8 – Flow curve of lyophilized hydrolysed egg yolk granules mayonnaise and commercial mayonnaise.

Table 2 – Parameters calculated from Fig. 2. k is the consistence index, n is the flow index.

	A	B	C
k (Pa s^n)	145(0.7)a	43(1.4)b	57(1.4)c
n	0.1(0.02)a	0.16(0.02)b	0.1(0.01)a
R^2	0.99	0.99	0.99

Within rows, values followed by the same letter do not differ significantly from each other ($p > 0.05$).

Newtonian fluids, meanwhile the lower values are considered related to more structured samples. The consistence and the flow behaviour indexes deduced from the flow curves are presented in Table 2.

In the mayonnaises tested, the lowest n values belong to the non-hydrolysed and the commercial recipes, showing them a more structured system than the hydrolysed mayonnaise. In the case of the non-hydrolysed protein recipe, this is in agreement with the comments made for the stress sweeps (Section 3.2.1.1), whereby the non-hydrolysed granular protein maintain a high capacity to structure the system compared to the hydrolysed protein. Furthermore, the use of non-hydrolysed granules produces mayonnaises with the highest consistence index value (k) too. This increment in the consistence index value produces a more viscous mayonnaise, with a high resistance to flow in comparison with the other two samples tested. On the other hand, the recipe elaborated with hydrolysed protein maintains a consistence index value similar to that obtained for the commercial reference, although the structure degree of the system was significantly reduced. It could be understood as a decrease in the protein-based interactions performed by the hydrolysis treatment in relation to the non-hydrolysed recipe. In the case of the commercial mayonnaise, it shows a flow behaviour index similar to that of the non-hydrolysed recipe, but the consistence index and the apparent viscosity is closer to that obtained from the hydrolysed recipe, particularly at high shear rates.

4. Conclusions

At the experimental conditions of the enzymatic proteolysis of egg yolk granules, a hydrolysis degree of 12% is obtained after one and a half hour operation time, producing peptides of 4990, 960 and 562 Da, mainly. The peptides spectrum reached was 50 ± 1.8% larger than 7000 Da, 5 ± 0.1% of 2500–5000 Da, 10 ± 0.9% of 1000–2500 Da and 30 ± 2% of less than 1000 Da, and allows the final lyophilized product to maintain good emulsifying qualities.

Rheological assays show that mayonnaise made with non-hydrolysed granules is stronger, with a higher resistance to flow and less stable to temperature changes than that elaborated using hydrolysed protein. It could be possible because the interactions between non-hydrolysed proteins enhance the structure of the system and the viscosity of the emulsion. The hydrolysis treatment could reduce these interactions, and therefore, varying the features of the obtained mayonnaise. In this sense, the use of the hydrolysed granular protein approximates the behaviour of the mayonnaise to that found in the commercial one, according to the results previously presented.

In broad terms, the enzymatic hydrolysis of the egg yolk granular fraction resulted in a product, that used in the mayonnaise formulation, provides rheological properties more

similar to those found in the commercial reference, maintaining the low-cholesterol feature of the granules.

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4.2. HIDRÓLISIS DE PROTEÍNA INSOLUBLE DE YEMA DE HUEVO CON AGUA EN CONDICIONES SUBCRÍTICAS, PROPIEDADES FUNCIONALES Y COMPARACIÓN CON LA HIDRÓLISIS ENZIMÁTICA

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Article

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Inert and Oxidative Subcritical Water Hydrolysis of Insoluble Egg Yolk Granular Protein, Functional Properties, and Comparison to Enzymatic Hydrolysis

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ABSTRACT: The use of enzymes to recover soluble peptides with functional properties from insoluble proteins could prove to be very expensive, implying high reaction times and low yields. In this study, the insoluble granular protein, previously delipidated, was hydrolyzed using enzymes (trypsin) as a comparison to the proposed alternative method: subcritical water hydrolysis (SWH) using both nitrogen and oxygen streams. The result of the hydrolysis was characterized in terms of the yield and peptide size distribution as well as different functional properties. The SWH of the delipidated granules resulted in a higher recovery yield than that obtained by enzymatic hydrolysis in half of the time. The foaming capacity of the peptides obtained by SWH was higher than that obtained by trypsin hydrolysis, although the foam stability was lower. Slight differences were detected between these peptides in terms of their emulsifying properties.

KEYWORDS: *egg yolk, functional properties, insoluble protein, subcritical water hydrolysis, trypsin hydrolysis*

1. INTRODUCTION

The use of proteins in the food industry is important, not only because of their nutritional value but also because proteins are capable of interacting among themselves and with other biopolymers in the food matrix, altering their organoleptic and functional properties.

Proteins used in the preparation of a food product can be from animal sources, such as the albumen of egg white, milk casein, or gelatin obtained from the connective tissue of cows and pigs. Vegetable proteins, such as soy protein, are used as well.

While all of these proteins are used to alter the features of food, their resultant peptides can extend these protein-dependent properties or produce other alterations that may be useful and profitable for the food industry. Protein hydrolysis has been broadly studied by Adler-Nissen,[1] and an extensive bibliography on a great variety of proteins has been published. Changes in the functional properties, protein recovery from food industry wastes, and others have been documented.[2−6] The main advantage of enzymatic over non-enzymatic hydrolysis is that the enzymatic reactions require mild conditions that prevent chemical alterations in the processed proteins. Furthermore, the size of the peptides can be adjusted by taking into account the hydrolysis time. This is important because the average size of the peptides is related to their functional properties. However, enzyme use can be considered expensive, and the targeted proteins must be accessible to enzymatic action.

In addition to enzymatic hydrolysis, other hydrolysis reactions can be considered, such as water hydrolysis under subcritical conditions. Water is considered to be subcritical when its temperature varies in the range from 100 to 370 °C, and it is maintained in a liquid state by application of high pressures. In this state, noticeable changes occur in its properties, increasing the ion concentration product (K_w) at 250 °C to 10^{-11}, as well as its decreasing dielectric constant at elevated temperatures, thereby exhibiting higher solvation behavior.[7] In fact, subcritical water has been used previously to recover phenolic compounds[8] and amino acids from biomass discards.[9−11] Furthermore, this method can be used in the hydrolysis of soluble proteins, allowing, with adjustment of the treatment parameters, the control of the end products.[12]

Egg yolk can be easily separated into plasma and granular fractions. The first one is the lipid-rich fraction, and it is mainly composed of lipoproteins. It has been described as having emulsion[13] and gelation properties,[14,15] similar to those found in the whole egg yolk. On the other hand, the second one is the protein-rich fraction, and it requires an ionic strength ≥0.3 M NaCl to solubilize its protein constituent. Below 0.3 M NaCl, the granules from egg yolk show a low solubility[16] and, therefore, decreased functional properties, which reduces their applications.

In this work, the hydrolysis of non-soluble egg yolk proteins has been studied using enzymes and the subcritical water method. For that purpose and because it has a high protein content and limited utility, the egg yolk granular fraction was obtained, delipidated, and hydrolyzed. The hydrolysis yield, average size of the peptides, and their functional properties were evaluated and compared in this study.

2. MATERIALS AND METHODS

2.1. Production of Delipidated Granules. The egg yolk granules were obtained using the Laca et al.[17] method. Egg yolk was separated from the albumen manually, and the vitelline membrane was discarded using tweezers. The egg yolk was diluted (1:1.5, v/v) with water, and the pH was adjusted to 7. Finally, the diluted egg yolk was kept overnight at 4 °C and centrifuged at 4 °C and 10000*g* for 45 min to obtain the granules in the sediment. Granules were lyophilized and mixed with ethanol (96%, 1:12.5, w/v) at 40 °C for 2 h with gentle agitation. Finally, the granules were recovered using a vacuum pump and Whatman No. 1

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filter paper. The delipidated egg yolk granules were then washed with 200 mL of fresh ethanol and dried overnight in a heater at 50 °C to obtain a dehydrated and ethanol-free powder.

2.2. Enzymatic Hydrolysis. The hydrolysis was carried out in a 400 mL bioreactor with a pH-STAT automatic titration (pH-Buret 24 2S, Crison) connected to an isothermal shaker. Delipidated egg yolk granules were mixed with water (1:20, w/v) and preheated at 90 °C for 7 min to enhance the dispersion of the granules. Two enzyme−substrate ratios were tested, 1:50 and 1:25, using trypsin from porcine pancreas EC 3.4.21.4 (93610, Sigma-Aldrich), 18 136 U/mg. The dispersed granules were heated at 37 °C, and the pH was adjusted to 7.5 using 0.1 M sodium hydroxide solution. Finally, the enzyme was added to the reactor, and after 6 h, the enzymatic reaction was stopped by heating the hydrolysate for 15 min in a water bath at 90 °C.

The degree of hydrolysis (DH) was evaluated using the pH-STAT method according to Adler-Nissen.[1] In this method, the amount of base solution consumed along the reaction is proportional to the amino groups liberated during the enzymatic hydrolysis. The DH was calculated using the Adler-Nissen equation (eq 1)

$$\mathrm{DH}\ (\%) = \frac{100 V_B N_B}{\alpha M_p h_{tot}} \tag{1}$$

where α is the degree of dissociation of the α amino group calculated by following eq 2, M_p is the amount of protein in the assay (g), and h_{tot} is the total number of peptide bonds in the protein (mequiv g^{-1} of protein). The value of h_{tot} for egg yolk protein was assumed to be 8 based on a suggestion found in ref 18, which perfectly fits with the h_{tot} values reported by Nielsen et al. for raw materials.[19] V_B is the volume (mL) of the base (NaOH) added during the course of the enzymatic reaction, and N_B is the normality of the base.

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{p}K}}{1 + 10^{\mathrm{pH}-\mathrm{p}K}} \tag{2}$$

The pK value used for the hydrolysis procedure is calculated approximately using the equation by Guadix et al.[20] (eq 3).

$$\mathrm{p}K = 3.80 + 0.45\mathrm{pH} \tag{3}$$

2.2.1. Subcritical Water Hydrolysis (SWH). The delipidated granules were dispersed in water (1:20, w/v) to obtain 400 mL of solution and placed in the jacketed reactor shown in Figure 1. Different hydrolysis temperatures were tested, and the lowest temperature tested with a good peptide recovery in 4 h was 180 °C. At each tested temperature, a pressure of 40 bar was achieved using a non-reactive (nitrogen) or an oxidizing (oxygen) gas stream of 1 L/min. A back-pressure valve (Brooks mass flow controller 5850) allowed for pressure control in the system. The injected gas was previously saturated with water to avoid sample evaporation and heated to keep the reactor temperature constant. In this study, the reaction time specified in each case includes the heating time. This heating time was short in each case; therefore, its influence on the overall process is negligible.

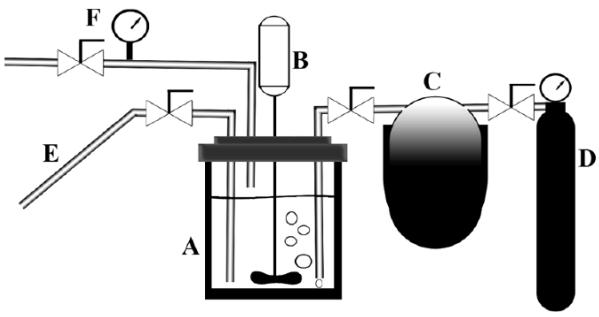

Figure 1. Setup of the SWH reactor: (A) pressure reactor, (B) agitation, (C) humidifier, (D) nitrogen or oxygen gas bottle, (E) sample taker, and (F) back-pressure valve.

2.3. Size-Exclusion Chromatography. An AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare, Sweden) was employed to determine the peptide size distribution using a size-exclusion column (Superdex Peptide 10/300 GL), which can separate peptides from 100 to 7000 Da. The buffer used was composed of 0.1 M Tris at pH 7.6. The elution flow was adjusted to 1 mL/min. The FPLC system used was dotted with an ultraviolet (UV) detector, and the wavelength employed was 214 nm. Previously, the column was calibrated with weight markers supplied by Sigma-Aldrich: lysozyme (14 307 Da), cytochrome *c* (12 384 Da), aprotinin (6512 Da), cianocobalamin (1355 Da), and tryptophan (204 Da) (R^2 = 0.99). The molecular size distribution and the average molecular size of the obtained peptides were analyzed to determine the total concentration. Subsequently, the area of the chromatograms obtained was divided into ranges of molecular size, according to previous calibration. The area of each zone was measured, employing the Unicorn 5.1 analysis software, and transformed into protein concentration. The frequency of each molecular weight range was calculated with regard to the total amount of soluble peptides.

2.4. Amino Acid Quantification. The amounts of amino acids produced during the SWH and the enzymatic hydrolysis were calculated by the Rosen method.[21] This method is based on the interaction of ninhydrin with the amino groups, resulting in a colored compound.

2.5. Functional Properties. To test the functional properties, after the hydrolysis processes, the solutions were centrifuged at 10000*g* for 30 min. After that, the supernatants were separated from the sediments by decantation. Immediately, the supernatants were lyophilized, and the sediments were discarded. The protein content of the lyophilized product was determined using the Dumas combustion method.[22] For that purpose, the nitrogen content was calculated using a CNHS/O Elementar Vario EL analyzer (Elementar, Germany). The conversion factor employed was 6.25 mg of protein/mg of N.

2.5.1. Solubility. The solubility of the hydrolyzed and non-hydrolyzed delipidated egg yolk granules was determined using a modified method by Wang et al.[18] Briefly, they were diluted in distilled water to obtain a protein concentration of 1 mg/mL. The pH of these solutions was adjusted with 1 N HCl or 1 N NaOH and equilibrated at room temperature for 60 min. The pH values were readjusted at 30 and 60 min to correct possible variations. Finally, the diluted samples were centrifuged at 10000*g* and 30 min, and the sediment was discarded. The protein content of the samples was determined according to Markwell et al.,[23] both before centrifugation and in the resulting supernatant. A calibration curve was carried out using bovine serum albumin. The protein solubility was calculated using the following equation:

$$\mathrm{PS}\ (\%) = \frac{\text{protein in supernatant (mg)}}{\text{protein in the initial solution (mg)}} \times 100 \tag{4}$$

2.5.2. Foaming Capacity (FC) and Foam Stability (FS). FC and FS were determined according to ref 24, with slight modifications. Peptide solutions of 5 mg/mL protein in water were prepared, and the pH was adjusted to 7 using 1 N NaOH or 1 N HCl. After 30 min of equilibration at room temperature, 30 mL of the solutions were taken and stirred at 15 000 rpm using a Heidolph SilentCrusher for 1 min. The volume of the foam formed was immediately calculated using a cylinder measure, and the FC was determined. To calculate the FS, foam volume changes in the cylinder measure were recorded at 30 min of storage.

$$\mathrm{FC}\ (\%) = \frac{\text{foam volume after whipping (mL)}}{\text{initial liquid volume (mL)}} \times 100 \tag{5}$$

$$\mathrm{FS}\ (\%) = \frac{\text{foam volume after 30 min(mL)}}{\text{foam volume after whipping (mL)}} \times 100 \tag{6}$$

2.5.3. Emulsifying Properties. Emulsifying properties were measured according to the study by Pinciroli et al.[25], with slight modifications. Peptides were diluted in a NaCl solution (0.075%, w/v) to obtain a final concentration of 10 mg/mL protein. The pH of these solutions was adjusted to pH 7 using 1 N HCl or 1 N NaOH and equilibrated for 30 min with continuous stirring at room temperature. A total of 15 mL of these solutions were mixed with 10 mL of cottonseed oil at 15 000 rpm

using a Heidolph SilentCrusher (Heidolph, Germany). Immediately, the emulsions were put into a glass measurement cell, and the stability against creaming was measured using a Turbiscan apparatus (Formulaction, France). Backscattered light was monitored every 2 min for 1 h as a function of the sample height. Clarification was measured at the bottom of the measured cell from 0 to 25 mm ($B\%_{0-25\ mm}$), and creaming was determined at the top of the cell ($B\%_{25-40\ mm}$). Furthermore, the initial backscattering value in the medium zone of the measurement cell ($B\%_{ini}$), stability parameter ($t_{0.1}$), and kinetic constant ($K_{0.1}$) were determined too. The stability parameter $t_{0.1}$ was calculated as the time in which the backscattering in the bottom of the measured cell decreased to 10% of its initial value. The kinetic constant $K_{0.1}$ was calculated according to the following equation:

$$K_{0.1}\ (h^{-1}) = (B\%_{ini,0-25\ mm} t_{0.1})^{-1} \quad (7)$$

2.6. Statistical Analysis. The assays shown in this work were carried out in triplicate. Mean and standard deviation are shown in each case.

3. RESULTS AND DISCUSSION

3.1. Enzymatic Hydrolysis Characterization. The DH is a parameter used with respect to enzymatic hydrolysis that is frequently referred to in the literature.[26−29] The DH is directly related to the solubility of the peptides obtained from the insoluble proteins.[30] Figure 2 shows DH versus enzymatic reaction time. It can be seen that, as one would expect, an increase in the hydrolysis time and enzyme concentration lead to an increase in the DH.

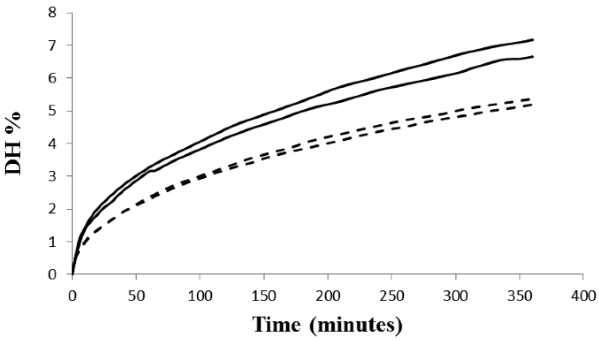

Figure 2. DH (%) of delipidated egg yolk granules treated with trypsin. Continuous lines correspond to a 1:25 enzyme/substrate ratio, and discontinuous lines correspond to a 1:50 enzyme/substrate ratio. Reproducibility is shown in each case.

As expected, the low solubility of the delipidated egg yolk granules at any pH impedes the cleavage of the peptide bond by the enzyme. This can be observed in Figure 2, where after 360 min of operation, the DH values reached 5.2 ± 0.12% (enzyme/substrate ratio of 1:50) and 6.9 ± 0.3% (enzyme/substrate ratio of 1:25).

According to the hydrolysis time, these DH values can be considered low compared to others found in the bibliography. In previous studies,[3] the non-delipidated granules were dissolved in a 0.55 M NaCl solution. In this NaCl solution, the phosphocalcic bridges between the granular proteins are disrupted and these proteins became soluble. In this case, the enzyme can interact with the peptide bonds efficiently and the DH increases to 12.0 ± 0.5% after 90 min. In other studies and using different enzymes, the hydrolysis of ethanol-delipidated whole egg yolk resulted in a DH of 6% in 80 or 100 min as a function of the enzyme tested.[18] However, the comparison of the results presented in this work to those obtained by these authors is difficult given the methodology differences. In any case, the trypsin hydrolysis of delipidated egg yolk granules resulted in a significantly longer reaction time compared to those reported by other authors.

The DH is an indication of the peptide sizes obtained; therefore, the higher the DH, the lower the size of these peptides. To calculate their average size with more precision, aliquots obtained at different times during the enzymatic hydrolysis were analyzed by size-exclusion chromatography (SEC). The chromatogram is shown in Figure 3A, and the peaks identified in this figure are detailed in Table 1.

In the hydrolysis using trypsin, five peaks were detected (Figure 3A), which maintain their relative importance during the period of hydrolysis. The main peak identified is number 4, which corresponds to peptides with an average size of 3500 Da (Table 1). Furthermore, in Table 2, the peptides were analyzed according to their size at each hydrolysis time. In enzymatic hydrolysis, the average peptide size was similar at each time tested, with no significant variations during the process.

3.2. SWH. In the SWH method, the aliquots obtained at different times were analyzed by SEC. In these non-enzymatic hydrolyses, the peptide size profiles vary according to the reaction time, with their sizes decreasing progressively from 23 000 Da in the initial stages to around 1000 Da at longer times. This can be observed in panels B and C of Figure 3, where at short reaction times, the main peak obtained is the number 1, while at longer reaction times, the SEC chromatograms obtained were progressively moving to the right, with the main peak appearing around the 1000 Da in these cases (peak numbers 3 and 4 in panels B and C of Figure 3). Therefore, in this type of hydrolysis, the temperature and reaction time that are chosen are important to obtain the greatest amount of soluble peptides, because the reaction progression leads to full protein breakdown. According to Abdelmoez et al.,[31] some amino acids are sensitive to temperatures around 230 °C. This can be observed in Table 2, where in both nitrogen and oxygen SWH, the population of peptides of more than 6 kDa decreases with the time of hydrolysis and those peptides of less than 6 kDa increase in a similar way.

3.3. Process Yield. The process yield in each case was calculated and shown in Figure 4. For enzymatic hydrolysis, the yield obtained rises with the reaction time. After 360 min of operation, the recovery of soluble peptides corresponds to 50% of the original protein when an enzyme−substrate ratio of 1:25 is used.

In the SWH under nitrogen stream, the maximum yield was obtained at 240 min. After that, the yield decreased slowly until it reached 70% at 580 min. According to ref 32, some additives, such as sodium bicarbonate, can be used to enhance the protein hydrolysis, although it produces a decrement in the molecular weight of final peptides. With regard to the SWH under oxygen stream, the yield rose quickly until 120 min and then decreased quickly too. Using these gas streams, the yield obtained was around 95%, almost doubling the value obtained by the enzymatic hydrolysis and with significantly shorter operation times as well. Between the nitrogen and oxygen treatments, the latter was more efficient, reaching the maximum recovery rate in half of the time required to achieve that value in the hydrolysis under a nitrogen stream. This is because, in the SWH under nitrogen stream, the non-reactive nitrogen has no effect on peptide breakdown, with hydrolysis being possible thanks to the increase in the amount of hydrogen and hydroxide ions in the subcritical water. However, using oxygen, in addition to the

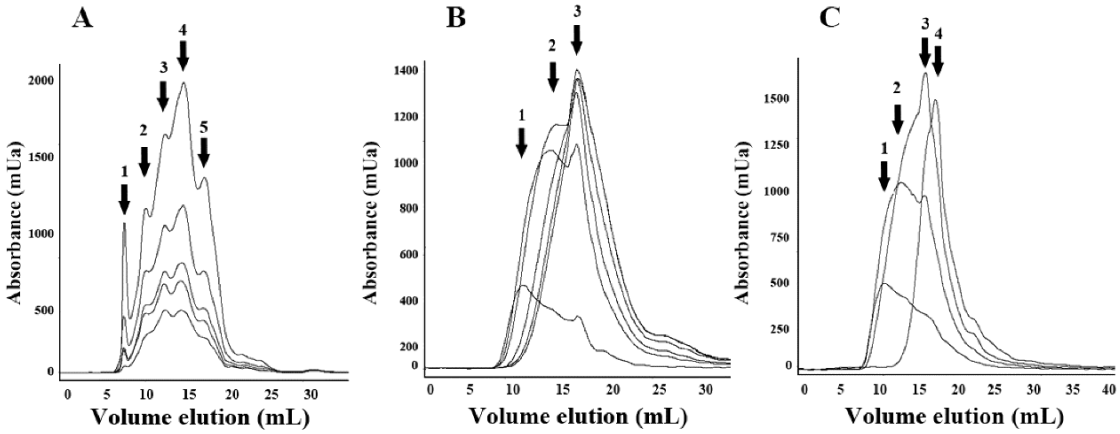

Figure 3. SEC chromatograms of the peptides obtained during hydrolysis: (A) peptides obtained from enzymatic hydrolysis, (B) peptides obtained from SWH under nitrogen stream, and (C) peptides obtained from SWH under oxygen stream.

Table 1. Size of the Main Peaks Detected in Figure 3

	trypsin		nitrogen		oxygen	
	volume elution (mL)	size (Da)	volume elution (mL)	size (Da)	volume elution (mL)	size (Da)
1	7.98	112171	10.97	23836	11.03	23106
2	10.39	32189	13.96	5065	13.98	5012
3	12.49	10846	16.86	1127	16.8	1163
4	14.68	3488			17.95	641
5	17.06	1016				

increase in the ion concentration product of the water, the dissolved oxygen produces a rise in the oxidization of the organic matter. According to the study by Kelvin et al.,[33] the exposure of proteins to the hydroxyl radical and the superoxide anion radical promotes changes in their structure. This could increase their susceptibility to proteolytic action and lead to the rise in peptide bond cleavage that was detected.

During the SWH reaction, loss of protein in the form of amino acids, ammonium compounds, and other volatile products was expected.[34] Amino acids are considered to be the main decomposition compound in both enzymatic and SWH. For this reason, amino acids were measured in the reaction medium at each hydrolysis time (Figure 5). In the enzymatic hydrolysis (Figure 5A), the production of amino acids was the lowest, and after 360 min of reaction, the loss of protein was around 2% (w/w). In the SWH under nitrogen stream, the rise in amino acid production was more noticeable and continuous as the reaction proceeded. However, at the minute of optimum peptide yield (240 min), the loss of protein was 6%, owing to the formation of amino acids. The progression was similar in the SWH under oxygen stream, but because of the fact that the maximum yield was obtained at 120 min, the production of amino acids was reduced to 3%. According to these results, in every hydrolysis method, the loss of protein in the form of amino acids and in the maximum yield times was not relevant.

3.4. Functional Properties. For the functional properties assays, the highest yields in each case were chosen according to section 3.3.

3.4.1. Solubility Profile. The solubility profiles of the peptides obtained by hydrolysis are shown in Figure 6. The ethanol-delipidated egg yolk granules were used as a reference for the improvement in solubility obtained. Solubility could be considered as the most important functional property of proteins, because other functional properties, such as, for example, their water holding, gelling, foaming, and emulsifying capacities, are dependent upon it.

The low solubility of the delipidated egg yolk granules at any tested pH was expected because of the ethanol treatment that they had undergone, which produces the protein denaturation. In food proteins, denaturation is usually associated with protein insolubilization and loss of other functional properties.[35] An increase in the solubility properties of the resultant peptides is expected after the hydrolysis process.[27,36]

In the case of the enzymatic hydrolysis, the peptides obtained were highly soluble. However, in this type of hydrolysis, it is very difficult to recover all of the initial insoluble protein in the form of soluble peptides. In previous studies using soy protein, a DH of 8% or higher was required and the 100% conversion from protein to soluble peptides was not achieved.[37] As previously commented, in this work, a DH of 7% was obtained and only 50% of the protein was recovered as soluble peptides. This is explained by the remaining hydrophobic protein aggregations that cannot be hydrolyzed by the enzymes.

In nitrogen and oxygen SWH and also in the enzymatic hydrolysis, in the tested pH range, the isoelectric point of the peptides was not detected and their solubility maintains a relative flat profile, instead of the characteristic U-shaped profile expected. In the two SWH tested, in addition to the high solubility of the peptides obtained, 95% of the protein was recovered.

3.4.2. Foaming Properties. The FC of the peptides is shown in Figure 7. The FC of the delipidated egg yolk granules was tested too, and it was considered to be 0 because of their low solubility.

As observed in Figure 7, all of the peptides showed foaming properties but differences in the FC and FS were found. Peptides obtained by enzymatic hydrolysis had the lowest values for FC, but their FS was the highest. In comparison, the peptides obtained by SWH under nitrogen and oxygen streams showed a higher FC, but their FS was poor compared to that obtained with the peptides derived from enzymatic hydrolysis.

These differences could be explained by the differences in the average size of the peptides that have been described previously. According to Sijtsma et al.[38] and Addler-Nilsen and Olsen,[37] the optimum peptide size varies from 1650 to 3850 Da. In enzymatic

Table 2. Peptide Size Distribution Obtained from Figure 3[a]

	trypsin					nitrogen					oxygen				
	20 min	40 min	60 min	120 min	240 min	360 min	120 min	180 min	240 min	300 min	420 min	480 min	60 min	90 min	120 min	150 min	180 min	240 min
>10 kDa (%)	30.1	36.6	32.7	33.3	32.8	31	15.7	15.1	11.3	9.58	7.17	5.01	35.8	23.4	9.8	35	2.67	0.81
10−6 kDa (%)	11.9	12.7	12.9	13.2	12.0	12.2	9.54	10.0	8.61	8.06	6.41	5.56	10.7	17.5	7.84	4.36	2.77	1.17
6−1 kDa (%)	39.2	39.4	40.6	40.3	41.2	42	39.7	41.8	43.6	43.8	44.8	42.3	32.8	44.0	47.1	46.8	44.7	36.6
<1 kDa (%)	16.2	12.3	13.5	13.6	14.0	13.8	37.0	34.3	36.8	38.3	43.4	47.4	20.5	21.7	35.7	45	49.8	63.3
average size (kDa)	7	7.9	7.4	7.5	7.4	7.1	4.6	4.7	4.1	3.8	3.37	2.9	6.4	6.5	3.9	2.8	2.4	1.8

[a]Standard deviations were lower than 10% in each case.

hydrolysis, the average peptide size obtained was around 7 kDa, while in SWH, the average size was around 4 kDa. In this case, it is likely that the lower size peptides obtained by SWH diffuse rapidly and adsorb at the interface.[39] However, the number of interactions at the air−water interface will be larger in the case of bigger proteins, and therefore, although SWH leads to more FC, the bigger size of the peptides derived from enzymatic hydrolysis gives greater FS.[40]

In addition to the peptide size, the adequate distribution of the hydrophobic groups and the charged nuclei in the peptide structure are another important feature for obtaining good interfacial qualities.[35] They are important for creating interactions with the interface and providing the electrostatic repulsions that give FS. The size distribution of the peptides obtained by SWH in oxygen and nitrogen was similar; however, the FC and FS were higher in the peptides obtained under the nitrogen stream than those obtained in the oxygen stream. This could be because, although the oxygen promotes protein hydrolysis, the oxidizing conditions provided could be altering the amino acid residues. According to Stadtman and Levine,[41] the oxidation of proteins leads to the hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, and conversion of some amino acid residues to carbonyl derivates. It could be interesting to further investigate if these amino acid residue modifications affect the ability of the peptides to interact with the air−water interface and, in particular, to stabilize the foams produced.

3.4.3. Emulsifying Properties. The emulsifying properties of the peptides obtained are shown in Figure 8. Figure 8A shows the variation in backscattering at the bottom of the measurement cell, which was measured to study the emulsion creaming. The peptides obtained by enzymatic hydrolysis showed a lower decrease in emulsion backscattering with time when compared to the values obtained for the SWH peptides. In fact, according to the values shown in Table 3, enzymatic hydrolysis produced the peptides whose emulsions were the most stable, with the highest $t_{0.1}$ and the lowest $K_{0.1}$ values. Peptides obtained by SWH under a nitrogen stream showed values closer to those found in the enzymatic hydrolysis, although their creaming was more noticeable. On the other hand, peptides obtained by SWH under an oxygen stream showed the poorest emulsifying properties.

This was confirmed by the $B\%_{ini}$ values. Higher initial values of backscattering are related to emulsions that contain a high number of oil droplets and low size. Therefore, a high value in this parameter is considered indicative of good emulsifying properties.

With regard to stabilization in the cream phase (Figure 8B), the decrease in the backscattering profile detected in the emulsions elaborated using peptides from the two SWH processes showed that coalescence is the main component in the destabilization, reversing the expected rise in backscattering caused by the creaming process.[25] However, the emulsion elaborated using peptides from enzymatic hydrolysis was more stable, which indicates that it is likely that coalescence is not the main cause of emulsion destabilization in this case.

According to the study by Wang et al.,[18] hydrolyzed ethanol-delipidated whole egg yolk protein resulted in improved emulsifying capacity and stability, in particular with the hydrolysates obtained at the highest DH tested (DH = 6%). In our study for delipidated egg yolk granular protein, the

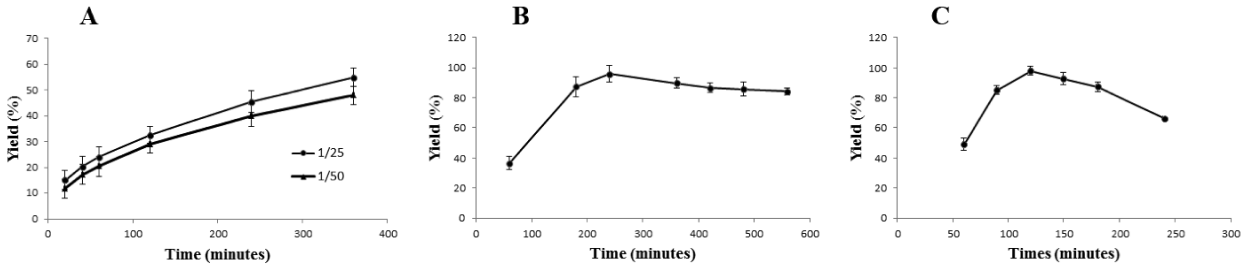

Figure 4. Yield of the hydrolysis process against the reaction time: (A) enzymatic hydrolysis process, (B) SWH under nitrogen stream, and (C) SWH under oxygen stream.

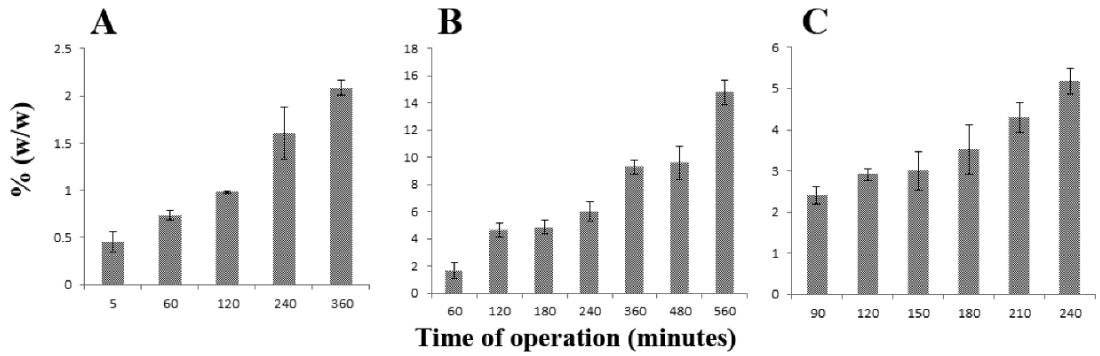

Figure 5. Amino acid residue production during the hydrolysis processes: (A) enzymatic hydrolysis process, (B) SWH under nitrogen stream, and (C) SWH under oxygen stream.

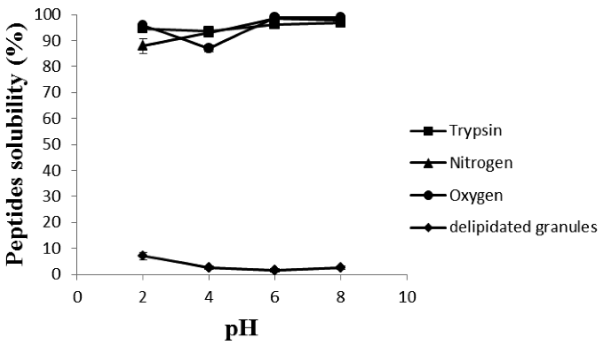

Figure 6. Solubility of delipidated granules and peptides at different pH values.

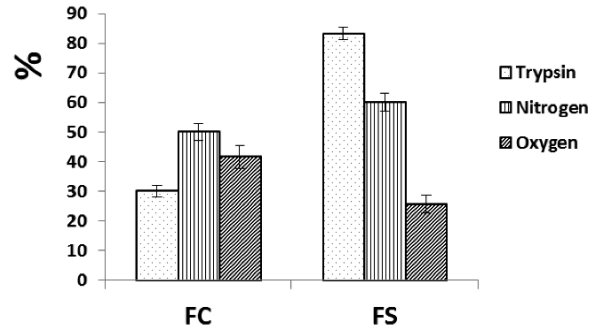

Figure 7. FC and FS of the peptides.

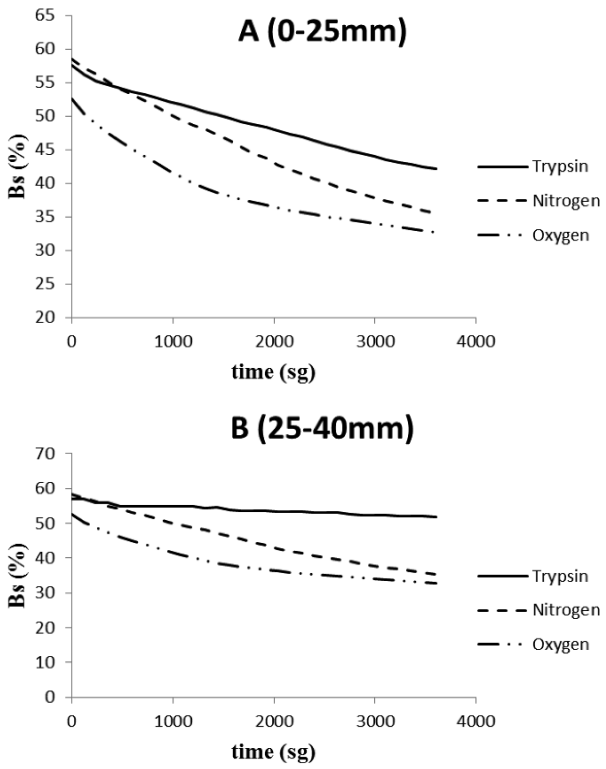

Figure 8. Variations in backscattering over 60 min in the emulsions elaborated using the peptides obtained by different means: (A) backscattering measured at the bottom of the cell and (B) backscattering measured in the upper part of the cell.

hydrolysates obtained after the enzymatic hydrolysis, with DH of 7%, had the better emulsifying properties. The SWH method

Table 3. Parameters Obtained from the Backscattering Variations Shown in Figure 8

	trypsin	SWH nitrogen	SWH oxygen
$t_{0.1}$ (min)	17.5 ± 0.6	12.25 ± 0.4	5.9 ± 0.11
$K_{0.1}$ (h^{-1})	0.06 ± 0.002	0.08 ± 0.002	0.19 ± 0.003
$B\%_{ini}$	57 ± 1.5	58.3 ± 2	52 ± 1

resulted in significantly smaller peptides, and they gave less stable emulsions. Therefore, as with foaming properties, the emulsifying properties were highly dependent upon the peptide size. This result is consistent with another study, which suggest that extensive hydrolysis of whey proteins reduces the emulsifying activity of the obtained products.[39]

Finally, the results suggest that the modifications caused by SWH under an oxygen stream had a negative effect on the interfacial properties of the peptides, while SWH under a nitrogen stream resulted in hydrolysates with foaming and emulsifying properties close to those obtained by enzymatic hydrolysis.

In conclusion, the delipidated and non-hydrolyzed egg yolk granular protein used as a control was highly insoluble at any tested pH. Using enzymatic hydrolysis (trypsin), 50% of the initial protein content was recovered in the form of soluble peptides in 360 min of reaction. However, using the SWH method, the yield increased until 95% and the reaction time decreased significantly. The functional properties of the peptides obtained using trypsin and those using SWH under a nitrogen stream showed slight differences in their foam and emulsifying capacities. Furthermore, when a SWH under oxygen pressure was carried out, probably because of the protein modifications caused by the oxidizing gas stream, a decrement in the functional properties of the peptides was detected.

According to these results, the use of SWH to obtain peptides from insoluble proteins can be considered as a good alternative to the enzymatic hydrolysis, being a faster and reactive-free method.

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Notes

The authors declare no competing financial interest.

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4.3. ACTIVIDAD ANTIOXIDANTE DE HIDROLIZADOS DE GRÁNULOS DE YEMA DE HUEVO DELIPIDADOS OBTENIDOS MEDIANTE HIDRÓLISIS CON AGUA EN CONDICIONES SUBCRÍTICAS. COMPARACIÓN CON LA HIDRÓLISIS ENZIMÁTICA

ANTIOXIDANT ACTIVITIES OF DELIPIDATED EGG YOLK GRANULES HYDROLYSATES OBTAINED USING INERT OR OXIDATIVE SUB-CRITICAL WATER HYDROLYSIS. COMPARISON WITH THE ENZYMATIC HYDROLYSIS.

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ABSTRACT

The obtaining of peptides from proteins with antioxidant properties by enzymatic hydrolysis has been widely described in the literature, and different protein sources and enzymes have been studied. However, the use of non-enzymatic methods to obtain peptides with antioxidant capacities has been poorly investigated. In this work, non-soluble proteins obtained from delipidated egg yolk granules were hydrolyzed with an enzyme (trypsin), and with a non-enzymatic method using sub-critical water and a pressure of nitrogen or oxygen alternatively. The antioxidant capacities of the hydrolysates were evaluated using the $ABTS^{\cdot+}$ scavenging assay, the DPPH radical scavenging activity assay, the peptides reducing power, the peptides ferrous ion chelating capacities, and the antioxidant effect of the peptides on beef homogenates. Finally, an antimicrobial test was performed too.

The sub-critical water hydrolysis under nitrogen stream hydrolysate gave similar or better results in the antioxidant tests than those obtained using Trypsin hydrolysis, except in the ferrous chelating capacity, where the trypsin hydrolysate showed the better performance. The oxidant environment promoted by the oxygen in the other sub-critical water hydrolysis method tested produced the peptides with the lowest antioxidant capacities, likely due to variations in the primary structure of the peptides. Furthermore, any hydrolysate showed antimicrobial activity. These results suggest that the sub-critical water hydrolysis method under nitrogen stream, in comparison with the enzymatic hydrolysis, is a reliable method to obtain peptides with good antioxidant capacities.

1. INTRODUCTION

In food preservation is important to maintain the oxidation processes under control. The lipid peroxidation is one of the most important causes of deterioration in precooked foods and meats, altering their organoleptic and nutritional properties (Wilson, Pearson, & Shorland, 1976). In order to avoid these oxidation problems the study of antioxidants has been broadly investigated in recent years, being incorporated to the food industry additives as for example butylated hydroxyanisole, butylated hydroxytoluene and propyl gallate. However, the research of natural substances with similar or better antioxidant properties is interesting and persists in the bibliography, being investigated these features in tea extracts, isoflavones, licopeno, vitamin C or peptides obtained from food proteins. In this case, the obtaintion of peptides with antioxidant properties is particularly fascinating due to their other possible bioactivities, as for

example antihypertensive (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004) or cholesterol lowering. Furthermore, the raw sources of these peptides can be proteins which came from wastes of the food industry, or proteins with limited functional properties which can be recovered using hydrolysis methods and revalued.

The antioxidant properties of these hydrolyzed varies with the raw protein used and with the process selected to obtain the peptides. According to this, many proteins have been investigated, as for example soya protein, rapeseed, porcine hemoglobin, porcine myofibrillar or egg yolk and egg white proteins among others. Concerning the methods used in the peptides obtention, the most commons include enzymatic hydrolysis, fermentation of foods by the microbial activity or through the action of enzymes derived from proteolytic micro-organisms (Korhonen & Pihlanto, 2003).

The main methods to obtain peptides from a source of proteins use the enzymatic hydrolysis and pancreatic enzymes, like trypsin. They have been quite refered in the bibliography to make hydrolyzed with bioactive and antioxidant capacities (Memarpoor-Yazdi, Asoodeh, & Chamani, 2012; Ranathunga, Rajapakse, & Kim, 2006; Rival, Boeriu, & Wichers, 2001; Rival, Fornaroli, Boeriu, & Wichers, 2001; Tanzadehpanah, Asoodeh, & Chamani, 2012; Zambrowicz, Pokora, Eckert, Szołtysik, Dąbrowska, Chrzanowska, et al., 2012). In the case of the enzymatic hydrolysis the digestion conditions have to be carefully selected and maintained during the reaction to obtain peptides with the desirable functional and antioxidant properties.

In addition to the enzymatic hydrolysis, other hydrolysis reactions can be considered, as the water hydrolysis in sub-critical conditions. The sub-critical water is considered when temperature varies in the range from 100°C to 370°C and the water is maintained in liquid state by high pressures. In this state, noticeable changes occur in the water properties, increasing the product of the ions concentration (Kw) at 250°C until 10^{-11} and decreasing its dielectric constant from 80 at room temperature to 27, which is appropriate to solubilize non-in water soluble compounds (Miller & Hawthorne, 2000). In fact, sub-critical water has been used previously to recover useful compounds from biomass discards (Esteban, García, Ramos, & Márquez, 2010; Pourali, Asghari, & Yoshida, 2010; Sereewatthanawut, Prapintip, Watchiraruji, Goto, Sasaki, & Shotipruk, 2008; Zhu, Zhu, Zhao, & CHENG, 2008).

Egg yolk can be easily separated to produce plasma and granular fractions. The plasma is mainly constituted by lipids while the granular fraction is protein-rich. The plasma fraction shows similar features to those found in the whole egg yolk, with similar emulsion (Le Denmat, Anton, & Beaumal, 2000) and gelation properties (Paraskevopoulou, Boskou, & Kiosseoglou, 2005). However, the granular fraction is formed by the interaction of different proteins through phophocalcic bridges. This protein association is highly insoluble, and it requires an ionic strength ≥ 0.3M NaCl (Causeret, Matringe, & Lorient, 1991) to solubilize its protein constituent and therefore, in solutions with low ionic strength, the functional properties of this fraction are reduced and its range of applications is limited.

Previously, other authors obtained peptides from egg yolk proteins and egg albumen, exhibiting antioxidant properties, as for example, inhibiting the lipid oxidation in a linoleic acid oxidation sytem (Sakanak, 2006; Sakanaka, 2004) and testing them in real food models as beef and tuna muscle homogenates too (Sakanaka & Tachibana, 2006), behaving them as hydroxyl radicals and DPPH scavenging. In all these cases the peptides were obtained using enzymes.

In this work, with the aim of extending the interest of the egg yolk fractionation in the food industry, and therefore, the usefulness of the egg yolk, its fraction of the granules was hydrolysed using water in sub-critical conditions under an oxygen or nitrogen stream. The antioxidant properties of the peptides obtained were tested in comparison with that of the peptides obtained by enzymatic methods (trypsin) in order to determine the possibilities of this non-enzymatic method in the obtaining of food-grade antioxidants.

2. MATERIALS AND METHODS

2.1. Granules separation

The egg yolk granules were obtained using the (Laca, Paredes, & Díaz, 2010) method. Egg yolk was separated from the albumen manually and the vitelline membrane was discarded using tweezers. The egg yolk was diluted (1:1.5 v/v) with water and the pH was adjusted to 7. Finally, the diluted egg yolk was kept overnight at 4°C and centrifuged at 4°C and 10,000g for 45 min to obtain the granules in the sediment. Granules were lyophilized and mixed with ethanol (96%, 1:12.5 w/v) at 40°C for 2 hours with gentle agitation. Finally, the granules were recovered using a vacuum pump and Whatman nr. 1 paper. The delipidated egg yolk granules were washed with 200 mL of fresh ethanol and dried overnight in a heater at 50°C to obtain an ethanol-free powder. The delipidated granules resulted highly insoluble in distilled water.

2.2 Hydrolysates obtaining.

The hydrolysates obtaining was developed according to Marcet et al. (2014). Briefly, in the case of the sub-critical water hydrolysis method, the delipidated granules were dispersed in water (1:20 w/v) to obtain 400 mL of solution and placed in the jacketed reactor. Different temperatures were tested and the best hydrolysis values were obtained at 180°C. A non-reactive gas stream (nitrogen) or an oxidizing one (oxygen) were evaluated using a back-pressure valve to maintain the pressure at 40 bar. Furthermore, a gas stream of 1000 mL/min was selected, and it was controlled by a precision electrovalve (Brooks mass flow controller 5850). The injected gas was previously saturated with water to avoid sample evaporation and heated to keep the reactor temperature constant.

Furthermore, in the case of the enzymatic hydrolysis, it was carried out in a 400 mL bioreactor with a pH-STAT automatic titration (pH-Burette 24 2S, Crison, Spain) connected to an iso-thermal shaker. Delipidated egg yolk granules were mixed with water (1:20 w/v) and preheated at 90°C for 7 min to enhance the dispersion of the granules. The enzyme-substrate ratio tested was 1/25, using trypsin from porcine pancreas E.C 3.4.21.4 (93610, Sigma-Aldrich) 18136 U/mg. The dispersed granules were heated at 37°C and the pH adjusted to 7.5 using 0.1 M sodium hydroxide solution. Finally, the enzyme was added to the reactor and after 6 h the enzymatic reaction was stopped by heating the hydrolysate for 15 min in a water bath at 90°C. The degree of hydrolysis (DH) was calculated according to (Orcajo, Marcet, Paredes, & Díaz, 2013).

2.3 $ABTS^{\cdot+}$ scavenging assay.

$ABTS^{\cdot+}$ (2,2´-azinobis-(-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging capacity was calculated in each case according to a modified method described by (Watchararuji, Goto, Sasaki, & Shotipruk, 2008). An initial stock solution of ABTS composed by an aqueous solution of 7 mM $ABTS^{\cdot}+$ and 2.45 mM potassium persulfate was prepared. This stock was kept in darkness at room temperature for 16 h before use. Following, it was diluted with distilled water until reaching an absorbance of 0.70±0.04 at 734 nm in a 1cm cuvette. Lyophilized samples were diluted at several concentrations (from 0.5 to 0.015%(w/v)) and aliquots of 166 mL were mixed with 4830 mL of the $ABTS^{\cdot+}$ solution and incubated in darkness at room temperature for 20 min, finally, the absorbance of the samples was measured at 734 nm. Positive (GSH) and negative (water) controls were performed too. The percent of the scavenging capacity (SC%) at each concentration and the concentration necessary to decrease the absorbance to 50% of the initial value (IC_{50}) were calculated. The SC% was calculated using the following equation:

$$SC\% = [1 - (A_t/A_r)] \times 100.$$

Where A_t is the absorbance of the sample and A_r is the absorbance of the negative control.

2.4 DPPH radical scavenging activity assay

The DPPH radical Scavenging activity was determined by the method of (Tanzadehpanah, Asoodeh, & Chamani, 2012) with slight modifications. The sample solution contained 0.6 mL of 1.0 mM DPPH radical solution, 0.6 mL of peptide solution at different concentrations (0.5-2% (w/v)) and 4 mL of ethanol (95%). The control solution contained 0.6 mL of distilled water, 4.0 mL of ethanol and 0.6 mL of 1.0 mM DPPH. In any case, the assay mixture was shaken vigorously using a mixer and incubated for 30 min in darkness at room temperature. After that, the absorbance was measured at 517 nm. In addition to the sample and the control solutions, the absorbance of the blank sample, and the blank control were considered too. The results were calculated as the percentage inhibition according to the following formula:

$$\%\, inhibition = [(A_{sample-blank} - A_{control-blank}) / A_{sample-blank}] \times 100$$

2.5 Peptides reducing power (RP)

According to (Oyaizu, 1986), the hydrolysates reducing power was calculated with slight modifications. Lyophilized peptides were dissolved in phosphate buffer (pH 6.6, 0.2 M) at several protein concentrations. 4 ml of the test sample were mixed with 2 ml of 1% (w/v) potassium ferric cyanide. The mix was maintained at 50°C during 20 minutes and cooled quickly. After that, 2 ml of tricloroacetic acid (10% w/v) were added and the solution was centrifuged at 10000 g for 10 minutes. The sediment was discarded and 2 ml of the supernatant were mixed with 2 ml of water and 2 ml of ferric chloride (0.1% w/v). After 10 min the absorbance was measured at 700 nm. A high absorbance was indicative of strong reducing power.

2.6 Ferrous ion chelating assay

The ferrous ion chelating ability of the peptides was assayed according to the method of (Decker & Welch, 1990). Briefly, 5 ml of the sample solutions were mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine solution. After incubation at room temperature for 10 min, the absorbance was measured at 562 nm. The complex Fe^{2+}/ferrozine presents a high absorbance at this wavelength, so high chelating ability is showed as a low absorbance. The chelating ability in percentage was calculated as follows:

$$Ferrous\ chelating\ ability\ (\%) = (A_{blank} - A_t / A_t) \times 100$$

Where A_t is the absorbance of the test sample.

2.7 Antioxidant effect of the peptides on beef homogenates

Ground beef homogenates (20% fat) was homogenized (20% w/v) in a 50 mM HEPES buffer solution at pH 7 using a grindomix homogenizer (model GM300, Retsch, Germany). Buffered systems have been widely used to study oxidation reduction reactions in meat systems (Lee & Hendricks, 1997). The hydrolysates were tested at several peptide concentrations. The test solution contains 0.8 ml of the meat homogenate and 0.2 ml of distilled water or the peptides solution. These assay solutions were incubated at 37°C for 60 min for then be tested in the TBARS formation. Butylated hydroxytoluene (20 µl, 0.2%) was added after the incubation to the reaction mixture to stop the oxidation.

A 0.25 M of HCl solution containing Trichloroacetic acid (TCA) 15% (w/v) and 2-thiobarbituric acid (TBA) 0.375% (w/v) was prepared using agitation and mild temperature to dissolve the components. 2 ml of this HCl solution were added to 1 ml of the incubated test mixture and cooked for 10 min in a boiling water-bath. The solutions were cooled at room temperature and centrifuged for 20 min al 5000 g. Finally, the pink colour developed was measured at 532 nm. TBARS were calculated from a standard curve of malonaldehyde (MDA), a breakdown product of tetraethoxyprop ane (TEP).

2.8 Antimicrobial test

Two species, L. innocua (gram positive) and E. coli (gram negative) were selected to test the antimicrobial effect of the peptides. Both were grown in PBS liquid medium at 37°C for 24 h. The final bacterial concentration was diluted until $1x10^7$ UFC/ml. 200 µl of these samples were spread over the surface of a PBS agar medium. In this agar medium three wells were made in every plate using a sterile punch and 50 µl of the test sample were disposed in each one. The peptide concentration assayed in each case was from 2 to 40 mg/mL. The plates were incubated for 24 h at 37°C and after that, the inhibition zone was measured. A negative control was performed using distilled water as well.

3. RESULTS AND DISCUSSION

3.1 Hydrolysates characteristics.

The characteristics of these hydrolysates were discussed in previous works (Marcet, Álvarez, Paredes, & Díaz, 2014). In summary, in the SWH under nitrogen pressure, the reaction time was 240 minutes, and in the SWH under oxygen stream it was 120 minutes. These times were necessary to solubilize the 95% of the granular protein in form of soluble peptides in each case. The peptides average size was of 4.1±0.1 and 3.9±0.2 KDa respectively. In the case of the enzymatic hydrolysis, the 50%of the initial granular protein was recovered in form of soluble peptides. The average size of these peptides was of 7.1±0.3 KDa.

3.2 $ABTS^{\cdot+}$ scavenging assay.

In the tested samples, the $ABTS^{\cdot+}$ scavenging capacity of the hydrolysates at several peptides concentration is shown in Figure 1. Furthermore, the IC_{50} for the peptides obtained by SWH under nitrogen stream was 0.5±0.02 mg/ml, for the peptides obtained using enzymatic hydrolysis (trypsin) was 0.6±0.01mg/ml, and in the case of the SWH under oxygen stream was 1.1±0.02mg/ml. The peptides obtained by SWH methods are smaller than those obtained using trypsin, however, according to (Tang, He, Dai, Xiong, Xie, & Chen, 2009), the $ABTS^{\cdot+}$ scavenging feature is not dependent of the peptide size.

Aliaga et al. (2000), studied the mechanism of the antioxidant activity between the $ABTS^{\cdot+}$ radical and several aminoacids, and they found that the most reactive amino acid was cysteine and a hydrogen atom from a thiol, amino or hydroxyl group was necessary along the reaction. In this sense, the use of an oxygen stream in the case of the SWH likely produce an oxidizing environment which can modify some amino acids. According to Stadtman and Levine (2003), the oxidation of proteins leads to the hydroxylation of aromatic groups and aliphatic amino acid side chains, nitrosylation of sulfhydryl gropus and the chlorination of aromatic groups and primary amino groups among other alterations. These changes in the primary peptide structure resulted in a low $ABTS^{\cdot+}$ scavenging activity in comparison with the results of the enzymatic hydrolysis method and the SWH under an inert gas stream (nitrogen).

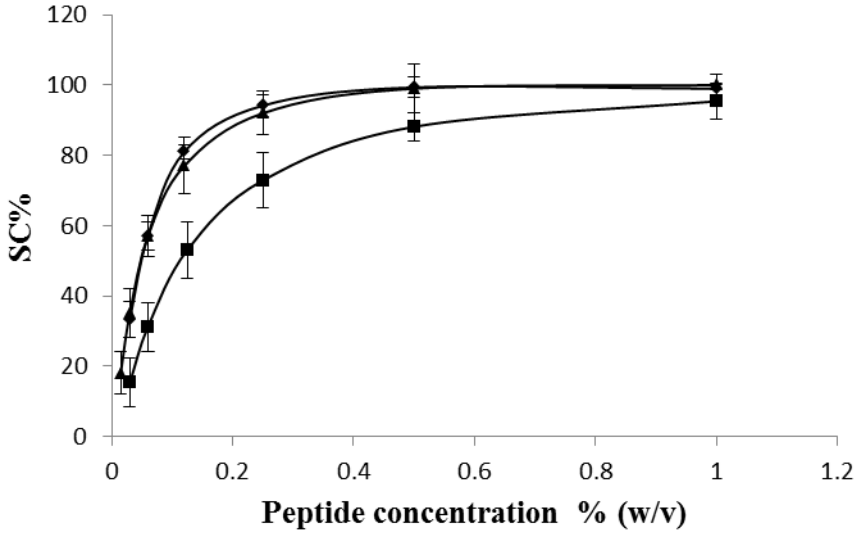

Figure 1. $ABTS^{\cdot+}$ scavenging capacity of the hydrolysate obtained by trypsin hydrolysis (diamond shape), SWH under nitrogen stream (triangle shape) and SWH under oxygen stream (square shape).

3.3 DPPH radical scavenging activity assay

DPPH is an organic compound frequently applied in many studies to evaluate the radical scavenging capacity of different compounds and extracts. This organic molecule is stable and it is unaffected by metal ion chelation (A. Chandrasekara & Shahidi, 2010; N. Chandrasekara & Shahidi, 2011). In this case, the antioxidant substance is a proton-donating, scavenging the DPPH radical and decreasing the absorbance at 517 nm. In the Figure 2, the inhibition of the DPPH radical by the peptides obtained by SWH and trypsin hydrolysis is showed. Peptides obtained using trypsin hydrolysis resulted with a very low antioxidant capacity compared with those obtained by the SWH method. It is possible because the ethanol media decreased the solubility of these peptides, preventing the quench of the DPPH molecule. However, peptides obtained with the SWH under nitrogen stream showed the highest percentage of inhibition. The differences observed between both SWH methods could be explained, like in the $ABTS^{\cdot+}$ scavenging section, due to modifications in some amino acids under the oxygen pressure. Similar DPPH scavenging values to the SWH under nitrogen stream were observed by other authors, using egg yolk protein and enzymes (Sakanaka & Tachibana, 2006). These authors obtained around 90% of percentage of DPPH inhibition using a mix of two enzymes, 6 hours of hydrolysis (orientase, EC 3.4.21.62; and protease, EC 3.4.11.12), and an egg yolk protein hydrolysates concentration of 1%.

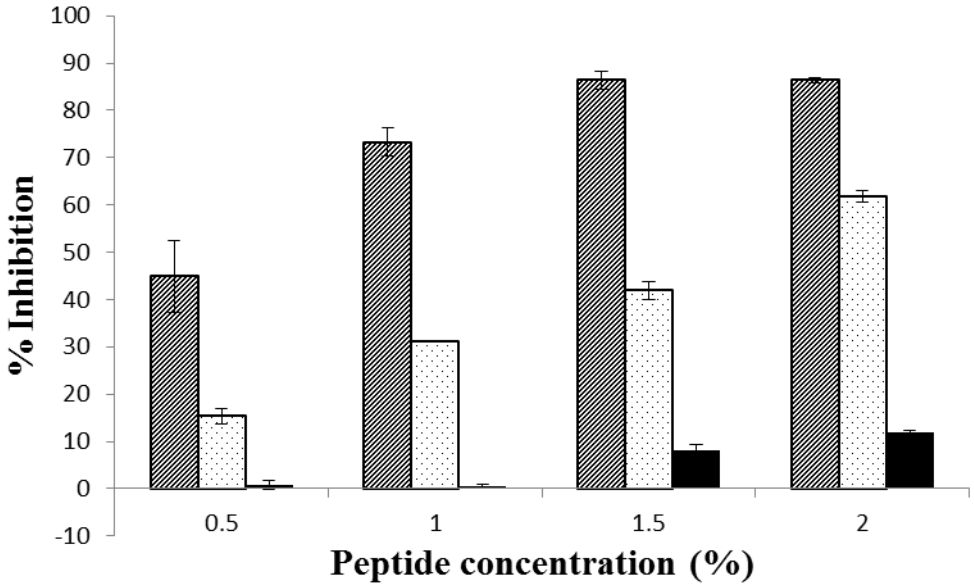

Figure 2. Inhibition of the DPPH by the hydrolysates obtained using trypsin hydrolysis (black columns), SWH under nitrogen stream (hatched columns) and SWH under oxygen stream (dotted columns).

3.4 Peptides reducing power (RP)

This method measures the capacity of the tested sample to reduce Fe^{3+}-ferricyanide complex to ferrous form (Fe^{2+}) and therefore, the antioxidant activity of the assessed compounds. The reducing power of the hydrolysates was calculated and shown in Figure 3. In all of the cases, a correlation in the peptide concentration and the reducing power was observed, although this trend is less marked in the peptides obtained by trypsin hydrolysis. According to Chang et al. (2007) there is a relation between the peptides size in haemoglobin hydrolysates and their reducing power. These authors suggested that the higher the peptide average size, the higher is this feature. However, in this case this correlation is not confirmed and the peptides obtained using the enzymatic hydrolysis had the lowest antioxidant activity. On the other hand, the smallest peptides obtained by SWH method resulted significantly more effective in this test. He et al. (2013) studied the trypsin hydrolysates of sarcoplasmic protein, myofibrillar protein and stromal protein from Paphia undulate, obtaining RP values of 0.58±0.05, 0.57±0.01 and 0.43±0.01 respectively using peptides concentration of 1% (w/v). In this work similar values were obtained in the SWH method under oxygen stream (0.54±0.09) and better results were obtained under nitrogen stream (0.83±0.08), however, using the same enzyme that these authors, the RP value was the lowest one (0.13±0.02). It could be possible for this case due to the small size of the SWH peptides obtained, enhancing the interaction of the reducing amino acids and the substrate, and increasing their RP.

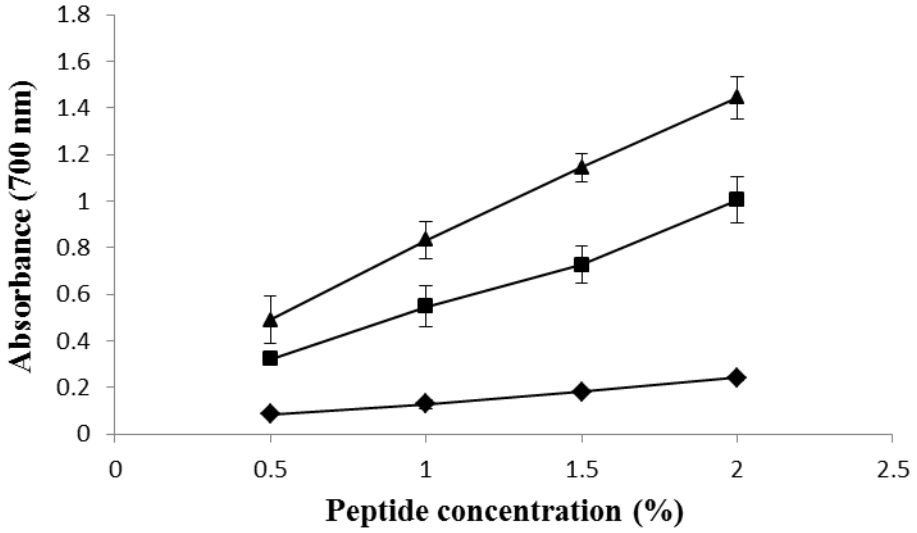

Figure 3. Reducing power of the peptides obtained by trypsin hydrolysis (diamond shape), SWH under nitrogen stream (triangle shape) and SWH under oxygen stream (square shape).

3.5 Ferrous ion chelating assay

In Figure 4, the chelating properties of the different hydrolysates are shown. According to other authors (Zambrowicz, et al., 2012) the tryptic hydrolysates obtained from egg yolk protein have more chelating properties than those obtained from egg white protein. It is

possible because one important protein present in the egg yolk is phosvitin. Phosvitin is concentrated in the egg yolk granules and it is the most phosphorylated protein found in the nature, being its chelating properties broadly studied (Y Mine & Kovacs-Nolan, 2006). Furthermore, the hydrolysates obtained from phosvitin maintain their chelating abilities (Xu, Katayama, & Mine, 2007). In Figure 4, can be observed such as the tryptic hydrolysate of the egg yolk granules maintain high levels of chelating capacities even at the lowest peptide concentrations tested, likely due to the presence of phosvitin oligopeptides. The high temperature and pressures necessaries in the SWH could produce the dephosphorilation of the phosvitin, and the reduction of the chelating capacities of the hydrolysates obtained. Furthermore, other authors have detected a decrement in the ferrous chelating ability of the peptides obtained by the SWH method. Specifically, Álvarez et al.(2012) studied the chelating capacity of the native hemoglobin in comparison with the hemoglobin hydrolysate obtained using SWH under nitrogen stream, showing the latter a reduction in their ferrous chelating ability. Other authors (Chang, Wu, & Chiang, 2007) have related the peptides size with their chelating properties and they concluded that the peptides with high molecular weight are more effective as ferrous chelating than those smaller. According to this, the lower size of the peptides obtained under nitrogen and oxygen stream, and the dephosphorilation of the phosvitin, can explain their limited ferrous chelating ability.

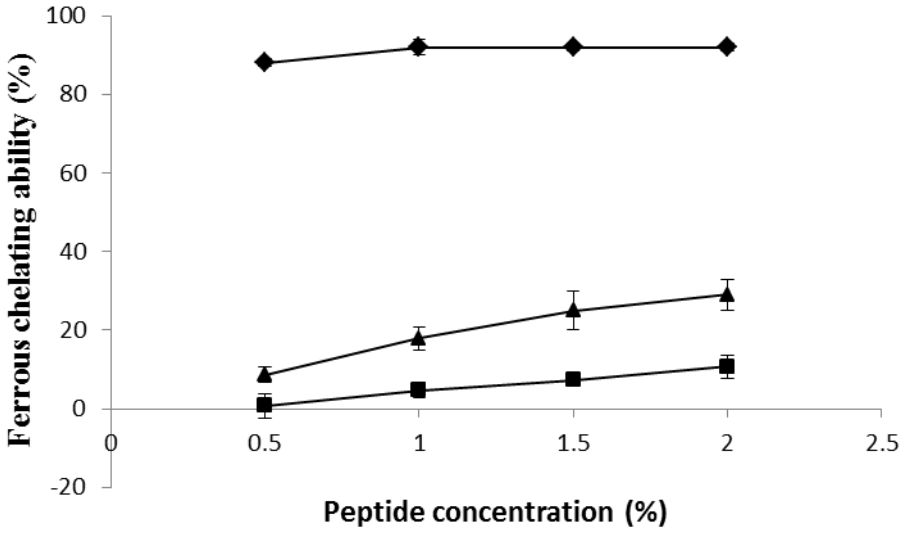

Figure 4. Ferrous chelating ability of the peptides obtained by trypsin hydrolysis (diamond shape), SWH under nitrogen stream (triangle shape) and SWH under oxygen stream (square shape).

3.6 Antioxidant effect of the peptides on beef homogenates

This test involves the generation of a pink chromophore measured at 532 nm due to the reaction between the thiobarbituric acid and the malondialdehyde produced during the lipid oxidation. Other authors (Lee & Hendricks, 1997) have related the antioxidant effect in beef homogenates of some substances to their capacity to chelate iron. In this sense, the peptides obtained by tryptic hydrolysis showed the best iron chelation capacity, as has been previously exposed, and the best antioxidant effect in this assay (Figure 5). The peptides obtained by SWH under nitrogen stream showed similar antioxidant effect than those obtained by tryptic

hydrolysis at high peptide concentration, although the ferrous chelating abilities of the former resulted decreased in relation to the latter as has been previously discussed. However, at the lowest peptides concentration tested, slight differences between the tryptic hyrolysate and the SWH under nitrogen stream were detected. According to these results, these two hydrolysates showed noticeable differences in their ferrous chelating capacities, which were not corresponding with the slight differences observed in their antioxidant effect on beef homogenates. For this reason, likely the antioxidant effect here presented was due to other mechanism not elucidated, at least in part.

Regarding to the peptides obtained by the SWH method under oxygen stream, their antioxidant effect was undetected, showing values similar to those found in the control sample (4.3±0.2).

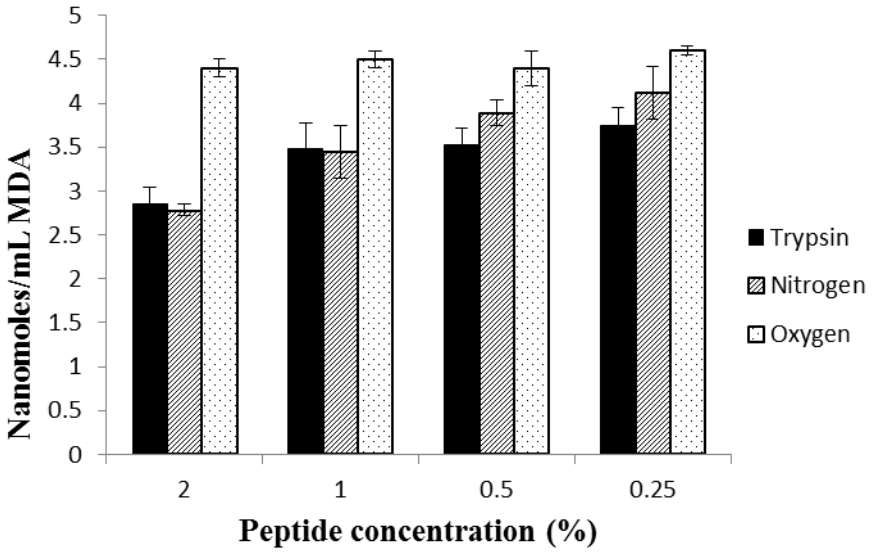

Figure 5. Effect of the hydrolysates on formation of TBARS in beef homogenates. Peptides obtained by trypsin hydrolysis (black columns), SWH under nitrogen stream (hatched columns) and SWH under oxygen stream (dotted columns).

3.7 Antimicrobial test

Antimicrobial peptides obtained by enzymatic hydrolysis of hen egg white have been described by other authors (Memarpoor-Yazdi, Asoodeh, & Chamani, 2012; Yoshinori Mine, Ma, & Lauriau, 2004). Furthermore, antimicrobial activity of hydrolyzed egg yolk proteins using digestive enzymes have been investigated too (Zambrowicz, et al., 2012Zambrowicz, et al., 2012), although according to these authors, the antimicrobial activity of the hydrolysates was weaker and only appears using trypsin. However, in the antimicrobial test assayed in this work, including trypsin, antimicrobial activity could not be detected in any case, even when the highest peptide concentrations (40 mg/ml) were tested. These differences with the results described in the bibliography could be possible due to methodology and hydrolysates variations.

4. CONCLUSIONS

The antioxidant properties of the hydrolysates obtained using SWH under a nitrogen stream resulted higher than those found in the hydrolysates obtained using trypsin. Only in the ferrous chelanting capacity the enzymatic hydrolysis resulted a better choice, and this can be mainly explained by the dephosphorilation of the phosvitin during the SWH. In the others antioxidant tests, the differences observed are explained by the smaller size of the SWH hydrolysates.

The SWH under oxygen stream is the faster method to obtain peptides from insoluble egg yolk proteins, however, its antioxidant capacities were significantly decreased in comparison with the other two methods, likely due to variations in the primary structure of the peptides. In any case, the SWH under nitrogen stream resulted a non-enzymatic method to consider in the obtaining of peptides with antioxidant properties.

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4.4. Hidrolizados de proteínas obtenidos mediante hidrólisis química y con agua en condiciones subcríticas. Revisión

CHEMICAL AND SUB-CRITICAL WATER HYDROLYSIS TO PRODUCE PROTEIN HYDROLYSATES: A REVIEW

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Abstract: The use of protein hydrolysates is a key field in biotechnology research, even more so in the food industry, where such products can be applied with very different objectives; the most important is to provide a source of peptides and amino acids in the diets of those who cannot digest proteins. Other important uses for hydrolysates are as antioxidants, antimicrobials, and antihypertensives; as foaming, emulsifying and chelating agents or as an iron supplement or even a protein source in animal feeding.

Proteases are undoubtedly the method that is most commonly used in industry to cleave peptide bonds. Recently, however, alternative non-enzymatic methods have been developed (or have been improved) that could be applied to produce peptides (of a wide range of molecular weights) and amino acids: supercritical water hydrolysis (SWH); microwave assisted hydrolysis (MWAH); chemical hydrolysis (CH) or even combinations of the above techniques. It is very important to choose the most suitable production method according to the final application of the hydrolysates. This present study deals precisely with such non-enzymatic methods, showing the advantages and the possibilities of these techniques which, thanks to new advances and discoveries, have been demonstrated to be a good alternative for the industrial production of hydrolysates.

Keywords: protein; peptides; hydrolysis; chemical hydrolysis; sub-critical water hydrolysis; bioactivity.

Index

1.- Introduction: from nutritional protein source to bioactive peptides

Traditionally, proteins were used as a dietary source of energy and essential amino acids, which are needed for growth and to maintain the physiological functions of the organism. The use of proteins in the food industry is important, not only because of their nutritional value, but because they have certain characteristics that are indispensable to the food engineering industry. Proteins are commonly used to create emulsions, foams and gels or to promote an increase in protein intake in the diet. But some individuals are unable to digest proteins; so since the 1940s, protein hydrolysates have been used for the nutritional management of such individuals, by increasing the digestibility of native proteins. The digestibility of di- and tri-peptides is at least greater than that of amino acids, which are in turn easier to digest than intact dietary proteins [1], due to the existence of specific receptors that transport these compounds into enterocytes [2]. It has been reported that, depending on the molecular size of the peptides, they have different properties that may render them useful for different technological applications; for example, it was published [1, 3] that low-molecular-weight-peptides (LMWP), especially di- and tripeptides, have high nutritional and therapeutic properties [4, 5]. Meanwhile, high-molecular-weight-peptides (HMWP) (more than 20 amino acid residues) are believed to be associated with an improvement in the functional properties

of hydrolysates. Some authors [6, 7] reported that peptides smaller than 1 kDa presented the best antioxidant properties; so production of low molecular weight peptides is desirable. When the generation and scavenging of oxidative compounds is not balanced, antioxidant molecules are able to reduce the damage caused [8], so including antioxidant peptides in diets could be beneficial. As a consequence of the above properties, it has been found that food protein hydrolysates have a wide range of applications as ingredients in the areas of nutrition, the food industry, health-care and cosmetics.

Because of these new properties, interest in peptides has increased in recent years, focusing mainly on peptides that present biological activity. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health [9]. Such peptides are inactive within the sequence of the parent protein and can be released by a variety of hydrolysis techniques. Some of these bioactivities have been widely reported: chelating [10], antioxidant [11], antibacterial [12] or antihypertensive [13]; or even to create hypoallergenic food products [14-17]. Upon oral administration, bio-active peptides may affect the cardiovascular, nervous, gastrointestinal and immune systems, depending on their amino acid sequence [18]. Also, in recent years, the production of synthetic, modified, constrained or conjugated peptides [19-23] has become significant. These can be used as antimicrobial agents, drug carriers, peptide vaccines, glycaemic regulators, to create new protein-peptide scaffolds or even to improve the bioactivity of already known peptides.

Besides, from an environmental point of view, the use of proteins recovered from industrial wastes to produce hydrolysates could help, and in fact already does, to minimize the impact of food industries and to achieve more sustainable processes. Wastes from meat (blood, skin, hair, heart, intestines)[13, 24-28], poultry (feathers, carcasses, internal organs)[29-33], fisheries (heads, guts, fins, tails)[34-41] and vegetables [42-44] are being used as sources of bio-active peptides.

The following diagram shows the evolution of protein use, from a simple energy resource to the use of peptides released by hydrolytic methods and finally, artificial peptides.

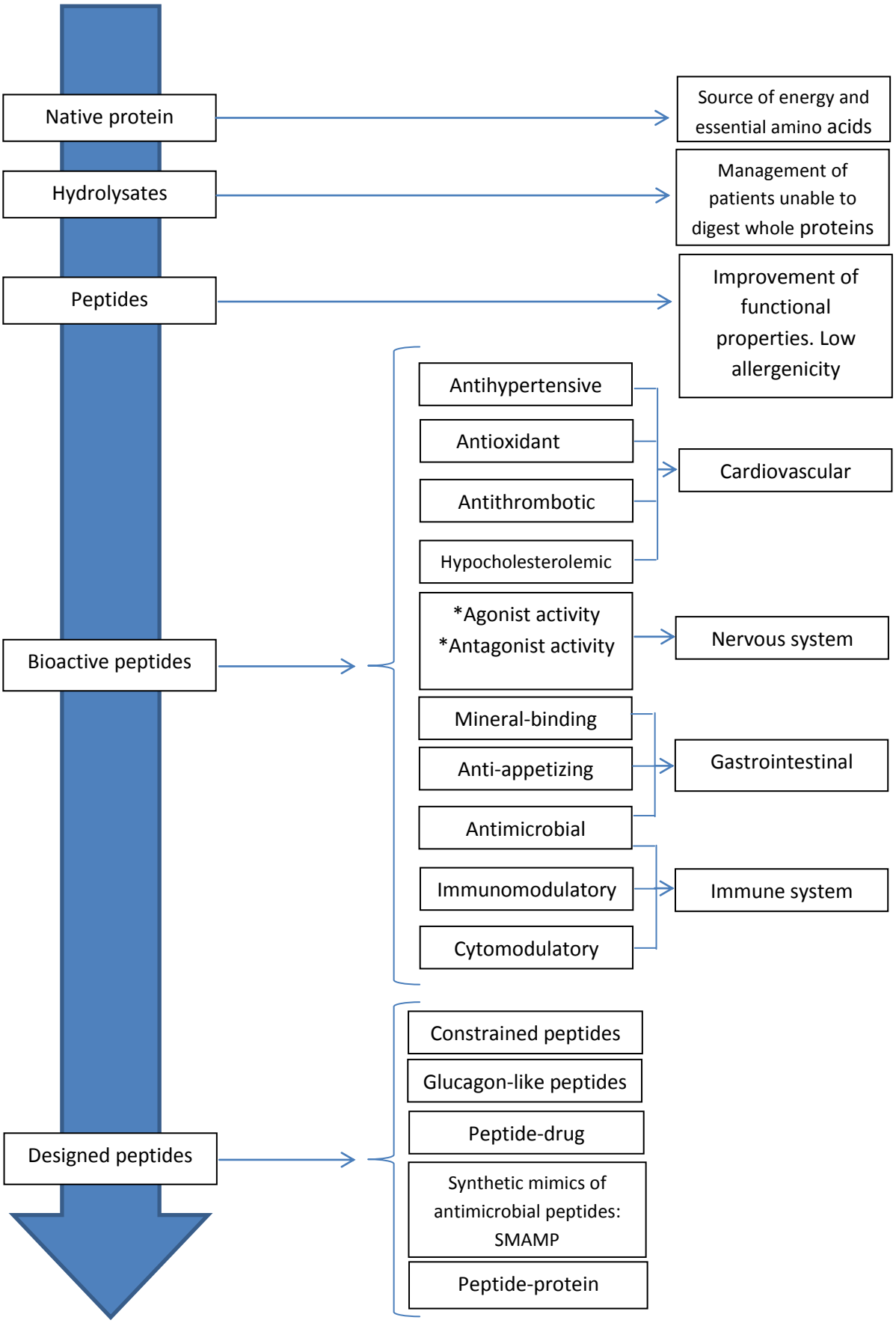
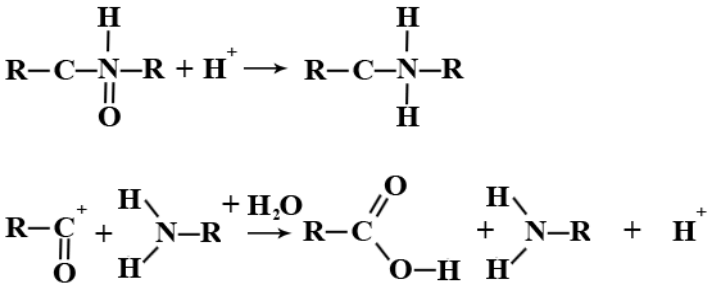


Figure 1. Evolution of protein use. From energy source to synthetic designed peptides.

2 HYDROLYSIS METHODS

Briefly, the mechanism for protein hydrolysis is as follows: first a proton is attached to the nitrogen atom of the peptide bond; this leads to a splitting of the bond, forming a carbo-cation and an amino group. After this stage, a hydrozide ion, from a dissociated water molecule, attaches to the carbo-cation, resulting in the formation of a carboxyl group. This mechanism was described by Brunner [45] and is schematized in Scheme 1. The hydrolysis process can be catalysed by enzymes, metal salts, acids or alkalis.



Scheme 1. Hydrolysis reaction of peptide bonds.

Depending on the method employed, the main factors that exert an influence on the process may change. However, temperature and residence time have been revealed as the main common factors when proteins are hydrolysed without enzyme participation [27, 28, 40, 46-48]. In general, the higher the temperature and residence time employed, the higher is the degree of hydrolysis achieved. A decrease in amino acid yield may occur if temperature and residence time are excessive, especially when SWH methods are used. The cause is a rapid conversion of amino acids into degradation compounds. It has been demonstrated that temperature and residence time are inversely related factors; i.e. the same yield rate can be achieved by decreasing temperature and increasing residence time or vice versa. If acids or alkalis are used, long residence times and high temperatures have a positive effect on amino acid production, despite the loss of certain amino acids by transformation or racemization [49, 50]; it must be borne in mind that free amino acids are continuously produced during chemical hydrolysis. So, if the objective of using this method is to produce peptides instead of amino acids, short times and moderate temperatures must be used in order to preserve the peptides.

2.1 Enzymatic hydrolysis

Enzymatic protein hydrolysis has been broadly studied by Adler-Nissen [51]. It is the most widely employed method of hydrolysing proteins in order to obtain bioactive compounds, and is carried out by using commercial enzymes such as Alcalase, trypsin or pepsin. These methods require stringent control of reaction parameters such as pH, temperature and the enzyme/protein ratio. Furthermore, the amount of protein processed

does not usually exceed 50 g/L. The main advantage of enzymatic compared to non-enzymatic hydrolysis is that the first produces peptides in a predictable way, regarding their size and sequence; and besides, requires mild conditions, which prevent chemical alterations in the hydrolysates. Besides, the degree of hydrolysis (DH), which is defined as the proportion of cleaved peptide bonds in protein hydrolysates, can be adjusted by controlling the hydrolysis time and enzyme/substrate ratio. However, its utilization can be considered expensive and it requires that the cleavage points of the target proteins remain accessible to enzymatic action. Furthermore, the enzymes are usually irreversibly inactivated during the stop phase and they can be inactivated likewise during the reaction phase by a wide range of phenomena [52].

However, obtaining LMWP by enzymatic hydrolysis is not always possible, partly because the DH obtained is completely dependent on the presence/absence of target. In fact, the employment of two different enzymes consecutively is usually necessary to obtain a high degree of conversion of protein into LMWP [11].

In this review, it is intended to describe the latest developments in alternative methods for the creation of protein hydrolysates avoiding the use of enzymes. The most frequent methods employed are supercritical water hydrolysis and chemical hydrolysis. These methods have been presented as realistic alternatives for the production of peptides and amino acids at industrial scale. Each one presents advantages and handicaps compared to enzymatic processes. Table 1 summarizes the advantages and disadvantages of each method reviewed in the present study.

Table 1. Comparison between enzymatic and non-enzymatic methods

	Enzymatic hydrolysis	**Acid or basic hydrolysis**	**Acid/basic parallel hydrolysis**	**SWH**
Specificity	Protein is broken at specific locations: the peptides produced are predictable.	New peptides can be produced and new functions can be discovered.		Some degree of specificity could be found depending on temperature employed.
Stopping reaction	Inactivation step can lead to a loss of functionality: thermal precipitation, unfolding.	Neutralization of the reaction mixture is required		Cooling and reduction of pressure

Amino acids loss	There is no loss of amino acids.	Some amino acids are destroyed depending on the method used.	More amino acids are preserved than when using only acids or alkalis.	No degradation under 250 ºC at short-medium times.
After-processing	Enzyme inactivation by heat or pH is swift. Purification by filtration, chromatography, etc.	Neutralization by acid or alkali. Salt removal by ultrafiltration or selective precipitation.	Neutralization of each other. Salt removal by ultrafiltration or selective precipitation.	Cooling. Remove insoluble particles and purification.
Amino acid production	Few free amino acids are produced.	Whole protein can be converted into free amino acids.		All protein can be converted in peptides or amino acids.
Cost of reagents	Peptidases are an expensive material.	Acid and alkalis are cheap reagents.		Investment in equipment and energy costs could be high.
Environmental impact	Very low impact	Medium impact	Low-medium impact	Low impact
Process control	Parameters of hydrolysis (pH, T, etc.) must be carefully controlled.	Easy control of process.		Temperature, pressure and flow injection.
Amount of protein processed	100 g/L	500 g/L		400-600 g/L
Yield of process	Close to 100% for soluble protein. Insoluble proteins are poorly processed.	70-85% for soluble or insoluble proteins.	80% for soluble or insoluble proteins.	80-95% for soluble or insoluble protein.
Time of process	1-12 h	6-24 h		2-4h

2.2 Chemical hydrolysis

Acid-alkaline hydrolysis methods are simple in operation, have a low cost and are applicable to industrial processes [53]. However, the use of strong acids or strong bases makes the hydrolysis process ecologically unacceptable [54].

According to the mechanism proposed by Brunner [45], protons are needed to break down the peptide bond. So, adding proton donors allows the reaction to occur faster, and for this purpose acids (organic or inorganic) are used as catalysers. The use of several different acids has been reported: hydrochloric or sulphuric acid [47, 55, 56]; acetic acid, citric acid, phosphoric acid or a mixture of these acids [57]; propionic acid, formic acid [58]; lactic acid, ascorbic acid, malic acid [59] or even sodium sulphite [60]. Alkaline hydrolysis uses sodium hydroxide [48, 61], barium hydroxide, potassium hydroxide, calcium hydroxide [24, 62] or calcium carbonate [63] as the main reagent to catalyse the hydrolysis of proteins. As a consequence, sodium, calcium or potassium salts of free amino acids are generated, while oligopeptides are generated as reaction intermediates. Alkaline and acid hydrolysis leads to the random breaking of nearly 40% of all peptide bonds in proteins.

One problem is that the amino acid sequence of the substrate is critical for the outcome of chemical hydrolysis [64]. The bonds that are the most difficult to cleave are those between aliphatic residue-containing amino acids, such as in the sequences Ile–Ile, Val–Val and Ile–Val. Furthermore, in the first steps of both methods (acid and alkaline hydrolysis), the appearance of aggregates and non-hydrolysed fragments is common and these can remain in the hydrolysis media for a long time. These handicaps must be borne in mind when considering the suitability of chemical hydrolysis for industrial application. For example, it has been reported [65] that globin hydrolysed for 30 minutes with citric acid forms large aggregates (around 800 kDa), composed of several subunit chains. Non-covalent bonding, especially hydrophobic interactions, is essential in forming these aggregates. Other authors [66, 67] related that low values of pH during hydrolysis, in the case of using haemoglobin as protein, leads to weak haem-peptide interactions, which promotes an intense polymerisation of the haem group and formation of large insoluble haem polymers. The same authors reported that the initial concentration of these aggregates is directly related to the alkaline or acid concentration employed; but its amount decreases as the hydrolysis process advances.

2.2.1 Amino acid degradation

From a nutritional point of view, it is well known that acid hydrolysis has different effects on the amino acids that form part of the protein used as raw material; asparagine and

glutamine are completely transformed into aspartic acid and glutamic acid, respectively; and tryptophan is completely destroyed. In the case of basic hydrolysis, serine, threonine, arginine and cysteine are destroyed [49]. Some amino acids (e.g. arginine, asparagine, glutamine and serine) are completely destroyed while others are racemized (i.e., structurally modified from a left-handed configuration to a mixture of left-handed and right-handed molecules). Besides, toxic substances such as lysine-alanine may be formed [1]. Recent studies [68] have demonstrated how the use in parallel of acid and alkaline hydrolysis can avoid this disadvantage; the amino acids destroyed by acid are preserved by alkalis and vice versa, so a mixture of the hydrolysates produced by each method independently contain all the amino acids present in the original protein.

This amino acid degradation is the greatest disadvantage of using acids or alkalis to produce peptides or amino acids as a nutritional source for human or animal feeding. Nevertheless, the hydrolysates produced are widely applied as peptones as ingredients in culture media for microorganisms [63]. Coward-Kelly [24, 62] reported that feather hydrolysis using $Ca(OH)_2$ as hydrolysis agent produced a decrease in arginine, threonine and serine, but an increase in the rest of the amino acids. The same author reported how by using shrimp head as raw material, a final product suitable for feeding monogastric animals could be obtained; i.e., the initial protein source, regardless of the method of hydrolysis employed, determines the final use of hydrolysates.

Racemization is the second major handicap that chemical hydrolysis may present. Various studies have reported that the degree of racemization depends on the type of protein and amino acid background [69, 70]. Such studies showed how free amino acids were racemized more slowly than those included in peptides. Due to this, faster hydrolysis processes reduce the amount of D-amino acids formed during hydrolysis. It has been reported [50] that the degree of racemization is increased by two or three times when the hydrolysis time is extended from 15 to 60 minutes.

As an additional advantage, the use of alkaline or acid hydrolysis to process high-risk biodegradable waste or to produce sterile products has been reported [71]. The protein coats of viruses are destroyed and the peptide bonds of prions and bacteria are broken, thus achieving a total elimination of tested bacteria [72, 73].

2.2.2 Operational parameters

Reagent concentration, temperature and time are the main parameters that must be controlled in order to obtain a certain degree of hydrolysis. In general, the higher these

parameters, the greater is the degree of hydrolysis. The control of these factors allows the final average molecular weight of the hydrolysates to be predicted. Preferably, short reaction times and high temperatures should be selected in order to reduce racemization and amino acid degradation. Several reaction time/temperature/concentration combinations which finally yield free amino acids have been published: 15 minutes/180 ºC/6M HCl; 24 hours/110 ºC/6M HCl [50]; 180 minutes/150 ºC/ 0.8 M KOH [71]; 240 minutes/110 ºC/2M H_2SO_4 [56]; 24 hours/50 ºC/6M H_2SO_4 [47]; or even 9 days/room temperature/1.5 M propionic acid and 2.5 M formic acid [58].

Once hydrolysis has finished, the reaction must be stopped; for this purpose, the reagent must be neutralized or removed. The most common methods are dilution by adding water or neutralizing acid with alkalis and vice versa. The first method does not produce salts, but nevertheless, the peptides and amino acids produced are diluted several times, so it makes the subsequent step of drying, concentration or purification more expensive; the typical processes used are spray-drying or freeze-drying. On the other hand, in order to remove salts from solutions on an industrial scale ultrafiltration [74] or nanofiltration [75]could be used. The latter is also useful for obtaining peptide concentrates. Unfortunately, these techniques do not prevent the loss of small peptides and amino acids, so the yield is decreased. Furthermore, such processes are energetically expensive, and are only cost-effective when the peptides produced have a high added value.

2.3 Sub-critical water hydrolysis

When high hydrostatic pressure (HHP) is used, a loss of spatial conformation takes place in proteins, the first step being the loss of the quaternary structure (if it exists) which is the most pressure-sensitive interaction [76]. Subsequently, the tertiary structure is unfolded in direct relation to pressure and temperature. Employing HHP at room temperature is known to produce the denaturation of proteins without any change in molecular size [77]. The same unfolding effect with no change in protein size is observed when the temperature is increased under atmospheric pressure [78]. Only combined use of pressure and temperature allows the unfolding and hydrolysis of the protein, which is carried out by the water under subcritical conditions. If the temperature is raised to the subcritical range, it leads to protein breakdown and a reduction in the size of the peptides produced. During the hydrolysis of proteins under SW conditions, the process culminates in the release of amino acids and their subsequent degradation to organic acids.

2.3.1 Water properties

The sub-critical water (SW) is maintained in liquid state at relatively high temperatures due to the high pressure. Water is considered to be sub-critical from 100°C and 0.10 MPa to 374°C and 22 MPa [79] and in this state, its properties change drastically. At ambient temperatures water shows a high capacity to dissolve and extract ionic and polar compounds. However, when the temperature increases until near to its critical temperature, non-polar compounds can also be extracted [80-82]. This can be explained by alterations in its electrochemical properties: the dielectric constant decreases from 78 Fm^{-1} at 25°C and 0.1 MPa to 14.07 Fm^{-1} m^{-1} at 350°C and 20 MPa [83], which allows water to interact with non-polar substances, thus decreasing their binding force and dissolving them. Furthermore, the ionic product of water increases a thousand times at high temperatures compared to that of water at ambient temperature (from 10^{-14} to 10^{-12} in sub-critical conditions). This increment in H^+ and OH^- in the aqueous medium raises the reactivity of water and, therefore, its activity as an acid- or base-like catalyst.

2.3.2 Amino acid decomposition

Amino acids are decomposed at high reaction temperature and longer reaction times. In this sense, Kang et al. [84] studied the variations in the concentrations of glycine, alanine, serine and aspartate during the SWH of the silk fibroin at several temperatures and times. In this study the highest concentration of the simplest amino acids (glycine and alanine) were found at relatively high temperatures (250°C) and times (more than 60 minutes of reaction time). In the same conditions the concentrations of serine and aspartate were near to zero, although at the lowest temperature tested (200°C), the presence of these amino acids was noticeable. These experimental results suggest that more complex amino acids are more thermally sensitive than those of low-molecular ones such as Gly and Ala.

Kingler et al. [85] studied the hydrothermal decomposition of glycine and alanine from 250ºC to 450°C at low residence times of 2.5-35 s. For alanine, the main reactions were decarboxylation to ethylamine and deamination to lactic acid. Glycine decomposes faster than alanine at temperatures from 300°C to 450°C and its decarboxylation to methylamine was the main reaction. A small degree of deamination of Gly to glycolic acid was also found. The decomposition of amino acids into organic acids at high temperatures in SW conditions was

studied by Kang et al. [86]. According to these authors, after 60 minutes at 250°C the main organic acids found were mostly propionic, lactic, acetic, formic, succinic and malic acids. After 60 minutes at 380°C there only appeared acetic, propionic and succinic acids and in a lower proportion than at 250°C, which suggests the decomposition of these acids into CO_2, CO and H_2O. Tavakoli et al. [87] studied the SWH of scallop viscera wastes to produce valuable compounds. They observed the concentrations of several organic acids after 5 minutes of reaction at different temperatures. Pyroglutamic acid was the most abundant organic acid from 226°C to 376°C, with a peak at around 300°C. At low temperatures, phosphoric acid was the predominant acid, showing a peak at around 220°C. Other organic acids such as lactic, formic and acetic acid were detected too. Rogalinski et al. [27] studied the amino acid degradation products from the SWH of BSA at 250°C and 290°C and residence time from 4 s. to 300 s. The main products found were carboxylic acids like acetic acid, propanoic acid, n-butyric acid and iso-butyric acid. Acetic acid was found at short reaction times, propanoic acid at medium times, and iso-butylic and n-butylic acid could only be detected after 300 s. Ethanolamine and ornithine amino acid and different alcohols and aldehydes were also found, but only as traces.

2.3.3 Operation parameters and yield

The operation parameters which resulted in the greatest yields have been summarized in Table 2. As can be observed, the SWH method has been widely used to recover amino acids and peptides from organic wastes. In this way, industry-derived protein sources whose removal implies expensive treatments, such as poultry wastes [88], hog hair [89], baker's yeast cells [90] or blood from slaughterhouses, can be revalorized and utilized for animal feed or human food purposes. Another novel application of the SWH method is its use to hydrolyse insoluble food protein to obtain peptides with increased functional properties [28, 91]. These peptides obtained by SWH could increase the economic revenue of the source.

The optimum temperature and reaction time to obtain the best yields vary greatly in the bibliography. The temperature and the time of operation are closely related, so the longest reaction times correspond with the lowest temperatures tested [28, 91]. In a general way, an increase in the temperature of operation involves an increment in the velocity constants and therefore a decrease in the reaction time. However, this increase in the temperature can result in the decomposition of amino acids into organic acids and a poor final yield. Another important aspect to consider is the protein source, since animal wastes require higher temperatures or longer reaction times than vegetable wastes, as can be observed in Table 3. In

fact, in the bibliography, it can be seen that different authors required similar operational conditions for similar protein sources [92, 93].

Finally and according to Table 2, the operation parameters are also conditioned by the final product. So, some authors hydrolyse organic wastes to produce amino acids [87, 94], while other authors search for the optimum conditions to produce peptides [91, 93, 95, 96]. To optimize the temperature and reaction time to produce the highest amount of either amino acids or peptides from the same source usually required different operational parameters [90, 97]. This makes it necessary to study each case in detail, according to the source and to the desired product.

Table 2. Operation parameters and yields found in the bibliography.

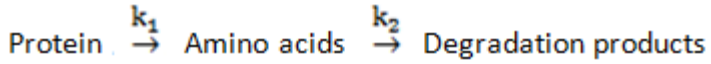
Source	Type of reactor	Optimum temperature (°C)	Reaction time (s)	Peptide recovery	Amino acid recovery
Microalgae (Scenedesmus sp.) [96]	Continuous flow reactor	240	10	60% [a]	Not relevant.
Bean dregs [98]	Closed reactor	200	1200	Not calculated	52.9%[a]
Deoiled rice bran [92]	Closed reactor	200	1800	219±26 mg/g dry bran	8.0±1.6 mg/g dry bran
Fish entrails [94]	Closed reactor	250	3600	Not calculated	137 mg/g dry fish
Scallop viscera [87]	Closed reactor	240	3000	Not calculated	150 mg/g dry viscera
Raw rice bran [93]	Closed reactor	220	1800	75% [a]	5% [a]
Deoiled rice bran [93]	Closed reactor	220/210		84% [a]	6% [a]
Raw soybean [93]	Closed reactor	210/200	1800	50% [a]	5% [a]
Deoiled rice bran [93]	Closed reactor	200		50% [a]	5% [a]

Yeast cells [90]	Closed reactor	100	900	Not calculated	63mg/g dry yeast
		250	1200	160 mg/g dry yeast	Not calculated
Shrimp Shells [95]	Closed reactor	250	3600	Not calculated	70mg/g dry shrimp shell
Sericin (silk waste) [97]	Closed reactor	160	3600	Not calculated	0.203 mg/mg raw silk
		120	600	0.466 mg/mg raw silk	Not calculated
Fibroin (silk waste) [97]	Closed reactor	220	3600	Not calculated	0.755 mg/mg silk fibroin
		220	600	0.455 mg/mg silk fibroin	Not calculated -
Porcine haemoglobin [28]	Closed reactor	180	10800	80% [a]	Not relevant
Granules from egg yolk [91]	Closed reactor	180	14400	95% [a]	Not relevant
Hog hair [89]	Closed reactor	250	3600	Not calculated	0.325mg/mg protein
Poultry wastes [88]	Closed reactor	260 (H2SO4; 0.02wt.%)	1680	Not calculated	0.114 mg/mg poultry waste

[a] percentage referring to the total protein content. Yield *not calculated* means that the goal of the process was to produce peptides or amino acids.

2.3.4 Hydrolysis kinetics

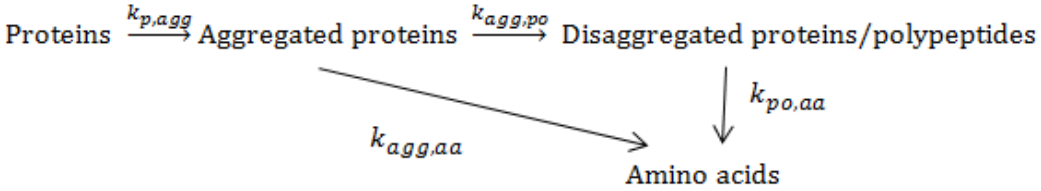
Authors generally assume irreversible first order reactions to describe the breakdown of protein and the degradation of amino acids, if it takes place. Rate constants as well as activation energy values have been summarized in Table 3. Rogalinski et al. [27] proposed a simplified empirical reaction model (Scheme 2). They calculated K_2 rate constant values higher than those for K_1, so the amino acid degradation process is enhanced at all the tested temperatures in comparison to protein breakdown. Specifically at temperatures higher than 270°C, a decrease in the amino acid yield was detected due to an increase in the decomposition reactions.



Scheme 2. Rogalinski et al. [27] simplified empirical reaction model. K_1 and K_2 are the rate constants of protein breakdown and amino acid decomposition respectively.

In this sense, Rogalinski et al. [99] found that the resulting amino acids were sensitive to thermal degradation if the temperature is high enough. In fact, at 310ºC and 330 ºC all amino acids were completely decomposed after 140 s. The best temperature they tested for preserving the amino acids in the medium was 230°C. The rate constants for amino acid formation were found to be significantly lower in the case of the hydrolysis of BSA in comparison to the rate constants for glucose formation from starch or cellulose, which indicates the resistance of the peptide bonds to high temperatures.

A similar simplified reaction scheme was suggested by Zhu et al. [98] for amino acid production from bean dregs. In this case the temperatures tested were 200°C, 220°C and 240°C, resulting in K_1 values higher than those for K_2 at 200°C, similar K_1 and K_2 values at 220°C and higher K_2 values than those for K_1 at 240°C. In a similar way, Zhu et al. [40] and Zhu et al. [88] studied the hydrolysis of fish proteins and poultry wastes respectively. In these cases, the degradation of amino acids was not taken into account. From the experimental results obtained with SWH of rice bran protein, Sunphorka et al. [100] observed a decrease in the protein concentration in the first 5 minutes of operation due to aggregation processes. In this case, the simplified reaction scheme is shown below (Scheme 3).



Scheme 3. Where $K_{p,agg}$ is the rate constant of protein aggregation, $K_{agg,po}$ is the rate constant of protein disaggregation or of polypeptide formation, $K_{agg,aa}$ is the rate constant of the direct conversion from aggregated protein to amino acids and *Kpo,aa* is the rate constant of the formation of amino acids from disaggregated proteins or polypeptides.

Table 3. Velocity constants at several temperatures and energy of activation values calculated from the SWH of proteins of different sources.

Source	Rate constant	Temperature (°C)					E_A ($KJ \cdot mol^{-1}$)
BSA [27]	(s^{-1})	**250**	**270**	**290**	**310**	**330**	
	k_1	0.0006	0.0037	0.0035	0.0186	0.02	114.8
	k_2	0.0135	0.0756	0.0552	0.4232	0.6	122.2
Bean dregs [98]	(s^{-1})	**200**	**220**	**240**			
	k_1	0.00127	0.0014	0.0017			14.6
	k_2	0.0005	0.0011	0.0024			NC
Rice bran [100]	(g/L·s)[a]	**150**	**175**	**200**	**225**	**250**	
	$k_{p,agg}$	0.01408	0.01769	0.0237	0.0363	0.07	NC
	$k_{agg,po}$	0.0000466	0.0017	0.0016	0.002	0.003	82.2
	$k_{agg,aa}$	0.00002	0.0000416	0.00016	0.00035	0.00037	NC
	$k_{po,aa}$	0.000073	0.0001033	0.000473	0.000835	0.001	NC
Fish protein [40]	(s^{-1})	**220**	**240**	**260**			
	k_1	0.0000283	0.000075	0.000161			145.1
Poultry [88]	(s^{-1})	**200**	**230**	**260**	**280**		
	k_1	0.0152	0.0335	0.115	0.14		64.44

[a]Values refer to experiments with an initial protein concentration of 4.16 g/L. NC: Not calculated.

4. Conclusions

Although enzymes are the most commonly used technique for creating hydrolysates, they have several disadvantages (high reagent cost, not suitable for high protein concentrations or high-tech controls needed) that could be avoided by the use of other hydrolysis techniques such as chemical and sub-critical water hydrolysis. Chemical hydrolysis can handle large amounts of protein in a low-cost process suitable for animal feeding, while SWH has been revealed as a useful tool for producing peptides with interesting bioactivity without adding chemicals or reagents. This quality is seen by the food industry as being highly desirable.

Further research regarding the mechanism of hydrolysis has to be carried out in order to be able to predict the resulting hydrolysates; to develop of new products suitable for human

consumption and to assess the optimal conditions and develop pilot scale or industrial scale processes.

Conflicts of Interest

The authors declare no conflict of interest.

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5. SEPARACIÓN DE FOSVITINA

A NOVEL METHOD FOR EXTRACT PHOSVITIN FROM EGG YOLK USING CITRIC OR CHLORIDRIC ACID.

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Abstract

In this study, a new phosvitin separation process using natural substances legally allowed for its use in the food industry (NaCl and citric acid) has been developed, employing egg yolk granules as the raw material. The granules were diluted in different NaCl solutions and the pH was adjusted to obtain, after centrifugation, a phosvitin-rich supernatant. This supernatant was desalted using dialysis membranes and the effect of the desalination process at several pHs was evaluated. In addition, phosvitin-rich fraction desalination by ultrafiltration was tested, being an alternative to the dialysis step.

The separation efficiency was investigated by electrophoresis, in denaturing and non-denaturing conditions, and using ion-exchange chromatography. The N/P atomic ratio was calculated too. This N/P ratio is considered a quality parameter, obtaining a value of 3.6±0.2. Furthermore, a yield of 3.48±0.2 g of lyophilized phosvitin-rich fraction/100 g of dried egg yolk was obtained, with a phosvitin recovery rate of 76±3%.

Introduction

Egg yolk is a cheap source of substances of interest for food, cosmetic, pharmaceutical and other applications, which is also obtained without animals suffering. It is known to contain molecules with a broad range of scientific interest such as immunoglobulin “Y” and phosvitin. Besides, egg yolk is composed of phases: a high water content and low density lipoproteins phase, called plasma, which presents agglomerations of a second phase, namely granules, which are aggregates of proteins and high-density lipoproteins mainly.

Egg yolk granules are composed of 70% high-density lipoproteins (HDLs), 16% phosvitin (Burley & Cook, 1961) and 12% low density lipoproteins, all of which are bound to themselves and to one another via phosphocalcium bridges (Burley et al., 1961; Causeret et al., 1991). HDLs and LDLs have emulsifying capacities, with a lipid content ranging from 22% in HDLs to 89% for LDLs (Kamat et al., 1972). These emulsifying capacities accordingly allow granules to be used as egg yolk substitutes in the elaboration of dietary products with a lower presence of cholesterol than when using whole egg yolk (Laca et al., 2010).

As regards phosvitin, this protein has two major compounds, a large one and a small one (Taborsky & Mok, 1967), known as the alpha and beta components, although five proteins of 40000, 33000, 18000, 15000, and 13000 Daltons, respectively, have also been reported

(Wallace & Morgan, 1986a). Phosvitin is also a protein with a large amount of serine residues, most of which are phosphorylated, making it one of the most phosphorylated naturally occurring proteins, with a 10% phosphorous content. Therefore, phosvitin is more hydrophilic than HDLs and LDLs, as these proteins contain 2% phosphorous (Itoh et al., 1986; Causeret et al., 1991). The amount of phosphate groups in phosvitin must be related to its biological properties, grouping the 95% of iron that appears in egg yolk, making it less available to microorganisms (Greengard et al., 1964). Furthermore, due to its major capacity to bind divalent cations, phosvitin is an effective inhibitor of oxidation mediated by Fe^{+2} and Cu^{+2} (Lu & Baker, 1986). On the other hand, the partial digestion of phosvitin using hydrolytic enzymes results in peptides with enhanced complexing properties compared to those of the original protein, making it more effective than other molecules such as ferritin, transferrin and EDTA (Ishikawa et al., 2007). Phosvitin also has emulsifying and emulsion stabilizing properties, these qualities being superior to those of bovine whey albumin, soya protein or β-casein (Chun & Ferrier, 1991; Khan et al., 1998).

On account of all its aforementioned qualities, phosvitin has been studied and purified in different ways: for example, separating it by means of its precipitation with magnesium sulfate (Mecham & Olcott, 1949) or using organic solvents (Joubert & Cook, 1958a; Sundararajan et al., 1960; Wallace & Morgan, 1986b; Belhomme et al., 2007) for subsequent purification by chromatography (Connelly & Taborsky, 1961) and gel filtration (Connelly & Taborsky, 1961; Clark, 1970 and Abe et al., 1982).

The extraction and purification of phosvitin using organic solvents has negative consequences for the structure of the proteins. Moreover, the manipulation of organic solvents has other disadvantages, including its environmental aspects and constituting undesired agents which have to be removed from the medium before being able to use the protein. Regarding the use of magnesium sulfate, the result is a phosvitin-rich sediment which is difficult to solubilize in water, with extractions of this type generally involving a poor scalable dialysis phase.

With the aim of broadening the potential range of applications, this paper presents a non-aggressive organic solvent-free extraction method for obtaining a phosvitin-rich fraction (PRF). The procedure employs the route of egg yolk granules as raw material, as phosvitin is previously concentrated in these granules. The final product has been broadly characterized.

Materials and Methods

Phosvitin Separation

Egg yolk granules were extracted following the procedures developed by Laca et al., (2010). Granules were diluted (1:1 w/w) with a 0.16M NaCl solution and mixed with a magnetic stirrer. After 1 hour, the solution was centrifuged for 45 minutes at 10000g at 4ºC, the supernatant was discarded and the precipitate (washed granules) was subsequently collected. The washed granules were resuspended (2% w/v) in different NaCl solutions until they were completely solved. NaCl concentrations of 0.5M, 1M, 1.4M and 1.72M were specifically tested and the pH was adjusted to 2, 2.5, 3 and 4 using 1M citric acid in each solution. Citric acid is an organic acid broadly used in the food industry which allows the pH adjustment with accuracy. Finally, the solutions were centrifuged at 10000g for 45 minutes at 0ºC, obtaining a pellet, the HDL-

rich fraction (HRF), and a supernatant, the phosvitin-rich fraction (PRF). The supernatant was filtered using Whatman paper number 1. The separation method is summarized in Figure 1.

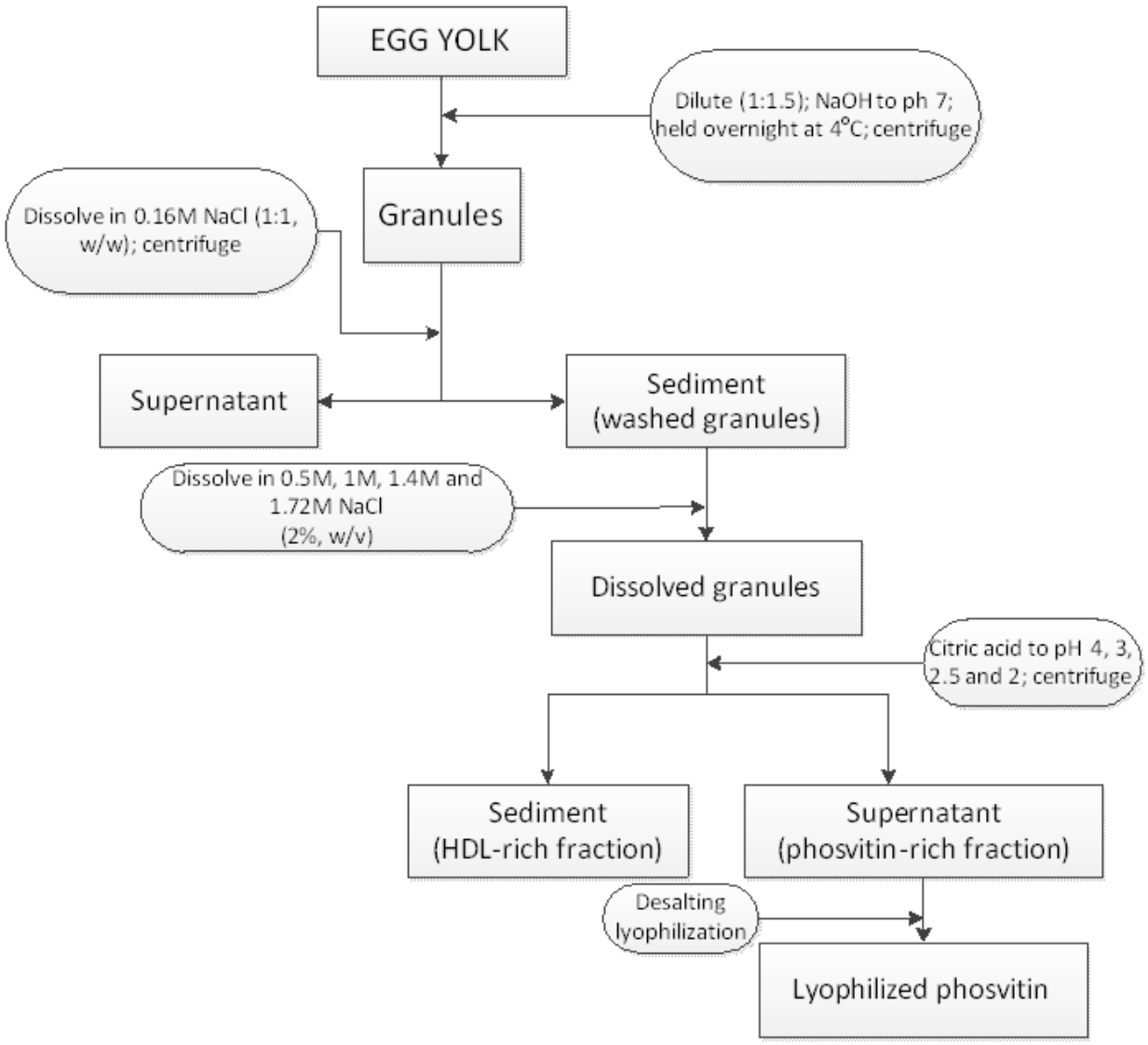

Fig. 1. General scheme for granule fractionation.

Chemical Analysis

Sample pH was measured at 20ºC. Dry matter was determined employing an HR73 Halogen Moister Analyzer (Mettler-Toledo, Switzerland) using 0.5 grams of sample. Total lipids were evaluated by acid hydrolysis according to Toschi et al., (2003). The amount of nitrogen was estimated using a CNHS/O Elementar Vario EL analyzer (Elementar, Germany). The percentage of organic phosphorus was determined using a colorimetric method according to King (1932) using 1-amino-2naphthol-4sulfonic acid and ammonium molybdate as reagents. All measurements were carried out in triplicate, calculating average values and standard deviations.

Detection and Quantification of Sodium

This method was employed in order to evaluate the amount of sodium chloride in samples. Lyophilized samples were diluted to 1mg/mL in ultrapure water. Eight mL of nitric acid (1%) were added to 1mL of the sample solution which was then heated in an Ethos One microwave digestion system (Milestone, USA) at 800W and 160ºC for 30 minutes. The result of the digestion was diluted with ultrapure water to a final volume of 15 mL. Finally, 1 mL of the digested solution was prepared by adding 9 mL of nitric acid 1% (v/v). An ICP-MS 7500ce (Agilent Technologies, USA) was used in its normal configuration to measure the samples employing 5 ppb of Rh as internal standard in each sample.

Phosvitin-rich Supernatant Desalting

Desalting by dialysis. Desalting was performed using dialysis membranes (D7884-10FT, Sigma Aldrich, USA), previously the pH of the PRF was adjusted employing 1M citric acid or 1N NaOH. The dialysis pH values tested were 3 (below the pI of phosvitin), 5 (above the pI of phosvitin), 7 and 8.5, both at the supposed pI of apo-HDLs (Le Denmat et al., 2000). Distilled water was used in each experiment and conductivity was monitored employing a conductivimeter (Model HI98129, Hanna Instruments, USA) until a value lower than 1 microsiemens was obtained in the dialysis water.

Desalting by ultrafiltration. A polyethersulfone ultrafiltration membrane was alternatively used to desalt the PRF, employing a 10 KDa MWCO membrane (Model PXB010A50, Millipore, USA) for this purpose. The transmembrane pressure was fixed at 1.4 atm and pH was adjusted to 8 using 1N NaOH, according to Chay Pak Ting et al., (2010) method. The operational temperature was then maintained at 20ºC and the initial volume of PRF to dialyze was 150 mL. It was diafiltrated with 6L of distilled water. The phosvitin lost was calculated by measuring phosphorous, before and after the operation. Desalted PRF was lyophilized and its content in sodium was calculated as explained previously.

Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE. SDS-PAGE was performed using a 12% w/v acrylamide separation gel in a Tris-Glycine buffer. Proteins were stained with two different solutions. The first was normally used to specifically stain phosphoproteins (Coomassie blue 0.05%, acetic acid 10%, triton 1%, ethanol 25%, aluminum nitrate 0.1M). The second was a standard Coomassie stain, which stains total protein (0.1% Coomassie blue, 50% methanol, 10% acetic acid and 40% water). SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad) were used as protein standards.

PAGE non-denaturing conditions. The separation gel was contained 10% w/v acrylamide. SDS was not added to any solution. Gel staining was performed using “stain all” supplied by Sigma Aldrich, following the supplier’s instructions. Phosvitin from egg yolk acquired from Sigma was used as protein standard.

Anion Exchange Chromatography

Chromatographic analyses were carried out on an FPLC ÄKTA system (Amersham Biosciences, UK). The chosen column was a Ceramic HyperD® Q (Life Biosciences, USA) anionic

resin, column volume was 5 mL and the elution flow rate employed was 1mL/min. These analyses were performed in order to determine the chromatographic profile of compounds in the PRF obtained. Samples were desalted by dialysis and then filtered using 0.45-µm pore size filters, subsequently lyophilized and finally dissolved in a Trizma®pH 8.0, 0.1M solution, to a final concentration of 3mg/mL.

Before injection, the column was equilibrated with 5 column volumes of a 0.1M Trizma® hydrochloride pH 8.0 solution (buffer A). The elution buffer (buffer B) was composed of 1M NaCl in 0.1M Trizma® hydrochloride pH 8.0. The gradient was performed from 0% of B to 60% for 60 minutes. The peaks obtained were monitored at 280 nm.

The detected peaks were collected and then analyzed using PAGE conditions.

Results and Discussion

Phosvitin Separation

Granules were diluted in several sodium chloride concentrations. These solutions were adjusted to different pH values and centrifuged. After that, a sediment (HRF) and a supernatant (PRF) were obtained and studied by electrophoresis (Figure 2). Sediments were dissolved to the original volume with distilled water prior to electrophoretic analysis.

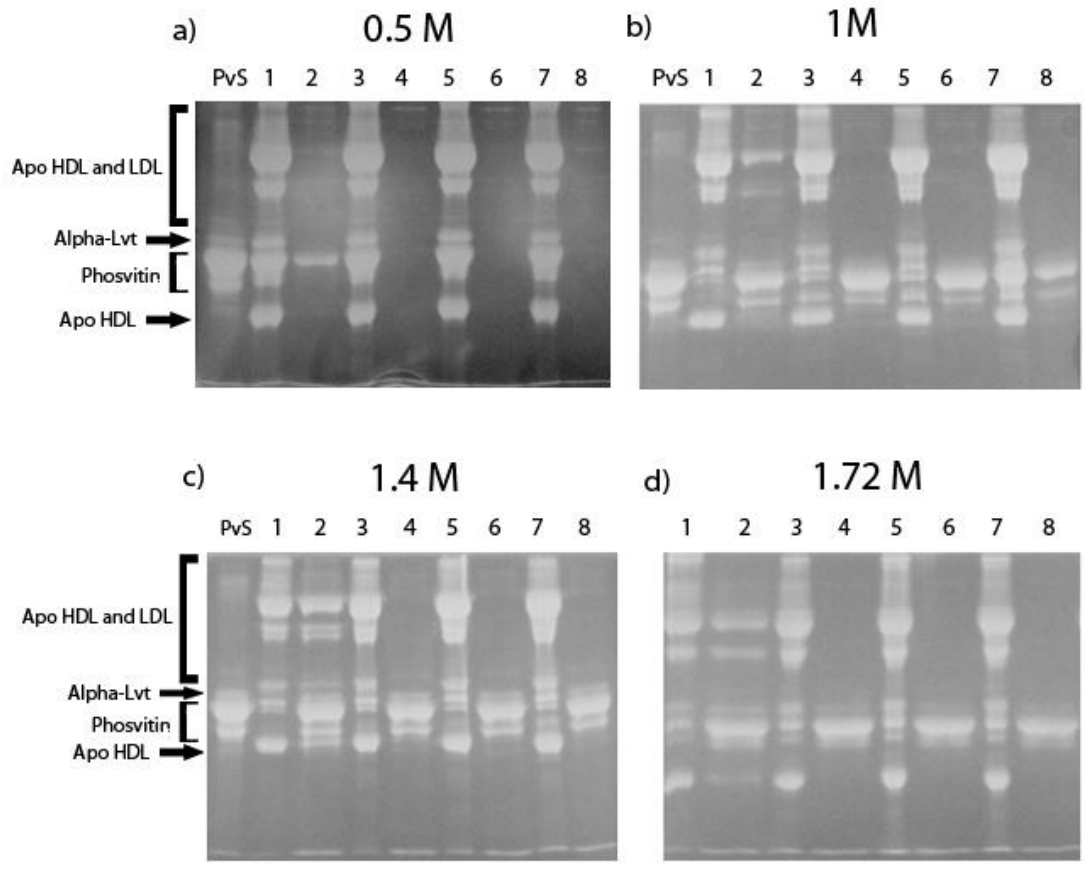

Fig. 2. Granules dissolved in NaCl at different pHs and later centrifuged. 0.5M NaCl (a), 1M NaCl (b), 1.4M NaCl (c) and 1.72M NaCl (d),. PvS: Standard phosvitin, 1 mg/mL (Sigma-Aldrich). Lane 1: pH 4 sediment. Lane 2: pH 4 supernatant. Lane 3: pH 3 sediment. Lane 4: pH 3 supernatant. Lane 5: pH 2.5 sediment. Lane 6: pH 2.5 supernatant. Lane 7: pH 2 sediment. Lane 8: pH 2 supernatant.

Figure 2a shows how the total precipitation of the granular protein was achieved at 0.5M NaCl and pH values of 3, 2.5 and 2. This fact can be observed due to the absence of protein in the supernatant after pH adjustment and centrifugation (lanes 4, 6 and 8, respectively). However, at 0.5M NaCl and pH 4, a part of the phosvitin remained in the supernatant (Figure 2a, lane 2).

The electrophoresis gel 2b shows supernatants and sediments when the NaCl concentration in the experiment was adjusted at 1M, with the same pH variation as in 2a. A reduced precipitation of HDLs to the sediment was observed at pH 4, seeing that HDL bands can be detected in the supernatant (Figure 2b, lane 2). However, mainly phosvitin bands can be appreciated in the supernatants at pH 3, 2.5 and 2 (Figure 2b, lanes 4, 6 and 8). Similar results to those of the 1M NaCl experiment were obtained at 1.4M (Figure 2c) and 1.72M NaCl (Figure 2d), with no variations in the electrophoretic bands obtained.

Results show that NaCl concentration is important to obtain an isolate solely through acid precipitation from dissolved granules. The low phosvitin solubility at 0.5M NaCl and pH 3, 2.5 and 2 can be due to a "salting in" effect. Therefore, as has been shown in Figure 2, at acidic pH the increasing of NaCl to 1M and more will also lead to maintain the phosvitin in solution.

The highest phosvitin recovery and the mildest condition were the parameters chosen to select an optimal pH and NaCl concentration to carry out the purification process. According to electrophoresis gels, differences were not detected among 1M, 1.4M, and 1.72M NaCl concentrations and pH values of 3, 2.5 and 2. To verify this finding, and as phosvitin represent about 90% of the egg yolk phosphorous (Samaraweera et al., 2011), its recovery rate was measured analyzing the phosphorus content in each sample (Figure 3).

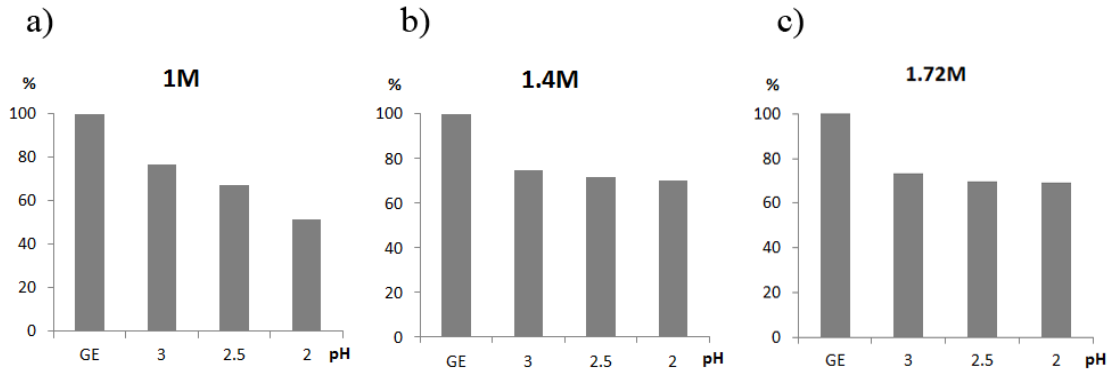

Fig. 3. Percentage of phosphorus recovery in the PRF. GE: Granules from egg yolk diluted (2% w/v). a) 1M, NaCl; b) 1.4M, NaCl; and c) 1.72M, NaCl.

At 1M NaCl, pH values of 2.5 or 2 lead to a decrease in the amount of phosphorous in the supernatant with respect to the same samples at 1.4M and 1.72M NaCl. That means that a greater amount of NaCl was required at lower pH to keep phosvitin in dissolution. According to the phosphorous content in the samples, the parameters for optimal phosvitin separation under the studied conditions correspond to 1M NaCl and pH 3. Employing these conditions, a recovery rate of phosvitin of 76±3% was measured.

Desalting of the Phosvitin-rich Fraction by Dialysis

Desalting was carried out at different pHs employing 2000 MWCO membranes to avoid the loss of phosvettes. Phosvettes are phosphorylated egg yolk proteins which are of as much interest as phosvitin. Phosvettes have molecular weights between 13000Da and 18000Da, lower values than phosvitin.

The pH of PRF was adjusted before dialysis at 3, 5, 7 and 8.5 values. When the pH was adjusted to 3 and 5 a milky solution was obtained after desalting due to protein aggregation. PRF dialyzed at pH 7 and 8.5 remained clear.

The amount of aggregates at pH 3 and 5 was calculated: Firstly, supernatants were dialyzed and subsequently lyophilized. Then, similar volumes of PRF were dialyzed, filtered at 0.45-µm to remove aggregates and lyophilized. Differences in weight correspond to aggregates eliminated by the filtration process (Figure 4). If dialysis was carried out at pH 3, aggregates represent 47% of the lyophilized weight. On the other hand, dialysis at pH 5 produces an aggregate formation of 10% of the final lyophilized weight.

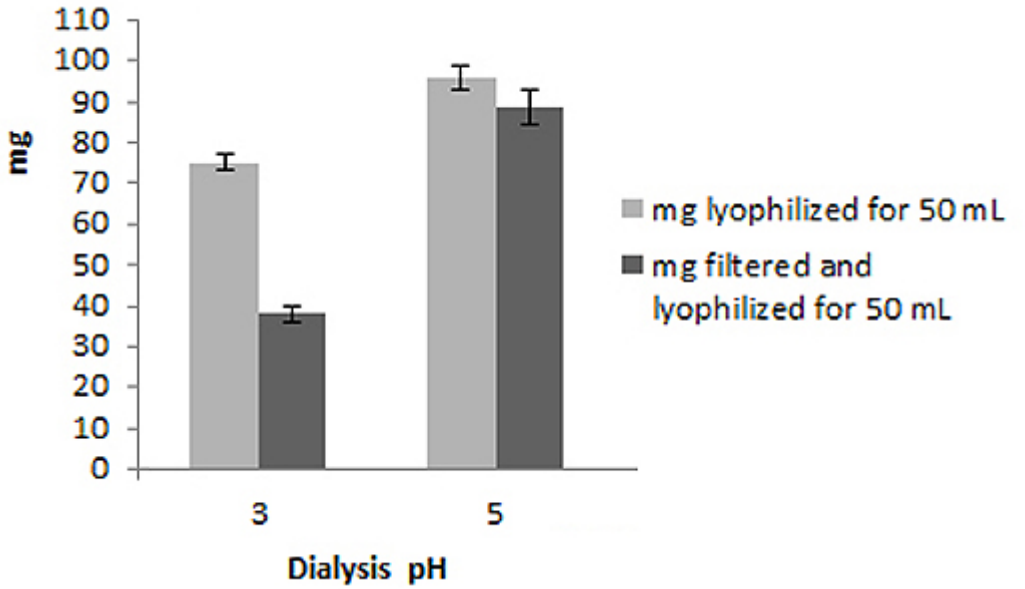

Fig. 4. Quantification of the aggregates formed in the dialysis at pH 3 and 5. 50 mL of PRF dialyzed at several pHs and lyophilized before and after the filtration process.

This means that as the pH of dialyzed sample increases, a lower amount of aggregates were formed. However, desalting becomes more difficult and consequently, the amount of lyophilized obtained at pH 5 was higher compared to that obtained at pH 3. This fact was confirmed by the results shown in Figure 5. It can be seen in this Figure, the nitrogen content in the lyophilized sample decreased when the pH of the PRF was increased before dialysis, due to the presence of more residual sodium. This could be the Donnan effect, according to which, with increments in the pH of the solution, phosvitin will behave like a polyelectrolyte with negative charges that prevents the free movement of ions through the membrane.

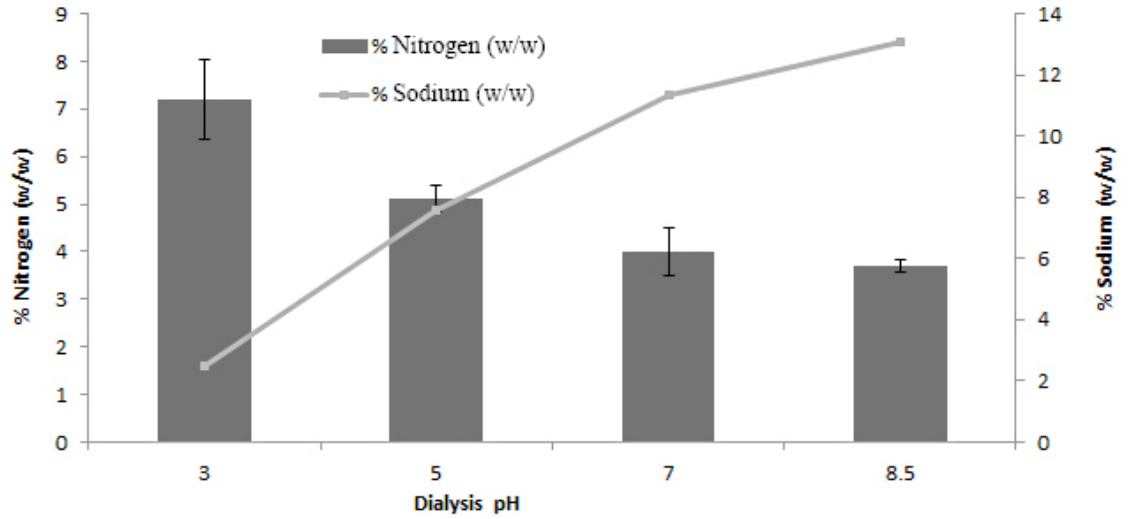

Fig. 5. Percentage of sodium and nitrogen in the phosvitin-rich fraction dialyzed at several pHs.

Furthermore, PRF dialyzed at pH 3, 5, 7 and 8.5 were centrifuged at 10000g for 30 minutes. The sediments obtained at pH 3 and 5 (aggregates) and the supernatants were analyzed by SDS-PAGE (Figure 6). Figure 6a shows how in the supernatants, except those obtained at pH 3, lipoproteins remained along with the phosvitin. At pH 3, the solubility of HDLs decreased and they then precipitate in distilled water together with part of the total phosvitin. In addition, aggregates (Figure 6b) show phosvitin with other granular proteins that persist in the supernatant.

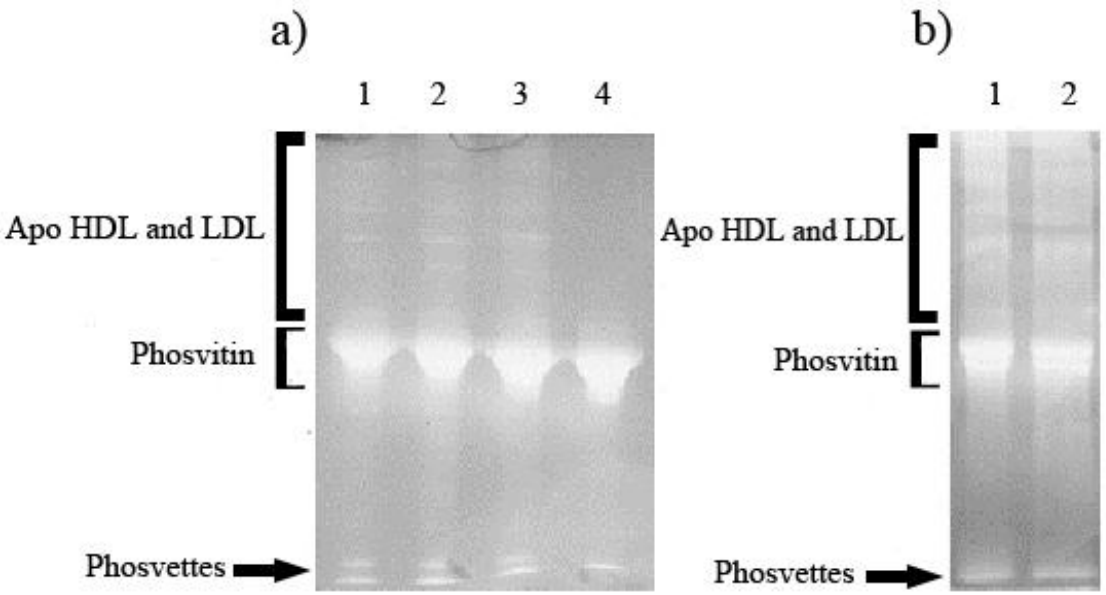

Fig. 6. SDS-PAGE of the PRF dialyzed at different pHs and of the aggregates formed at pH 3 and 5. 6a) PRF adjusted to several pHs, dialyzed and filtered at 0.45 microns. Lane 1: pH 8.5. Lane 2: pH 7. Lane 3: pH 5. Lane 4: pH 3. 6b) Lane 1: Aggregates from the PRF adjusted to pH 3 and dialyzed. Lane 2: Aggregates from the PRF adjusted to pH 5 and dialyzed.

Changes in the secondary structure of phosvitin due to pH media may modify its solubility. It has been reported that the secondary structure at pH 7 is in random coil, whereas if the medium is adjusted to a pH near to a value of 3, this structure changes and a beta sheet conformation is mainly originated. A similar change occurs when phosvitin is lyophilized (Renugopalakrishnan et al., 1985). These changes in secondary structure of the protein lead to its aggregation (Taborsky, 1974), thus allowing its precipitation in a desalted medium.

Furthermore, the charges of phosvitin phosphoserines at pH close to neutral keep the protein soluble. Under these conditions, it can be observed that by adjusting the PRF to pH 7 or 8.5 and then desalting by dialysis, all the protein remains soluble.

Anion Exchange Chromatography

To evaluate the purity and the anion-exchange chromatography performance of the phosvitin extracted using citric acid precipitation, the PRF was adjusted to different pH values: 3, 5, 7 and 8.5, and then dialyzed, filtered using 0.45-µm pore size filters, and lyophilized. The chromatograms obtained are shown in Figure 7, except for the PRF at pH 8.5, due to its similarity with the PRF at pH 7.

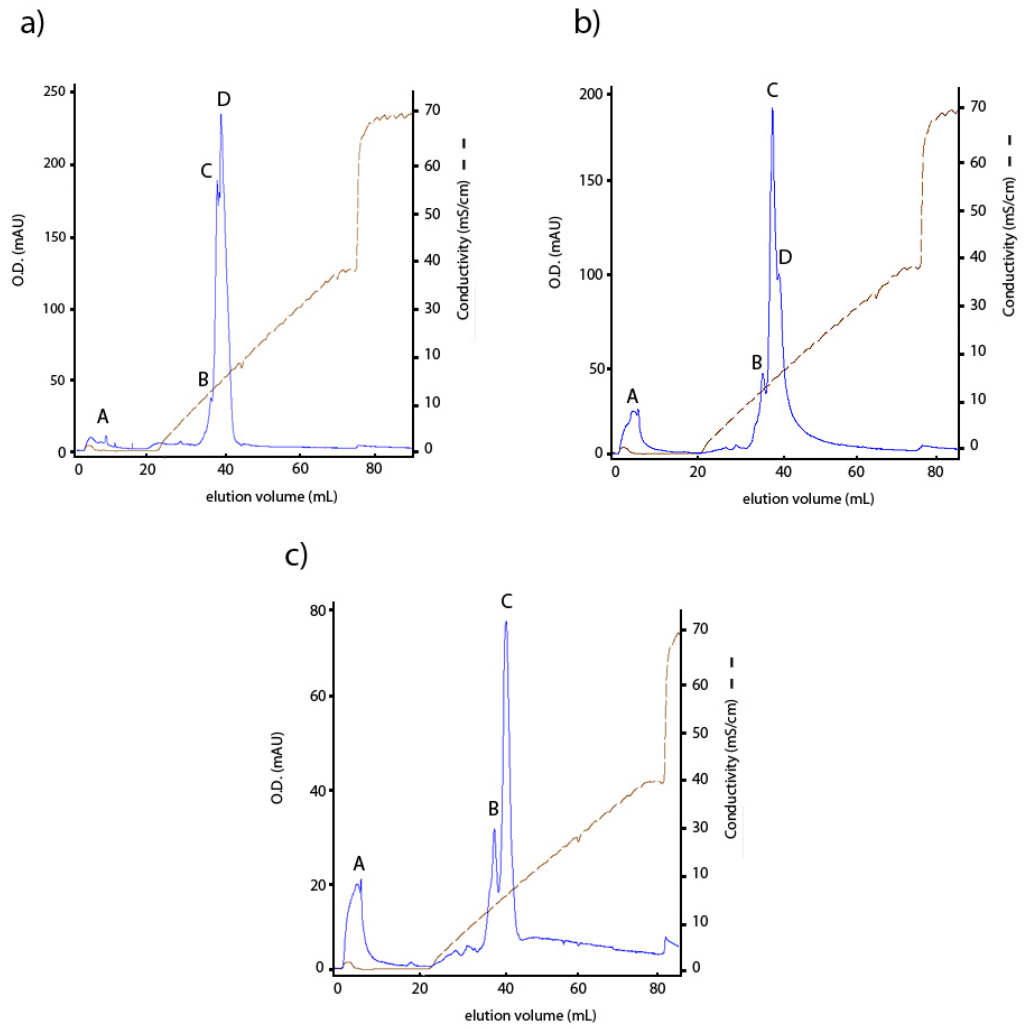

Fig. 7. Chromatography of the PRF dialyzed at several pHs, filtered and lyophilized. a) Dialyzed at pH 3; b) dialyzed at pH 5; and c) dialyzed at pH 7.

As it can be seen in the chromatograms, protein not bounded to the column was detected (area denoted as “A”). It could be explained by the presence of residual NaCl in the samples as has been previously commented. This NaCl could prevent the bounding of protein to the column matrix. Furthermore, a mean peak was detected in each chromatogram, and a reduction in the total area takes place with increasing pH of the PRF before dialysis. In fact, the area under the main peak from samples dialyzed at pH 5 is 72% of that of the peak obtained from samples dialyzed at pH 3. In the case of samples dialyzed at pH 7, this percentage is 35%. This is in agreement with the Donnan effect explained previously.

Several aliquots were collected in each chromatogram for PAGE analysis. The main peak was divided into two or three aliquots in order to detect the coelution of the different phosvitin fractions and the HDLs.

The results are shown in Figure 8. Electrophoresis was performed under denaturing conditions and revealed using a Coomassie staining specific for phosphoproteins. Electrophoresis was also performed under native conditions and the results were revealed with “stain all”, which is a specific coloration for acidic proteins (Wallace & Morgan, 1986b). Employing these stains enables the detection of all kind of impurities, particularly HDLs.

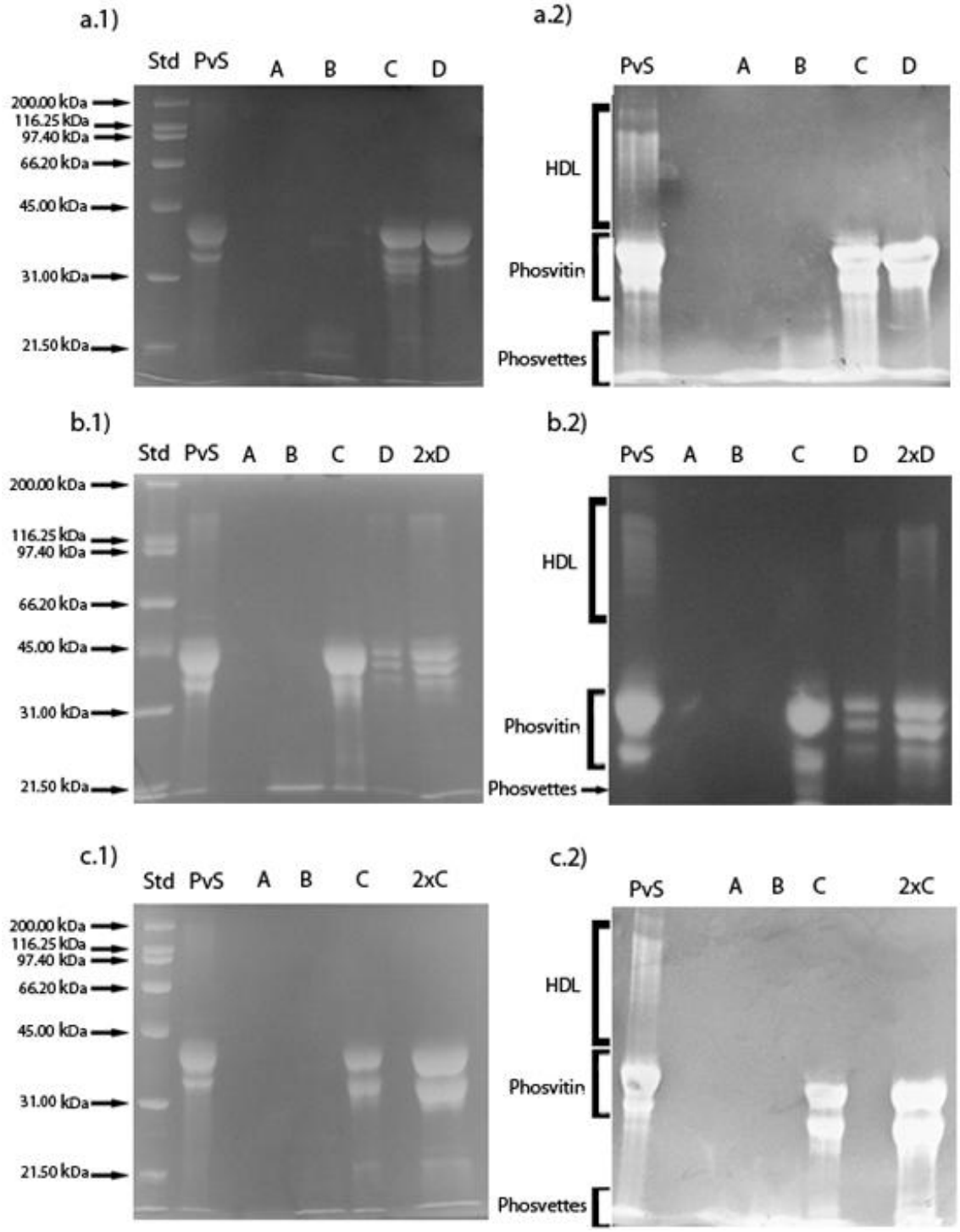

Fig. 8. Electrophoresis from chromatograms shown in figure 7. (a1) SDS-PAGE and (a2) PAGE under native conditions from Figure 7a); (b1) SDS-PAGE and (b2) PAGE under native conditions from Figure 7b), lanes marked with "2xD" correspond to lane D at a twofold concentration; (c1) SDS-PAGE and (c2) PAGE under native from Figure 7c), lanes marked with "2xC" correspond to lane C at a twofold concentration.

In the electrophoresis, no protein was detected in area "A". This may be because the proteins are diluted more than 3.5-fold after chromatography, so their concentration could be too low to be detected in the electrophoresis gel, even when aliquots were concentrated twofold prior to analysis.

Figure 8a corresponds to aliquots from Figure 7a, where the pH of the PRF was previously adjusted to 3 and then dialyzed and filtered. Only phosvettes were identified in aliquot "B" of the mean peak. β-phosvitin (38 kDa) and α-phosvitin polypeptides (35 and 36 kDa) were detected in aliquot "C". Aliquot "D" is similar to aliquot "C", though only one α-phosvitin is present. HDLs were not detected in any aliquot because the filtering operation removes HDL aggregates during sample preparation, as previously explained. This is in agreement with the results shown in Figure 6.

Figure 8b corresponds to aliquots obtained from the chromatogram shown in Figure 7b. The pH of the PRF was previously adjusted to 5 and then dialyzed and filtered. Contamination with apo-HDLs was detected at the end of the peak (aliquot "D") when employing both staining methods: Coomassie blue (8b.1) and "stains all" (8b.2). Coelution of different fractions of phosvitin likewise takes place.

Finally, Figure 8c shows the electrophoresis of aliquots from Figure 7c. In this electrophoresis, no apo-HDLs were detected in the "B" and "C" chromatograms regions nor in samples twofold concentrated before electrophoretic analysis and stained with "stain all". When the concentration of the lyophilized sample to be analyzed by FPLC was increased to 8 mg/mL, phosvitin-HDL coelution was detected in the main peak, as also detected at pH 5 (data not shown).

Under the tested conditions, the behavior of phosvitin extracted by means of our procedure was similar to the phosvitin extracted using magnesium sulfate (Castellani et al., 2003). The same coelution of high-density lipoproteins and phosvitin was detected. However, when the aggregates formed after dialysis at pH 3 were removed, the purest phosvitin was obtained. On the other hand, according to Wallace & Morgan (1986a), the separation of the different subfractions of phosvitin is not possible because its phosphorylation is a heterogeneous post-translational process and hence its binding capacities to the column are also heterogeneous.

Chemical Analysis

Table 1 shows the composition of the fractions obtained from egg yolk granules. This study was completed analyzing fresh granules washed with 0.16M NaCl solution. The analysis presented in Table 1 shows that washed granules have less lipids and higher protein content than unwashed granules. This is because the residual plasmatic fraction is partially removed, resulting in a lipid count reduction in the granular fraction.

Table 1. Composition of the fractions obtained compared with whole unwashed and washed granules.

	Unwashed granules[a]	Washed granules	HDL-rich fraction	PRF (lyophilized)
Dry matter	41.4 ±1.5[a]	41.3±0.2	28.5±1.23	87±0.1
Proteins	24.0±0.9[a]	27.6±0.5	18,71±0.2	52.4±5.1
Total lipids	16.6±2.8[a]	12.65±1.34	6.51±0.11	24.95±0.16
Sodium	0.12±0.04	0.34±0.04	1.4±0.01	2.65±0.15

[a] Values adapted from Laca et al. (2010).

On the other hand, the HRF is poor in dry matter compared to the washed and unwashed granules. Whereas the granular fraction is made up of a protein agglomerate with a hydrophobic nucleus, the HRF is a lipoprotein precipitate from dissolved granules in which the hydrophobic agglomerates are broken down, resulting in a sediment with a high capacity to retain water.

Finally, the pH of the PRF adjusted to pH 3 and dialyzed through a dialysis membrane and subsequently lyophilized presents a yield of 3.48±0.2 g lyophilized/100 g dried egg yolk, which is similar to that obtained by sulfate magnesium extraction (Castellani et al., 2003). This lyophilized PRF has 52.4% w/w protein content and an amount of lipids that represents the remains of lipoproteins in the supernatant. Furthermore, a 2.65% of sodium was detected. However, the amount of sodium may vary in relation to the PRF pH before dialysis, as was discussed previously.

Desalting by Ultrafiltration

An alternative ultrafiltration method in order to remove NaCl from the PRF was also investigated. This method offers the advantage of a low-cost alternative to membrane dialysis, as well as being easily scalable.

In order to avoid protein precipitation during the process, the pH of the PRF was adjusted to 8. At this pH, phosvitin is negatively charged, as the polyethersulfone ultrafiltration membrane, so interaction between both is avoided. A 10 KDa polyethersulfone membrane was used, keeping the final retentate volume at 150 mL. This retentate was then lyophilized and the phosphorus and sodium content subsequently determined. Results showed that, after desalting, the recovery of phosphorus was 89±2% of that of the original phosvitin content in PRF. The product lyophilized contained a 8%±1 of sodium.

Purity Criterion

The nitrogen/phosphorous atomic ratio (Wallace & Morgan, 1986b; Chay Pak Ting et al., 2010) is normally assumed as the purity grade. The results of these ratios are shown in Table 2, comparing the samples studied with those of an extraction method via precipitation with magnesium sulfate and with that of the phosvitin acquired from Sigma.

Table 2. N/P atomic ratio for different phosvitin preparations.

Sample	Sigma	Magnesium sulfate Castellani	PRF at pH 3 dialyzed.	PRF at pH 3 dialyzed and filtered.
N/P	2.95±0.3	3.4±0.1	3.6±0.2	2.5±0.1

The values for Sigma sample may be assessed as being close to 2.8 as has been previously reported (Castellani et al., 2003; Chay Pak Ting et al., 2010). The value for the sample precipitated with magnesium sulfate may also be considered to be close to 3.5±0.2, as reported by other authors (Castellani et al., 2003).

Separation with citric acid results in an N/P atomic ratio similar to that obtained with magnesium sulfate. PRF dialyzed at pH 3 through a dialysis membrane and subsequently lyophilized presents a yield of 3.48±0.2 g lyophilized/100 g dried egg yolk and a phosvitin recovery rate of 76±3%.

If the PRF is dialyzed at pH 3 and filtered at 0.45-µm prior to lyophilization, a N/P atomic ratio of 2.5±0.1 is obtained. It results in a purer grade of phosvitin, what has been shown by chromatography and electrophoretic analysis (Figure 6a, 7a and Figure 9). This fact can be explained because, as previously discussed, dialysis at pH 3 causes protein precipitation, being removed with the filtration at 0.45-µm the remaining lipoprotein. In each case, the nature of the impurities can be appreciated in the Figure 9, being basically HDLs for the magnesium sulfate extraction procedure and the phosvitin supplied by Sigma. More bands can be detected in the extraction with citric acid, although they are less intensive. This can be because granular LDLs, or part of them, remain in the supernatant with part of the HDLs.

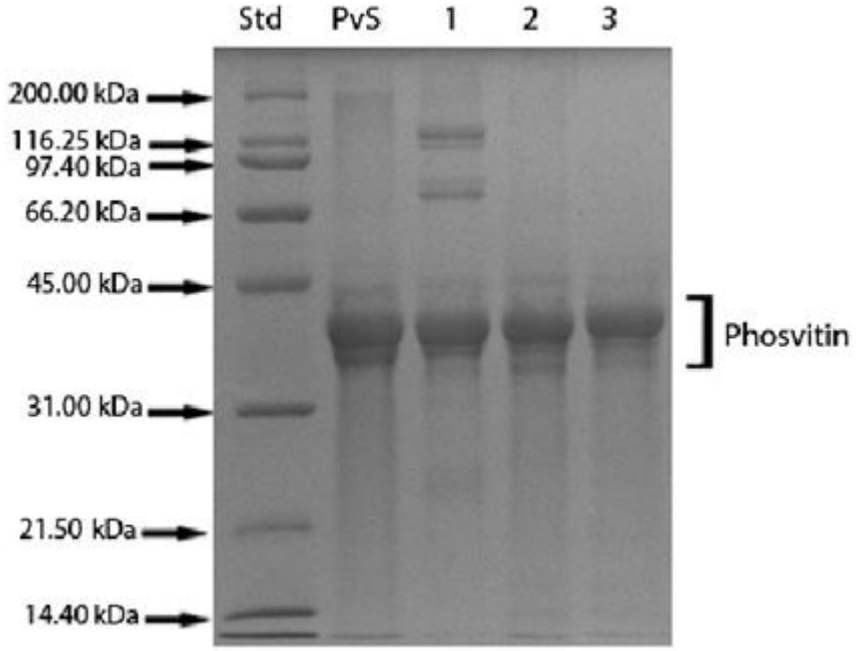

Fig. 9. SDS-PAGE of lyophilized samples shown in Table 2 at 1 mg/mL. Std: size standard. PvS: phosvitin standard (Sigma-Aldrich). Lane 1: Phosvitin extracted by means of the magnesium sulfate method (Castellani et al., 2003). Lane 2: PRF dialyzed at pH 3 (our procedure). Lane 3: PRF at pH 3, dialyzed and filtered.

Conclusions

Two fractions are obtained by acidic treatment of previously dissolved granules of egg yolk. One is a high-density lipoprotein-rich fraction, while the other one is a phosvitin-rich fraction.

Phosvitin remains soluble in the phosvitin-rich fraction (PRF). It allows the application of different technologies for its purification and for its possible applications, without the use of magnesium sulfate, giving rise to a difficult-to-solubilize pellet, or organic solvents, avoiding its environmental consequences.

The pH of the PRF is important in order to desalt the protein through dialysis membranes. A Donnan effect was detected when the pH is increased prior to desalting. When the PRF at pH 3 was desalted, protein aggregations were detected and a purer grade of phosvitin is obtained. Desalting the PRF at pH 7 and 8.5 avoids the aggregation of proteins. The chromatographic behavior of the phosvitin obtained was similar to that extracted using magnesium sulfate.

Given that the dialysis results in a poor scalable technique, an ultrafiltration alternative was tested for desalting the PRF.

The phosvitin-rich fraction shows a purity grade, measured as the nitrogen/phosphorous atomic ratio, similar to that obtained by means of the sulfate magnesium method. The yield is also similar.

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6. APROVECHAMIENTO DE LOS GRÁNULOS DE YEMA DE HUEVO EN PRODUCTOS DE HORNEADO

6.1. GRÁNULOS DE YEMA DE HUEVO COMO SUBSTITUTO BAJO EN COLESTEROL DE LA YEMA DE HUEVO ENTERA EN LA PREPARACIÓN DE MADALENAS SIN GLUTEN

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Egg yolk granules as low-cholesterol replacer of whole egg yolk in the preparation of gluten-free muffins

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ABSTRACT

In this work, the preparation and evaluation of a low-cholesterol gluten-free bakery product has been investigated. For that purpose, in a muffin recipe, the full egg yolk was replaced by a low-cholesterol granular yolk fraction.

In addition to the egg yolk recipe, three other formulations were elaborated: In the first preparation egg yolk was completely substituted by its weight in fresh granules (100% granules recipe). In the second one, the granules were lyophilized (lyophilized recipe) and reconstituted before their use. In another preparation, to further reduce the cholesterol content, all the egg yolk has been substituted by a lower amount of granules (60% granules recipe).

Rheological batter characterization at 25 °C has shown that the viscoelastic properties of the recipes elaborated using 100% egg yolk granules and whole egg yolk were similar. Rheological characterization at 100 °C was performed too and differences were observed, with variations in the strength of the interaction in the batter produced by the granular components. Texture profile analysis has shown that all the recipes made using granules were harder than the one made with egg yolk.

1. Introduction

In some bakery products whole egg is used in large quantities and replacing it is difficult because of its properties: the addition of whole egg to sweet bakery products gives color, flavor and functional properties, including the enhancement of the product's emulsifying, foaming and gelation qualities (Mine, 2002). Besides, egg yolk specifically allows bakery products to obtain an optimal crumb, increasing the batter volume and the sponginess of the final product (Kamat, Lawrence, Hart, & Yoell, 1973). Furthermore, egg yolk is a high caloric ingredient made up of 33% of fat, of which 62% are triglycerides, 28% phospholipids and around 5% cholesterol (Anton, Le Denmat, & Gandemer, 2000). The essential fatty acids that can be found in egg yolk are around 90% $n-6$ PUFA and 10% $n-3$ PUFA. It results in a coefficient about 9:1 of $n-6/n-3$, a long way from the healthier proportion of 5:1 that may reduce the risk of cardiovascular diseases (Lands, 2000; Leaf, Kang, & Xiao, 2000). For this reason, in order to make dietary recommendations, the total amount of fat and cholesterol consumed must be taken into consideration (Seuss-Baum, 2007) and therefore the elaboration of low-fat and cholesterol egg-dependent recipes is desirable.

Egg yolk can be separated in two fractions with different nutritional and functional properties. One of them is the plasmatic fraction, which is the lipid-rich one and contains the highest proportion of fats and cholesterol and has a low protein content. The second fraction is the granular one. Granules represent 19–23% of yolk dry matter and they are mainly constituted by proteins (64%) and lipids (31%). This granular fraction has a low cholesterol content (Anton, 2007) and maintains good emulsifying properties (Anton & Gandemer, 1997). The major components of the granular fraction are the high density lipoproteins (HDL, 70%) and phosvitin (16%). HDLs are constituted by 75–80% of proteins and around 20–25% of lipids. They are not lipoproteins with a spherical micelle-like structure, and their appearance is more similar to that of globular proteins. This fraction has been used previously in the elaboration of mayonnaise recipes as a substitute for whole egg yolk, maintaining the functional and organoleptic properties and reducing the cholesterol and saturated-fat content (Laca, Sáenz, Paredes, & Díaz, 2010).

Finally, it is also known that over 1% of the general population suffers from celiac disease. This is an autoimmune disorder caused by intolerance to gluten and characterized by small bowel villous atrophy. Treatment for celiac disease requires a strict gluten-free diet to allow disease remission, so the food industry produces a special category of products labeled as "gluten-free". In bakery,

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these products have been widely studied in order to propose high-quality alternatives, and some of them include egg in their formulations.

The aim of the present study is to prepare and evaluate a low-cholesterol gluten-free egg-based bakery product. A muffin recipe containing whole egg from a local supplier has been used, and the whole egg yolk was entirely substituted by the granular fraction in several formulations. Muffins batters were analyzed using rheological techniques and the baked muffins were tested employing texture and color analysis.

2. Materials and methods

2.1. Egg yolk granules

Egg yolk granules were obtained according to Laca et al. (2010).

2.2. Lyophilization

Granules were freeze-dried at −70 °C and 0.1 mbar in a Telstar Cryodos Lyophilizer (Terrassa, Spain). Previous to lyophilization, they were frozen at −80 °C. Lyophilized granules contain 97 g of dry matter/100 g of product.

2.3. Muffin preparation

Egg yolk muffins were elaborated according to the recipe of a national manufacturer of gluten-free products. The basic formulation includes 17 g of apple pectins, 102.12 g of gluten-free corn flour (containing 1.2 g of proteins/100 g of flour, 4.4 g of lipids/100 g of flour, diphosphate disodium and sodium bicarbonate), 65.36 g of liquid pasteurized egg white, 36.76 g of fresh egg yolk, 93.6 g of refined sunflower oil and 85.08 g of sugar. Apple pectins and flour were a gift of a local supplier (Adpan Europa, Asturias, Spain). The other ingredients were acquired at a local market.

Egg yolk and egg white were whipped for 3 min employing a 180 Watt hand blender (Model HB01, Morphy Richards, Manvers, UK) at maximum speed. Sugar and gelatin were added and mixed for 2 min. Sunflower oil was then added and mixed for 1.5 min. Finally, flour was added and mixed for 2 min with a 200 W mixer (Model SM3827, Severin Eletrogeräte, Sundern, Germany) adjusting to "3" the speed control.

Three different muffins made with egg yolk granules were also elaborated. The egg yolk content was replaced by the same weight of egg yolk granules in the first preparation (100% granules recipe). In another formulation and in order to obtain a further reduction in the cholesterol content, the 36.76 g of fresh egg yolk per 400 g of batter were replaced by 22 g of granules (60% granules recipe). In the third formulation, the egg yolk was replaced by its equivalent in lyophilized granules previously reconstituted in water. Freeze-dried granules were prepared mixing 21.32 g with 15.43 g of lyophilized granules (lyophilized recipe). Lyophilized granules were tested because their relative imperishability provides clear technological advantages.

2.4. Muffin composition

Total lipids were calculated assuming that the sunflower oil has a lipid content of 100 g/100 g of oil. Flour lipids and proteins were considered too (content data provided by the flour manufacturer). Phospholipid values were obtained from previously published data for sunflower oil (Morrison, 1981). Egg yolk and granules protein, water and cholesterol content were calculated according to the egg yolk (Anton, 2007) and granules composition (Laca et al., 2010).

2.5. Rheological properties of batter mixes

Batters were kept for 60 min at 25 °C before the rheological test, in all the cases, samples were disposed at this temperature in the rheometer plate. Rheological properties of batter were determined employing a Haake MARS II rotational rheometer (Thermo Scientific, Waltham, USA) using a Peltier unit to control the temperature. A plate/plate measuring system (PP60) was used with a gap of 1 mm. Samples were left for 25 min to relax stress and stabilize the temperature. In the assays performed at 100 °C, this temperature was reached in 6 min, the remaining 19 min were long enough to reach the gelatinization, measuring an equilibrium property. The excess of sample was removed, and a glass hood and silicone oil were employed to protect against dehydration during the duration of each measuring procedure.

2.5.1. Flow properties

Flow properties were measured at 25 °C. Apparent viscosity was obtained as a function of shear rate from 0.01 1/s to 100.0 1/s. The experimental time was adjusted to 300 s 100 points were collected with a logarithmic distribution in each case. Duplicates presented differences lower than 10%. Data obtained were adjusted to the Ostwald de Waele model (Martínez-Cervera, Salvador, Mueguerza, Moulay, & Fiszman, 2011).

$$\eta = K \cdot \dot{\gamma}^{n-1}$$

Where η is the apparent viscosity, $\dot{\gamma}$ is the shear rate, K (Pa s^n) is the consistency index, and n is the flow behavior index.

2.5.2. Dynamic tests

Stress sweeps at 25 °C and 100 °C were conducted to verify the lineal viscoelastic range of the batters at both temperatures. G' and G'' functions were measured throughout the stress range of 0.1–100 Pa. Frequency was adjusted to 1 Hz.

Frequency-dependence tests were conducted at 25 °C and 100 °C in the range of linear viscoelasticity. Frequency range tested was from 10.0 Hz to 0.01 Hz. The experimental data obtained were adjusted to the following power law equation according to Gabriele, Cindio, and D'Antona (2001).

$$G^* = A \cdot \omega^{1/Z}$$

Where G^* is the complex modulus in Pa, ω the frequency in Hz, Z (dimensionless) the coordination number and A the proportional coefficient.

2.6. Physical measurement of baked muffins

2.6.1. Texture profile analysis (TPA)

TPA analysis was performed employing a TA.XT.plus Texture Analyser (Stable Microsystems, Godalming, UK). 2 cm of the lower half of each muffin was evaluated, the upper half was discarded. Texture parameters were set at 1.0 mm/s, and a double compression of 50% of the original height was carried out with a flat-ended cylindrical probe (P/75). The TPA parameters calculated were hardness (the maximum value in the first compression cycle), springiness (the distance until the detected height in the second compression divided by that in the first bite), cohesiveness (dividing the total area of the second compression cycle by the total area of the first compression cycle), chewiness (the product of the hardness and the springiness) and resilience (dividing the area during the withdrawal by the area of the first compression).

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2.6.2. Air cells number determination

Muffins were cut at the mold level, the upper half was removed and an image was obtained of the crumb using a flatbed scanner (Model HP PSC 1610, Hewlett Packard, Palo Alto, USA). Air cell number was calculated by image analysis employing ImageJ software (Schnedier, Rasband & Eliceiri, 2012).

2.6.3. Height, weight loss and color

Baked muffin height was measured with a caliper from the highest point to the bottom after 3 h of cooling at room temperature. Weight loss was calculated from the weight of the muffins before and after baking. Color properties were measured in the L^*, a^*, b^* system using an UltraScan VIS spectrophotometer (HunterLab, Reston, USA). Several points of the crust were analyzed and an average value was obtained for each muffin. The crust was removed and the crumb was homogenized employing a 180 W electric grinder (Model AR100G, Moulinex, Alençon, France). A fixed amount of crumb was poured into the measurement cell and analyses were conducted in specular exclusion mode. The color differences between the muffins made using granules were examined in relation with the egg yolk muffin. ΔE is the total color change calculated from:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

2.7. Statistical analysis

Analysis of variance (ANOVA) was applied. Fisher's test ($P < 0.05$) was used to calculate the least significant difference (LSD) using a statistical software (Statgraphics v.15.2.06, Warrenton, USA). In order to measure each rheology parameter, two batches were analyzed for each recipe and two replicates were carried out in each case. As regards the physical measurements, 8 muffins from 2 different batches per formulation were tested.

3. Results and discussion

3.1. Muffin composition

As can be observed in Table 1, the protein content in each recipe was modified by the amount of granular fraction added. The total lipid content was mainly provided by the sunflower oil. However, the granules recipes showed lower amounts of lipids due to the removal of the high-lipidic plasmatic fraction. Egg yolk substitution produced a decrease in the high-emulsifying phospholipids too. Furthermore, egg yolk substitution by the granular fraction allowed a reduction in the cholesterol content. In the 100% granules recipe, a decrease to one fourth of the initial value was obtained. In the 60% granules recipe, the reduction obtained was up to one seventh of the original value.

As regards water, in the muffins recipe the ingredients that provide it were principally egg yolk and egg white. Changes in the amount of water supplied due to egg yolk substitution could produce variations in the batter system at low temperatures.

Table 1
Muffins composition.

	Egg yolk	Granules 100%	Granules 60%	Lyophilized granules
Proteins (g/100 g batter)	3.55	4.3	3.62	4.3
Total lipids (g/100 g batter)	27.83	26.08	25.45	26.08
Phospholipids (g/100 g batter)	1.13	0.85	0.67	0.85
Cholesterol (mg/100 g batter)	463.17	107	64.182	107
Water supplied (g/400 g batter)	18	21.6	13	21.6

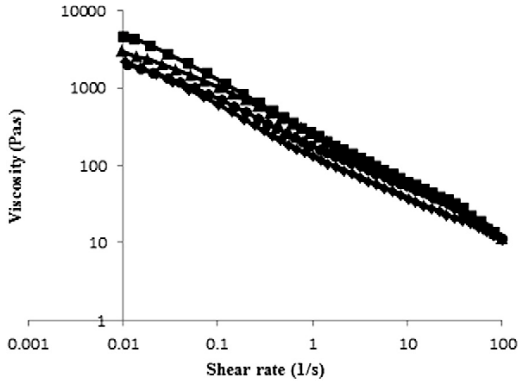

Fig. 1. Flow properties of the assayed batters. Values obtained from the egg yolk (◆), granules 100% (▲), lyophilized (●) and granules 60% (■) recipes.

3.2. Rheological properties of batter mixes

3.2.1. Flow properties of batters

In flow curves shown in Fig. 1, it can be observed that the apparent viscosity decreases with increments in the shear rate.

This behavior is a feature of shear thinning products. This fact was confirmed by the flow behavior indexes (n) presented in Table 2. A flow behavior index <1 is distinctive of pseudoplastic materials, but the closer the values approach 1, the less is the implied complexity of the structure, more similar to a Newtonian fluid ($n = 1$). Furthermore, in Table 2 the consistence coefficient (K) of each recipe is shown too.

As it can be observed in Table 2, the recipe elaborated with egg yolk presented a lower structural complexity (a high n value), compared with the 100% and 60% granules recipes, and the lowest consistence coefficient (K). This could be caused by its lower protein content (Table 1) and by the presence of the high emulsifying plasmatic fraction. In fact, decrements in K values in low fat content cake batters are related to the ability of the emulsifiers to increase the level of air incorporation and to decrease their apparent viscosity (Sakiyan, Sumnu, Sahin, & Bayram, 2004).

The recipe elaborated using lyophilized granules showed the highest flow behavior index value and a consistence coefficient between that of egg yolk and the other granules recipes. This low consistence, in relation to the batter made using fresh granules, could be explained because reconstituted lyophilized granules cannot provide a final product that is totally identical to the original one. It is also known that secondary structure changes and aggregation in some egg yolk protein molecules can be originated after lyophilization (Renugopalakrishanan, Horowitz, & Glimcher, 1985). Furthermore, this could produce a reduced degree of rehydration and the excess of water could increase the n value.

Finally, batter made using 60% granules showed similar K and n values to those obtained with fresh 100% granules. In the 60% granules recipe, the reduction in high-moisture granules content

Table 2
Parameters calculated from Fig. 1. K is the consistence index, n is the flow index.

Ostwald de Waele	K (Pa s^n)	n	R^2
Egg Yolk	130.6 (4.24)a	0.464 (0.006)a	0.9999
Granules 100%	250.5 (5.02)b	0.374 (0.03)b	0.9997
Granules 60%	270 (14)b	0.341 (0.04)b	0.9998
Lyophilized	180.4 (7.07)c	0.465 (0.004)a	0.9991

Values are expressed as means ± SD. Different letters in the same column indicate significant differences ($P < 0.05$).

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produced a decrease of water in the system. This is related to the fact that this was one of the most viscous and structured batters tested. Furthermore, in the 100% granules recipe, the high protein content and the removal of the plasmatic fraction affected the viscosity of the sample too.

3.2.2. Dynamic tests

Results of stress sweeps at 25 °C are shown in Fig. 2A. Tested batters held their linearity until 10 Pa at least. To verify the linear viscoelastic response at high temperatures, stress sweeps were also carried out at 100 °C (Fig. 2B). At high temperatures linearity extends above 10 Pa.

3.2.2.1. Mechanical spectra at 25 °C. Mechanical spectra at 25 °C show values of G' slightly higher than G'' (Fig. 3A).

Significant frequency dependence of both moduli within the frequency range studied denoted the existence of a soft-gel, with G' higher than G'' and $\tan\delta > 0.1$ in all cases. Similar results have been obtained previously using muffin batters and gluten-free dough and bread (Baixauli, Sanz, Salvador, & Fiszman, 2007, 2008; Lazaridou, Papageorgiou, Belc and Biliaderis, 2007; Witczak, Juszczak, Ziobro, & Korus, 2012).

Differences among samples in both moduli were detected and furthermore, some changes in the loss tangent value ($\tan\delta = G''/G'$) were found too (Fig. 3B).

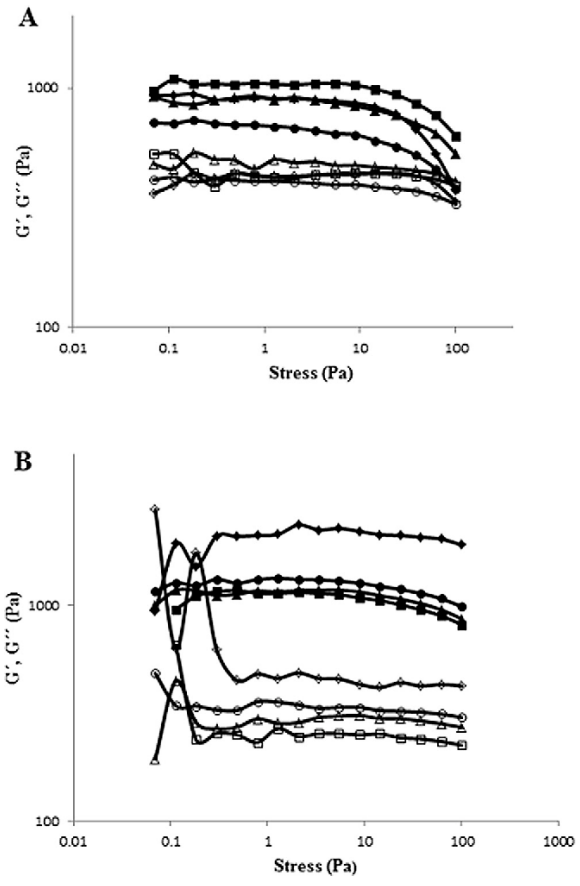

Fig. 2. Stress sweep at 25 °C (A) and 100 °C (B). G' (solid symbols) and G'' (open symbols) values obtained from the egg yolk (◆, ◇), granules 100% (▲, △), lyophilized (●, ○) and granules 60% (■, □) recipes.

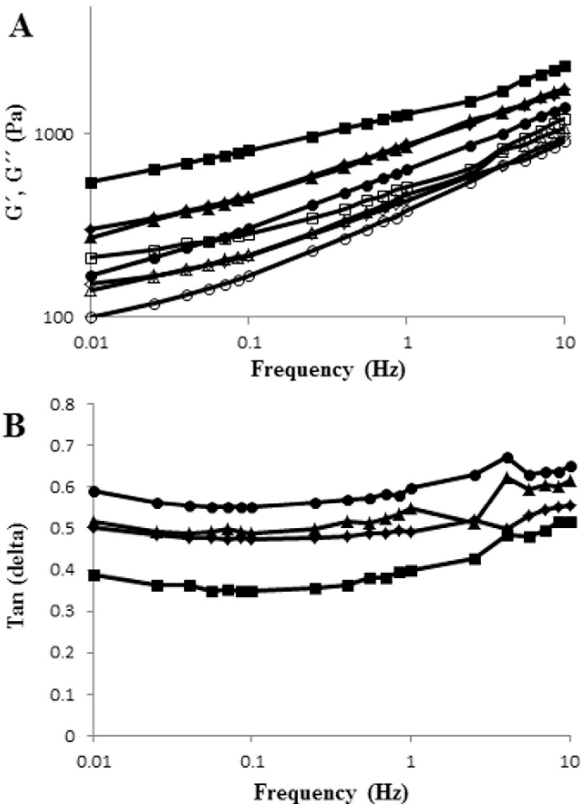

Fig. 3. Frequency dependence of the viscoelastic modules at 25 °C for the batters assayed. G' (solid symbols) and G'' (open symbols) values obtained from the egg yolk (◆, ◇), granules 100% (▲, △), lyophilized (●, ○) and granules 60% (■, □) recipes (A). Tangent delta of values obtained (B).

The 100% granules and egg yolk recipes showed similar values for both moduli. Therefore, the replacement of egg yolk by the same amount of fresh granules did not change the complexity of the system in the viscoelastic linear range. However, in the lyophilized recipe, a decrease in the G' and G'' values were detected compared with the other recipes values. In this case, imperfect rehydration could produce protein modifications and/or an increase in the free water provided by the granular fraction. This tendency for the modulus values to decrease when more water is available is in agreement with other studies (Autio, Flander, Kinnunen, & Heinonen, 2001). On the other hand, an opposite effect was detected in the 60% granules recipe owing to a reduction in the available water.

The loss tangent values showed variations in the proportional contribution of the viscous and elastic moduli. The lyophilization process produced a higher contribution of the viscous component. However, the reduction in water and protein content that takes place with the change from the 100% granules to the 60% granules recipe resulted in the most elastic batter.

The viscoelastic properties of each batter were measured within the frequency range with the complex modulus (G^*). These experimental data of G^* were adjusted to the power law equation presented in Section 2.5.2. Values obtained are shown in Table 3.

In global terms, the muffin batter is considered as a highly-aerated emulsion, and therefore, it is a three-dimensional network formed by dumps and strands. In this system, the

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Table 3
Coordination number (Z) and proportional coefficient (A) obtained from frequency sweeps at 25 °C.

	25 °C			
	Egg yolk	Granules 100%	Granules 60%	Lyophilized
A (Pa)	994 (38)a	1001 (30)a	1453 (32)b	759 (7)c
Z	3.4 (0.004)a	3.26 (0.07)a	4.35 (0.28)b	2.94 (0.02)c
R	0.991	0.995	0.993	0.992

Values are expressed as means ± SD. Different letters in the same line indicate significant differences ($P < 0.05$).

dimensionless parameter Z is related with the number of rheological units in the network, while the A (Pa) parameter shows the strength of the interaction between those units (Gabriele et al., 2001). The Z values ranged from 2.94 to 4.35, and the 60% granules recipe presented the most complex structure, while the lyophilized recipe exhibited the lowest complexity. It confirms the comments made previously, that the free water could be affecting the structuring degree of the sample. Besides, for the same reason, the network strength was greater in the recipe elaborated employing 60% granules, while the recipe elaborated with reconstituted lyophilized granules originated the weakest gel. Furthermore, egg yolk and 100% granules recipes presented statistically similar A and Z values. In this case, the amount of water is higher in the 100% granules recipe, but the increase in the protein content could be compensating this water difference.

3.2.2.2. Mechanical spectra at 100 °C. Temperature sweep of the tested samples were carried out (data not shown) and the onset gelatinization temperature was characterized approximately at 90 °C in each case. To study the effect of the granular protein on the resultant gel, mechanical spectra at 100 °C were performed. In this test, the time used to relax the stress and reach the temperature was long enough to reach the gelatinization. As can be observed in Fig. 4A, at 100 °C increments of both viscoelastic moduli were detected in comparison to the values found at 25 °C. G' remains higher than G'' values (tan $\delta < 1$) and the material can still be considered a soft gel, although variations in viscoelastic moduli throughout the frequency range were not as marked as were seen in Fig. 3A. These changes imply the prevalence of a stronger gel than that found at 25 °C. Furthermore, at 100 °C, although both viscoelastic functions rise proportionally, the increment was slightly higher in G'' than G', which means an increment in the viscous contribution to the viscoelastic behavior of the system. This can be observed in Fig. 4B, which shows changes in the contribution of both moduli.

Tan δ (G''/G') at 100 °C (Fig. 4B) was closer to 0 in each tested sample, showing a more structured system than that found at 25 °C.

In Table 4 the A (Pa) and Z parameters calculated from the experimental data at 100 °C are shown.

It was observed that at 25 °C the batter that showed the strongest interactions was elaborated employing 60% granules (Section 3.2.2.1), while at 100 °C it was that made using 100% granules recipe. These differences denote changes in the interactions between the compounds in the samples when influenced by the temperature. According to these results, the addition of granular protein increased the protein–protein and/or the protein–starch interactions. In this sense, the similar protein content of batters in the egg yolk and 60% granules recipes produced soft-gels with similar A values. The batter elaborated using lyophilized granules showed a lower A value than expected from its protein content. This could be explained by the changes in the granular protein associated to the lyophilization process.

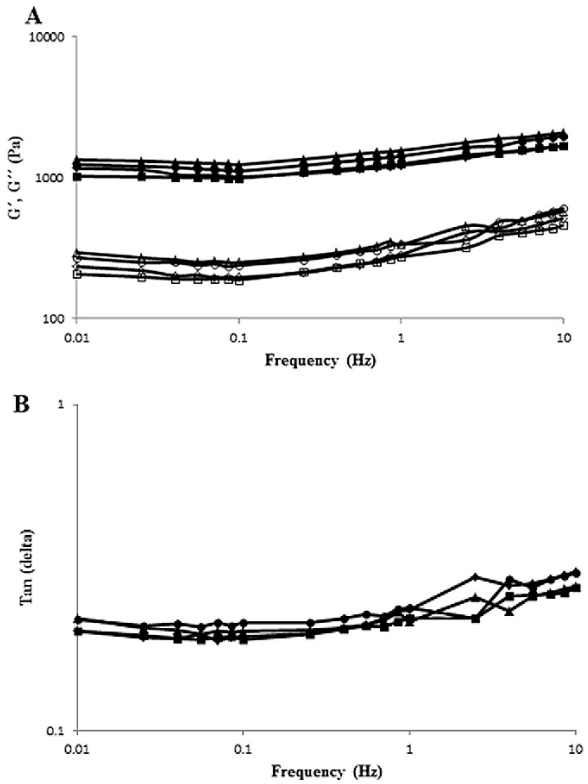

Fig. 4. Frequency dependence of the viscoelastic moduli at 100 °C. G' (solid symbols) and G'' (open symbols) values obtained from the egg yolk (◆, ◇), granules 100% (▲, △), lyophilized (●, ○) and granules 60% (■, □) recipes. (A). Tangent delta of the values obtained (B).

3.3. Physical measurement of baked muffins

3.3.1. Texture profile analysis (TPA)

Hardness is the most valued textural parameter in this kind of products. In Table 5, the hardness values and the number of bubbles calculated by image analysis for each recipe are shown. Muffins elaborated employing 100% granules presented the highest hardness value. This parameter is directly related to the density of the tested sample. Thus, a hardness increment corresponds to a muffin with fewer bubbles as well as to an increment in the protein-based interactions in the gel matrix (Section 3.2.2.2).

The second muffin in hardness was the one elaborated using lyophilized granules. In this recipe, the total amount of protein was the same as that seen when using 100% fresh granules. However, the lyophilization process could produce changes in the granular protein and therefore, a reduced involvement in the gel formation.

Table 4
Coordination number (Z) and proportional coefficient (A) obtained from frequency sweeps at 100 °C.

	100 °C			
	Egg yolk	Granules 100%	Granules 60%	Lyophilized
A (Pa)	1304 (92)a	1968 (80)b	1523 (130)a	1513 (119)a
Z	9.02 (0.5)ab	9.65 (0.3)b	9.17 (0.62)ab	8.3 (0.15)a
R	0.985	0.997	0.994	0.992

Values are expressed as means ± SD. Different letters in the same line indicate significant differences ($P < 0.05$).

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Table 5
Air cells number and hardness (TPA parameter) of baked muffins.

Air cells number						Hardness (g)
	<1 ($m^2 \times 10^{-9}$	1–4 ($m^2 \times 10^{-9}$	4–7 ($m^2 \times 10^{-9}$	7–25 ($m^2 \times 10^{-9}$	Total number	
Egg yolk	555 (39)a	58.8 (5)a	6.7 (2.1)a	5.2 (0.45)a	634 (34)a	1356 (41)a
Granules 100%	439 (28)b	60.4 (4.8)a	7 (2)a	6.4 (2.3)a	508 (23)b	2004 (86)b
Granules 60%	507 (51)c	60 (4.47)a	8.6 (2.8)a	5.4 (1.14)a	565 (20)c	1631 (102)c
Lyophilized	434 (41)b	50.2 (3.1)b	7.7 (0.8)a	8.9 (1.8)b	490 (35)b	1846 (80)d

Values are expressed as means ± SD. Different letters in the same column indicate significant differences ($P < 0.05$).

In the egg yolk muffin the amount of total protein is the lowest one, as it is shown in Table 1. Egg yolk contains less protein per gram than granules from egg yolk, and the amount of lipids is higher. According to Graham and Kamat (1977), neutral lipids and phospholipids have importance during the finishing stages of cooking, being released from the lipoproteins to the cake matrix and improving its quality. In fact, this lipid and protein content produced the least hard of all the muffins tested, with the greatest number of tiny bubbles.

In addition to the hardness parameter, springiness, cohesiveness and resilience were calculated too, although these texture parameters resulted similar in all the samples tested. In addition, chewiness was considered too, and its behavior was parallel to that of the hardness.

3.3.2. Air cells number

Bubbles are generated in the crude batter during mixing and they behave as growing nuclei while cooking. During baking, CO_2 production results in an increase in bubble size, allowing the batter to rise and giving a tender quality to the final product. Furthermore, oil lipids and egg yolk proteins and lipids are fundamental foam stabilizers. Therefore, substitution of the whole egg yolk by 100% granules resulted in a reduction in the tiniest bubbles (Table 5) and, as has already been mentioned, in the hardest muffins. This trend is also noticeable in the other recipes, where a reduction in the granular protein produced more air bubbles and less hardness. These results suggest that one of the effects of this granular fraction on the muffin texture is through the decrease in the air bubble population.

3.3.3. Color analysis and appearance

The plasmatic fraction contains the most fat-soluble carotenoids, to which the color of the whole egg yolk has been attributed (Laca et al., 2010). Therefore, if this plasmatic fraction is removed and replaced by the granular one, color alterations are expected.

In Table 6, the color properties of the cooked muffins are shown. Crust color is the result of the Maillard reaction between the proteins and the carbohydrates in the surface of the muffin, as well as the egg yolk pigments. In muffins elaborated employing granules (100%, 60% and lyophilized) an increase in the lighter parameter for the crust was detected. The ΔE values were higher than 3 for muffins elaborated using 100% granules and 60% granules. These color differences would be appreciated by the human eye. According to these results, 60% granules and 100% granules recipes were slightly more yellowish than egg yolk muffins. In the case of lyophilized granules, no crust color differences were appreciated.

These results suggest that more granular fraction is necessary to maintain the muffin crust color and compensate for the pigment loss. Finally, granules modifications during the lyophilization process produced a final muffin with the same crust color as the egg yolk product.

As regards the crumb and compared with the crumb of the whole egg yolk muffins, those elaborated employing granules (100%, 60% and lyophilized recipes) exhibited ΔE values higher than 3 units in each case. These color changes, due to the removal of the plasmatic fraction, produce less reddish and yellowish muffins than the egg yolk recipe.

Regarding weight loss during the cooking, no statistical differences were detected between the muffins analyzed. Concerning height, muffins with a higher content in granular protein (100% granules and lyophilized) showed the highest value. The harder structure formed in these muffins leads to them being higher. In the same way, 60% granules muffins have more granular protein than egg yolk recipe products and were third on the height scale. The egg yolk recipe was the least hard and produced the lowest muffins.

3.3.4. Lyophilized granules vs fresh granules

The use of lyophilized products gives industrial advantages such as improvements in their storage and shelf-life. However, the lyophilization can induce changes in the food macromolecules, as for example denaturation of proteins (Chang, Kendrick, & Carpenter, 1996), which could involve changes in the reconstituted product.

In the case of the lyophilized egg yolk granules, their use in the preparation of muffins implied rheological parameters values different to those obtained using fresh granules, resulting in batters with lower apparent viscosity and lower gel strength both at 25 °C and 100 °C.

In the cooked muffin, the lyophilization of the egg yolk granules produced a reduction in the hardness in comparison to the 100% granules recipe, and a crust color similar to that obtained using whole egg yolk. According to these results, the modifications induced in the granules by the lyophilization process and the subsequent reconstruction are positives, and a final muffin, more similar to that prepared using whole egg yolk, was obtained.

Table 6
Weight loss, height and crust and crumb color in baked muffins.

Crust	L^*	a^*	b^*	ΔE^*	Crumb	L^*	a^*	b^*	ΔE^*	Weight loss (g/100 g)	Height ($m \times 10^{-3}$)
Egg yolk	49.7 (1.03)a	20.7 (0.2)a	36.1 (1.3)a	0	Egg yolk	80.3 (0.2)a	4.56 (0.01)a	29.78 (0.15)a	0		
Granules 100%	52.6 (1.2)b	20.68 (0.34)a	38.5 (1.3)b	3.765	Granules 100%	82.5 (0.76)b	2.1 (0.02)b	23.87 (0.32)b	6.76	9.02a (0.55)	38.6bc (0.98)
Granules 60%	58.3 (0.8)c	18.9 (0.23)b	40.03 (0.65)c	9.625	Granules 60%	80.72 (0.35)a	2.45 (0.08)c	23 (0.185)c	7.11	9.02a (0.6)	37.83b (098)
Lyophilized	51.4 (1.5)d	20.3 (0.3)c	36.3 (1.3)a	1.75	Lyophilized	83.5 (0.110)c	2.26 (0.004)d	24 (0.04)b	7	9.73a (0.6)	40c (1.09)

Values are expressed as means ± SD. Different letters in the same column indicate significant differences ($P < 0.05$).

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4. Conclusions

The rheological behavior of the batter of a gluten free bakery product made using egg yolk granules resulted similar to that made using whole egg yolk. However, during the cooking process, the higher protein content and the reduction in lipids of this egg yolk fraction produced noticeable changes in the viscoelastic behavior of the batter due to the interactions promoted by these granular components. This effect was translated to the cooked muffin in form of a bakery product which exhibits more hardness and some color differences in comparison to the whole egg yolk recipe. Acceptability test performed with consumers should be developed to know if these differences in hardness are negatively perceived.

Finally, is also remarkable the fact that the obtainment of egg yolk granules for food applications will demand new research in order to develop other new applications for the residual egg yolk plasma as well.

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6.2. ESTUDIO DE LOS EFECTOS FÍSICOS INDUCIDOS POR LAS FRACCIONES DE YEMA DE HUEVO EN UN PRODUCTO DE HORNEADO. CORRELACIÓN ENTRE LOS PARÁMETROS OBTENIDOS

STUDY OF RHEOLOGICAL, TEXTURAL AND OTHER PHYSICAL EFFECTS INDUCED BY THE EGG YOLK FRACCTIONS IN A BAKERY PRODUCT. CORRELATION BETWEEN THE PARAMETERS OBTAINED

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ABSTRACT.

With the aim of widening the knowledge about the structural changes induced by the egg yolk granules components and their relevance on baked goods, rheological and other physical parameters have been studied in a real food system and correlated in function of the proportion of plasma/granules added in each case.

For this purpose, the whole egg yolk content was progressively substituted by its high protein-content granular fraction in a muffin recipe until obtaining a 100% granular recipe. In this research, five formulas, which correspond to different plasma/granules ratios, were elaborated. Furthermore, another formula with 100% granules and containing mono- and diglycerides of fatty acids (E471) was tested too.

Flow curves at 25°C and mechanical spectra at 90°C were obtained for each substitution in order to evaluate the effect of the granules on the structure of the batter. In addition, other physical parameters of the cooked muffins, such as the hardness, were determined. The effects of the granular fraction on the aeration of the batter and on the cooked muffins were also assessed by means of image analysis.

The progressive addition of granular proteins resulted in a non-lineal increase in the consistence coefficient, the strength of the interactions and the hardness of the baked muffins, particularly from a plasma/granules ratio lower than 0.75. The addition of emulsifiers reverted the effects observed.

1. Introduction

Egg yolk is a key ingredient in many food products, such as, for example, in sweet bakery formulations. These bakery products have a high content in fats, which require the addition of ingredients that act as emulsifiers, being this one of the main features of the egg yolk (Mine, 2002). Another functional property of the egg yolk is the ability of its proteins to coagulate forming gels that may affect the texture and other qualities of cakes (Paraskevopoulou &

Kiosseoglou, 1997). Furthermore, the egg yolk influence on others cake parameters as colour, flavour and appearance is well known.

Egg yolk can be easily separated in two fractions, the plasmatic and the granular ones. The former represents around 75-81% of egg yolk solids (Le Denmat, Anton, & Beaumal, 2000). It is constituted mostly by low density lipoproteins (LDL) and its content in lipids and cholesterol is high. The second fraction, the granular one, is constituted mainly by high density lipoproteins (HDL). These HDLs provides 70% of the granular content (McCully, Mok, & Common, 1962), and they are forming aggregates together and with other granular proteins through phosphocalcium bridges. This fraction presents high protein content and low levels of cholesterol. Furthermore, it shows functional properties that could allow its use as a whole egg yolk substitute in foods, like mayonnaises (Laca, Sáenz, Paredes, & Díaz, 2010), with a cholesterol content lowering effect.

In previous works the total substitution of the egg yolk by its granular fraction in a gluten-free muffin recipe was studied (Marcet, Paredes, & Díaz, 2014). However, how the different parameters in function of different plasma/granules ratios vary, and therefore to what extend these muffin parameters are affected for each sub-fraction remain unstudied. Hence, the aim of this work is to study, in a muffin recipe used as an egg-based bakery product model, the effect of different plasma/granules ratios on the structure of the batter and the cooked product. Furthermore, the study of a muffin recipe made with 100% egg yolk granules and with food additives (E471), to reverse the effect of the egg yolk granules on the physical properties of the batter and of the muffins, was performed too. Finally, the rheological and other physical parameters were modeled for a better understanding of the described phenomena

2. Materials and methods

2.1. egg yolk granules Obtaining

According to (Laca, Sáenz, Paredes, & Díaz, 2010), egg yolk and albumen were manually separated and the albumen residuals were eliminated from the yolk employing a blotting paper. Egg yolk material was mixed with water (1:1.5 v/v) and the pH of the diluted egg yolk was adjusted to 7 using NaOH (1N). Then, it was kept overnight at 4^{o}C and centrifuged later at 10000 x g for 45 min to separate it into plasma and granules fractions.

2.2. Muffins preparation.

Egg yolk muffins were elaborated according to a traditional muffin recipe. The basic formulation includes 100g of wheat flour containing 10.32% of proteins and 1.2% of lipids; 3g of baking powder containing disodium diphosphate (E-450i), sodium bicarbonate (E-550ii), sodium carbonate (E-500i) and calcium sulfate (E-516); 65g liquid pasteurized egg white; 35g of fresh egg yolk; 100g of refined sunflower oil and 100g of sugar. All the ingredients were acquired from a local market.

Egg yolk and egg white were whipped for 3 minutes employing a 180 WATT hand blender (Morphy Richards HB01 Hand blender, UK) at maximum speed. Sugar and oil were added and

mixed for 2.5 minutes with a 200 WATT mixer (Severin eletrogeräte, Germany) adjusting the speed control at level 3. Finally, flour and baking powder were added and mixed for 1.5 minutes using the 200 WATT mixer too.

Six different muffins were then elaborated. As it is shown in Table 1, in each muffin the whole egg yolk (35g) was progressively substituted by the granular fraction, which represent different plasma/granules ratios. Furthermore, in the F preparation 2.5g of the emulsifier E471 (mono- and diglycerides of fatty acids) were added. This emulsifier is a food grade additive and it is used in the industry to improve the mix of the ingredients and the tender feature of the bakery products.

Table 1. Percentage of egg yolk, granules and emulsifier added in each muffin recipe.

	A	B	C	D	E	F
Egg Yolk % (w/w)	100	75	50	25	X	X
Granules % (w/w)	X	25	50	75	100	100
Plasma/granules ratio	3.5	1.6	0.75	0.3	0	0
Diacilglicerol (g)	X	X	X	X	X	2.5

2.3. Specific gravity (SG) and image analysis of the batter.

The specific gravity of each sample was calculated in duplicate as follows. A standard container was filled with batter and its weight was recorded and divided by the weight of the same container filled with water. Regarding the image analysis of the batter, a method similar to that of (Gómez, Ruiz, & Oliete, 2011) was carried out. An amount of fresh batter was disposed on a microscope slide and a cover slip was used to create a thin layer of preparation. To maintain the same thickness in each case, two paperclips were used between the slips. The micrographs of the batters were obtained using a light microscope Olympus BX50 with 10x magnification. Bubbles number was calculated in a surface of 4 mm^2 of raw batter using the ImageJ software. Furthermore, bubbles were distributed in three groups in function of their size.

2.4. Rheological properties of batter mixes.

Batters were kept for 60 minutes at 25^{o}C before the rheological test. Rheological properties of batters were determined employing a Haake MARS II rotational rheometer using a Peltier unit to control the temperature. A plate/plate measuring system (PP60) was used with a gap of 1 mm where samples were left for 25 min to relax stress and stabilize the temperature. The excess of sample was removed, and a glass hood and silicone oil were employed to protect

against dehydration along experimental time in each measure. Rheological measures were calculated in duplicate of two different batches.

2.4.1. Flow properties.

Flow properties were measured at 25°C. Apparent viscosity was obtained as a function of shear rate from 0.01 1/s to 100.0 1/s. The experimental time was adjusted to 300 seconds. 100 points were collected with a logarithmic distribution and two flow curves of different batches of every formulation were obtained. Duplicates presented differences lower than 10%. Data obtained were adjusted to Ostwald model (Martinez-Cervera, Salvador, Muguerza, Moulay, & Fiszman, 2011)

$$\eta = K\ \dot{\gamma}^{n-1} \qquad (1)$$

Where η is the apparent viscosity, $\dot{\gamma}$ is the shear rate, K (Pa.s^n) is the consistency coefficient, and n is the flow behavior index.

2.4.2. Dynamic tests.

Frequency dependence tests were conducted at 90°C in the range of linear viscoelasticity. Frequency range tested was from 10.0 Hz to 0.01 Hz. The experimental data obtained were adjusted to the following power law equation according to (Gabriele, de Cindio, & D'Antona, 2001).

$$G^* = A \cdot \omega^{1/z} \qquad (2)$$

Where G^* is the complex modulus in Pa. The complex modulus is a measure of the overall resistance of the batter to deformation, ω the frequency in Hz, Z (dimensionless) the coordination number and A the proportional coefficient (Pa).

2.5. Physical measurements of baked muffins.

Physical measurements were made on 8 muffins from 2 different batches.

2.5.1. Height, weight and middle section area of baked muffins.

Baked muffins height was calculated from the highest point to the bottom employing a caliper, after 3 hours of cooling at room temperature. Weight loss in percentage was calculated weighting the muffins before and after baking at room temperature. To calculate the middle section area the higher half was removed and the muffin diameter was measured at the mold level. The area of the muffin surface at the cut level was calculated.

2.5.2. Air cells number determination.

Muffins were cut at the mold level, the higher half was retired and an image was obtained of the crumb using a flatbed scanner (Model HP PSC 1610, Hewlett Packard, USA). Air cell number was calculated in a 4 cm side square surface by image analysis using the ImageJ software.

2.5.3. Texture profile analysis (TPA).

Texture analysis was performed with a TA.XT.plus Texture Analyser (Stable Microsystems, UK). Two centimeters of the lower half of each muffin was evaluated, the upper halve was discarded. Texture parameters were set at 1.0 mm/s, and a double compression of 50% of the original height was carried out with a flat-ended cylindrical probe (P/75). The parameters obtained from the TPA graph were hardness, springiness, cohesiviness, chewiness and resilience.

Hardness is the force necessary to produce a given deformation. It was calculated as the maximum value in the first compression cycle. Springiness is the recuperation rate of a deformation after the applied force has been removed, and it was calculated as the distance until the detected height in the second compression divided by that in the first bite. The cohesiviness parameter shows how can be deformed the material before it ruptures. Cohesiveness value was obtained dividing the total area of the second compression cycle by the total area of the first compression cycle. Chewiness is the energy required to disrupt a solid material until it is ready for swallowing. It was calculated as the product of the hardness and the springiness. Finally, resilience is the initial effort of a product to recover its original position. It was measured dividing the area during the withdrawal by the area of the first compression.

2.6. Statistical analysis.

Analysis of variance (ANOVA) was applied. Least significant differences (LSD) were calculated by Fisher´s test to determine significant differences among the tested samples. These analyses were performed using a statistical software (Statgraphics® v.15.2.06). Fittings of the experimental data to models were carried out using the Micromath Scientist® Software.

3. Results and discussion

3.1. Raw batter

3.1.1. Flow curves, specific gravity, batter image analysis

Flow curves centered in the power-law region (shear rate from 0.01 to 100 1/s) are shown in Figure 1. In this Figure it can be observed how the viscosity decreased within the shear rate range for each formulation, which denotes the pseudoplastic behavior of all the batters. Furthermore, differences among the samples were detected when the flow curves were fitted

to the Ostwald model. All the data were successfully fitted (R^2>0.99), and the consistency coefficient (K) and the flow behavior index (n) obtained are showed in Table 2. The flow behavior index (n) is related to the degree of structuring of the sample; values close to 1 are a feature of newtonian fluids, while lower values are related to more structured samples. In the batters tested, no significant statistical differences were detected between the flow behavior index values, showing that all the samples had similar entangled structures and resistances to flow.

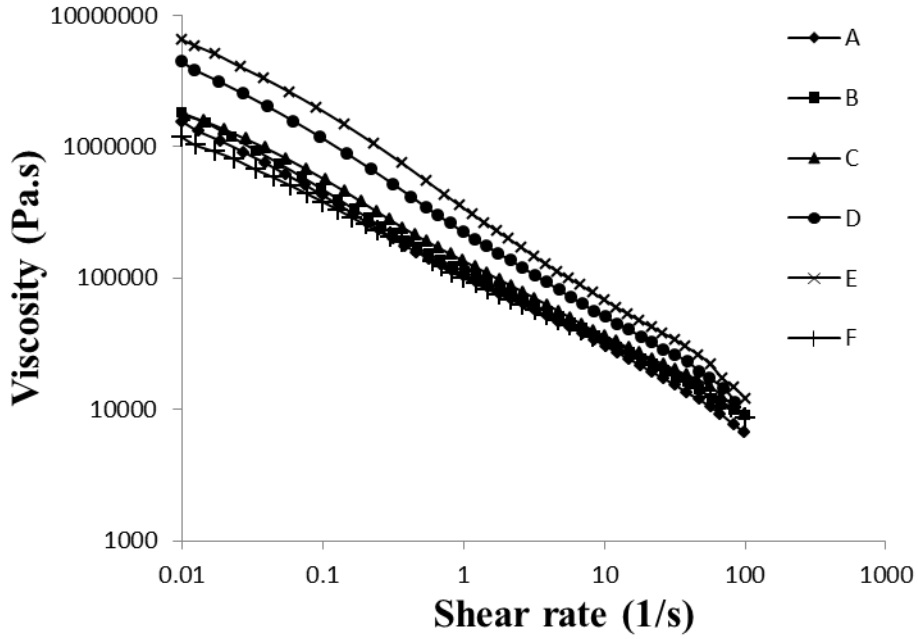


Figure 1. Plots of batter viscosity with different percentage of egg yolk substitution vs the shear rate at 25°C.

Table 2. Consistency coefficient (K) and flow behavior index (n) calculated from Figure 1 and specific gravity (SG) of each batter.

	A	B	C	D	E	F
K (pa.s^n)	111000 (700)a	118000 (3550)a	170000 (13742)b	275000 (24500)c	448000 (17600)d	108000 (1815)a
n	0.42 (0.007)a	0.405 (0.09)a	0.445 (0.036)a	0.4 (0.042)a	0.405 (0.01)a	0.47 (0.01)a
SG	0.907 (0.008)a	0.908 (0.004)a	0.946 (0.012)b	0.993 (0.011)c	1.005 (0.009)c	0.92 (0.004)d

Regarding the consistency coefficient (K), this parameter is related to viscosity, since increments in K values correspond to increments in the apparent viscosity (Rao & Kenny, 1975). In this assay, the increase in the K values associated to the progressive addition of granules, was produced, at least partially, by protein interactions that could be increasing the viscosity in the batter system, since structural associations are related to increments in this parameter (Damodaran, 1997; Meyers, 1989).

In other works about fat and flour replacements, the decrease in the apparent viscosity with the variations in the formulations has been associated to a more consistent crumb and a lower cake size (Baixauli, Sanz, Salvador, & Fiszman, 2008; Lakshminarayan, Rathinam, & KrishnaRau, 2006). This is probably because low viscosities correspond to unstable batters which encourage the buoyancy of the bubbles and their release to the surrounding atmosphere. In the tested formulations it was observed that the higher the egg yolk substitution the higher the K values were, which could be a positive indicative about the improvement of the capacity of the batter to retain the air during the cooking when the plasma/granules ratios are low(Tan, Chin, Yusof, Taip, & Abdullah, 2014).

Regarding the specific gravity data showed in Table 2, it is related to the capacity of air retention of the batters during mixing of the ingredients, with high values indicating poor air retention. This decrease in the air retention of the batters with the percentage of substitution was confirmed by image analysis (Figure 2). In the muffin batters, the incorporation of bubbles is promoted by the batter whisk during the mix of ingredients. Thus, the bubbles size and their quantities depend on the mixing time and energy. Furthermore, egg proteins have an important role in the foam formation and stabilization, since yolk lipoproteins align themselves in the interface (Cauvain & Young, 2008). In Figure 2, it can be observed that the reduction in whole egg yolk content and its substitution by granules resulted in a gradual decrease in the amount of bubbles (Table 3), particularly the smallest ones. The smallest bubbles in the raw batter provide nucleating sites for the CO_2 produced by the bakery powder, and after the starch gelatinization and protein denaturation, they give rise to the porous crumb. On the other hand, the biggest bubbles are more susceptible to coalescence phenomena, and they tend to disappear during the cooking. Therefore, the reduction in the population of the smallest bubbles should have some effects in the muffin texture and size. The emulsifying properties of the granular and plasmatic fraction have been broadly described by other authors (Anton & Gandemer, 1997; Anton, Martinet, Dalgalarrondo, Beaumal, David-Briand, & Rabesona, 2003; Le Denmat, Anton, & Beaumal, 2000; Martinet, Saulnier, Beaumal, Courthaudon, & Anton, 2003) being those higher in the plasmatic fraction than in the granular one. This fact corroborates the lower amount of bubbles found in the 100% egg yolk substitution formulation, in which the plasmatic fraction was completely removed. As can be observed in Table 3, this effect was neutralized by the addition of the high-emulsifier mono- and diglycerides (F preparation). Emulsifiers are largely used in the bakery industry because they provide aeration and gas bubble stability until the starch gelatinization (Turabi, Sumnu, & Sahin, 2008; Zhou, Faubion, & Walker, 2011).

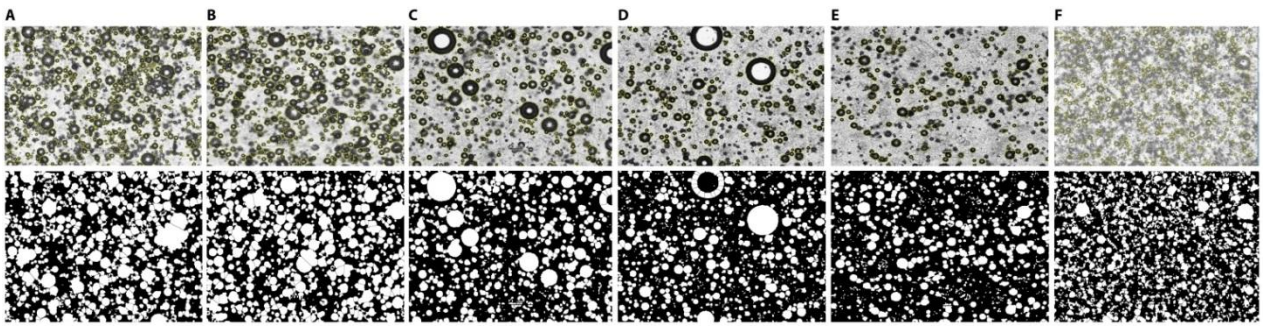


Figure 2. Batters images taken using confocal microscopy and analyzed by software.

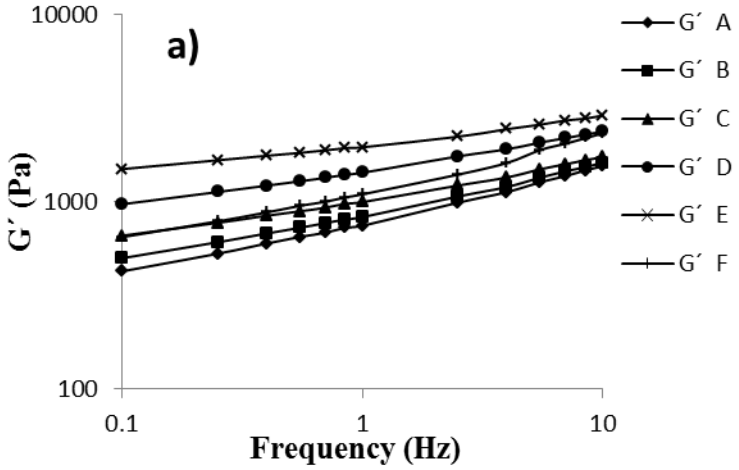
Table 3. Bubble size distribution and quantification in each batter calculated from images presented in Figure 2.

	Nº 0-4($10^3 \mu m^2$)	Nº 4-8($10^3 \mu m^2$)	Nº >8($10^3 \mu m^2$)	Total
A	237(24)a	39(8.2)a	5(2)a	297(23)a
B	198(18)b	65(5.4)b	7(3.4)a	280(21)b
C	135(19)c	44(6.4)a	6(2.5)a	192(18)c
D	106(9.4)d	32 (3.2)c	5(2.4)a	142(15)d
E	96(13)d	29(9.3)c	5(2)a	137(14)d
F	212(20)ab	48(5)a	4(1.2)a	282(35)ab

3.1.2. Mechanical spectra at 90°C

Stress sweeps at 90°C were carried out previously to verify the lineal viscoelastic range of the batters (not shown).

When the batter is heated in the oven above the starch gelatinization onset temperature, its rheological properties suffer great changes. In these conditions the batter constituents, mainly the flour starch and proteins, as well as the egg proteins, give rise to an increment in the viscosity, with a paste being formed. Therefore, the interactions among biopolymers at high temperatures are different than those observed for temperatures lower than the gelatinization one. For this reason, and in order to evaluate the rheological effect of the granular lipoproteins on the structure of the baked muffins, mechanical spectra were obtained at 90°C (Figure 3).



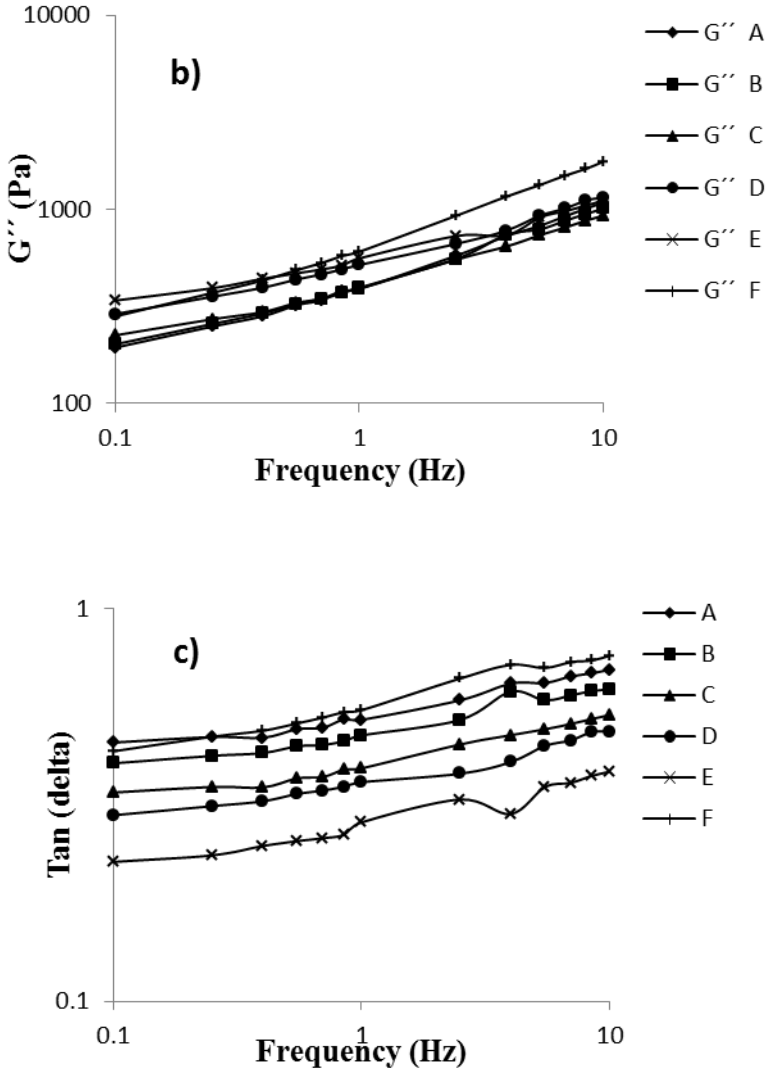


Figure 3. Mechanical spectra at 90°C of the tested batters. Storage modulus (3a) loss modulus (3b) and loss tangent values (3c) are plotted.

In Figure 3a) and 3b), within the frequency range from 0.1 to 10 Hz the batter behavior corresponds to a soft-gel, with a slight frequency dependence of G´and G´´, and tan δ values are higher than 0.1. In addition, in the frequency range studied it was observed that values for G´ were higher than for G´´ for all the formulations . This behavior is typical of cross-linked polymer networks. The substitution of egg yolk by granules causes an increase in the compacting degree, which results in a stronger and more structured batter. In fact, the highest viscoelastic modulus belongs to E preparation while the no substitution recipe (A preparation) showed the lowest degree of structuring. So, the results revealed a positive effect of the granules, since a batter with a high viscosity and elasticity at high temperatures is commonly associated to a better baking process. In this sense, a lower structure degree has been associated with the batter collapse during cooking (Sahin, 2008; Shelke, Faubion, & Hoseney, 1990).

Loss tangent values show the relative contributions of the elastic and viscous moduli to the batters characteristics (Figure 3c). In the tested formulations, the progressive substitution of egg yolk led to a reduction in tan δ values, and therefore, to increase the relative importance of the elastic moduli over the viscous one. This confirms the structuring effect of the granular

protein, approaching the system behavior to that found in solids (tan δ = 0). Accordingly, the substitution changed the viscoelastic behavior of the batters.

Data obtained from the mechanical spectra at 90°C were adjusted to equation 2 and presented in Table 4. The A parameter indicates the strength of the interactions in the network of the soft-gel, whereas Z value is related with the number of rheological units.

Table 4. Soft-gel model applied on data obtained from mechanical spectra at 90°C

	A	B	C	D	E	F
A(Pa)	884(49)a	960(42)ab	1028(51)b	1607(82)c	2008(125)d	1137(87)b
Z	3.34(0.22)a	3.55(0.01)ab	4.57(0.34)bc	4.84(0.11)c	6.68(0.36)d	3.42(0.05)a

The progressive substitution caused an increase in the strength of the interaction in the batters (A) as well as an increase in the network extension (Z). At 90°C the gelatinization of the egg yolk protein and the starch is expected. However, the gelatinization characteristics of the emulsions using whole egg yolk are different of those obtained using granules in their formulations: the use of the granular fraction produces a strengthening of the heated-emulsion gels compared with those elaborated with whole egg yolk only, since the granular lipoproteins are behaving as active fillers, taking them the hydrophobic/hydrophilic interface and interacting with the proteins of the food matrix. This fact was confirmed by the results of the F preparation, in which the addition of mono and diglycerides of fatty acids resulted in a reduction in the strength of the interactions with regarding to those values found using the whole egg yolk formula. This reduction in the strength is probably due to a displacement of the granular lipoproteins from the interface, taking this place the mono- and diglycerides molecules and, therefore, avoiding the granular protein interactions. The active filler behavior of the egg yolk granules has been described by other authors in more simple oil in water emulsions (Anton, Le Denmat, Beaumal, & Pilet, 2001). However, in a complex food matrix, these interactions between proteins could be eased by the presence of other ingredients. As can be observed in Table 4, it was required a significant substitution of the whole egg yolk by granules in order to obtain an important increment in the strength of the interactions (preparation D and E).

3.2. Baked muffins

3.2.1. Physical properties and image analysis

Physical measurements of baked muffins are presented in Table 5. As regards the muffins height, it incremented as the egg yolk substitution was higher. This means that the 100% granules recipe (E preparation) was the highest one, while the 100% egg yolk (A preparation) was the smallest one. This increase in the height was statistically significant, although the difference between the highest and the lowest ones is around of 6 mm, being barely perceptible to the eye. Regarding the surface area of the central section of the muffin, the granules addition produced a decrease in this parameter. This fact can be explained as a crumb compaction.

To confirm this latter, crumb images were taken and they are shown in Figure 4. Images from preparation A to E showed changes in the distribution of the air cells that can be visually appreciated particularly from preparation C to E. To characterize the air cell population in each formulation, software image analyses were carried out (Table 5). As regards the most numerous smallest air cells, they were progressively reduced when the granules content increased, which caused a reduction in the air cells total number too. This effect was more marked for the smaller air cells, which were initially the most numerous, whereas the changes on the other distributions were less noticeable. In this sense, the decrease in the smaller bubbles have undesirable effects on the textural parameters of the bakery products (Bennion, Bent, & Bamford, 1997).

These results are in accordance with the bubble size distribution in raw batter showed in section 3.1.1. In conclusion, the high addition of granules from egg yolk produced muffins with less middle section surface area, more compact crumb and likely with a harder texture. As in previous analyses, the addition of emulsifiers (F preparation) again produced variations in the measured parameters, giving values of the height, middle section area and air cells distribution closer to those found in the A and B preparations (Table 5).

Table 5. Physical properties of baked muffins. Height, middle-section area and air cells distribution.

	Height (mm)	**Middle-section area (mm^2)**	**Air-cells number, mean area (mm^2)**				
			Nº<1	Nº 1-4	Nº 4-7	Nº 7-25	TOTAL
A	37(0.81)a	3981(123)a	688(17)a	40(7)a	4.6(2.7)ab	3(1.2)a	736(17)a
B	36.4(1.27)a	3855(132)b	625(42)b	39(9)a	4(2.3)a	3.3(1.7)a	654(55)b
C	40.1(0.92)b	3666(93)c	549(28)c	47(6)ab	4.5(2)a	6.2(2.2)b	616(17)b
D	42.2(0.6)c	3356(84)d	473(44)d	46(12)ab	4.6(2)a	10.6(1.5)c	534(38)c
E	43(1.3)c	3094(74)e	484(40)d	51(4)b	7.8(1)b	6.5(1.9)b	532(42)c
F	35(0.6)	3811(34)	653(53)	45(9)ab	6(1.2)ab	2.3(1.2)a	707(45)ab

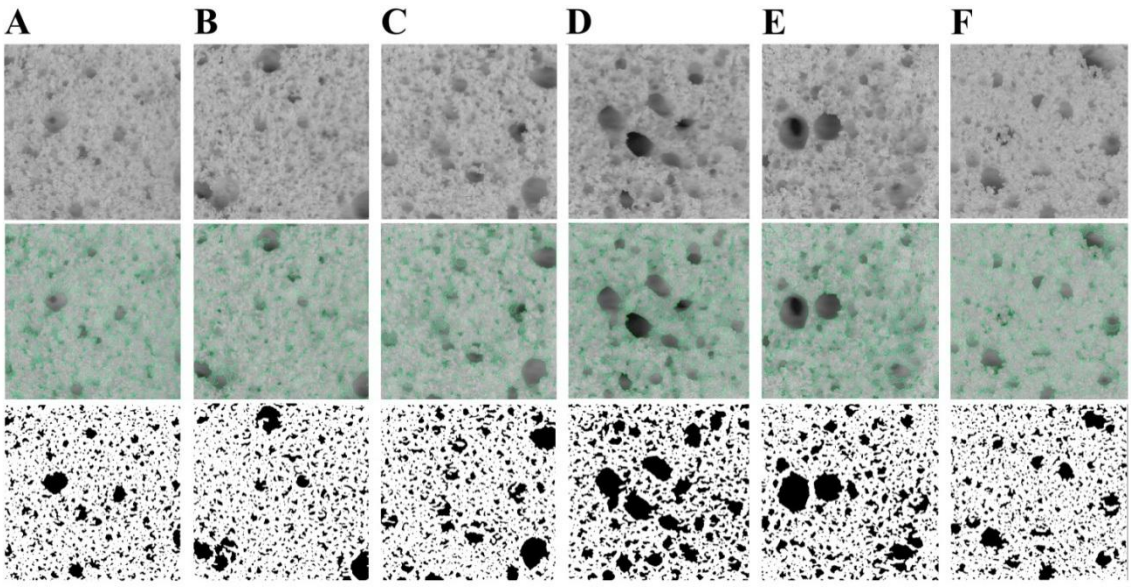


Figure 4. Digital images of muffins, with different levels of egg yolk substitution, analyzed by software.

3.2.2. Texture profile analysis

The texture profile analyses of baked muffins are shown in Table 6. As can be observed, the addition of granules from egg yolk produced a large increment in the muffin hardness, specifically in the E preparation, where the hardness was more than twice the value obtained for A preparation. In the preparation of gluten-free muffins, this textural parameter can be increased or decreased in function of the protein source used in the recipe (Matos, Sanz, & Rosell, 2014), so in this case, the hardening of the muffins is an ability that can be associated with the increase in the granular lipoprotein amount from the recipe A to E. Furthermore, the hardness parameter is affected by the degree of aeration of the cooked batter too (Handleman, Conn, & Lyons, 1961), and the increase in the granular lipoproteins together the decrease in the plasma lipoproteins are affecting the aeration of the muffins, as has been shown in Figure 2 and 4. Regarding chewiness, this parameter increased with increments in the granules substitution, as it happens with the hardness parameter.

For springiness, the variations were not lineal respect the egg yolk replacement. However, the maximum value corresponded to the E preparation.

As regards the cohesiveness parameter, the addition of granules from C to E preparation made the muffins structure weaker than the 100% and 75% egg yolk recipe (A and B preparation).

Finally, the resilience behavior was similar to that found for cohesiveness parameters. In general, the granules substitution favors a decrease in this value respect the A preparation.

In broad terms, the progressive substitution of egg yolk by granules caused little changes on the textural parameters evaluated, except in the case of hardness and chewiness ones. Thus,

with high levels of substitution (C preparation and more) the muffins were harder and more difficult to chew.

Table 6. TPA parameters of baked muffins with different levels of egg yolk substitution.

	Hardness	Springiness	Cohesiveness	Chewiness	Resilience
A	1018 (77)a	0.865 (0.017)a	0.648 (0.017)ac	570 (31.5)a	0.239 (0.01)a
B	1100 (65)a	0.863 (0.016)b	0.654 (0.008)a	620.8 (31)a	0.236 (0.04)a
C	1319 (66)b	0.844 (0.015)c	0.602 (0.022)b	669 (12)a	0.213 (0.012)b
D	1707 (152)c	0.852 (0.009)d	0.635 (0.018)a	951 (90)b	0.225 (0.011)ab
E	2394 (143)d	0.87 (0.028)e	0.634 (0.011)c	1320 (103)c	0.223 (0.009)b
F	1073 (33)a	0.89 (0.03)cd	0.656 (0.005)a	627 (23)a	0.25 (0.003)d

3.3. Modelling effects of the plasma/granules ratio on the physical parameters of the raw batter and of the cooked muffins

According to Figure 5, the progressive substitution of the egg yolk by the granular fraction causes an increase in the total protein content in the formula. This increment is mainly due to granular protein (HDL mainly), while the plasma protein content (LDL mainly) is reduced. In addition, the progressive substitution produces a decrease in the total lipid content. In fact, in the formula A, the amount of lipids provided by the egg yolk is 71% of the total egg yolk dry matter (lipids of the granular and the plasma fraction) whereas, this amount of lipids is decreased to 42% of the dry matter in the formula E and it is provided only by the granular fraction. These variations in the total protein and lipid content probably are the main responsible of the changes on the different parameters observed in previous sections for both, raw batter and cooked muffin.

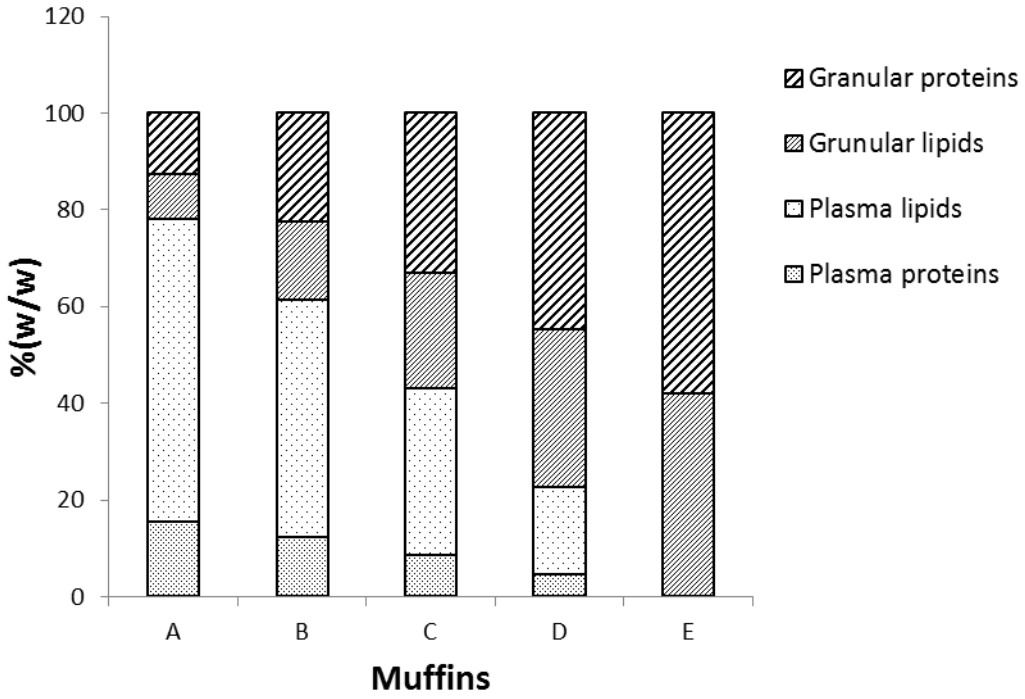


Figure 5. In the egg yolk ingredient, the percentage of proteins and lipids from granules and plasma for each muffin recipe.

In order to modelling the effect of the plasma and granules on the different parameters, models according to equation 3 (if the parameter increases with the amount of granules) or to equation 4 (if the parameter decreases) have been proposed.

$$\frac{Y_{i,100\%\,substitution}-Y_i}{Y_{i,100\%\,substitution}-Y_{i,egg\,yolk}}=\frac{X_i}{C+X_i} \qquad (3)$$

$$\frac{Y_i-Y_{i,100\%\,substitution}}{Y_{i,egg\,yolk}-Y_{i,100\%\,substitution}}=\frac{X_i}{C+X_i} \qquad (4)$$

Being Y_i the parameters obtained in sections 3.1 and 3.2 and detailed in Table 7; X_i the plasma(g)/granules(g) ratio and C the constant value calculated in each case.

In the case of the raw batter, the strength of the interactions (A) and the consistence index (K) values were successfully fitted according to the equation 3 in function of the ratio plasma/granules in each formula. The results obtained from these equations and the experimental values obtained are shown in Figure 6 and 7. According to Figure 6, in the muffin A, the proportion of plasma/granules is 3.6, and when it decreases to 1.5 the K and A values only increased a 10%. In a same way, with a proportion of 0.75, the values of these parameters increased a 20% of the initial values. However, for plasma/granules ratios lower than 0.75, little increments in the amount of the granular fraction involved great changes in the consistence index and in the strength of interactions values.

The bubbles in the batter (b_i) can be adjusted in function of the amount of plasma and granules too, using in this case the equation 4. The number of the bubbles in the raw batter change quickly when the ratio plasma/granules is lower than 1.6.

In a similar way, the height and hardness of the muffins (h and H respectively) values adjust to the equation 3. In the case of the hardness (H, Figure 7) its behavior is similar to that found for the K and A parameters. However, the height of the muffins is also affected by the plasma/granules ratio of 1.6 in a similar way to the number of bubbles in the raw batter. In this case, it must be taken into account that the maximum variation in the height was only of around 6 mm, so the effect of the substitution on the height is less marked.

The middle section surface area (M) and the number of bubbles in the cooked muffin (b_f) were also successfully fitted to the equation 4. As can be observed in Figure 7, the theoretical muffin height and middle section surface area vary in a similar way, which denotes the changes in the muffin shape when the degree of substitution progress until the 100% granules recipe. In regards to the number of bubbles in the cooked muffin (b_f), it varies in all the range plasma/granules tested, and the behavior of the reduction in the bubble number is similar to that of the b_i parameter. However, from 3.6 (recipe A) to 0.3 (recipe D) plasma/granules ratio, the 53% of the bubbles in batter were lost, and this reduction was of 27% in the case of the cooked batter. This means that the air retention of the granules during the mixing of ingredients is low, although its capacity of air retention is higher than in the case of the plasma.

Table 7 shows the values of C obtained in the fitting and the regression coefficients. It can be easily deduced from equation 3 and 4 that the lower the C value, the higher the effect of the degree of substitution on the parameter. According to this, the parameters most seriously affected by the presence of granules in the formulation were, in descending order, the consistence index (K), the hardness (H) and the interactions strength (A). On the other hand, the effect of formulation was less marked on the number of bubbles of the raw and cooked batter.

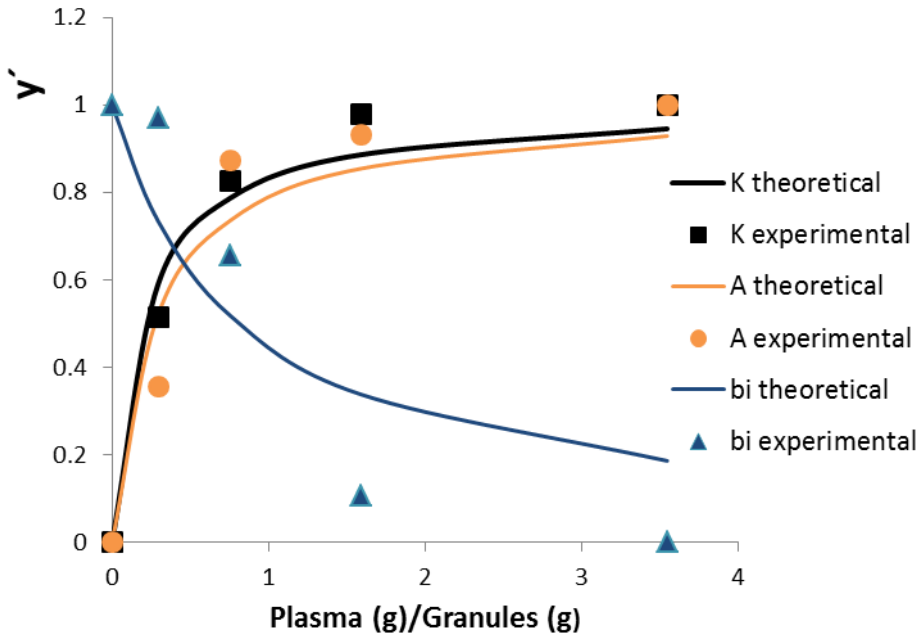


Figure 6. Experimental and theoretical values obtained from the batter.

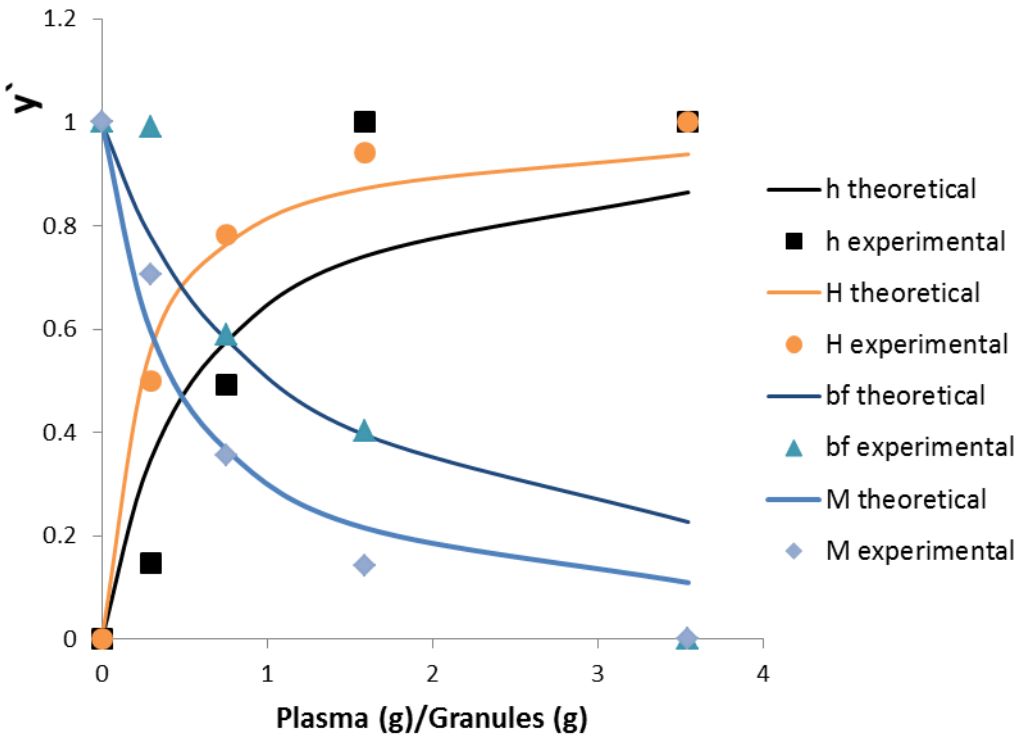


Figure 7. Experimental and theoretical values obtained from the cooked muffins.

Furthermore, in order to obtain a deeper knowledge of the effect of proteins and lipids on the parameters, the parameters calculated in the raw batter and in the cooked muffins were successfully fitted in function of the ratio granular protein/lipids and plasma protein/lipids according to equation 5. The values of the constants J and B in each case are shown in Table 7. These J and B values reveal the weighted effect on the parameter Y_i of the proteins from the plasma and the granules, respectively. That is, a high J value indicates that a gram of proteins from granules have a more intense effect on the parameter than a gram of proteins from plasma, and vice versa. The progressive substitutions tested in this work involve in each step the reduction of a little amount of plasma protein and the addition of larger amount of granular protein. Keeping this in mind, it is possible to find equations where the coefficient J is lower than the coefficient B and the parameter Y_i increases with the degree of substitution, because the amount of granular protein added offsets the low value of its coefficient. This can be observed in the cases of the number of bubbles in the raw batter (b_i), the middle section area (M), the height of the muffins (h) and the bubbles in the baked muffins (b_f). Furthermore, there are other parameters with similar coefficients J and B, such as the strength of interactions (A), and the hardness (H). In these cases, the effect of the granular protein is more evident, since the amount of granular protein added is not offset by its coefficient, and its relevance have to be greater than that of the plasma protein. Finally, in the case of the consistence index (K), the coefficient J is greater than the coefficient B, so the granular protein resulted with a higher ability to affect this parameter in comparison with the plasma protein.

$$Y_i = J\left(\frac{Gp(g)}{Gl(g)+Ll(g)}\right) + B\left(\frac{Pp(g)}{Gl(g)+Ll(g)}\right) \quad (5)$$

Table 7. Fitting parameters obtained from equation 3, 4 and 5.

Y_i	C	r^2		J	B	r^2
$K(Pa \cdot s^n)$	0.204	0.99		312630	114547	0.99
A(Pa)	0.27	0.98		1454	2501	0.99
b_i	0.812	0.93		78	1222	0.98
h(mm)	0.55	0.94		31.8	143	0.99
H(N)	0.233	0.99		1693	2890	0.99
$M(mm^2)$	0.43	0.99		2210	16391	0.99
b_f	1.04	0.94		360	2895	0.99

4. Conclusions

The progressive substitution of the plasmatic fraction by the granular one, produces a non-lineal variation in the rheological and textural parameter values of muffins. Therefore, it is possible a substitution of 50% of the whole egg yolk by egg yolk granules in bakery products without significant changes in the physical properties and with an average increment of 4 mm in the muffin height. This also supposes a reduction by half in cholesterol content. Furthermore, it is possible to determine the degree of substitution and to know with precision the variations produced in each case using the equations presented in this work. Regarding these physical changes produced by the substitution on the muffins obtained, they are due likely to the active filler behavior of the granular lipoprotein, which induce the compacting of the batter during cooking. The addition of mono- and diglycerides of fatty acids in the 100% granules recipe reverses all the changes induced by the granules fraction.

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7. CONCLUSIONES

CONCLUSIONES

1. El grado de hidrólisis que se obtiene cuando se tratan los gránulos de yema de huevo disueltos en agua salada (NaCl) con tripsina durante 90 minutos fue del 12%, observándose en el hidrolizado la permanencia de péptidos de gran tamaño. La elaboración de mayonesa con este hidrolizado resulta en un producto más estable a los cambios de temperatura y con menos resistencia a fluir que la misma receta elaborada con gránulos no hidrolizados. En este sentido, el comportamiento de la mayonesa elaborada con gránulos hidrolizados es más similar a una mayonesa comercial tomada como referencia.
2. La proteína de gránulos de yema de huevo delipidada y no hidrolizada resulta altamente insoluble. Mediante la hidrólisis enzimática con tripsina durante 6 horas se consigue recuperar el 50% de la proteína granular en forma de péptidos solubles. Utilizando hidrólisis con agua en condiciones subcríticas incorporando presión con nitrógeno se recupera el 95% de la proteína granular y el tiempo de reacción disminuye a 4 horas. Utilizando oxígeno en lugar de nitrógeno el tiempo disminuye aún más. Los hidrolizados obtenidos enzimáticamente y mediante hidrólisis en condiciones subcríticas con corriente de nitrógeno presentan ligeras diferencias en cuanto a sus propiedades espumantes y emulsionantes.
3. Las propiedades antioxidantes de los péptidos obtenidos mediante hidrólisis en condiciones subcríticas con corriente de nitrógeno resultan superiores en comparación con el hidrolizado con tripsina. El hidrolizado con tripsina sin embargo mantenía unas propiedades quelantes de hierro superiores. Las capacidades antioxidantes del hidrolizado obtenido mediante hidrólisis con agua en condiciones subcríticas y usando una corriente de oxígeno fueron muy disminuidas en comparación con los otros hidrolizados, probablemente debido a variaciones en la estructura primaria de la proteína.
4. La fosvitina puede ser separada de los gránulos disolviéndolos en agua salada (NaCl) y ajustando el pH. En el proceso de separación los factores importantes a tener en cuenta son la concentración de NaCl y el pH final que se va a alcanzar. De esta manera se obtiene una fosvitina que se mantiene soluble. La desalación del medio se puede llevar a cabo por ultrafiltración o por diálisis, en este último caso, el pH al cual se lleva a cabo la operación resulta determinante para mantener el rendimiento o disminuirlo pero aumentar la pureza.
5. Elaborando un producto de horneado, al sustituir toda la yema de huevo por gránulos la reología de la masa cruda no se ve alterada antes del cocinado. Sin embargo durante el cocinado, la reducción en el contenido de lípidos y el aumento de proteína granular afecta a las cualidades viscoelásticas de la masa. El resultado es una madalena más dura y con algunas diferencias en el color con respecto al mismo producto elaborado con yema de huevo.
6. Cuando elaborando el mismo producto de horneado se tienen en cuenta diferentes proporciones de fracción plasmática/granular, siempre y cuando dicha proporción sea igual o mayor a 0.75, se observa como los diferentes parámetros reológicos y texturales se mantienen muy similares a aquellos observados con el mismo producto elaborado sólo con yema. Además, es posible desarrollar ecuaciones empíricas que describan el comportamiento reológico de la masa de madalenas y la dureza del

producto cocinado en función de la cantidad relativa de plasma y gránulos. En cualquier caso, si se retira toda la yema de huevo y se sustituye completamente por gránulos, la compensación de la receta añadiendo nono- y diglicéridos de ácidos grasos devuelve los parámetros evaluados a los valores encontrados en el producto elaborado con 100% yema de huevo.

8. APENDICES

A continuación se incluye el artículo “IgY isolation from a watery by-product obtained from an egg yolk fractionation process”, elaborado durante el trabajo de laboratorio e incluido en la Tesis de la Dra. Amanda Laca (“Fraccionamiento y aprovechamiento de la yema de huevo”) y que se ha referenciado varias veces en el cuerpo principal del presente documento.

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Short communication

IgY isolation from a watery by-product obtained from an egg yolk fractionation process

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ABSTRACT

The possibility of recovering IgY from a watery by-product produced during an egg yolk fractionation process was evaluated. The protocol employed for the extraction of IgY was the polyethylene glycol precipitation method. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to confirm the presence of IgY at the different steps of the IgY purification process. Finally, the amount of IgY obtained was quantified by means of anion exchange chromatography. Native egg yolk was employed as IgY reference source, and results showed that the by-product could be at least as suitable as egg yolk as an IgY source. Additionally, the use of the by-product as a source of biotechnological compounds, such as IgY, leads to an increase in the value added during the egg yolk fractionation process.

Keywords: IgY; SDS-PAGE; Egg yolk; By-product; PEG; Anionic exchange chromatography

1. Introduction

Egg is broadly recognised as containing many substances with biological functions that extend beyond basic nutrition (López-Fandiño et al., 2007). Yolk, particularly, represents a major source of active principles which may be used in the medical, pharmaceutical, cosmetic and biotechnological industries (Anton, 2007).

Egg yolk is a so-called liquid emulsion of proteins and lipids, consisting of 48% water, 34% lipids and 17% protein (Li-Chan and Kim, 2008). Specifically, livetins constitute 10% of egg yolk dry-matter (Anton, 2007). This is a relatively non-homogeneous protein fraction including α-, β- and γ-livetin (Schade and Chacana, 2007) and the IgY is the predominant and also the most important fraction of γ-livetin (Kovacs-Nolan et al., 2005). Thus, hen eggs are presently considered as a potential source for large-scale production of IgY (Li-Chan and Kim, 2008).

IgY technology is a highly innovative and expanding branch of biotechnology which offers many advantages: it is produced by a non-invasive method which does not cause pain to animals or lead to their death, since it is based on the simple act of collecting eggs. Hens cost less to keep than rabbits and, furthermore, the IgY production of a hen corresponds to that of a large mammal (Schade et al., 2007). IgY is successfully used in immunochemistry for the detection of antigens of viral, bacterial, plant and animal origin, to assess the incidence of intestinal parasites in domestic animals (Schiniering et al., 1996) and the contamination of foods with toxins or drugs (Pichler et al., 1998). During the past decade, IgY has increasingly been used in therapy or disease prophylaxis as well as in the context of the so-called functional foods (Schade et al., 2007).

In general, IgY extraction methods can be divided into three groups: precipitation methods, chromatographic methods and ultrafiltration (Schade and Chacana, 2007). All these methods have in common that the IgY source is native egg yolk, so as IgY is the only compound recovered, many egg yolk compounds are wasted.

An egg yolk fractionation process reported recently (Laca et al., 2010a) allows two fractions to be obtained which are suitable for use as potential ingredients in foods (Laca et al., 2010b)

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and cosmetics (Laca et al., 2010a). However, in addition to these two fractions, a watery by-product also originates from this fractionation process and no application has been developed until now for this by-product. Thus, one way of increasing the added value of this fractionation process is to use the obtained by-product as a source of different compounds for biotechnological purposes. As IgY is a water-soluble protein (Li-Chan and Kim, 2008), the aim of this work was to evaluate the possibility of recovering IgY from this by-product. Since it is well known (Schade and Chacana, 2007) that Polson's polyethylene glycol precipitation method (Polson et al., 1985) is a commonly used and very effective protocol for IgY isolation, this method was adapted to purify IgY from the watery by-product. SDS-PAGE was carried out to confirm the presence of IgY at the different steps of the isolation process. Finally, the amount of IgY purified was quantified by means of anion exchange chromatography. Native egg yolk was also assessed as source of IgY as a reference to evaluate the success of IgY isolation from the by-product.

2. Materials and methods

2.1. *Fractionation of egg yolk*

The fractionation method was conducted according to Laca et al. (2010a). Egg yolks were prepared from fresh eggs and the shelling of the eggs and the separation of the yolk from the albumen were performed manually. The albumen residues were eliminated from the yolk using blotting paper, and the removal of the vitelline membrane was achieved using tweezers. Next, the egg yolk material was mixed with water (1:1.5, v/v). Then the pH of the diluted egg yolk was adjusted to 7 by the addition of NaOH (1N) and it was kept overnight at 4 °C before being centrifuged at 4 °C and 10,000 × *g* for 45 min to separate plasma (supernatant) and granules (fraction 1).

Sodium alginate 1% (w/v) solution was added to the plasma to achieve a final concentration of alginate of 0.1% (w/v). This mixture was centrifuged at 20 °C and 10,000 × *g* for 15 min to obtain a lipidic paste (fraction 2) and a watery by-product.

2.2. *Isolation of the IgY*

The isolation of the IgY was conducted according to Polson et al. (1985).

Firstly, as an IgY reference source, native egg yolk was employed to isolate IgY. Egg yolk was separated from white, pooled and mixed well, and then 10 mL of yolk were taken from the pool and processed as follows. Two volumes (20 mL) of 0.1 M Tris–HCl buffer, pH 7.6 (Trizma® Hydrocloryde, Sigma) was added to the yolk and mixed thoroughly. After that, 3.5% (w/v) of polyethylene glycol (PEG6000) was added and mixed until the PEG was completely dissolved. The mixture was centrifuged at 4500 × *g* for 20 min. The precipitate was discarded and the supernatant was filtered through a cotton wool plug placed in a funnel. Aliquot 1 was removed from this filtrate and then the PEG concentration of this sample was increased to 12% by adding 8.5% (w/v) PEG. The PEG was completely dissolved and the mixture was centrifuged at 12,000 × *g* for

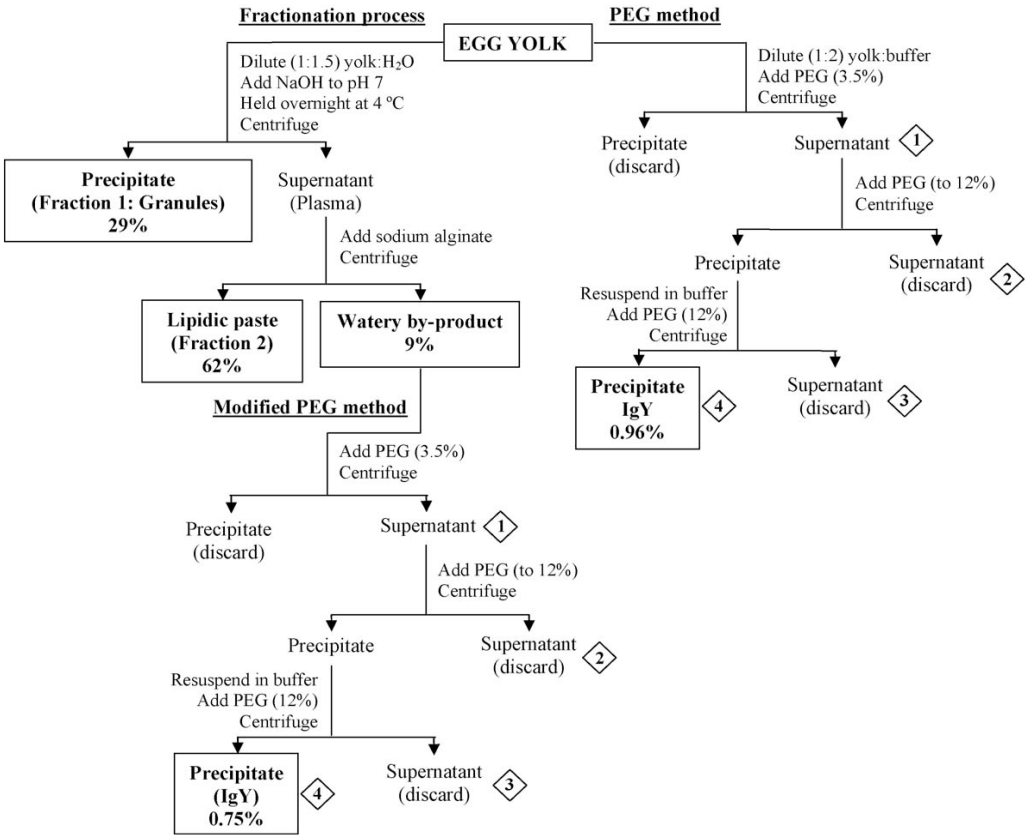

Fig. 1 – A general outline of IgY isolation from the watery by-product (left) and from egg yolk (right). Numbers in rhombi correspond with aliquots taken at different steps of the purification method. Percentages are on dry egg yolk basis.

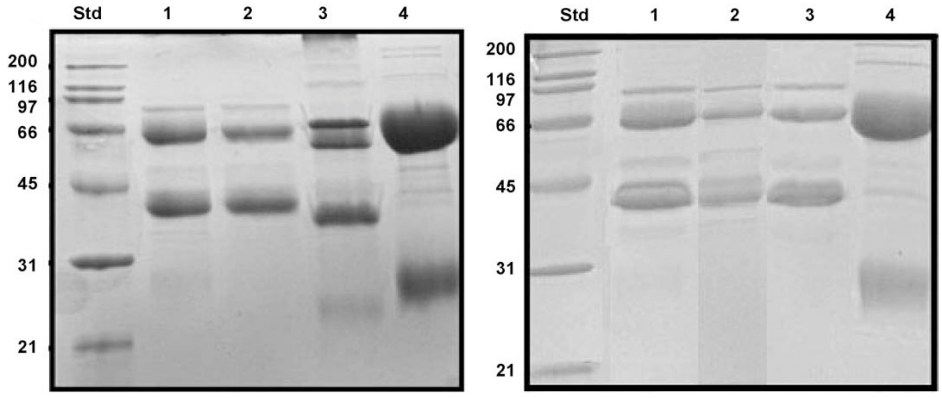

Fig. 2 – SDS-PAGE of aliquots obtained from the watery by-product (left) and from egg yolk (right); lane 1, Aliquot 1; lane 2, Aliquot 2; lane 3, Aliquot 3; lane 4, Aliquot 4. All the aliquots, except 3, were diluted 1:15 (v/v). Std, molecular weight standards (kDa).

10 min. Aliquot 2 was taken from this supernatant and the pellet was resuspended in 30 mL of Tris–HCl buffer and, again, 12% (w/v) of PEG was added, mixed thoroughly, and centrifuged at 12,000 × *g* for 10 min. Aliquot 3 was removed from this supernatant and the final pellet was resuspended in 2 mL Tris–HCl buffer and aliquot 4 was taken from this final dilution.

The watery by-product was treated in a similar way to the process described above. An amount of 3.5% (w/v) PEG was added and mixed thoroughly with an initial volume of 30 mL of watery by-product. After that, the method was the same as described for egg yolk.

A general outline of these procedures is shown in Fig. 1.

2.3. *Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

The presence of IgY at the different steps of the isolation method was assessed by means of SDS-PAGE. The aliquots were diluted 3:1 (v/v) in a dissociation buffer consisting of a 0.5 M Tris–HCl pH 6.8, 0.05% bromophenol blue, 35% glycerol, 5% β-mercaptoethanol, 8% (w/v) SDS solution, and heated in boiling water for 5 min. Electrophoresis was carried out on polyacrylamide gels (stacking: 3.5% and resolving: 12%) with a migration buffer consisting of a 0.02 M Tris(hydroxymethyl)aminomethane, glycine 5 M, SDS (w/v) 0.1% solution. The proteins were stained with the usual staining procedure (Coomassie blue 0.1%, methanol 50%, acetic acid 10% and water 40%). The gels were destained in a solution containing acetic acid (10%), methanol (40%) and water (50%). SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad) were used as protein standards.

2.4. *Anion exchange chromatography*

Chromatographic analyses were performed on an ÄKTA FPLC apparatus (Amersham Biosciences). After several tests with different resins to determine the best option, an anionic resin, Ceramic HyperD® Q (Life Technologies, Inc.) was employed to quantify IgY. The column volume was of 5 mL and the flow employed was 1 mL/min.

Before being charged with protein, the column was equilibrated with 5 volumes of 0.1 M glycine–NaOH solution, pH 9.6 (buffer A). The sample was then charged and, finally, the retained protein was eluted by an increasing gradient of 1 M NaCl 0.1 M glycine–NaOH solution, pH 9.6 (buffer B). The gradient was conducted in two steps: first step at 35% of the buffer B and second step at 100% of this buffer.

The performance of the anionic resin was first tested using a solution (1 mg/mL) of γ-globulins (99% purity; 80% IgG, 10% IgM and 10% IgA) (Sigma) in buffer A. The amount of IgY was calculated in relation to the peak area obtained for this standard protein using the ÄKTA Unicorn 5.10 Software.

The final aliquots obtained from the polyethylene glycol method were diluted 40 times before being injected in the FPLC apparatus. All analyses were conducted at least in duplicate.

3. Results and discussion

3.1. *IgY determination*

The SDS-polyacrylamide gel electrophoresis of aliquots obtained using egg yolk as source of IgY is shown in Fig. 2 (right). In agreement with Le Denmat et al. (2000) and as can be seen in the figure, in lanes 1, 2 and 3 a band of approximately 83 KDa, which possibly corresponds to the α-livetins, appears.

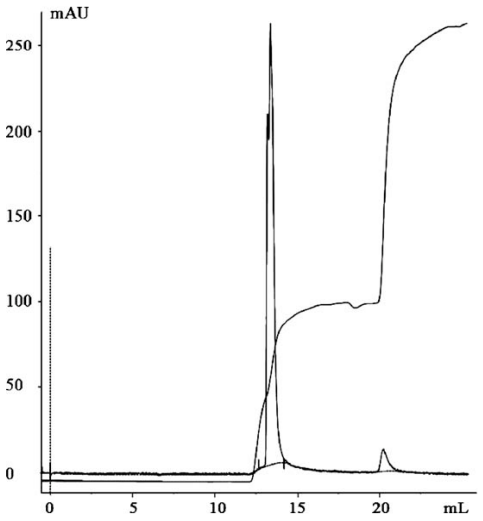

Fig. 3 – Chromatogram of γ-globulins dilution used as standard (1 mg/mL). Line over the peak represents the conductivity evolution.

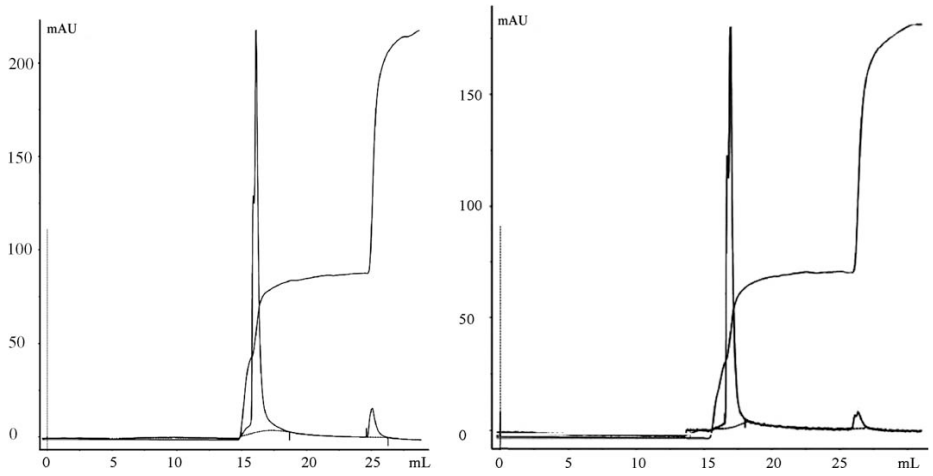

Fig. 4 – Chromatogram of aliquot 4 obtained from the watery by-product (left) and from egg yolk (right). Line over the peak represents the conductivity evolution.

In these lanes also can be observed a band around 38–40 KDa which corresponds to the β-livetins. Neither α-livetins, nor β-livetins clearly appear in the final aliquot as can be observed in lane 4. Nevertheless, between 60 and 70 KDa, a band corresponding to the γ-livetins is evident in lanes 1, 2, 3 and 4. Specifically, and in agreement with results reported by Schade and Chacana (2007), in lane 4 there clearly appear two bands, which correspond to the heavy chain of the IgY (65 kDa) and to the light one (less stained and with a size of 25 kDa). It is also important to remark that the last supernatant protein profile (lane 3) is nearly identical to that reported by Akita and Nakai (1993) for IgY purification from egg yolk by the PEG method.

As can be seen in Fig. 2 (left), the protein profile of by-product aliquots is very similar to that shown by egg yolk, so the PEG method seems to be a suitable protocol for purifying IgY from the by-product.

3.2. *IgY quantification*

Firstly, chromatograms of 1 mg/mL γ-globulins used as standard were obtained: an example is shown in Fig. 3. Aliquots 4 obtained from egg yolk and from watery by-product using the polyethylene glycol method were also analysed by FPLC and an example of each is shown in Fig. 4. As can be seen, and as was also shown by SDS-PAGE, chromatographic profiles of the samples from egg yolk and watery by-product were very similar.

Taking the γ-globulin chromatograms as reference, recoverable IgY corresponds to 4.8 ± 0.8 mg/mL in egg yolk and 2.5 ± 0.2 mg/mL in the by-product. This amount of IgY recovered from egg yolk agrees with Polson's procedure results reported by other authors (Akita and Nakai, 1993; Bizhanov and Vyshniauskis, 2003).

In agreement with the results mentioned above, since approximately 150 mL of residual watery fraction are obtained from 120 mL of egg yolk in the fractionation process, a maximum amount of IgY of 375 mg could be isolated from this volume of the by-product; while a maximum amount of 576 mg can be isolated from 120 mL of egg yolk. Thus, the amount of IgY recovered from the by-product is approximately 65% the quantity that can be isolated from egg yolk. The amounts of IgY recovered using egg yolk and watery by-product are compared in Fig. 1.

In addition, results show that in the dry extract of the watery by-product the IgY is much more concentrated (as a proportion of the total dry-matter) than in the dry extract of egg yolk. The dry extract content of the by-product is approximately 3% (w/w) (Laca et al., 2010a), so 8.3% of this dry extract could be recovered as IgY; while, since egg yolk contains approximately 50% solids (Anton, 2007), less than an 1% of this dry extract could be recovered as IgY.

To sum up, results show that this by-product could be as suitable an IgY source as egg yolk, while increasing the added value of the egg yolk fractionation process used in the study.

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