Universidad de Huelva

Departamento de Ingeniería Química, Química Física y Química Orgánica

Natural pigments and microalgal biomass in colloidal food systems : rheological characterization

Incorporación de pigmentos naturales y microalgas en sistemas coloidales alimentarios : caracterización del comportamiento reológico

Memoria para optar al grado de doctora
presentada por:

Ana Paula dos Santos Batista

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José María Franco Gómez
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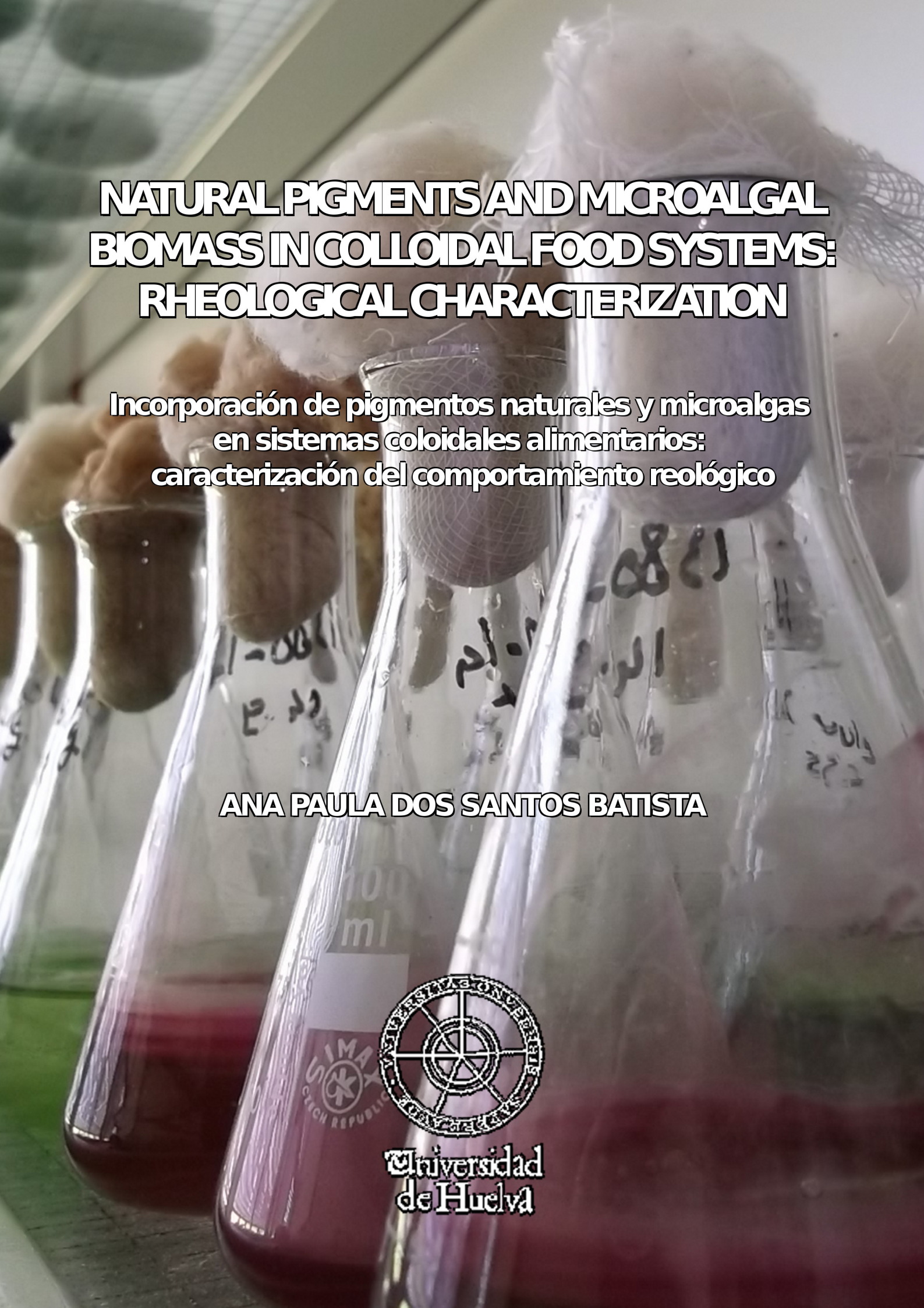

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ANA PAULA DOS SANTOS BATISTA

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Tesis Doctoral

Presentada por: Ana Paula dos Santos Batista

Dirigida por: Dr. José María Franco Gómez
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UNIVERSIDAD DE HUELVA
Departamento de Ingeniería Química, Química Física y Química Orgánica

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Memoria presentada por Ana Paula dos Santos Batista, licenciada en Ingeníeria Agro-Industrial, para aspirar al grado de Doctor en Ingeniería Química con Mención Europea por la Universidad de Huelva.

Fdo: Ana Paula dos Santos Batista

La presente Tesis ha sido realizada en el Departamento de Ingeniería Química, Química Física y Química Orgánica de la Universidad de Huelva, bajo la dirección del Dr. José María Franco Gómez y Dr.ª Anabela Cristina da Silva Naret Moreira Raymundo, los cuales autorizan su presentación:

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Incorporación de pigmentos naturales y microalgas en sistemas coloidales alimentarios: caracterización del comportamiento reológico

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La presente Tesis Doctoral ha recibido la ayuda de la *"Fundação para a Ciência e a Tecnologia"* del *"Ministério da Ciência e do Ensino Superior de Portugal"* mediante la concesión de la beca de doctorado SFRH/BD/21388/2005, y forma parte del proyecto de investigación PTDC/AGR-ALI/65926/2006. Se agradece por la financiación aportada.

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RESUMEN

Las microalgas representan una fuente alternativa e innovadora de ingredientes y colorantes naturales que pueden ser utilizados en el desarrollo de nuevos productos alimenticios. En las células de microalgas están naturalmente encapsulados compuestos biológicamente activos (ej.: carotenoides), capaces de resistir las severas condiciones implicadas en los procesos tecnológicos de la industria alimentaria. La adición de biomasa de microalgas puede también impartir algunas modificaciones microestructurales en el producto alimenticio, que se reflejan en cambios de textura y otras propiedades sensoriales.

El objetivo de esta tesis fue estudiar la incorporación de pigmentos naturales y biomasa de microalgas como agentes colorantes en sistemas coloidales alimentarios - emulsiones y geles. El impacto de estos colorantes naturales en los diferentes sistemas coloidales se estudió, además, desde un punto de vista reológico y su relación con las características microestructurales y físico-químicas. La incorporación de esto colorantes naturales y/o biomasa de microalgas, beneficiosos para la salud, resultó ser un procedimiento eficaz e innovador, que al mismo tiempo permite el desarrollo de productos con coloraciones originales y estables y un fortalecimiento microestructural del sistema coloidal, como se observa a través de su respuesta reológica.

Se llevó a cabo una caracterización de la composición de la biomasa de seis microalgas diferentes: *Spirulina maxima*, *Chlorella vulgaris* (verde y carotenogénica - naranja), *Haematococcus pluvialis* (carotenogénica), *Diacronema vlkianum* y *Isochrysis galbana* (*Sm*, *Cv*, *CVo*, *Hp*, *Dv* y *Ig*, respectivamente). Las principales diferencias en la composición de las diferentes biomasas están relacionadas con la inducción de procesos de carotenogénesis en *CVo* y *Hp*. Sin embargo, cada microalga presenta un perfil de nutrientes típico, que permite la selección de unas características físico-químicas para aplicaciones alimentarias específicas.

Se adicionaron pigmentos naturales, luteína y ficocianina, y biomasa de microalgas - *Sm*, *Cv*, *CVo* y *Hp* - a emulsiones estabilizadas con proteína de guisante. Luteína y ficocianina causaron efectos marcadamente diferentes, conforme a su afinidad por la fase acuosa u oleosa, respectivamente. La luteína modifica la naturaleza de la fase oleosa originando,

emulsiones con propiedades reológicas características de microestructuras más débiles, mientras que la ficocianina tiene una influencia significativa sobre la Reología de la emulsión, con parámetros reológicos que aumentan linealmente con su concentración. La adición de biomasa de microalgas fue beneficiosa en términos de permitir una reducción del contenido en aceite de las emulsiones, sin alterar sus propiedades reológicas y texturales. Estos resultados sugieren que las microalgas pueden utilizarse como agente mimético de grasa, además de las ventajas que presentan como agentes colorantes.

Los geles de proteína de guisante/κ-carragenato/almidón fueron utilizados para estudiar el impacto de la adición de biomasa de microalgas (*Sm*, *Cv*, *CVo*, *Hp* y *Dv*) y pigmentos naturales (astaxantina, β-caroteno, cantaxantina, luteína y ficocianina). Los geles obtenidos tienen colores interesantes, evidenciando además una buena resistencia térmica de los pigmentos naturales y ácidos grasos. La incorporación de biomasa de microalgas parece beneficiosa, especialmente de *Hp*, en cuanto que produce un fortalecimiento estructural de los geles, con propiedades reológicas mejoradas. Este efecto se ha relacionado con su alto contenido en grasa, teniendo en cuenta que las gotas de grasa pueden actuar como "fillers" activos incorporados en la matriz de gel. Por el contrario, *Sm* produce una drástica reducción en los parámetros reológicos de los geles. Esta alga parece inhibir el proceso de gelatinización del almidón, lo que podría estar relacionado con una competición por el agua por parte de las moléculas de proteínas *Sm* que impiden la hidratación de los gránulos de almidón. Las propiedades viscoelásticas lineales de los geles fueron altamente dependientes de las condiciones de gelificación, combinación de tiempos y temperaturas aplicadas y velocidades de calentamiento/enfriamiento. Para todos los sistemas microalga/biopolímero estudiados, se observó que proteínas y polisacáridos, solos o en combinaciones binarias, son los principales responsables de la formación de la estructura del gel y del comportamiento reológico resultante. Las microalgas parecen estar embebidas en la red de gel originando microestructuras más densas con mejores parámetros reológicos en la mayoría de los casos.

Palabras clave: microalgas; colorantes naturales; sistemas coloidales; emulsiones aceite-en-agua; geles; Reología; viscoelasticidad lineal.

ABSTRACT

Microalgae represent an alternative and innovative source of natural colouring ingredients that can be used for developing novel food products. Biologically active compounds (*e.g.* carotenoids) are naturally encapsulated within microalgal cells being able to resist harsh technological conditions involved in food technology processes. Microalgal biomass can also impart some microstructural modifications on the food product, reflected by texture and sensorial changes.

The aim of this thesis was to study the addition of natural pigments and microalgal biomass as colouring agents in model colloidal food systems – emulsions and gels. The impact of these natural colourings on different colloidal systems was studied through a rheological perspective *i.e.* by monitoring changes in the rheological behaviour, related to the microstructural and physicochemical characteristics. This proved to be an efficient and innovative procedure which simultaneously enables the development of products with original and stable colourations, via health-beneficent natural pigments, and microstructural reinforcement as observed through the rheological response of these colloidal systems.

The biomass profile of six microalgae was studied: *Spirulina maxima*, *Chlorella vulgaris* (green and after carotenogenesis - orange), *Haematococcus pluvialis* (carotenogenic), *Diacronema vlkianum* and *Isochrysis galbana* (*Sm*, *Cv*, *Cvo*, *Hp*, *Dv* and *Ig*, respectively). The major differences in physicochemical composition were found to be related to inducting carotenogenesis process in *Cvo* and *Hp*. Nevertheless, each microalga presented a typical biomass nutrient profile allowing the selection of desired physicochemical characteristics for specific food technology applications.

Pea protein-stabilized oil-in-water emulsions were coloured with natural pigments – lutein and phycocyanin – and microalgal biomass – *Sm*, *Cv*, *Cvo* and *Hp*. Lutein and phycocyanin imparted markedly different effects, according to the affinity to the aqueous or to the oil phase. Lutein addition modified the oil phase nature originating emulsions with rheological properties typical of much weaker microstructures, while phycocyanin exerted a significant influence on the emulsions rheology, with rheological parameters increasing linearly upon its concentration. The addition of microalgal biomass

was beneficial in terms of enabling lesser oil contents for the emulsions, without disturbing their rheological and textural properties. These results support the potential use of microalgae material as a fat mimetic besides the advantages as colouring agents.

Pea protein/κ-carrageenan/starch gels were used for studying the impact of adding microalgal biomass (*Sm*, *Cv*, *Cvo*, *Hp* and *Dv*) and natural pigments (astaxanthin, β-carotene, canthaxanthin, lutein and phycocyanin). Gels with interesting colours were attained also evidencing a good thermal resistance of natural pigments and fatty acids. Microalgal biomass incorporation seemed beneficial, especially for *Hp,* which promoted a structural reinforcement expressed by improved gel rheological properties. This could be related to its high fat content considering that fat droplets can act as active filler particles embedded in the gel matrix. However, *Sm* addition promoted a drastic reduction on the gels rheological parameters. This alga seems to inhibit the starch gelatinization process, which could be related to a competition for water binding sites by *Sm* protein molecules hindering starch granules hydration. Linear viscoelastic properties were highly dependent on gel setting conditions, i.e. time and temperature processing conditions and heating/cooling rates. For all the studied microalga/biopolymer systems, it was observed that protein and polysaccharide biopolymers, alone or in binary combinations, are the main responsible for the formation of the gel structure and resulting rheological behaviour. Microalgae seem to be embedded in the gel network originating denser microstructures with improved rheological parameters in most cases.

Keywords: microalgae; natural colourants; colloidal systems; oil-in-water emulsions; gels; rheology; linear viscoelasticity.

RESUMEN DE LA TESIS EN ESPAÑOL

I – Introducción

La industria alimentaria moderna actualmente tiende a un aumento de productos más baratos, sanos y aceptables por parte del consumidor. El uso de ingredientes naturales, como los ácidos grasos poliinsaturados (PUFA) y pigmentos antioxidantes, con un alto impacto en las propiedades funcionales, es importante a fin de reducir la incidencia de enfermedades crónicas, que son fuertemente considerados de importancia capital en Europa, donde los costos asociados al envejecimiento y bienestar de la población son cruciales para la gestión de los recursos públicos.

La apariencia de la comida es, entre otros, un factor que define la calidad y la primera impresión que el consumidor obtiene directamente de los alimentos. El color, como un aspecto de la apariencia, juega un papel importante en la aceptabilidad de un producto alimenticio, siendo determinante en su identificación, como un indicador de la calidad, frescura, estado de conservación, la expectativa de sabor y valor comercial. La consecución de un color adecuado, de acuerdo con las expectativas del consumidor sobre el producto, se logra generalmente mediante el uso de colorantes. En la industria alimentaria, estos compuestos se utilizan en productos alimenticios incoloros, así como en la compensación de la pérdida de color debido a las condiciones de procesado (por ejemplo, altas temperaturas y presiones).

Los colorantes sintéticos son percibidos por los consumidores como indeseables y perjudiciales, y algunos de ellos son considerados responsables de reacciones alérgicas o de intolerancia. En cuanto a estas preocupaciones, la Comisión Europea ha pedido a la EFSA (*European Food Safety Authority*) volver a evaluar todos los aditivos alimentarios autorizados en la actualidad en la Unión Europea, dando máxima prioridad a los 46 colorantes alimentarios que pueden ser legalmente añadidos a los alimentos de acuerdo con el Reglamento CE 94/36 / CE. Además, McCann y col. (2007) han publicado recientemente un documento, conocido como *"El estudio de Southampton"*, donde se llega a la conclusión de que la exposición a dos combinaciones de cuatro colores sintéticos (rojo allura AC, azorrubina, rojo Ponceau 4R, amarillo de quinoleína, amarillo

ocaso FCF y tartracina), además de un conservante (benzoato de sodio) en la dieta ha resultado en una mayor hiperactividad en niños con 3 y 8-9 años de edad.

Por consiguiente, existe una creciente demanda de fuentes naturales alternativas para colorantes de alimentos. Muchos pigmentos naturales, tales como los carotenoides y antocianinas, son fitoquímicos cuyos efectos nutracéuticos han sido resaltados, incluyendo la actividad antioxidante (Wilska-Jeszka, 2002). Por lo tanto, el uso de colorantes naturales puede ser una herramienta interesante en el desarrollo de nuevos alimentos funcionales, respondiendo a la creciente demanda del consumidor por productos alimenticios más naturales que presentan beneficios para la salud.

Las microalgas son una excelente fuente de pigmentos naturales, tales como los carotenoides, clorofilas y ficobiliproteínas, que presentan un gran potencial industrial para ser usados como agentes colorantes naturales. Estos organismos microscópicos pueden ser cultivados bajo ciertas condiciones controladas del medio ambiente (por ejemplo, temperatura, salinidad, luz, nutrientes) que pueden estimular o inhibir la biosíntesis y acumulación de compuestos bioactivos (por ejemplo, pigmentos) en grandes cantidades. La posibilidad, no sólo de cosecha, sino también su cultivo en condiciones diferentes, permite el uso de microalgas como reactores naturales a gran escala (Plaza *et al.*, 2009).

Además de colorear los productos alimenticios, la utilización de microalgas presenta otras ventajas nutricionales, ya que son ricas en antioxidantes y otros compuestos biológicamente activos como los ácidos grasos poliinsaturados de cadena larga – omega 3 (ω_3), que están naturalmente encapsulados dentro de las células de la biomasa. Actualmente, se piensa que los antioxidantes naturales, como los carotenoides, posiblemente previenen la formación de cánceres y enfermedades cardiovasculares o relacionadas con la edad, al proteger las células contra el daño oxidativo. Los ácidos grasos poliinsaturados (PUFA ω_3) pueden influir positivamente en la salud humana, especialmente en la prevención de enfermedades cardiovasculares y el cáncer, la reducción de colesterol en la sangre (Bønaa *et al.*, 1990), la arteriosclerosis (Thies, 2003), la diabetes, así como mejorar el correcto desarrollo y funcionamiento del cerebro y la retina (Connor, 2000; Crawford, 2000). Por lo general, estos compuestos son suministrados a través de algunos pescados y aceites obtenidos de éstos. Sin embargo, las existencias

mundiales de peces están disminuyendo debido a una pesca excesiva y a métodos de pesca excesivamente eficientes. Además, los aceites derivados del pescado a veces están contaminados con una variedad de metales pesados y toxinas. Así, las fuentes alternativas de ácidos grasos poliinsaturados son claramente deseables, y algunas microalgas, las cuales sintetizan estos ácidos grasos, son particularmente atractivas en este sentido.

Algunas especies de microalgas, como *Chlorella* y *Spirulina*, se han venido utilizado durante siglos como un alimento nutritivo en Asia, África y México. Sin embargo, la producción comercial a gran escala de microalgas sólo se inició en la década de 1960 (Japón) y, hoy en día, las microalgas son principalmente comercializados como complementos alimenticios, comúnmente vendidas en forma de tabletas, cápsulas, jarabes o bebidas. Además, hay un mercado cada vez más creciente de productos alimenticios que incluyen microalgas, tales como pastas, galletas, pan, bocadillos, dulces, yogures, bebidas, etc…, ya sea como suplemento alimenticio, o como fuentes de colorantes naturales de alimentos (Becker, 2004). En algunos países, como Alemania, Francia, Japón, USA, China o Tailandia, las empresas de producción y distribución de alimentos ya han comenzado seriamente a comercializar alimentos funcionales con microalgas y cianobacterias (Pulz y Gross, 2004). La explotación biotecnológica de los recursos de microalgas para la alimentación humana se limita a muy pocas especies, debido a las estrictas normas de seguridad alimentaria, factores comerciales, la demanda del mercado y procesamiento específico necesario (Pulz y Gross, 2004). Sin embargo, los alimentos suplementados con biomasa de microalgas pueden ser sensorialmente más convenientes y variados, combinando de este modo beneficios para la salud con el atractivo para los consumidores, por ejemplo, en términos de color. Así, cuando varias microalgas procesadas correctamente tienen un sabor agradable o picante podrían ser incorporadas en muchos tipos de alimentos, agregando no sólo el valor nutricional, sino también nuevos sabores, únicos y atractivos (Richmond, 2004).

Por otra parte, debido a la compleja composición fisicoquímica de las microalgas, se espera que la incorporación de biomasa de microalgas pueda impartir algunas modificaciones microestructurales en los alimentos, que pueden originar cambios de textura y en las propiedades sensoriales.

El objetivo de esta tesis doctoral es el estudio de la adición de pigmentos naturales y biomasa de microalgas como agentes colorantes en sistemas coloidales alimentarios - emulsiones y geles. Esta investigación forma parte de un proyecto multidisciplinar más amplio denominado "pigmentos, antioxidantes y ácidos grasos poliinsaturados en productos alimenticios a base de microalgas - implicaciones funcionales y estructurales" (PTDC/AGR-ALI/65926/2006). El impacto de la adición de microalgas y pigmentos naturales en diferentes sistemas coloidales se ha estudiado desde un punto de vista reológico, mediante el análisis de los cambios inducidos en el comportamiento reológico, así como su relación con sus características microestructurales y físico-químicas.

La mayoría de los productos alimenticios manufacturados son sistemas coloidales complejos resultantes de la combinación de numerosos componentes (por ejemplo, proteínas, carbohidratos y lípidos) organizados y dispuestos en complejas microestructuras de diversos tipos, tales como dispersiones, emulsiones, espumas, geles, etc. (Garti, 1999). La estabilidad y las propiedades estructurales de los sistemas coloidales no sólo dependen de las propiedades funcionales de los ingredientes individuales, sino también de la naturaleza y la fuerza de las interacciones entre ellos (Dickinson, 1995; Neirynck *et al.*, 2004). El uso de colorantes naturales y microalgas en los sistemas alimentarios está limitado por la naturaleza de la matriz del alimento en el que el pigmento se dispersa, y por las interacciones con otros componentes alimenticios (proteínas, polisacáridos, lípidos, azúcares, sales, etc.). El comportamiento reológico de estos sistemas refleja las modificaciones microestructurales que pueden resultar de la adición de estos colorantes naturales en alimentos tipo emulsiones y geles.

Se utilizó un aislado de proteína de guisante como base para formular emulsiones alimentarias y geles, basados en formulaciones optimizadas en estudios previos (por ejemplo, Raymundo *et al.*, 2002; Batista *et al.*, 2005; Nunes *et al.*, 2006). Esta elección está de acuerdo con la tendencia actual a sustituir productos de origen animal por otros vegetales, debido a la conciencia del consumidor sobre los beneficios de una dieta y estilo de vida saludables, junto con consideraciones éticas y medioambientales para la reducción de la explotación de recursos animales.

La tesis se divide en siete capítulos, incluyendo una introducción general (capítulo 1), una revisión de la literatura existente en esta temática (capítulo 2), las principales

conclusiones (capítulo 6) y referencias bibliográficas (capítulo 7). El procedimiento experimental y la discusión de los resultados se dividen en tres grandes capítulos (3-5), que se resumen a continuación.

II – Caracterización físico-química de la biomasa de microalgas

Las microalgas son una fuente potencial de compuestos naturales que pueden ser utilizados como ingredientes funcionales. Hay un gran número de especies de microalgas disponibles, y el conocimiento de la composición química es obligatorio como un primer paso en una metodología de detección, ya que ayudará a distinguir y orientar compuestos valiosos como antioxidantes, ácidos grasos poliinsaturados etc…, hacia el diseño de nuevos productos con propiedades funcionales determinadas.

Como con cualquier planta superior, la composición química de las algas no es un factor constante intrínseco, sino que varía en un amplio intervalo. Los factores ambientales, tales como la temperatura, la salinidad, la iluminación, el valor de pH, contenido de minerales o suministro de CO_2, afectan a la fotosíntesis y a la productividad de la biomasa celular. Estos factores también influyen en el patrón, la vía y la actividad del metabolismo celular y en la composición química celular, lo que tiene consecuencias e implicaciones biotecnológicas con mucho alcance (Hu, 2004). La mayoría de los parámetros ambientales varían según la temporada, y los cambios en las condiciones ecológicas pueden estimular o inhibir la biosíntesis de varios nutrientes (Plaza *et al.*, 2009). Por lo tanto, las condiciones de cultivo pueden ser optimizadas con el fin de maximizar la producción de biomoléculas de interés. Puesto que las algas deben adaptarse rápidamente a nuevas condiciones ambientales para sobrevivir, producen una gran variedad de metabolitos secundarios biológicamente activos, con estructuras que no se pueden encontrar en otros organismos (Plaza *et al.*, 2009).

La adición de biomasa de microalgas a productos alimenticios es una herramienta interesante que permite proporcionar suplementos nutricionales con compuestos biológicamente activos (por ejemplo, los antioxidantes), además de proporcionar color. En consecuencia, la selección de especies de microalgas con perfiles nutricionales equilibrados es fundamental para el desarrollo de nuevos alimentos. Una caracterización físico-química detallada de las microalgas es una etapa esencial que permitirá decidir qué algas son más adecuadas para diferentes aplicaciones y propósitos.

El objetivo del capítulo 3 fue determinar la composición aproximada, de cinco cepas de microalgas: *Spirulina* (*Arthrospira*) *maxima*, *Chlorella vulgaris* (verde y carotenogénica), *Haematococcus pluvialis* (carotenogénica), *Diacronema vlkianum* e *Isochrysis galbana*. Esta caracterización físico-química servirá de base para la comprensión del efecto de la adición de biomasa de microalgas en los sistemas alimentarios coloidales que se presentan en los capítulos 4 (emulsiones) y 5 (geles).

Las microalgas seleccionadas han sido ampliamente cultivadas en LNEG (*Spirulina*, *Chlorella*, *Haematococcus*) y IPIMAR (*Diacronema* e *Isochrysis*) desde hace varios años. Estas microalgas han sido utilizadas principalmente para la alimentación animal y acuicultura, debido a su capacidad para acumular pigmentos (por ejemplo, carotenoides y ficobiliproteínas) y ácidos grasos omega-3 (por ejemplo, EPA y DHA).

Las diferentes muestras de microalgas liofilizadas fueron analizadas en términos de humedad, contenido de minerales, proteína, lípidos totales, perfil de ácidos grasos, hidratos de carbono y perfil de pigmentos (clorofilas, carotenoides, ficocianina). De los seis tipos de microalgas estudiados, las mayores diferencias en la composición se relacionan con la inducción del proceso de carotenogénesis en *Chlorella* (naranja) y *Haematococcus*. No obstante, cada microalga presentó un perfil de nutrientes en biomasa típico, permitiendo la selección de características físico-químicas deseadas para aplicaciones específicas en tecnología de alimentos.

Spirulina maxima es una cianobacteria, que se caracteriza por un alto contenido en proteína (44% p/p) y bajo contenido en grasa (4% p/p), aunque presenta una proporción interesante de ácido γ-linolénico (0,5 g/100g). También presenta un alto contenido mineral (31% p/p), rico en sodio y potasio (8,5 y 2,6% p/p, respectivamente). En cuanto al contenido en pigmentos, su característica principal es la producción de ficocianina (7% p/p), una ficobiliproteína de color azul, que es soluble en agua, y está asociada a varios efectos beneficiosos para la salud.

Chlorella vulgaris verde también presenta un alto contenido en proteínas (38% p/p) y bajo contenido en grasa (5% p/p), y también es rica en calcio (4,7% p/p). Sin embargo, tras un proceso de carotenogénesis, *Chlorella* se vuelve naranja debido a la

acumulación de pigmentos carotenoides, principalmente cantaxantina, junto con una disminución de clorofila. Como resultado de esto proceso se observa también un aumento del contenido en grasa (28% p/p) y una disminución del contenido total de proteína (12% p/p). Esto debe estar relacionado con el hecho de que, cuando se privan de nitrógeno, las células son incapaces de dividirse y acumulan grasa dentro de ellas. También se observa un mayor contenido de cenizas totales, principalmente debido a la adición de NaCl. A pesar de que *Chlorella* verde es ampliamente utilizada como ingrediente o suplemento alimentario, especialmente en los países asiáticos, la microalga carotenogénica todavía no está autorizada para el consumo. Recientes ensayos de toxicidad no han revelado mortalidad o signos clínicos relevantes, ni cambios de conducta en ratas y ratones alimentados con *Chlorella* carotenogénicas (Gouveia *et al.*, 2011), del mismo lote de alga que el utilizado en el presente trabajo.

Haematococcus pluvialis, tras un proceso carotenogénesis acumula hasta el 3,0% (p/p) de carotenoides, principalmente astaxantina esterificada con ácido oleico (18:1ω9). Esta alga presenta un contenido en grasas muy alto (41% p/p), así como un alto contenido en hidratos de carbono (34% p/p) y bajo contenido en proteínas (10% p/p), junto con concentraciones de microelementos interesantes, tales como hierro (823 mg/kg), cobre (344 mg/kg) y zinc (232 mg/100kg).

Diacronema vlkianum e *Isochrysis galbana* son microalgas marinas de la familia Haptophyceae, que están mucho menos estudiadas en términos de su aplicación en alimentación humana pero, sin embargo, son ampliamente utilizadas en la acuicultura. Al igual que las otras algas verdes estudiadas, tienen un alto contenido en proteínas (38-40% p/p), pero también un alto contenido en grasa (18-24% p/p), rica en ácidos grasos poliinsaturados de cadena larga – omega 3, especialmente EPA (3,2 a 49 g/100 g) y DHA (0,8 a 1,2 g/100 g). Estas algas son también ricas en carotenoides (3,2% p/p), principalmente fucoxanthina, lo que les da una coloración marrón. Esta interesante composición química está asociada a una acción antioxidante superior a la detectada en un estudio previo para *Chlorella vulgaris* (Batista *et al.*, 2010).

La descomposición térmica de estas microalgas fue determinada mediante análisis termogravimétrico (TGA). Esta técnica analiza el cambio en la masa de la muestra mientras se somete a una alteración de temperatura (Hemminger y Sarge, 1998). De esta

forma, es posible evaluar la resistencia térmica de las microalgas después de un calentamiento, lo que puede ser útil considerando algunas tecnologías utilizadas en los procesos tecnológicos alimentarios. Para las microalgas carotenogénicas, se observan temperaturas más altas (254-258°C) para el inicio de la degradación de la biomasa, así como una segunda zona característica de degradación térmica (350-500°C), estando la temperatura de máxima descomposición térmica en esta zona, T_{max2}, estrechamente relacionada con su alto contenido en lípidos. Esto indica que las microalgas carotenogénicas que tienen altos contenidos de grasa, parecen ser capaces de resistir mejor los tratamientos térmicos.

Se aplicó un método estadístico de análisis factorial (PCA) a 38 de los parámetros físico-químicos analizados para las seis microalgas objeto de estudio, con el fin de determinar las variables que mejor las definen y diferencian. Es posible agrupar y correlacionar varias variables en cinco componentes principales (factores), de los cuales, los tres primeros describen el 85% de la variabilidad de los resultados. Al mostrar las variables y las microalgas en un plano bidimensional definido por los factores 1 y 2, es posible visualizar que cada una de las microalgas está fuertemente correlacionada con un grupo específico de variables. Sin embargo, es posible distinguir las principales diferencias que surgen de las algas verdes y carotenogénicas (bajo factor 1), y que *Diacronema* e *Isochrysis* se diferencian claramente de las otras algas verdes.

III – Biomasa de microalgas y pigmentos naturales en emulsiones aceite-en-agua estabilizadas por proteína de guisante

Las emulsiones aceite-en-agua son la base de muchos tipos de alimentos, como por ejemplo las mayonesas, las cuales son una de las salsas más antiguas y más ampliamente consumida hoy día. Estas emulsiones pueden ser un vehículo eficiente para ingredientes funcionales (por ejemplo, carotenoides), que pueden ser encapsulados, ya sea en la fase oleosa o acuosa.

Los pigmentos naturales pueden ser utilizados como una alternativa a los colorantes sintéticos en la industria alimentaria, que se perciben como indeseables y perjudiciales, algunos de ellos considerados como responsables de reacciones alérgicas y de intolerancia. Por otro lado, los pigmentos más naturales son los fitoquímicos que se han

asociado a efectos nutracéuticos (acción antioxidante, por ejemplo), con beneficios adicionales para la salud, además de su actividad como colorantes.

La utilización de biomasa de microalgas como una fuente de colorante en los productos alimenticios presenta ventajas adicionales. No sólo la extracción de los pigmentos y los costes de purificación son eliminados, sino que se espera una estabilidad del color mejorada, ya que los pigmentos están encapsulados dentro de las células de las microalgas. Además de la acción colorante, las microalgas también pueden proporcionar beneficios adicionales a través del enriquecimiento de alimentos con otros compuestos biológicamente activos como los ácidos grasos poliinsaturados omega 3.

La introducción de colorantes naturales (pigmentos y biomasa de microalgas) en emulsiones de aceite-en-agua puede también cambiar la microestructura y la textura percibida, ya que el comportamiento de las emulsiones durante su producción, procesado y almacenamiento es altamente dependiente de la composición (Granger *et al.* 2003). Las propiedades físicas de las fases acuosa y oleosa pueden ser modificadas de acuerdo con la naturaleza y afinidades polares o no polares de los colorantes y, en función de que posean propiedades tensioactivas, las moléculas de pigmento puede estar preferentemente situadas en la interfase. Las interacciones de los colorantes con los emulsionantes en la superficie de las gotas de la emulsión, así como con los agentes estabilizantes en el medio continuo (Dickinson, 2003; Klinkerson *et al.*, 2004) pueden contribuir a un fortalecimiento de la estructura de la emulsión a través de la formación de entramados físicos (Clark *et al.*, 1992; Riscardo *et al.*, 2003). Sin embargo, si estos componentes son incompatibles, la emulsión puede ser menos estable, pudiendo originar una pérdida de viscoelasticidad superficial inducida por el desplazamiento de la proteína de la interface (Dickinson y Hong, 1995).

El objetivo del capítulo 4 fue estudiar el efecto de la incorporación de pigmentos naturales y biomasa de microalgas a emulsiones aceite-en-agua estabilizadas por proteína de guisante. Las emulsiones fueron caracterizadas en términos de color, textura, comportamiento reológico y distribución de tamaños de gotas.

- *Emulsiones coloreadas con luteína y ficocianina*

La luteína (3,3 '-dihidroxi-α-caroteno) es un carotenoide xantofila con dos grupos hidroxilo en la cadena de polieno conjugado (Alves-Rodrigues y Shao, 2004). La luteína se encuentra en verduras de hojas verdes como el brócoli y las espinacas y se extrae comercialmente de la flor de caléndula (*Tagetes erecta*) para la producción de colorantes de color naranja para la industria de alimentos y bebidas y la producción de suplementos dietéticos (Sowbhagya *et al.*, 2004). Estudios epidemiológicos proporcionan evidencias de que la luteína puede proteger contra la degeneración macular relacionada con la edad ("*Age Related Macular Degenerescence*" ARMD), una de las principales causas de ceguera en personas mayores de 65 años (Chong *et al.*, 2007), así como otras enfermedades oculares como las cataratas. Este efecto se relaciona con el hecho de que la luteína (y su estereoisomero zeaxantina) se deposita en el cristalino y la mácula lútea de la retina, actuando como filtro de luz azul que protege los tejidos subyacentes del daño fototóxico (Alves Rodrigues y Shao, 2004). También hay evidencias que sugieren que la luteína puede proteger contra enfermedades del corazón cardiovasculares y ciertos tipos de cáncer (por ejemplo, de mama), a través de su papel como antioxidante (Johnson, 2000).

Estudios recientes han investigado la estabilidad de emulsiones y microemulsiones enriquecidas con carotenoides, como el licopeno (Ribeiro *et al.*, 2003; Spernath *et al.*, 2002), la astaxantina (Ribeiro *et al.*, 2005; Wackerbarth *et al*, 2009), β-caroteno (Neves *et al.*, 2008; Yuan *et al.*, 2008) y luteína (Amar *et al.*, 2004; Losso *et al.*, 2005; Santipanichwong y Suphantarika, 2007). Estos sistemas pueden ser utilizados como vehículos para los carotenoides en aplicaciones alimentarias nutracéuticas. Todas las moléculas de carotenoides presentan un carácter hidrófobo y, por consiguiente, se disuelven en la fase oleosa, no polar, de la emulsión. No obstante, debe considerarse que la presencia de grupos polares hidroxilo en el extremo de algunas moléculas de carotenoides xantofilas, tales como luteína y astaxantina, puede proporcionar un cierto carácter polar (Khachatryan, 2003; Shibata *et al.*, 2001) y una potencial actividad interfacial, así como cierta capacidad para unirse a proteínas a través de enlaces de hidrógeno (Bassi *et al.*, 1993; Moros *et al.*, 2002).

La ficocianina es un pigmento fotosintético azul extraído de cianobacterias, con reconocida actividad antioxidante *in vitro* (Romay *et al.*, 2003). Es una ficobiliproteína

hidrófila compuesta por una apoproteína ligada a grupos prostéticos coloreados (cromóforos tetrapirrólicos). Las biliproteínas son altamente solubles en agua, lo que hace que su aplicación en sistemas no polares sea difícil. Para superar esta limitación, algunos estudios experimentales han puesto a prueba la incorporación de estos compuestos en sistemas multifásicos, tales como micelas inversas y microemulsiones (Bermejo *et al.*, 2000; 2003). Sin embargo, debido a su naturaleza proteica, es posible que la ficocianina presente alguna actividad superficial (Chronakis *et al.*, 2000).

En el presente estudio, luteína y ficocianina se añadieron al aceite y a la fase acuosa, respectivamente, de emulsiones aceite-en-agua estabilizadas por proteína de guisante (3% p/p aislado de proteína de guisante, 65% p/p aceite vegetal) a diversas concentraciones (0,00%, 0,25%, 0,50%, 0,75%, 1,00%, 1,25% p/p), así como en forma de mezclas de los dos pigmentos en diferentes proporciones (100L:0P, 75L:25P, 60L:40P, 50L:50P, 40L:60P, 25L:75P, 0L:100P, con una concentración total de pigmento de 0,50% p/p).

La estabilidad del color de las emulsiones se analizó (sistema CIEL*a*b*) a lo largo de cuatro semanas de almacenamiento. Las emulsiones con luteína presentaron tonalidades de amarillo a naranja. Las emulsiones con ficocianina tienen una coloración azul intenso, que, aunque inesperado en un producto alimenticio, es muy valioso teniendo en cuenta que los colorantes azules naturales estables son difíciles de encontrar. Por lo tanto, este estudio abre una nueva perspectiva de uso de ficocianina en sistemas coloidales del tipo emulsión.

La estabilidad del color de las emulsiones a lo largo del tiempo se midió a través del parámetro de diferencia de color total (ΔE^*). Para todas las emulsiones estudiadas, al comparar el color presentado en función del tiempo con respecto al color inicial, $\Delta E^* < 3$, lo cual está por debajo del umbral que el ojo humano es capaz de distinguir entre dos colores (Gonnet, 1998; Castellar *et al.*, 2006).

Además de su capacidad para colorear emulsiones, se ha desarrollado un amplio estudio sobre la caracterización reológica de estos sistemas, que implica el análisis de la textura, la viscoelasticidad lineal, el flujo en estado estacionario y el flujo transitorio (experimentos de crecimiento del esfuerzo). Asimismo, se realizó un seguimiento de los

ensayos reológicos con un sistema de análisis óptico, con el fin de interpretar las modificaciones estructurales inducidas por estos pigmentos naturales durante la cizalla. Estos resultados se correlacionan con las mediciones de distribución de tamaño de gotas de las emulsiones.

Los dos pigmentos, luteína y ficocianina, causaron efectos marcadamente diferentes, según su naturaleza química, en particular la afinidad por la fase acuosa (continua) o para la fase de aceite (dispersa), y sus interacciones con las moléculas de emulsionante en la interfase parece ser de gran importancia.

Además, la adición de luteína provoca una modificación en la naturaleza de la fase oleosa de las emulsiones, que presentan valores de los parámetros reológicos y de textura mucho menores que la emulsión de control (0,00% luteína), pero con valores similares de tamaño de gota (d_{32}).

La adición de ficocianina tuvo una influencia significativa sobre la respuesta reológica de las emulsiones, atribuido a la interacción con la proteína de guisante en la capa interfacial. Como resultado, los parámetros reológicos y texturales aumentaron linealmente con la concentración de ficocianina, mientras que los valores del diámetro de Sauter (d_{32}) presentaron una variación polinomial, con un mínimo para 0,75% (p/p) de concentración de este pigmento.

Cuando se utilizan mezclas de ambos pigmentos, los parámetros reológicos y texturales analizados aumentan con la proporción de ficocianina, mientras que los valores de tamaño de gota (d_{32}) se mantienen similares. Se observó un efecto sinérgico cuando se utilizaron pequeñas cantidades de luteína (menos del 50% en la mezcla).

Fotografías de microscopía tomadas durante los ensayos reológicos (en RheoScope®) también proporcionaron información útil, especialmente para la emulsión control y las que contenían luteína, que son más fluidas, mientras que para las emulsiones que contienen ficocianina y la mezcla de pigmentos, más consistentes y opacas, fue más difícil obtener mediciones microscópicas precisas.

- *Emulsiones coloreadas con biomasa de microalgas*

Posteriormente, se evaluó la capacidad de la biomasa de microalgas para actuar como agente colorante en emulsiones alimentarias (sección 4.3.2). Se añadió biomasa de *Spirulina maxima*, *Chlorella vulgaris* (verde y carotenogénica) y *Haematococcus pluvialis* (carotenogénica), en diversas concentraciones (0,05 a 2,00% p/p), a la fase acuosa de emulsiones estabilizadas por proteína de guisante (3% p/p de aislado de proteína de guisante, 65% p/p de aceite). Se analizó, la estabilidad del color, a lo largo de cinco semanas, y la textura de las emulsiones (sección 4.3.2.1).

La biomasa de las microalgas *Chlorella vulgaris* (verde y naranja) y *Haematococcus pluvialis,* demostraron ser vehículos apropiados de pigmentos en las emulsiones aceite-en-agua estudiadas, impartiendo colores estables y atractivos, con ventajas adicionales, como la de proporcionar otras moléculas bioactivas, por ejemplo antioxidantes y ácidos grasos poliinsaturados omega 3. *Haematococcus pluvialis* demostró ser el más eficaz agente colorante, lo que está relacionado con su alto contenido en pigmentos (3,0% w/w). Mayores estabilidades de color, se alcanzaron para concentraciones de biomasa por debajo del 1,0% (w/w), para todas las microalgas estudiadas.

La adición de biomasa de microalgas originó emulsiones con mayor firmeza que la emulsión control (sin adición de biomasa). Para todos los tipos de microalgas estudiadas, se observó una relación lineal positiva (R^2>0,84) entre la concentración de biomasa y la firmeza. Este parámetro de textura discrimina en mayor medida entre las diferentes muestras, en comparación con otros parámetros obtenidos del ensayo de análisis del perfil de textura (TPA).

Spirulina maxima parece ser la microalga menos eficaz, ya sea como colorante o como agente texturizante. Se observaron coloraciones verdes más apagadas, incluso a altas concentraciones de biomasa, y efectos menos significativos sobre la firmeza.

El refuerzo estructural derivado de la adición de biomasa de microalgas puede ser muy ventajoso, teniendo en cuenta las aplicaciones tecnológicas y nutricionales. Uno de los principales aspectos de la tecnología de emulsiones es la reducción del contenido de grasa, ya que, por ejemplo, tradicionalmente, las mayonesas son emulsiones aceite-en-agua con 70-80% de grasa (Depree y Savage, 2001). Un alto consumo de grasa se asocia

con un mayor riesgo para la obesidad, colesterol arterial alto, enfermedades coronarias y algunos tipos de cáncer. Por esto, en los últimos años la industria alimentaria se ha esforzado en desarrollar alternativas de productos bajos en grasa. Además de los aspectos nutricionales, la grasa influye en las propiedades reológicas y características sensoriales de los alimentos, particularmente en sistemas coloidales en el cual se dispersa una fase de aceite. Por lo tanto, los tecnólogos de alimentos se han centrado en la investigación de sustitutos de grasa, tales como agentes miméticos de grasa es decir, sustancias que imitan las propiedades organolépticas o físicas de los triglicéridos (Akoh, 1998). Algunos agentes miméticos de grasa, tales como las proteínas del suero de la leche, almidón modificado, celulosa, pectina o inulina se han utilizado para estabilizar emulsiones y para aumentar la viscosidad de mayonesas bajas en calorías (Wendin *et al.*, 1997; Chouard, 2005; Liu *et al.*, 2007.; Murphy, 2007).

En la sección 4.3.2.2 se estudió la utilización de biomasa de microalgas como agente mimético de grasa en emulsiones aceite-en-agua estabilizadas por proteína de guisante. La concentración de emulsionante (proteína de guisante) se mantuvo constante (3% p/p), así como de la biomasa de microalgas (2% p/p). El contenido de aceite se redujo de 65% a 50% (w/w). Las emulsiones fueron analizadas en términos de su comportamiento reológico (en flujo estacionario y viscoelasticidad lineal), y se comparó con un sistema control (3% p/p proteína de guisante, 65% p/p aceite, 0% p/p microalga).

Se observó que, para una determinada concentración de proteína y de biomasa de microalga, un mayor contenido en grasas supuso un fortalecimiento de la estructura de la emulsión, para todas las microalgas estudiadas.

Para *Chlorella vulgaris* (verde y naranja), el efecto de la estructuración de la emulsión es más evidente, con valores de viscosidad limite (η_0) mucho mayores que los valores obtenidos para emulsiones preparadas con sólo el 55% (p/p) de aceite, y que la emulsión control (65% p/p aceite, 0% p/p biomasa).

Por el contrario, en las emulsiones que contienen *Spirulina maxima*, no se manifiesta un refuerzo efectivo de la microestructura de la emulsión después de la adición de biomasa, ya sea en el comportamiento de flujo o en ensayos dinámicos.

El efecto de *Haematococcus pluvialis* en la estructuración de la emulsión parece estar estrechamente relacionado con el contenido en aceite de las emulsiones. Esto parece estar relacionado con el hecho de que esta microalga carotenogénica acumula grandes cantidades de lípidos (41%) en forma de gotas de grasa dentro de las células.

En general, se demuestra que la adición de biomasa de microalga resulta ser beneficioso a la hora de permitir un menor contenido de aceite en las emulsiones, sin alterar su estructura y las propiedades texturales. Estos resultados apoyan el uso potencial de biomasa de microalgas como agente mimético de grasa, además de las posibles ventajas como agente colorante.

En conclusión, la adición de biomasa de microalgas, y pigmentos naturales, en emulsiones aceite-en-agua estabilizadas por proteína de guisante resultó ser un procedimiento eficaz e innovador, que logra, al mismo tiempo, coloraciones originales y estables, a través pigmentos naturales beneficiosos para la salud, y un fortalecimiento microestructural, como se observa a través del comportamiento reológico de las emulsiones.

IV – Biomasa de microalgas y pigmentos naturales en sistemas mixtos de gel de proteína de guisante

Biopolímeros naturales, tales como proteínas globulares y polisacáridos, han sido ampliamente utilizados en la formulación de productos alimenticios estructurados tipo gel (Doublier *et al.*, 2000). La mayoría de los productos alimenticios tienen una composición compleja, que comprende al menos tres tipos de biopolímeros cuya interacción determina las propiedades mecánicos y estructurales de estos productos (Tolstoguzov, 2003).

Recientemente, el aislado de proteína de guisante ha sido utilizado con éxito en combinación con κ-carragenato y almidón, para desarrollar postres gelificados estrictamente vegetales, como una alternativa vegetariana a los postres basados en productos lácteos (Nunes *et al.*, 2003; 2006a; 2006b). Estos geles de biopolímeros fueron utilizados como sistemas modelo para estudiar la adición de biomasa de microalgas en matrices gelificadas en el capítulo 5.

El impacto de la adición de microalgas en diferentes matrices refleja las interacciones con otros componentes alimentarios, tales como las proteínas y los polisacáridos (hidrocoloides), por ejemplo. Además del objetivo de colorear, desde un punto de vista de desarrollo de nuevos productos, es esencial definir las características de textura / sensoriales y reológicas de estos sistemas, ya que reflejan las modificaciones microestructurales que pueden surgir como consecuencia de la adición de biomasa de microalgas en matrices tipo gel, y que pueden debilitar la estabilidad del producto. Por lo tanto, el objetivo del capítulo 5 es estudiar el impacto de la adición de biomasa de diferentes microalgas en las propiedades reológicas de geles mixtos de proteína/polisacáridos.

- *Caracterización físico-química de geles con pigmentos naturales y microalgas*

Se llevaron a cabo algunos estudios preliminares con el propósito de desarrollar una formulación de postres gelificados a base de productos vegetales (con propiedades similares a los "postres lácteos") con diferentes tipos y concentraciones de biomasa de microalgas (sección 5.3.1). Los geles se prepararon sobre la base de una formulación optimizada previamente por Nunes *et al.* (2006a; 2006b): 4% (p/p) aislado de proteína de guisante, 0,15% (p/p) κ-carragenato, 2,5% (p/p) almidón nativo de maíz y 15% (p/p) de azúcar. A esta formulación se le adicionó biomasa de microalgas (*Spirulina maxima*, *Chlorella vulgaris* verde y naranja, *Haematococcus pluvialis* y *Diacronema vlkianum*) y pigmentos naturales (astaxantina, cantaxantina, β-caroteno, luteína y ficocianina), en concentraciones de 0,00% (control), 0,25% y 0,75% (p/p).

Los geles fueron analizados en cuanto a color y textura. En el caso de geles que contienen *Spirulina maxima* y *Diacronema vlkianum*, se llevó a cabo, además, un análisis del perfil de ácidos grasos y del contenido en pigmentos en función de la temperatura de procesado.

Tanto el color, como el contenido en pigmentos y ácidos grasos de los geles, presentan una buena estabilidad térmica al aumentar la temperatura de procesamiento de 75°C a 90°C, revelando una protección eficaz de los pigmentos dentro de las células de *Spirulina maxima* y *Diacronema vlkianum*. Se observó un efecto similar en un estudio previo de incorporación de microalgas en galletas (Gouveia *et al.*, 2008). La resistencia de las

moléculas bioactivas de microalgas a diferentes procesos de transferencia de calor ("secos" y "húmedos") en diferentes matrices de alimentos, pone en evidencia el potencial de las microalgas como ingredientes alimentarios y nutracéuticos.

La adición de microalgas y pigmentos resultó en un incremento de la firmeza de los geles, excepto en el caso de los geles de ficocianina y *Spirulina maxima*. Este es un resultado interesante, teniendo en cuenta que para emulsiones aceite-en-agua (sección 4.3.1), se observó una tendencia inversa, es decir, valores más bajos de firmeza fueron para las emulsiones con luteína, mientras que hubo un aumento significativo para las emulsiones que contenían ficocianina. Esto corrobora el hecho de que, un mismo pigmento natural para colorear productos alimenticios, puede producir efectos texturizantes o efectos de desestructuración, dependiendo del tipo de sistema coloidal.

- *Caracterización reológica de geles con microalgas*

En la sección 5.3.2 se ha realizado una caracterización reológica de geles enriquecidos con microalgas (0,75% p/p biomasa de microalgas), incluyendo curvas cinéticas de curado, controladas a través de ensayos en cizalla oscilatoria de pequeña amplitud (SAOS) en función del tiempo de barrido. También fueron analizados los espectros mecánicos (barridos de frecuencia) y el carácter termorreversible (barridos de temperatura) de los geles formados.

El comportamiento de maduración de estos geles (ensayos de curado) es el típico en procesos de gelificación de biopolímeros, con G' aumentando rápidamente al principio y luego más lentamente (Nunes *et al.*, 2003; Clark, 2001). Esta evolución permite una definición simple de un valor de equilibrio de G' (G'_{eq}), es decir, el valor de G' a un tiempo infinito, donde el gel llega a una estructura estable y plenamente desarrollada. En un calentamiento (de 5ºC a 80°C) y enfriamiento (de 80°C a 5°C) posterior de las muestras, se observó que todos los geles estudiados son sólo parcialmente termo-reversibles, demostrando una histéresis térmica.

La incorporación de microalgas en estos sistemas mixtos gelificados parece ser beneficioso, especialmente en el caso de *Haematococcus pluvialis* (con un atractivo color *"rosa fresa"*), que promovió una estructuración del sistema con propiedades

reológicas mejoradas. Esta acción de refuerzo estructural puede estar relacionada con su alto contenido en grasa (41% p/p), considerando que las gotas de grasa pueden actuar como "filler" activo incorporadas en la matriz del gel, tal como se observa en sistemas lácteos gelificados.

Sin embargo, la adición de *Spirulina maxima* produce una drástica reducción en los parámetros reológicos de los geles, lo que se relaciona con una incompatibilidad termodinámica entre la proteína de la microalga y los otros componentes del sistema gelificado mixto.

- *Efecto de las condiciones de gelificación en la viscoelasticidad lineal de geles con Spirulina maxima y Haematococcus pluvialis*

Teniendo en cuenta el distinto comportamiento reológico que presentan las microalgas *Spirulina maxima* y *Haematococcus pluvialis* en los sistemas gelificados de proteína de guisante/κ-carragenato/almidón, se decidió ampliar los estudios reológicos en estos sistemas concretos.

El proceso de gelificación de estos sistemas mixtos es inducido térmicamente, lo que implica una amplia desnaturalización y/o cambios de conformación de los biopolímeros y, posteriormente, el desarrollo de una red tridimensional (de gel) al enfriarse. El perfil térmico aplicado en la preparación de los geles es determinante para el desarrollo de la estructura de gel, lo que se refleja en sus propiedades reológicas (Nunes *et al.*, 2006a).

El efecto del tratamiento térmico sobre el comportamiento viscoelástico lineal de geles mixtos proteína-polisacárido con adición de biomasa de microalgas *Spirulina maxima* y *Haematococcus pluvialis* se estudió en la sección 5.3.3, mediante el establecimiento de diferentes condiciones de temperatura máxima y combinaciones tiempo/temperatura (70ºC/5min, 80ºC/5min, 90ºC/5min, 90ºC/15min y 90ºC/30min).

Las propiedades viscoelásticas lineales de sistemas mixtos gelificados de proteína de guisante/κ-carragenato/almidón son altamente dependientes de las condiciones de preparación del gel, incluyendo la combinación de temperatura y tiempo de tratamiento térmico. El aumento de temperatura (70-90°C, 5 min) produce geles más estructurados, como resultado del desdoblamiento de la proteína y la interacción entre

biopolímeros. El efecto del tiempo (5-30 min, 90°C) fue menos pronunciado. Los valores más altos de las funciones viscoelásticas son lograron con la velocidades de calentamiento y/o enfriamiento más bajas. Sin embargo, el tiempo requerido para el proceso, y los costes posteriores implicados, deben ser considerados.

La adición de biomasa de microalgas promueve algunas modificaciones en la estructura del gel, aunque la respuesta a las condiciones de preparación del gel sigue, en general, el comportamiento del gel control (sin microalgas). La temperatura de gelificación aumenta con la adición de biomasa de microalgas, lo que significa que la formación del gel se produce previamente, durante el proceso de enfriamiento. Esto permite el uso de condiciones más suaves de procesamiento térmico, que permiten la preservación de compuestos naturales, tales como los pigmentos naturales (por ejemplo, astaxantina y ficocianina).

Por otra parte, se estudió la gelificación de sistemas proteína de guisante/κ-carragenato/almidón, aplicando diferentes velocidades de calentamiento enfriamiento: ± 0,5ºC/min, ± 1,0ºC/min y ± 5,0°C/ min. Muchos estudios sobre biopolímeros han demostrado la influencia de la velocidad de enfriamiento en las propiedades mecánicas de los geles simples (por ejemplo, Lopes-da-Silva y Rao, 1995), así como sobre el proceso de separación de fases y las propiedades físicas de los sistemas mixtos (Loren *et al.*, 1999; Turgeon y Beaulieu, 2004).

La adición de *Haematococcus pluvialis* promueve una estructuración de la matriz del gel que es menos dependiente de las condiciones de preparación.

Para obtener un gel de *Spirulina maxima* con propiedades mecánicas similares a las del gel control es necesario un tratamiento térmico más amplio, es decir, deben aplicarse temperaturas y tiempos de exposición más altos. Se sugiere que las moléculas de proteínas de *Spirulina maxima* compiten por los sitios de fijación del agua, lo que dificulta la hidratación de los gránulos de almidón, ya que esta microalga (cianobacteria) carece de una pared celular rígida (según los resultados presentados en la sección 5.3.4). Por lo tanto, debe favorecerse el hinchamiento de los gránulos de almidón y de liberación de amilosa mediante el aumento de la intensidad del tratamiento térmico.

La desnaturalización de los componentes de *Spirulina maxima* y la formación de geles no se consideran fenómenos simples, como consecuencia de la formación de complejos de pigmentos (ficocianina) y proteínas (Chronakis, 2001). Los geles sometidos a una velocidad de enfriamiento más lenta, presentaron en general mayores valores de los parámetros reológicos analizados, por ejemplo G'_{24h} y G'_{eq}. De hecho, para los sistemas enfriados lentamente, hay más tiempo para alcanzar el equilibrio dinámico de la red estructural del gel, es decir, la maduración de la estructura de gel da lugar a una red más estructurada en el enfriamiento (Nunes *et al.*, 2006a). Sin embargo, para los sistemas de estudio, las propiedades reológicas (G'_{24h}, G'_{eq}, entre otros parámetros) de los geles enfriados a -1 y -5 ºC/ min no son sustancialmente diferentes. Esto presenta una ventaja considerando que serán necesarios tiempos más cortos para lograr un producto final similar.

➢ *Sistemas binarios – Interacción de la biomasa de microalgas en sistemas de biopolímeros gelificados*

En sistemas complejos mixtos, es difícil determinar la influencia de la adición de microalgas, ya que existen muchos factores y procesos que tienen lugar simultáneamente. En consecuencia, se decidió estudiar la interacción de la biomasa de microalgas (*Spirulina maxima* y *Haematococcus pluvialis*) con cada biopolímero en geles binarios simples (microalga/biopolímero) y en sistemas ternarios formados por microalga/proteína de guisante/κ-carragenato o bien microalga/proteína de guisante/almidón. El objetivo de este estudio es elucidar cómo estas microalgas se organizan durante la formación de la estructura del gel y la forma en la que interactúan con los biopolímeros presentes en el sistema complejo. Las concentraciones de biopolímeros tuvieron que ser incrementadas para alcanzar la concentración mínima de gelificación, pero la proporción microalga: biopolímero fue la misma que en la formulación del gel mixto.

Para todos los sistemas microalga/biopolímero estudiados, se observó que los biopolímeros (proteínas y polisacáridos), solos o en combinaciones binarias, son los principales responsables de la formación de la estructura del gel y del comportamiento reológico resultante. Las microalgas parecen estar embebidas en la red del gel,

originando microestructuras más densas, y un aumento de los parámetros reológicos analizados, en la mayoría de los casos.

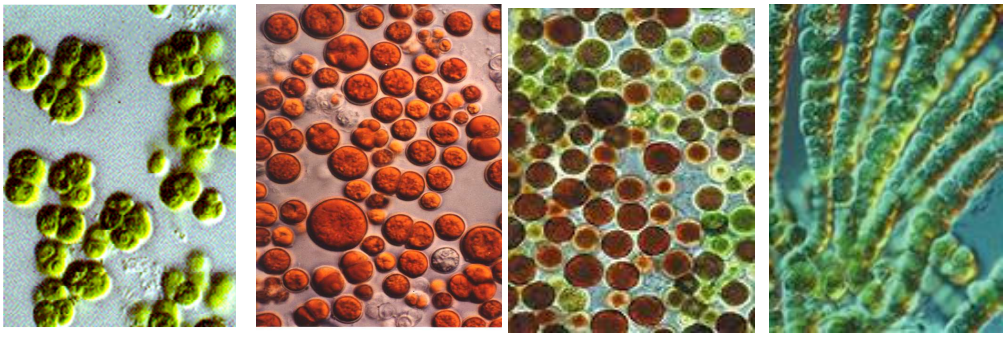
Spirulina maxima dio lugar a geles más fuertes, aunque siempre inferiores a los originados con *Haematococcus pluvialis*, excepto en el caso de sistemas con almidón, donde afecta negativamente, impidiendo, o dificultando, el proceso de gelatinización del almidón. Como ya se ha comentado, este efecto está relacionado con la influencia en el hinchamiento de los gránulos de almidón. La adición de *Haematococcus pluvialis* y *Spirulina maxima* causa un aumento en la temperatura de gelatinización de almidón, lo que indica que la biomasa de microalgas interfiere con el proceso de gelatinización de almidón, quizás compitiendo por los sitios de unión de agua durante el proceso de hidratación de los gránulos. En el caso de los sistemas *Haematococcus*/almidón este inconveniente fue superado, resultando geles fuertes, con valores de *G´* y *G"* más altos que el gel de almidón simple. En oposición, los sistemas de *Spirulina*/almidón presentan valores mucho más bajo de *G´* que el gel control.

Teniendo en cuenta que la biomasa de microalgas contiene cantidades significativas de iones, éstas también tienen una influencia significativa en la interacción de los biopolímeros, asociado con el mecanismo de gelificación, en particular, en el caso de la gelificación del κ-carragenato, donde se observa un aumento importante en los parámetros reológicos con la adición de microalgas.

La microscopía óptica de fluorescencia demostró ser una técnica simple y eficaz para visualizar las microestructuras de estos materiales. Las microalgas son fácilmente detectadas debido a la auto-fluorescencia de sus pigmentos naturales. Los resultados de microscopía se correlacionan bien y apoyan las conclusiones obtenidas de los ensayos reológicos.

CHAPTER 1

Introduction



1. INTRODUCTION

Modern food industry leads to an increase of cheaper, healthier and more convenient products. The use of natural ingredients, like polyunsaturated fatty acids (PUFA) and antioxidant pigments, exhibiting high impact on functional properties is important in order to reduce the incidence of chronic diseases, which are strongly considered of capital importance in Europe, where aging population and welfare costs are crucial for public resources management.

Food appearance is, among others, a factor that defines its quality and the first impression the consumer gets directly from foods. Colour, as one aspect of appearance, plays a major role in the acceptability of a food product, being determinant on its identification, as an indicator of quality, freshness, conservation state, flavour expectation and commercial value. The attainment of the adequate colour, according to the consumer expectations on the product, is usually accomplished through the use of colourings. In the food industry, these compounds are used in colourless food products as well as in the compensation of colour loss due to processing conditions (e.g. high temperature and pressure).

Synthetic colourings are perceived by the consumers as undesirable and harmful, and some of them are considered to be responsible for allergenic and intolerance reactions. Regarding these concerns, the European Commission has asked EFSA (European Food Safety Authority) to re-evaluate all currently permitted food additives in the EU giving highest priority to 46 food colours which may be legally added to food according to EU Regulation 94/36/EC. Additionally, McCann *et al.* (2007) recently published a paper, known as *"The Southampton Study"* which concluded that exposure to two mixtures of four synthetic colours (allura red AC, azorubine, ponceau 4R, quinoline yellow, sunset yellow FCF and tartrazine) plus a sodium benzoate preservative in the diet result in increased hyperactivity in 3-year old and 8-9 year-old children.

Consequently, there is an increasing demand for alternative colouring sources. Many natural pigments, such as carotenoids and anthocyanins, are phytochemicals for which nutraceutical effects have been claimed, including antioxidant activity (Wilska-Jeszka, 2002). Therefore, the use of natural colourings can be an interesting tool in the development of new functional foods, responding to the increasing consumer demand for more natural food products presenting health benefits.

Microalgae are an excellent source for natural pigments, such as carotenoids, chlorophylls and phycobiliproteins, presenting great industrial potential to be used as natural colouring agents. These microscopic organisms can be grown under certain controlled environmental conditions (*e.g.* temperature, salinity, light, nutrients) that can stimulate or inhibit the biosynthesis and accumulation of bioactive compounds (*e.g.* pigments) in large amounts. The possibility of not only harvesting them but also growing them at different conditions,

enables microalgae use as natural reactors at a large scale (Plaza *et al.*, 2009). Besides colouring purposes using microalgae in food products presents additional nutritional advantages since they are rich in antioxidants and other biologically active compounds such as long chain ω_3 polyunsaturated fatty acids, naturally encapsulated within biomass cells. Moreover, due to its complex physicochemical composition it is expected that the incorporation of microalgal biomass can impart some modifications on the food microstructural assembly, reflected by texture and sensorial changes.

The aim of the present thesis is to study the addition of natural pigments and microalgal biomass as colouring agents in model colloidal food systems – emulsions and gels. This was part of a broader multidisciplinary project "Pigments, antioxidants and PUFA's in microalgae-based food products - functional and structural implications" (PTDC/AGR-ALI/65926/2006). The impact of microalgae and pigment addition on different colloidal systems was studied through a rheological perspective, *i.e.* by monitoring changes in the rheological behaviour, also relating the rheological response with microstructural and physicochemical characteristics.

Most food products are complex colloidal systems resulting from the combination of numerous food components (*e.g.* proteins, carbohydrates and lipids) organised and arranged in complex internal microstructures with various types of assemblies such as dispersions, emulsions, foams, gels, etc. (Garti, 1999). The overall stability and structural properties of colloidal systems depend not only on the functional properties of the individual ingredients, but also on the nature and strength of the interactions between them (Dickinson, 1992; Neirynck *et al.*, 2004). The use of natural colourings and microalgae in food systems is limited by the nature of the food matrix in which the pigment is to be dispersed in, and to the interactions with the other food components (proteins, polysaccharides, lipids, sugars, salts, etc.). The rheological behaviour of these systems reflects the microstructural modifications that may have undergone through the addition of the colouring materials to colloidal food emulsions and gels.

Pea protein isolate was used as a basis to formulate model food emulsions and gels, as optimized in previous studies (*e.g.* Raymundo *et al.*, 2002; Batista *et al.*, 2005; Nunes *et al.*, 2003). This is in agreement with the current trend of replacing animal products by vegetable ones, due to consumer's awareness on the benefits of a healthier diet and lifestyle along with ethical and environmental considerations on reducing animal resources exploration.

The thesis is divided into seven chapters, including the current general introduction for contextualization purposes (chapter 1).

Chapter 2 comprises a literature review on the main subjects involved in the thesis, including Microalgae (2.1); Colloidal systems (food emulsions and gels) (2.2); and general rheology concepts and methods (2.3).

Chapter 3 deals with the characterization of the microalgae (freeze-dried biomass) that were added in the model emulsions and gels: *Spirulina maxima*, *Chlorella vulgaris* (green and carotenogenic), *Haematococcus pluvialis* (carotenogenic), *Diacronema vlkianum* and *Isochrysis galbana*. This includes a physicochemical characterization in terms of protein, fat, fatty acids profile, pigment profile, ashes, mineral profile and thermal analysis (TGA).

Chapter 4 deals with the incorporation of natural pigments and microalgal biomass to oil-in-water pea protein stabilized emulsions. Lutein (hydrophobic) and phycocyanin (hydrophilic) pigments were added to the oil and aqueous phase, respectively, of the emulsions at various concentrations (4.1). The effect of microalgal biomass addition on the emulsion stability (colour, texture) was studied (4.2.1), as well as the microalgae potential as fat replacers (4.2.2). Emulsions were characterized in terms of colour, texture, rheological behaviour and droplet size distribution.

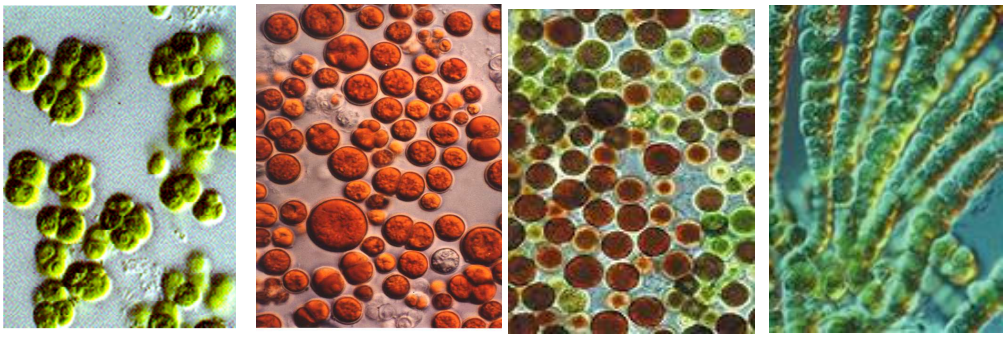
Chapter 5 deals with the incorporation of microalgal biomass in pea protein/κ-carrageenan/starch mixed gels. Some preliminary studies on the addition of natural pigments and microalgal biomass to gel matrices and repercussion on colour and texture were taken (5.3.1). A rheological characterization of microalgae-enriched gels, including maturation kinetics, was performed thereafter (5.3.2). *Spirulina* and *Haematococcus* gels were selected and studied in more detail, regarding the effect of gel setting conditions on the gels rheological behaviour (5.3.3), as well as the interactions between microalgae and the biopolymers present in the gelled systems (5.3.4).

Finally, chapter 6 reports the main conclusions of the thesis and literature references, cited along the text, are listed in chapter 7.

The resulting publications of this work are collected in appendix A at the end of the thesis: two book chapters (one review and one original paper), seven articles published in scientific journals with peer-review (plus one submitted), along with several communications (around 30) in scientific conferences (listed in appendix B).

CHAPTER 2

Literature review



2.1. MICROALGAE

2.1.1. Introduction

Microalgae are an enormous biological resource, representing one of the most promising sources for new products and applications (Pulz and Gross, 2004). They can be used to enhance the nutritional value of food and animal feed due to their well-balanced chemical composition. Moreover, they are cultivated as a source of highly valuable molecules such as polyunsaturated fatty acids, pigments, antioxidants, biologically active compounds, with application in the feed, food, nutritional, cosmetic, pharmaceutical and even fuel industries.

Microalgae are an extremely heterogeneous group of organisms, described as a life-form, not a systematic unit. They are regarded as unicellular photoautotrophic (contain chlorophyll *a*) microorganisms that can be eukaryotic or prokaryotic. These amazing life forms have evolved over billions of years being mainly distributed in waters, but also found on the surface of all types of soils, including earth's harshest environments.

Microalgal biotechnology is similar to conventional agriculture, but presents some advantages and appears to be more photosynthetically efficient than terrestrial plants. Microalgae can reach higher biomass productivities, faster growth rate, highest CO_2 fixation rates and O_2 production rates when compared to higher plants. They are feasibly grown in liquid medium which can be handled easily, and can be cultivated in variable climates and non-arable land, including marginal areas unsuitable for agricultural purposes (*e.g.* desert and seashore lands); in non-potable water or even as a waste treatment purpose; use far less water than traditional crops and do not displace food crop cultures; avoid environmental impacts, like soil desertification and deforestation (Chisty, 2007). Moreover, their production is not seasonal; there is no need for pesticides or herbicides and they do not produce contaminants. Microalgae can also effectively remove nutrients or pollutants (*e.g.* nitrogen and phosphorus) from water and are useful in soil bioremediation and N_2-fixing biofertilizers.

Microalgae production is an important natural mechanism which helps to reduce the excess of atmospheric CO_2 by bio-fixation and recycling of fixed carbon in products, ensuring a lower greenhouse effect, reducing the global environmental heating and climate changes. Microalgae can fix CO_2 efficiently from different sources, including the atmosphere, industrial exhaust gases, and soluble carbonate salts, which combined with biofuel production, and wastewater treatment may provide a very promising alternative to current CO_2 mitigation strategies according to the Kyoto Protocol (Wang *et al.*, 2008).

In fact, microalgae can provide several types of renewable biofuels, including: methane produced by anaerobic digestion of algal biomass; biodiesel derived from microalgal oil; bioethanol from microalgal sugars fermentation; and photobiologically produced biohydrogen (Chisti, 2007; Dutta *et al.*, 2005). Presently, one can witness a growing interest

and awareness on microalgae biodiesel as a renewable fuel that has the potential to completely displace petroleum-derived transport fuels without adversely affecting supply of food and other crop products (Chisti, 2008). Moreover, the concept of microalgal biorefineries is a sustainable production strategy, where every component of the biomass raw material can be used to produce useable products. The residual biomass from biodiesel production processes can be potentially used as animal feed, and/or to produce methane by anaerobic digestion, for generating the electrical power necessary to run the microalgal biomass production facility (Chisti, 2007).

Currently, the major application in commercial microalgalculture is the production of dietary supplements for animal (*e.g.* poultry) and aquaculture feed, as well as for human nutrition. Microalgae play a vital role in the rearing of many aquatic animals, especially the larvae of molluscs, shrimps, and rotifers which in turn are used to rear the larvae of marine fish and crustaceans, being of strategic economic interest for aquaculture (Borowitzka, 1997).

Commercial production of phototrophic microbial biomass is limited to a few microalgal species that are cultivated in open ponds (Figure 2.1) by means of a selective environment (*e.g.* high pH or salinity) or a high growth rate (Tredici, 2004).

Figure 2.1. Microalgae cultivated in open ponds systems. a) *Dunaliella* by Cognis at Hutt Lagoon (Australia); b) *Arthrospira* in raceway ponds by Cyanotech (Hawaii, USA); and Earthrise Farms (California, USA).

Closed photoautotrophic culture systems (*e.g.* tubular, flat, vertical cylinders and sleeves photobioreactors), with transparent walls (glass or plastic), have been developed in the last years, to overcome the limitations of open systems, mainly the risk of contamination (*e.g.* microorganisms, heavy metals) and to enable the culture of specific microalgae that do not grow in highly selective environments (Borowitzka, 1999). Some attempts have been made to develop commercial-scale photobioreactors, but most were closed after a few months of operation, including Photo Bioreactors Ltd plant in Santa Ana (Murcia, Spain). The first truly successful large-scale industrial production of microalgae in a photobioreactor has been accomplished by the system developed by Prof. Otto Pulz (2001) at a plant built in Klötze (Germany) by Ökologische Produkte Altmark GmbH (ÖPA) and run by IGV Ltd. The plant consists of a 700 000 l glass tubular reactor (500 km total length), divided in 20 subunits, installed in a 12 000 m^2 greenhouse (Figure 2.2), with an expected productivity of 150 ton/year. The aim is to produce *Chlorella* sp. biomass (sold at 50€/kg DW as food additive for

poultry) through the use of CO_2 evolved during the composting process of pine wood chips for artificial peat production by ÖPA in the same location (Tredici, 2004).

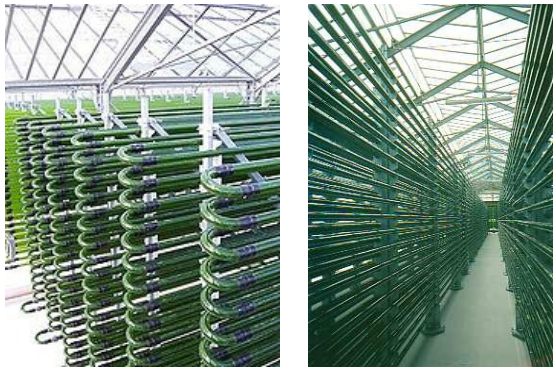


Figure 2.2. *Chlorella* sp. growing in tubular photobioreactors (Klötze, Germany).

Production of heterotrophic microalgae in fermentation reactors presents significant economic advantages over photoautotrophic production. The first production lines have been developed by MARTEK (USA) for the production of polyunsaturated fatty acids (PUFAs), mainly docosahexaenoic acid (DHA), which has recently won Novel Foods approval by the European Union.

2.1.2. Some microalgae species with potential application in the food industry

Microalgae use as natural food by indigenous populations has occurred for centuries. However, the cultivation of microalgae is only a few decades old (Borowitzka, 1999) and among the thousands of species that are believed to exist (Chaumont, 1993; Radmer and Parker, 1994), only a few thousand strains are kept in collections, a few hundred being investigated for chemical content and just a handful being cultivated in industrial quantities (Olaizola, 2000). Some of the most biotechnologically relevant microalgae are the Cyanobacteria *Arthrospira* (*Spirulina*) and the green algae (Chlorophycea) *Chlorella vulgaris, Haematococcus pluvialis* and *Dunaliella salina* which are already widely commercialized and used, mainly as nutritional supplements for humans and as animal feed additives.

2.1.2.1. Arthrospira (Spirulina)

Arthrospira is an ancient microscopic filamentous cyanobacterium (prokaryotic) that belongs to the Class Oscilatoriacea (Figure 2.3). It is classified as a microalga (blue-green alga) due to its chlorophyll *a* content and ability to do photosynthesis (photoautotrophic). It is currently known as *Spirulina*, although *Arthrospira* and *Spirulina* have been recognized as separate genera, which refers mainly to two species: *A. maxima* and *A. platensis* (Belay, 2008).

Arthrosphira (*Spirulina*) grows profusely in certain alkaline lakes in Mexico and Africa, forming massive blooms, and has been used as a staple food by local populations since ancient times (Yamaguchi, 1997).

Since the late 1970s, when the first large-scale *Spirulina* production plant was established in Mexico, it has been extensively produced around the world (Hawaii, California, China, Taiwan, Japan) using open raceway ponds, (Borowitzka, 1999). It is estimated a total production of 3000 tons/year, being broadly used in food and feed supplements, due of its high protein content and its excellent nutritive value, such as high γ-linolenic acid (GLA; 18:3ω6) and vitamin B_{12} level (Ötles and Pire, 2001; Shimamatsu, 2004).

In addition, this microalga has various possible health promoting effects: the alleviation of hyperlipidemia, suppression of hypertension, protection against renal failure, growth promotion of intestinal *Lactobacillus*, suppression of elevated serum glucose level (Spolaore *et al.*, 2006), anticarcinogenic effects and hypocholesterolemic properties (Reinehr and Costa, 2006). The inhibition of several pathogenic viruses (*e.g.* Herpes simplex virus, influenza A, HIV-1) has also been reported, as related to the presence of a sulphated polysaccharide (calcium spirulan) (Hu, 2004).

Spirulina is also the main source of natural phycocyanin, a valuable blue pigment used as a natural food and cosmetic colouring and as biochemical tracer in immunoassays, among other uses (Ötles and Pire, 2001; Kato, 1994; Shimamatsu, 2004), that also present high free-radical scavenging activity and potential therapeutic effects (Romay, 2003).

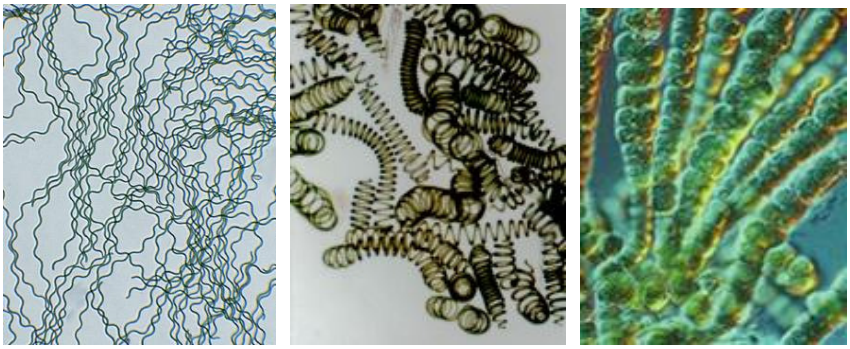


Figure. 2.3. *Arthrospira* (*Spirulina*) sp.

2.1.2.2. Chlorella

Chlorella was the first microalga to be isolated and cultivated in laboratory by Beijerinck in 1890. Belongs to the Chlorophyta (green algae) family, and presents Chlorophyll *a* and *b* and several carotenoids that may be synthesized and accumulated outside the chloroplast under conditions of nitrogen deficiency and/or other stress, colouring the alga orange or red (Figure 2.4).

Chlorella has been used as an alternative medicine in the Far East since ancient times and it is known as a traditional food in the Orient. The commercial production of *Chlorella* as a novel health food commodity started in Japan in the 1960s, under the scientific supervision of the Microalgae Research Institute of Japan (*Chlorella* Institute), and by 1980 there were 46 large-scale factories in Asia (Borowitzka, 1999). Nowadays, *Chlorella* is widely produced and marketed as a health food supplement in many countries, including China, Japan, Europe and the US, being estimated a total production around 2000 ton/year in the 1990s (Lee, 1997).

Chlorella is considered a potential source for a wide spectrum of nutrients (*e.g.* carotenoids, vitamins, minerals) being widely used in the health food market as well as for animal feed and aquaculture. *Chlorella* is important as a health promoting factor on many kinds of disorders such as gastric ulcers, wounds, constipation, anemia, hypertension, diabetes, infant malnutrition and neurosis (Yamaguchi, 1997). It is also attributed a preventive action against atherosclerosis and hypercholesterolemia by glycolipids and phospholipids, and antitumor actions by glicoproteins, peptides and nucleotides (Yamaguchi, 1997). However the most important substance in *Chlorella* seems to be a beta-1,3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore *et al.*, 2006).

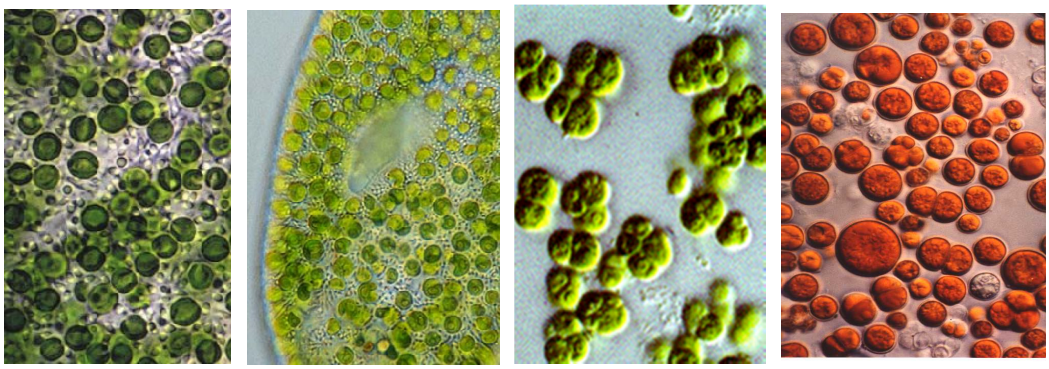


Figure. 2.4. *Chlorella vulgaris* green and orange (carotenogenic) microalga.

2.1.2.3. Haematococcus pluvialis

Haematococcus is a freshwater, unicellular, green alga (Chlorophyceae, order Volvocales) that is extensively used for the production of the orange-red pigment astaxanthin (Figure 2.5). When green vegetative cells come across stress conditions (*e.g.* nitrogen deficiency, high light intensity, salt stress) the alga rapidly differentiates into encysted cells that accumulate the ketocarotenoid astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) in globules located outside the chloroplast. It has been suggested that the accumulated astaxanthin might act as a protective agent against oxidative stress damage (Kobayashi *et al.*, 1997). This

carotenoid pigment is a potent radical scavenger and singlet oxygen quencher, with increasing amount of evidence suggesting that it surpasses the antioxidant benefits of β-carotene, vitamin C and vitamin E (Todd-Lorenz and Cysewski, 2000).

Astaxanthin is responsible for the pinkish-red hue of some marine animals, such as salmonids, trouts, shrimps, lobsters and crayfish, as a result of the consumption of microorganisms that are able to synthesize this pigment. Only few microorganisms can produce astaxanthin, including the red yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) and the marine bacteria *Agrobacterium auratium* yeast, but *Haematococcus pluvialis* has been identified as the organism which can accumulate the highest level of astaxanthin in nature (1.5-3.0% dry weight) which is currently the prime natural source of astaxanthin for commercial exploitation, (Todd-Lorenz and Cysewski, 2000).

During the 1990s in the USA and India, several plants started with large-scale production of *Haematococcus pluvialis,* particularly as pigmentation source in farmed salmon, trout and poultry industries, as well as for nutraceutical and pharmacological applications (Spolaore *et al.*, 2006).

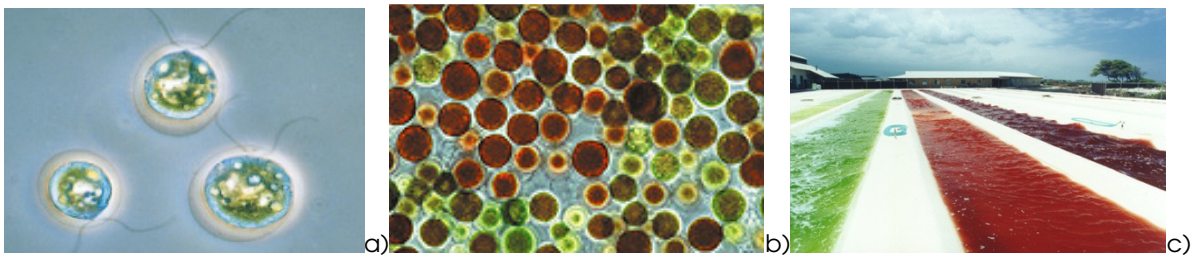
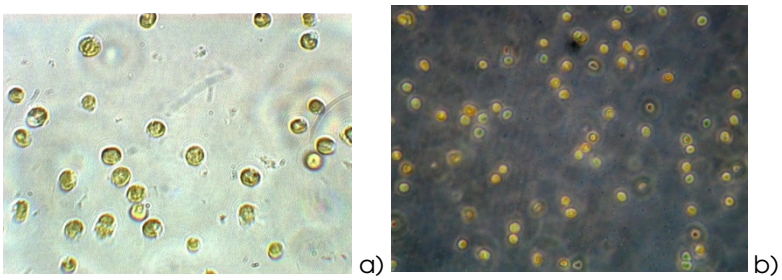

a) b) c)

Figure. 2.5. *Haematococcus pluvialis* a) green vegetative cells; b) encysted carotenogenic cells; c) cultivated in open raceway ponds.

2.1.2.4. Diacronema vlkianum and Isochrysis galbana

Recently, attention has been drawn on the marine microalgae *Isochrysis galbana* (Figure 2.6) and *Diacronema vlkianum* (Haptophyceae) due to their ability to produce long chain polyunsaturated fatty acids (LC-PUFA), mainly eicosapentaenoic acid (EPA, 20:5ω_3) and also docosahexaenoic acid (DHA, 22:6ω_3), that are accumulated as oil droplets in prominent lipid bodies in the cell (Liu and Lin, 2001). These microalgae are widespread in marine environment, forming a major part of marine phytoplankton, and have been used as feed species for commercial rearing of many aquatic animals, particularly larval and juvenile molluscs, crustacean and fish species (Fidalgo *et al.*, 1998). For example, in a relative ranking of microalgal diets for clam *Mercenaria mercenaria*, the microalga *I. galbana* was shown as the most suitable source of nutrition for rapid growth (Wikfors *et al.*, 1992), while *D. vlkianum* resulted in high growth rates and low mortality for the Pacific oyster *Crassostrea gigas larvae* (Ponis *et al.*, 2006). These microalgae are also potentially promising for the food industry as a

valuable source of LC-PUFA's, in alternative to fish oils, supplying also sterols (mainly sitosterol), tocopherols, colouring pigments and other nutraceuticals (Bandarra *et al.*, 2003; Donato *et al.*, 2003).



a) b)

Figure. 2.6. *Isochrysis galbana* (a) and *Diacronema vlkianum* (b).

2.1.3. Microalgae as food colouring agents

One of the most obvious and arresting characteristic of the algae is their colour. In general, each phylum has its own particular combination of pigments and an individual colour. Aside chlorophylls, as the primary photosynthetic pigment, microalgae also form various accessory or secondary pigments, such as phycobiliproteins and a wide range of carotenoids. These natural pigments are able to improve the efficiency of light energy utilization of the algae and protect them against solar radiation and related effects. Their function as antioxidants in the plant shows interesting parallels with their potential role as antioxidants in foods and humans (van den Berg *et al.*, 2000). Therefore, microalgae are recognized as an excellent source of natural colourings and nutraceuticals and it is expected that they will surpass synthetics as well as other natural sources due to their sustainability of production and renewable nature (Dufossé *et al.*, 2005). Three major classes of photosynthetic pigments occur among the algae: chlorophylls, carotenoids (carotenes and xanthophylls) and phycobilins.

2.1.3.1. Chlorophyll

All algae contain one or more type of chlorophyll, being chlorophyll-*a* the primary photosynthetic pigment in all algae (Figure 2.7) and the only chlorophyll in cyanobacteria (blue-green algae) and Rhodophyta. Like all higher plants, Chlorophyta and Euglenophyta contain chlorophyll-*b* as well; chlorophylls -*c*, -*d* and –*e* can be found in several marine algae and fresh-water diatoms. Chlorophyll amounts are usually about 0.5-1.5% of dry weight (Becker, 2004).

Chlorophylls are widely used as natural colorants in the food and pharmaceutical industry, being primarily extracted from vegetable sources, although there is a growing interest in the

production of chlorophylls from microalgae sources such as *Spirulina* (Rasmussen and Morrissey, 2007).

Chlorophyll derivatives, such as chlorophyllins, can exhibit health promoting activities. These compounds have been traditionally used in medicine due to its wound healing and anti-inflammatory properties as well as control of calcium oxalate crystals and internal deodorization (Ferruzi and Blakeslee, 2007). Recent epidemiological studies from The Netherlands Cohort Study (Balder *et al.*, 2006) have provided evidence linking chlorophyll consumption to a decreased risk of colorectal cancer.

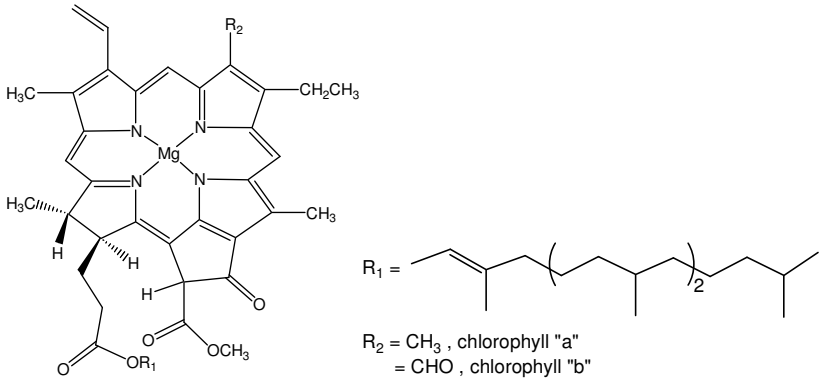


Figure 2.7. Chemical structures of chlorophyll *a* and *b*.

2.1.3.2. Carotenoids

Carotenoids are naturally occurring pigments that are responsible for the different colours of fruits, vegetables and other plants (Ben-Amotz and Fishler, 1998). Carotenoids are usually yellow to red pigments derived from lycopene, with a long polyene chain (Figure 2.8), which determines light absorption properties and phytochemical properties (Astorg, 1997). They can be classified in two types of compounds: carotenes, which are unsaturated hydrocarbons, *e.g.* licopene, α- and β-carotene; and xanthophylls, more abundant in nature, which present one or more functional groups containing oxygen, *e.g.* lutein, astaxanthin and cantaxanthin.

Carotenoids are synthesized *de novo* by photosynthetic organisms (plants and some other microorganisms, such as bacteria, algae and fungi (Borowitzka, 1988). In animals, the carotenoids ingested in the diet are accumulated and/or metabolized in the body, being present in meat, eggs, fish skin (trout, salmon), the carapace of Crustaceans (shrimp, lobster, Antarctic krill, crawfish), and in the subcutaneous fat, skin, egg yolks, liver, integuments, and feathers of birds (poultry) (Breithaupt, 2007).

In the algae, carotenoids seem to function primarily as photoprotective agents and as accessory light harvesting pigments, thereby protecting the photosynthetic apparatus against photo damage (Ben-Amotz *et al.*, 1987). They also play a role in phototropism and

phototaxis (Borowitzka, 1988). Some microalgae can undergo a carotenogenesis process in response to various environmental and cultural stresses (*e.g.* light, temperature, salts, nutrients), where the alga stops growing and changes dramatically its carotenoid metabolism, accumulating secondary carotenoids as an adaptation to severe environments (Bhosale, 2004).

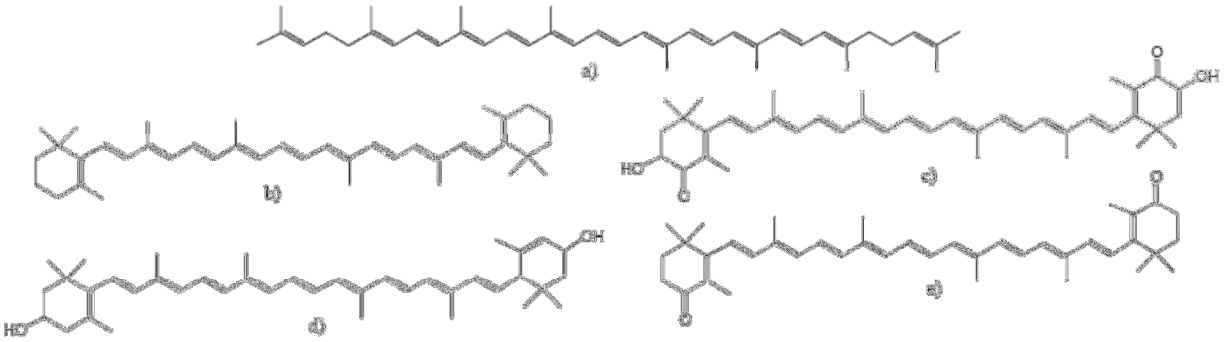


Figure 2.8. Chemical structures of some carotenoids. a) lycopene, b) β-carotene, c) astaxhanthin, d) lutein, e) canthaxanthin.

Carotenoids play different biological functions, such as provitamin A activity (α-carotene, β-carotene, β-cryptoxanthin), antioxidant action, UV skin and macular protection. Accordingly, the consumption of a carotenoid rich diet has been epidemiologically correlated with a lower risk for several diseases such as atherosclerosis, cardiovascular disease, cataracts, age-related macular degeneration, certain types of cancer such as breast, prostate and lung, multiple sclerosis and other major degenerative diseases, as well as to an enhanced immune resistance to viral, bacterial, fungal and parasitic infections (Astorg, 1997; Cooper *et al.*, 1999; Engelhart *et al.*, 2002; Klipstein-Grobusch *et al.*, 2000; Stahl and Sies, 2005; Tapiero *et al.*, 2004). The mechanism by which carotenoids exert their health benefits is, as yet, not well understood. In part, it may be due to its antioxidant activities (Mathews-Roth, 1991).

Nevertheless, large-scale intervention trials (ATBC, 1994; Omenn *et al.*, 1996) with β-carotene supplementation, showed higher incidence of both lung-cancer and mortality in high risk heavy smokers and asbestos workers in US and Finland, suggesting that the threshold between the beneficial and adverse effects of some carotenoids is low and provides a strong stimulus to further understand the functional effects of specific carotenoids (van den Berg *et al.*, 2000).

More than 600 known carotenoids are reported in nature, but only very few have commercial use: β-carotene, astaxanthin and, of lesser importance, lutein, zeaxanthin, lycopene and bixin which are used in animal feeds, pharmaceuticals, cosmetics and food colourings. The main carotenoids commercially produced by microalgae are β-carotene from *Dunaliella salina* and astaxanthin from *Haematococcus pluvialis*.

Dunaliella is an halotolerant green microalga, naturally occurring in salted lakes (seasonal orange coloured blooms), that is able to accumulate very large amounts of β-carotene (up to 14% DW), under nutrient-stressed, high salt and high light conditions (Oren, 2005). *Dunaliella* is produced in large scale pond systems in Australia (Betatene Ltd), Israel (Nature Beta Technologies) and USA (Cyanotech Corp), being natural β-carotene widely commercialized as food colorant (1-20% oil extracts), nutritional supplements (tablets and capsules) and for feed applications. β-carotene serves as an essential nutrient and has high demand in the market as a natural food colouring agent, as an additive to cosmetics and also as an health food (Raja *et al.*, 2007). β-carotene is routinely used in soft-drinks, cheeses and butter or margarines. It is well regarded as being safe, and indeed positive health effects are also ascribed to these carotenoids due to a pro-vitamin A activity (Baker and Gunther, 2004).
The benefits of astaxanthin are said to be numerous, and include enhancing eye health, improving muscle strength and endurance and protecting the skin from premature ageing, inflammation and UVA damage. Astaxanthin is also a strong colouring agent and has many functions in animal health, such as growth, vision, reproduction, immune function, and regeneration (Blomhoff *et al.* 1992; Tsuchiya *et al.* 1992; Beckett and Petrovich, 1999). Some reports support the assumption that daily ingestion of astaxanthin may protect body tissues from oxidative damage as this might be a practical and beneficial strategy in health management. It has also been suggested that astaxanthin has a free radical fighting capacity worth 500 times that of vitamin E (Dufossé *et al.*, 2005).

2.1.3.3. Phycobiliproteins

Besides chlorophyll and carotenoid lipophilic pigments, Cyanobacteria (blue-green algae), Rhodophyta (red algae) and Cryptomonads algae contain phycobiliproteins, deep coloured water-soluble fluorescent pigments, which are major components of a complex assemblage of photosynthetic light-harvesting antenna pigments - the phycobilisomes (Glazer, 1994). They absorb energy in portions of the visible spectrum (450-650 nm) that are poorly utilized by chlorophyll and, through fluorescence energy transfer, convey the energy to chlorophyll at the photosynthetic reaction centre (Arad and Yaron, 1992). Phycobiliproteins are formed by a protein backbone covalently linked via one or two tioether links through cysteine residues to linear tetrapyrrole chromophoric prosthetic groups, named phycobilins (Figure 2.9). In many algae the phycobiliproteins are arranged in subcellular structures called phycobilisomes; these allow the pigments to be arranged geometrically in a manner which helps to optimize the capture of light and transfer of energy (Arad and Yaron, 1992). The main natural resources of phycobiliproteins are the cyanobacterium *Spirulina* (*Arthrospira*) for phycocyanin (blue) and the rhodophyte *Porphyridium* for phycoerythrin (red).

This group of pigments has a large spectrum of applications, evidenced by the recent work of Sekar and Chandramohan (2007) which screened 297 patents on phycobiliproteins from global patent databases. Phycobiliproteins are extensively used for fluorescence applications, as highly sensitive fluorescence markers in clinical diagnosis and for labelling antibodies used in multicolour immunofluorescence or fluorescence-activated cell-sorter analysis (Becker, 1994; Eriksen, 2008). Due to their limited distribution, and to the difficulties in their purification, these pigments are rather expensive, and obtaining them as pure compounds is a potentially attractive endeavour (Reis *et al.*, 1998).

Phycocyanin is currently used in Japan and China as a natural colouring, in food products like chewing gums, candies, dairy products, jellies, ice creams, soft drinks (*e.g.* Pepsi® blue) and also in cosmetics such as lipstick, eyeliner and eye shadow (Sekar and Chandramohan, 2007). In a recent study, phycocyanin was considered to be a more versatile blue colorant than gardenia and indigo, providing a bright blue colour in jelly gum and coated soft candy, despite its lower stability towards heat and light (Jespersen *et al.*, 2005). A rising number of investigations revealed several pharmacological properties attributed to phycocyanin including, high free-radical scavenging capacity, anti-inflammatory, neuroprotective and hepatoprotective effects (Romay *et al.* 2003; Benedetti *et al.*, 2004; Bhat and Madyastha, 2000).

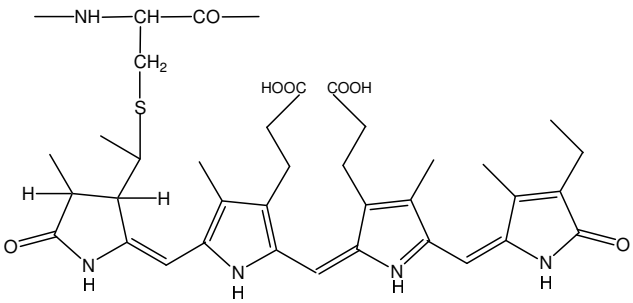


Figure 2.9. Chemical structure of a phycocyanobilin attached by thioether linkage to the apoprotein.

2.1.4. Microalgae as source of bioactive molecules

Microalgae can biosynthesize, metabolize, accumulate and secrete a great diversity of primary and secondary metabolites, many of which are valuable substances with potential applications in the food, pharmaceutical and cosmetics industries (Yamaguchi, 1997).

Like any other organism, the microalgae respond to changes in external environment with changes in their intracellular environment. Thus, the manipulation of cultivation parameters (presence or absence of certain nutrients, temperature, light intensity, photoperiod and the microalgae growth phase), stimulates the biosynthesis of compounds ranging from drugs to enzymes and natural antioxidants, some of high commercial value. In this sense, microalgae can be regarded as natural bioreactors since they can grow under specific controlled

conditions manipulated in order to produce and accumulate some biologically active compounds in large amounts (Plaza *et al.*, 2009).

2.1.4.1. Fatty Acids

Polyunsaturated fatty acids (PUFAS) are essential for human development and physiology being structural components of cell and organelle membranes. Long-chain (LC) PUFAs with more than 18 carbons (Figure 2.10) require specific synthesizing enzymes, present in microalgae and fresh water fish species. In healthy adult humans, it is estimated that the conversion rate from α-linoleic acid (ALA, 18:3ω_3) to eicosapentaenoic acid (EPA, 20:5ω_3) is <5%, and <0.05% for docosahexaenoic acid (DHA, 22:6ω_3) (Wang *et al.*, 2006; Burdge and Calder, 2005), so these LC-PUFAs have to be obtained through diet.
Microalgae are believed to be the primary producers of these LC-PUFAs in the marine food chain, being able to accumulate high levels of fatty acids with particular interest namely GLA (*Arthrospira*), arachidonic acid (AA, 20:4ω_6) (*Porphyridium*), EPA (*Nannochloropsis, Phaeodactylum*, *Nitzschia*, *Isochrysis*, *Diacronema*) and DHA (*Crypthecodinium*, *Schizochytrim*) (Bandarra *et al.*, 2003; Donato *et al.*, 2003; Chini-Zittelli *et al.*, 1999; Molina-Grima *et al.*, 2003; Spolaore *et al.*, 2006).

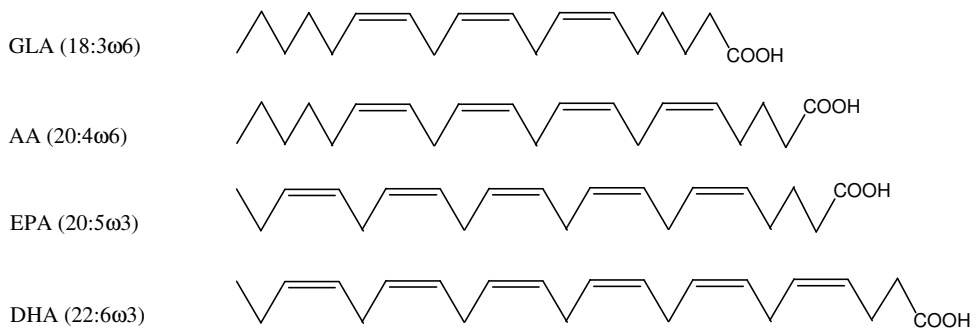


Figure 2.10. Chemical structure of polyunsaturated fatty acids of high pharmaceutical and nutritional value.

Fish and fish oils are the main sources for LC-PUFA; still global fish stocks are declining due to general fishing methods and over-fishing, and the derived oils are sometimes contaminated with a range of pollutants, heavy metals, toxins and typical fish odour, unpleasant taste and poor oxidative stability (Certik and Shimizu, 1999; Luiten *et al.*, 2003). The production of LC-PUFA from microalgae biotechnology is an alternative approach, and currently microalgal DHA from *Crypthecodinium*, *Schizochytrium*, and *Ulkenia* is commercially available by Martek Biosciences (USA) and Nutrinova (Germany) companies, for application in infant formulas, nutritional supplements and functional foods (Pulz and Gross, 2004; Spolaore *et al.*, 2006).

Dietary fatty acids can influence cognition by altering the lipid composition of cell membranes, cell membrane fluidity and neuronal cell signalling. There has been particular research interest in the role of ω3 long-chain polyunsaturated fatty acids (LC-PUFA ω3) which have been recognized due to its therapeutic action, with beneficial effects upon human health. PUFA's ω3, especially DHA, are essential in infant nutrition, being important building blocks in brain development, retinal development and ongoing visual, cognitive, as well as important fatty acids in human breast milk (Ghys *et al.*, 2002; Wroble *et al.*, 2002; Arteburn *et al.*, 2007; Crawford, 2000). LC-PUFA ω3 consumption has been associated with the regulation of eicosanoid production (prostaglandins, prostacyclins, tromboxanes and leucotrienes) which are biologically active substances that influence various functions in cells and tissues (*e.g.* inflammatory processes), being important in the prophylaxis and therapy of chronic and degenerative diseases including reduction of blood cholesterol, protection against cardiovascular, coronary heart diseases, atherosclerosis, diabetes, hypertension, rheumatoid arthritis, rheumatism, skin diseases, digestive and metabolic diseases as well as cancer (Simopoulos, 2002; Bønaa *et al.*, 1990; Sidhu, 2003; Thies *et al.*, 2003). Other important role is attributed to gene expression regulation, as well as cholesterol and fasting triacylglycerol (TAG) decreases (Calder, 2004). The evidence of a dietary deficiency in LC-PUFA ω3 is firmly linked to increased morbidity and mortality from coronary heart disease.

2.1.4.2. Tocopherols and Sterols

Tocopherols are lipid-soluble antioxidants synthesized only by photosynthetic organisms, widespread in nature. α-Tocopherol (Figure 2.11) represents about 80% of total tocopherols and tocotrienols and is the main compound responsible for vitamin E activity in microalgae and other organisms of vegetable origin (Chen *et al.*, 1998).

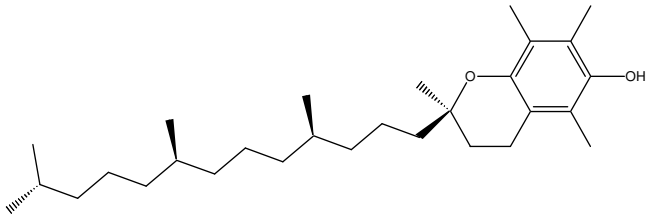


Figure 2.11. Structure of α-tocopherol (vitamin E).

The variability of vitamin E in microalgae is inherent to the species and environmental conditions (*i.e.*, light and temperature). *Euglena* microalga has the highest tocopherol content among the several genera of yeast, moulds and algae tested (Kusmic *et al.*, 1999). The benefits of vitamin E on human health have been subject of several studies that confirm its beneficial effect in preventing degenerative disorders, such as cardiovascular diseases,

arteriosclerosis, certain types of cancer and light-induced pathologies of skin and eyes (Clark, Eitenmiller, 1997; Gómez-Coronado *et al.*, 2004). For this reason, it has been suggested that its use as a supplement or nutraceutical could have a positive impact on health (Rasool and Wong, 2007), being currently widely used in food as natural lipophilic antioxidant (Chen *et al.*, 1998).

Sterols are a group of triterpenoids, a family of natural products derived from biosynthetic squalene which can easily take over a cyclic structure, as shown in Figure 2.12 (Goodwin, 1980). These biomolecules are an important family of lipids and are found in most eukaryotic cells, mainly in the plasma membrane. Animal cells and fungi usually contain one major sterol (cholesterol and ergosterol, respectively) while more than 100 types of phytosterols have been reported in plant species (mainly sitosterol, campesterol and stigmasterol). The microalgae contain different types of sterols that are characteristic of each species, being useful as biomarkers to support taxonomic classification (Volkman, 2003). In green algae there is no single major sterol; the dominant sterol seems to vary within the order and within the family. Studies covering a wide range of phytoplankton have suggested that the growth rates of bivalves are related to the kind and amount of sterols present in the diet phytoplankton (Wikfors *et al.*, 1992). On the other hand, it has been found that many polyhydroxysterols from marine organisms have anticancer, cytotoxic and other biological activities (Cui *et al.*, 2000; Tang *et al.*, 2002; Volkman, 2003).

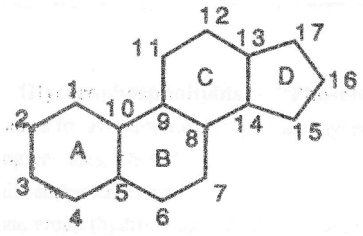


Figure 2.12. Basic structure of sterols.

2.1.4.3. Proteins

Since the early fifties, intense efforts have been made in order to explore new and unconventional protein sources and/or food supplements, primarily in anticipation of an increasing world population and a repeatedly predicted insufficient protein supply. Microalgae, as well as yeast, fungi and bacteria, have been described as promising sources of single cell protein (SCP) (Becker, 2004; Soletto *et al.*, 2005). A limiting factor for SCP use is their high content in nucleic acids, since purines are metabolized to uric acid that can cause adverse health effects (*e.g.* gout, kidney stones). Compared to other sources of SCP algae proteins contain a low amount of nucleic acids (Chronakis, 2000), and it has been shown that taking up to 50 g of *Chlorella* per day does not increase the level of uric acid in humans

(Waslein *et al.*, 1970), since it is far below the 2 g/day maximum recommended daily intake of nucleic acids for a healthy adult.

The nutritive value of algae proteins is comparable to that of most conventional protein feed supplements, in terms of gross protein content, amino acid quality and composition as well as nutritional acceptability (Chronakis, 2000). Protein content in algal biomass depends on the availability of nutrients, such as potassium, sodium and nitrogen, and is determined by growth phase as well as by light quality (Gantar and Svircev, 2008).

The amino acid pattern of almost all algae compares favourably with that of other food proteins, especially of vegetable origin, being generally deficient in the sulphur containing amino acids cystine and methionine (Chronakis, 2000). Since the cells are capable of synthesizing all amino acids, they can provide the essential ones to humans and animals (Guil-Guerrero *et al.*, 2004). As other bioactive compounds synthesized by microalgae, the composition in amino acids, especially free amino acids, varies greatly between species as well as with growth conditions and growth phase (Borowitzka, 1988). Protein or amino acids may therefore be by-products of an algal process for the production of other fine chemicals, or with appropriate genetic enhancement, microalgae could produce desirable amino acids in sufficiently high concentrations (Borowitzka, 1988).

2.1.4.4. Polysaccharides

Polysaccharides are widely used in the food industry primarily as gelling and/or thickening agents. Many commercially used polysaccharides like agar, alginates and carrageenans are extracted from macroalgae (*e.g. Laminaria*, *Gracilaria*, *Macrocystis*) (Borowitzka, 1988). Nevertheless, most microalgae produce polysaccharides and some of them could have industrial and commercial applications, considering the fast growth rates and the possibility to control the environmental conditions regulating its growth.

Polysaccharides account for the bulk of microalgae carbohydrates, and are often divided into three groups: reserve glucans, extracellular polysaccharides and cell wall polysaccharides.

Branched starches and β-1,3-glucans are the most common cell reserve materials in microalgae, being present in concentrations up to 50% (Gantar and Svircev, 2008). Due to the nature of the glycosidic linkages of these polysaccharides, they are commonly referred as interesting for bioethanol production, by saccharification and subsequent or simultaneous fermentation (Ogaki, 2009).

Microalgae can secrete extracellular polymeric substances, mainly exopolysaccharides (EPS), which can be formed as capsular material around the cell, or as loose-slime matrices that are released more widely into the surrounding environment. *Porphyridium cruentum,* one of the most promising microalga for commercially purposes, produces sulphated galactan

EPS that can replace carrageenans in many applications. These heteropolymers are composed of about 10 different sugars (mainly xylose, glucose and galactose), glucuronic acid and half-ester sulphate groups, and dissolve continuously into the medium (Arad, 1988; Geresh, 2009). *Porphyridium* EPS are considered to have relevant biological activity, exhibiting anti-retroviral, anti-inflammatory, hypocholesterolaemic and anticell proliferation activities (Dvir, 2000; Talyshinsky, 2002) related to the sulphate groups (Keidan 2006).
The cell wall of eukaryotic algae is predominantly composed of cellulose polysaccharides, while cyanobacteria such as *Spirulina* lack a rigid cell wall. Peptidoglycan is the major polysaccharide in the cyanobacteria cell membranes which makes their biomass more easily digestible (Gantar and Svircev, 2008), and glycogen their principal storage product.

2.1.4.5. Vitamins and Minerals

Microalgae biomass represents a valuable source of nearly all essential vitamins (*e.g.* A, B_1, B_2, B_6, B_{12}, C, E, nicotinate, biotin folic acid and pantothenic acid) and balanced mineral content (*e.g.* Na, K, Ca, Mg, Fe, Zn and trace minerals) (Becker, 2004). The high levels of vitamin B_{12} and Iron in some microalgae, like *Spirulina*, make them particularly suitable as nutritional supplements for vegetarian individuals. The vitamin content of an alga depends on the genotype, the stage in the growth cycle, nutritional status of the alga and light intensity (photosynthetic rate). The vitamin content is therefore amenable to manipulation by varying the culture conditions as well as by strain selection or genetic engineering. However, the vitamin's cell content fluctuates with environmental factors, the harvesting treatment and the biomass drying methods (Brown *et al.*, 1999; Borowitzka, 1988).

2.1.4.6. Antioxidants

Antioxidants are thought to possibly prevent the incidence of many diseases such as cancer, cardiovascular and age related diseases, by protecting cells against oxidative damage.
Microalgae are photoautotrophic organisms that are exposed to high oxygen and radical stresses, and consequently have developed several efficient protective systems against reactive oxygen species and free radicals (Pulz and Gross, 2004).
Synthetic antioxidants, such as BHT (butylated hydroxytoluene) and BHA (buthylated hydroxyanisole), are believed to be carcinogenic and tumorigenic if given in high doses (Aruoma 2003), so there is an increasing interest in using alternative natural antioxidants for cosmetics (*e.g.* sun-protecting) and functional food/nutraceuticals.
Natrah *et al.* (2007) reported a stronger antioxidant activity exhibited by methanolic microalgal crude extracts (from *e.g. Isochrysis galbana*, *Chlorella vulgaris*, *Nannochloropsis oculata*, *Tetraselmis tetrathele*, *Chaetoceros calcitrans*) when compared with α-tocopherol,

but lower than the synthetic antioxidant BHT. Rodriguez-Garcia and Guil-Guerrero (2008) also observed that extracts from *Chlorella vulgaris* and *Phaedactylum tricornutum* showed higher antioxidant activity than synthetic BHA and BHT.

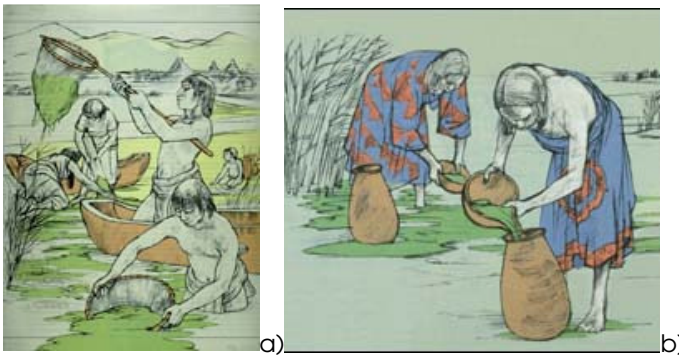
2.1.4.7. Pharmaceuticals and other biologically active compounds

Microalgae represent a very large, relatively unexploited reservoir of novel compounds, many of which are likely to show biological activity, presenting unique and interesting structures and functions (Yamaguchi, 1997). These secondary metabolites (*e.g.* organic acids carbohydrates, amino acids, peptides, vitamins, antibiotics, enzymes, pigments, toxic compounds) are part of the microalgal normal cell growth, related to the interactions with the environment, and their production is usually favoured by sub-optimal growth conditions.
In the last decades marine microorganisms, particularly Cyanobacteria, have been screened for new pharmaceuticals such as antibiotics, antiviral, anticancer, enzyme inhibitory agents and therapeutic applications in the treatment of cancer. Published data until 1996 revealed 208 cyanobacterial compounds with biological activity while in 2001 the number of compounds screened was raised to 424, including lipoproteins (40%), alkaloids, amides and others (Burja *et al.*, 2001). The reported biological activities comprise cytotoxic, antitumor, antibiotic, antimicrobial (antibacterial, antifungal, antiprotozoa), antiviral (*e.g.* anti-HIV) activities as well as biomodulatory effects like immunosuppressive and anti-inflammatory (Burja *et al.*, 2001; Singh *et al.*, 2005). The cytotoxic activity, important for anticancer drug development, is likely related to defence strategies in the highly competitive marine environment, since usually only organisms lacking an immune system are prolific producers of secondary metabolites such as toxins (Burja *et al.*, 2001).

2.1.5. Microalgae in Human Nutrition

Utilization of algae as a human food source began inconspicuously. Edible blue-green microalgae, including *Nostoc*, *Spirulina*, and *Aphanizomenon* species, have been used as a nutrient-dense food for many centuries in Asia, Africa and Mexico (Hallman, 2007). The first traceable use of microalgae by humans dates back 2000 years to the Chinese, who used *Nostoc* to survive during famine (Spolaore *et al.*, 2006; Hallman, 2007). Spanish chroniclers from the 16th century described Aztecs collecting blue coloured *"techuitlatl"* (*Spirulina*) from Lake Texcoco (Mexico) and producing a type of bread from this sun-dried material (Figure 2.13). For Kanembu tribal people living in deserted areas around Lake Chad (Central Africa), *Spirulina* biomass is a precious food resource from ancient times until present. The wet biomass is dried in the sun, and then cut into small squares (*Dihé*) that are crumbled and

mixed with a sauce of tomatoes and peppers, being consumed in 70% of their meals (Abdulqader *et al.*, 2000).



a) b)

Figure 2.13. Aztecs harvesting algae from lakes in the Valley of Mexico (a); Kanembu women gathering *Spirulina* from the area around Lake Chad (b). (Source: Furst (1978)).

In the early 1950's microalgae were considered to be a good supplement and/or fortification in diets for malnourished children and adults as a single cell protein (SCP) source.

Although microalgae are consumed since ancient times, they are considered as unconventional food items and have to undergo a series of toxicological tests to prove their harmlessness. In fact, some algae have been tested under all possible aspects much more carefully than most of any conventional food commodities (Becker, 2004).

Some of the prerequisites for the utilization of algal biomass for human and animal consumption include the determination of proximate chemical composition; biogenic toxic substances; non-biogenic toxic compounds; protein quality studies; biochemical nutritional studies; supplementary value of algae to conventional food sources; sanitary analysis; safety evaluations (feeding trials with animals); clinical studies (test for safety and suitability of the product for human consumption) and acceptability studies (Becker, 2004).

Some human nutritional studies were done with humans and the authors suggest that the algae daily consumption should be restricted to about 20 g, with no harmful side effects occurrence, even after a prolonged period of intake (Becker, 1988). While some studies report that people have lived solely on algae for prolonged periods of time without developing any negative symptoms, in other studies, discomfort, vomiting, nausea, and poor digestibility of even small amounts of algae were reported.

Powell *et al.* (1961) performed one of the first studies, in which a meal containing up to 500 g of a mixture of *Chlorella* and *Scenedesmus* was given to young healthy men. Subjects tolerated well 100 g incorporation levels, but above this some gastrointestinal disorders were observed.

Gross *et al.* (1982) performed a study feeding algae (*Scenedesmus obliquus*) to children (5 g/daily) and adults (10 g/daily), incorporated into their normal diet, during four-week test period. Haematological data, urine, serum protein, uric acid concentration and weight

changes were measured, and no changes in the analysed parameters were found, except a slight increase in weight, especially important for children. The same authors also carried out a study (Gross *et al.*, 1978) with slightly (group I) and seriously (group II) malnourished infants during three weeks. The four-year-old children of group I (10 g algae/daily) showed a significant increase in weight (27 g/day) compared with the other children of the same group who received a normal diet, and no adverse symptoms were recorded. The second group was nourished with a diet enriched with 0.87 g algae/kg body weight, substituting only 8% of the total protein and the daily increase in weight was about sevenfold (in spite of a low protein contribution) and all anthropogenic parameters were normal. The authors concluded that the significant improvement in the state of the health was attributed not only to the algal protein but also to therapeutic factors.

Almost no adverse symptoms have been revealed so far in connection to the consumption of microalgae and unwanted side effects appear to be extremely rare (Becker, 2004). However, there are still some health concerns remaining regarding the ingestion of microalgae. Several strains of cyanobacteria have been identified with the production of biogenic toxins (Cox *et al.*, 2005). However, these cases are associated with wild algal blooms and no such cases have ever been reported in connection to mass cultured algae (Becker, 2004). Non-biogenic toxins, such as heavy metals and other contaminants, can also be avoided by proper cultivation techniques and non-polluted cultivation areas. The content in nucleic acids (RNA and DNA) is another concern, since these are sources of purines which are uric acid precursors that when accumulated in the serum may increase the risk of gout and kidney stones. In fact, this has been the major limitation for SCP use as food or food ingredient. However, microalgae have relatively low nucleic acid contents (4-6%) when compared to yeasts (8-12%) and bacteria (20%), so an intake below 20 g of algae per day or 0.3 g of algae per kg of body weight should present no harm (Becker, 2004).

2.1.5.1. Novel Foods Regulation

Authorization of novel foods and novel food ingredients is harmonised in the European Union (EU) by the Regulation EC 258/97. This Regulation applies to foods and food ingredients which present a novel primary molecular structure (*a*); consist of micro-organisms, fungi or algae (*b*); consist of or are isolated from plants or isolated from animals (*c*); or whose nutritional value, metabolism or level of undesirable substances has been significantly changed by the production process.

Foods commercialised in at least one Member State before the entry into force of the Regulation on Novel Foods on 15 May 1997, are on the EU market under the *"principle of mutual recognition"*. This is the case of the microalgae *Arthrospira* (*Spirulina*) *platensis, Chlorella pyrenoidosa*, and *Aphanizomenon flosaquae* (filamentous blue-green algae from

Klamath Lake, Oregon USA), according to the DG Health and Consumer Protection, Novel Foods Catalogue (http://ec.europa.eu/food/food/biotechnology/novelfood/)

In order to ensure the highest level of protection on human health, novel foods must undergo a safety assessment before being placed on the EU market. Some of the key aspects that should be considered are, among others, the history of use of the organism; predicted levels of ingestion; nutritional microbiological data; toxicological studies (animal and human); allergenic potential; substantial equivalence to other foods; and possible implications on human nutrition. The production of Lycopene from *Blakeslea trispora* fungus by the spanish Company Vitatene Antibiots S.A.U. (2006/721/EC) is an example of an approved novel food ingredient under Regulation 258/97. The application of DHA-rich algal oil from *Schizochytrium* sp. for additional food uses by Martek Biosciences Corporation (USA) is currently under evaluation.

Novel foods or novel food ingredients may follow a simplified procedure (article 5th), only requiring notifications from the company, when they are considered by a national food assessment body as *"substantially equivalent"* to existing foods or food ingredients (regarding their composition, nutritional value, metabolism, intended use and the level of undesirable substances contained therein). This was the case of the Microalga *Odontella aurita* from Innovalg (France), considered substantially equivalent to other authorized algae in December 2002; or the DHA (docosahexahenoic acid)-rich microalgal oil (DHActive™) from Nutrinova (Germany) in November 2003. Novel foods notifications of Astaxanthin-rich extracts derived from *Haematococcus pluvialis* have been approved for several companies such as US Nutra (USA), AstaReal AB (Sweden), Alga Technologies Ltd (Israel) and Cyanotech (USA). The successful authorization of these microalgal based foods and food ingredients broaden perspectives for a wider inclusion of these valuable microorganisms in the human diet.

The European Commission has adopted a proposal to revise the Novel Foods Regulation (COM(2007)872) with a view for improving the access of new and innovative foods to the EU market, while maintaining a high level of consumer protection and ensuring food safety. A notification procedure is introduced for foods which have not been traditionally sold in the EU but have a safe history of use in third countries. This will be particularly interesting for the widespread use of microalgae as foods considering that these organisms have been used for human nutrition in Far East countries since ancient times.

2.1.5.2. New Trends in Microalgae Food Applications

Commercial large-scale production of microalgae started in the early 1960s in Japan with the culture of *Chlorella* as a food additive, which was followed in the 1970s and 1980s by expanded world production in countries such as USA, India, Israel, and Australia (Spolaore *et al.*, 2006; Borowitzka, 1999). In 2004, the microalgae industry had grown to produce 7000

tonnes of dry matter per year (Table 2.1) with *Chlorella, Spirulina* and *Dunaliella* dominating the market (Pulz and Gross, 2004).

Table 2.1. Major microalgae commercialized for human nutrition purposes (adapted from Pulz and Gross, 2004; Spolaore *et al.*, 2006 and Hallmann, 2007).

Microalga	Major Producers	Products	World Production (ton/year)
Spirulina (*Arthrosphira*)	Hainan Simai Pharmacy Co. (China) Earthrise Nutritionals (California, USA) Cyanotech Corp. (Hawaii, USA) Myanmar Spirulina factory (Myanmar)	Powders, extracts tablets, powders, extracts tablets, powders, beverages, extracts tablets, chips, pasta and liquid extract	3000
Chlorella	Taiwan Chlorella Manufacturing Co. (Taiwan) Klötze (Germany)	tablets, powders, nectar, noodles Powders	2000
Dunaliella salina	Cognis Nutrition and Health (Australia)	Powders β-carotene	1200
Aphanizomenon flos-aquae	Blue Green Foods (USA) Vision (USA)	Capsules, crystals powder, capsules, crystals	500
Haematococcus pluvialis	Cyanotech (USA), Mera Pharmaceuticals (USA), Parry's Pharmaceuticals (India), Algatech (Israel)	Astaxanthin	300
Chrypthecodinium cohnii	Martek (USA)	DHA oil	240
Schizochytrium	Martek (USA)	DHA oil	10

Nowadays, microalgae are mainly marketed as health food or food supplement and commonly sold in the form of tablets, capsules, and liquids (Chacón-Lee and González-Mariño, 2010). Much attention is being diverted to algae as ingredient factories, particularly of nutritional ingredients such as omega-3 fatty acid DHA and astaxanthin carotenoid. In fact, while *Chlorella*, *Spirulina* and *Haematococcus* biomass are sold at 36-50€/kg for human and animal nutrition (aquaculture), fine chemical compounds reach far most expensive prices: β-carotene 215-2150 €/kg, DHA oil 43€/g, astaxanthin 7 €/mg and phycocyanin 11€/mg (Brennan and Owende, 2010).

Additionally, there is an increasingly growing market for food products with microalgae addition (Figure 2.14) such as pastas, biscuits, bread, snack foods, candy bars or gums, yoghurts, drink mixes, soft drinks, etc., either as nutritious supplements, or as a source of natural food colorant (Becker, 2004). In some countries (Germany, France, Japan, USA, China, Thailand), food production and distribution companies have already started serious activities to market functional foods with microalgae and cyanobacteria (Pulz and Gross, 2004). However, biotechnological exploitation of microalgae resources for human nutrition purposes is restricted to very few species due to strict food safety regulations, commercial factors, market demand and specific preparation (Pulz and Gross, 2004). Potential consumers

may have some reluctance to use algal or algal related products due to conservative ethnic factors including religious and socio-economic aspects (Becker, 2004), but foods supplemented with microalgae biomass may be sensorially more convenient and variable, thus combining health benefits with attractiveness to consumers, namely in terms of colour. Also, several microalgae when correctly processed have an attractive or piquant taste and can thus be well incorporated into many types of foods, adding not only nutritional value, but also new, unique and attractive tastes (Richmond, 2004).

Figure 2.14. Examples of commercial microalgae based food products.

In the last few years, some research has been carried out regarding the development of a range of novel attractive healthy foods, prepared from microalgae biomass, rich in carotenoids and polyunsaturated fatty acids with antioxidant effect and other beneficial properties. Traditional foods, like biscuits, pasta, mayonnaises and breakfast cereals, largely consumed on a daily basis in different European diets, can be used as vehicles for those nutraceuticals.

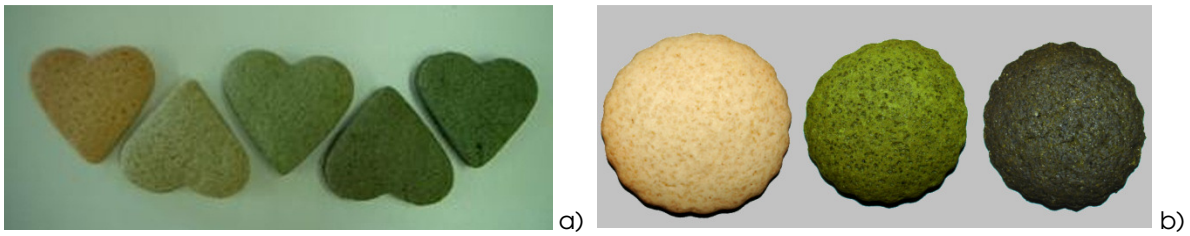
The viability of incorporating microalgal biomass in food systems is conditioned by the applied processing type and intensity (*e.g.* thermal, mechanical), by the nature of the food matrix (*e.g.* emulsion, gel, aerated dough systems) and by the interactions with other food components (*e.g.* proteins, polysaccharides, lipids, sugars, salts). Besides colouring and nutritional purposes, introducing microalgal ingredients in food systems can also impart significant changes in its microstructure and rheological properties.

Gouveia and co-workers published some studies on the development of short-dough biscuits with *Chlorella vulgaris* (Gouveia *et al.*, 2007) and *Isochrysis galbana* (Gouveia *et al.*, 2008) microalgal biomass addition.

Chlorella vulgaris biscuits presented an accentuated green tonality (Figure 2.15a), that remained stable along the storage period, and an increase of added microalgal biomass reflected a significant increase on the biscuits' firmness, evidencing the positive effect of the alga in the biscuit structure, reinforcing the short dough system. Biscuits are considered solid emulsions of sucrose, lipids and non-gelatinized starch, and the main factor affecting their structure and texture is the moisture content and water mobility, which is highly affected by

the interaction with hydroxyl groups present in the matrix (Hoseney *et al.*, 1988). The replacement of a small amount of flour by microalgae biomass resulted in the inclusion of a complex biomaterial, rich in different proteins and polysaccharides. These molecules have an important role on the water absorption process, which promotes the increase of biscuit firmness, resulting in more compact structures.

Similar results were observed for *Isochrysis* biscuits (Figure 2.15b) which presented high colour and texture stability along time. Due to *Isochrysis* high levels of LC-PUFA-ω_3, especially EPA and DHA (Bandarra *et al.*, 2003), and in spite of the drastic thermal processing (high temperatures) during the biscuit manufacturing, the addition of microalgal biomass leads to the presence of ω_3 fatty acids (EPA+DHA=100-300 mg/100 g) which remain stable along storage time. The thermal resistance of fatty acids should be due to its presence in an encapsulated form inside the microalga cell. These values reflect an important source of PUFA-ω_3 with a moderate biscuit consumption, as the recommendations for dietary intake in healthy adults is 500 mg/day (ISSFAL, 2004).



a) b)

Figure 2.15. Short-dough biscuits with microalgal biomass addition - *Chlorella vulgaris* (a) and *Isochrysis galbana* (b) (Gouveia *et al.*, 2007; 2008).

Recently, Fradique and co-workers (2010) studied the incorporation of microalgal biomass to durum wheat semolina pastas. *Spirulina* and *Chlorella* (green and carotenogenic) pastas presented very appealing colours, such as green and orange, similar to pastas produced with vegetables (Figure 2.16). The use of microalgae biomass can enhance the nutritional and sensorial quality of pasta, without affecting its cooking and textural properties.

Pastas prepared with *Isochrysis galbana* and *Diacronema vlkianum* biomass incorporation, presented a fatty acid profile containing EPA and DHA ω_3-fatty acids that did not suffer a significant change during the pasta cooking procedure (Fradique *et al.*, submitted).

The sensorial evaluation revealed that *Spirulina* and *Chlorella* pastas had higher acceptance scores than the control pasta, while in *Diacronema* and *Isochrysis* pastas a slight depreciative fish flavour for 2% microalgal biomass was detected. Nevertheless, this can prove to be an advantage when using a fish-based culinary preparation/product.

Another example of emerging applications of microalgae in novel food products is the study of Valencia and co-workers (2007) that developed dry fermented sausages rich in docosahexaenoic acid with oil from the microalgae *Schizochytrium* sp. The influence on nutritional properties, sensorial quality and oxidation stability was evaluated, with promising

results. However, in this study only the oil fraction was added while in the above cited studies with short-dough biscuits and durum wheat semolina pastas full microalgal biomass was added. This approach allows saving costs related to microalgae cell rupture and extraction, and at the same time provides natural encapsulation of the bioactive compounds (*e.g.* pigments, antioxidants, PUFAs) that could otherwise be degraded throughout food processing operations.

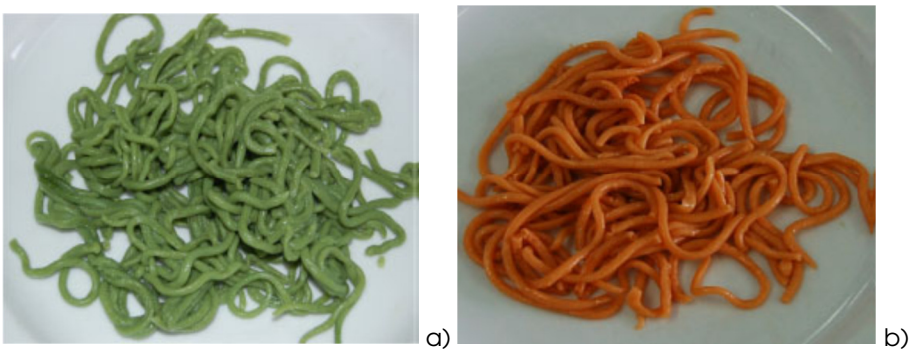


Figure 2.16. Durum wheat semolina pastas with *Chlorella vulgaris*, green (a) and carotenogenic (b), microalgal biomass addition (Fradique *et al.*, 2010).

2.2. COLLOIDAL SYSTEMS

2.2.1. General Concepts

Most food products, especially processed foods, are complex materials composed by small particles and molecules of different sizes and shapes. The stability and physical properties of these complex materials are ultimately determined by the nature of the interactions between the constituent microparticles and the type of microstructures they form (Dickinson, 2007).
Colloidal systems (or colloids) are the most common systems found in food materials, as well as in a wide range of chemicals from pharmaceuticals and cosmetics to agricultural chemicals, paints, cement, rubber, etc…
Colloids are a class of materials lying between bulk and molecularly dispersed systems, in which, although one component is finely dispersed in another, the degree of subdivision does not approach that of simple molecular mixtures (Everett, 1988). A colloidal dispersion is a system in which particles of colloidal size (at least one dimension within the nanometre to micrometre range) are distributed uniformly in a finely divided state (dispersed or discontinuous phase) in a dispersion medium (or continuous phase) of a different composition (or state).

Table 2.2. Main types of colloidal systems (adapted from Everett, 1988 and Shaw, 1992).

System	***Continuous phase***	***Dispersed phase***	***Examples***
Liquid aerosol	Gas	Liquid	Fog, liquid sprays
Solid aerosol	Gas	Solid	Industrial smokes, dust
Foam	Liquid	Gas	Whipped cream, whisked egg white
Emulsion	Liquid	Liquid	Milk, mayonnaise, asphalt
Sol*	Liquid	Solid	Uncooked custard, unset jelly, toothpaste, paints
Gel	Liquid	Macromolecules	Jelly, jam
Solid foam	Solid	Gas	Meringue, bread, cake, ice cream
Solid emulsion	Solid	Liquid	Butter, margarine
Solid suspension	Solid	Solid	Pigmented plastics, pearl

**Colloidal suspension or paste (high solid concentration).*

The main types of colloidal dispersions as well as some examples are presented in Table 2.2. One of the most common types of colloidal system is the emulsion, in which two immiscible liquids are dispersed in one another. Common examples are oil-in-water (o/w) emulsions in which small oil droplets are dispersed through water (*e.g.* milk) or water-in-oil (w/o) emulsions in which small water droplets are dispersed through oil (*e.g.* butter). Emulsions are

thermodynamically unstable and need to be stabilized by an emulsifying agent (amphiphilic molecule) that prevents phase separation.

Foams are also very common colloids, formed by the dispersion of a large proportion of gas by volume in the form of gas bubbles in a liquid. The colloidal dimensions are associated not with the bubbles (>1μm) but with the thickness of the interstitial lamella between the bubbles (IUPAC, 2001).

Gels are considered macromolecular colloids since they are composed of polymeric materials (*e.g.* proteins, polysaccharides) dispersed in a liquid (Everett, 1988). These systems are sometimes described as dispersions of liquids in solids, since the continuous phase usually presents an elastic behaviour upon polymer dispersion. According to the IUPAC (2001) a gel is defined as a colloidal system with a finite, usually rather small, yield stress. They can also be classified as network colloids where both phases consist of interpenetrating networks, the elements of each being of colloidal dimensions (Everett, 1988).

Many biological structures are colloidal, *i.e.* are biocolloids. This is the case of blood, which is a dispersion of corpuscles in serum, and bone, which is a dispersion of calcium phosphate embedded in collagen (Everett, 1988).

Simple colloids, such as emulsions, which are formed by surface active molecules, can be associated together to form small aggregates (*micelles*) in water that are thermodynamically stable. The aggregates (association colloids) formed by certain substances may adopt an ordered structure and form liquid crystals (Everett, 1988).

Multiple colloids involve the co-existence of three phases of which two (and sometimes three) phases are finely divided. This is the case of multiple emulsions in which finely divided droplets of an aqueous phase are contained within oil droplets, which themselves are dispersed in an aqueous medium. Much attention has been drawn on these colloid systems as effective vehicles for reducing fat content in emulsified food products (Muschiolik, 2007).

A characteristic feature of colloidal systems is the large area-to-volume ratio of the particles involved, so their nature is closely linked to surface science. At the interfaces between the dispersed phase and the dispersion medium, characteristic surface properties play a very important part in determining the physical properties of the system as a whole. Colloidal dispersions are thermodynamically unstable due to their high surface free energy and are irreversible systems in the sense that they are not easily reconstituted after phase separation (Shaw, 1992).

Colloidal systems give structure, texture and mouthfeel to many different products. Their properties, specifically particle size, shape and flexibility, surface properties, particle-particle and particle-solvent interactions, underlie the way many foods are built up (Shaw, 1992; Dalgleish, 1996).

2.2.2. Food Emulsions

2.2.2.1. Introduction

An emulsion can be defined as an heterogeneous system, thermodynamically unstable, consisting of at least one immiscible liquid intimately dispersed in another in the form of droplets whose diameters, in general, exceed 0.1 μm. Such systems possess a minimal degree of stability, which may be accentuated by such additives as surface active agents, finely divided solids, etc. (Bassi *et al.*, 1993). The phase presented as finely divided droplets is called the disperse, internal, or discontinuous phase and the phase that forms the matrix in which these droplets are suspended is called the external or continuous phase (Bennion, 1980).
Food emulsions are usually dispersions of oil and water, comprising droplets with diameters between 0.1 and 100 μm. If oil is dispersed as small droplets in an aqueous continuous phase the emulsion is oil-in-water (o/w) type (*e.g.* mayonnaise, milk, cream, soups and sauces) whereas if water is the dispersed phase and oil the continuous phase, the emulsion is a water-in-oil (w/o) type (*e.g.* margarine, butter and spreads). Moreover, research has been carried out to create stable multiple emulsions (o/w/o or w/o/w), in which the dispersed phase contains additional globules of other phases which that can be used to control the release of certain ingredients, reduce the total fat content of emulsion-based food products, or isolate one ingredient from another (McClemments, 1999). The concentration of droplets in an emulsion is usually described in terms of volume fraction of the disperse phase (Φ), which is equal to the volume of emulsion droplets divided by the total volume of the emulsion.
The long term stability of an emulsion can be achieved by using efficient emulsifier and stabilizing agents. Emulsifiers are surface active molecules that facilitate the formation of emulsions by lowering the oil-water interfacial tension and imparting stability by forming a protective film around the dispersed droplets. Stabilizers are components that are able to improve the stability of an emulsion, usually by increasing the viscosity of the continuous phase (thickening agents) or by a colloidal protective action (Franco, 1995).

2.2.2.2. Droplet size distribution

Many of the most important properties of food emulsions, such as the shelf life, appearance, texture and flavour, are determined by the size of the droplets (Dickinson, 1992).
The number of droplets in most emulsions is extremely large, and so their size can be considered to vary continuously from a minimum to a maximum value, following a statistical distribution. Usually, the most stable emulsions have a monodisperse droplet distribution, *i.e.* the droplets are all approximately of the same size, with a maximum situated in small diameters. Ideally, in these cases the size of the droplets can be characterized by a single

number, such as the droplet diameter or radius. Nevertheless, food emulsions always contain a distribution of droplet sizes, which can be more or less disperse depending on the global composition of the emulsion system. Along the destabilization process the emulsions tend to be more polydisperse, with a maximum located in larger diameters (Dickinson, 1992).

The shape of the droplets may be irregular, but in general it is assumed that when Φ<0.74 they are approximately spherical; above that value the droplets are compacted and distorted into polyedric faced shapes (Rahalkar, 1992; Halling, 1981). Either way, the size of an irregular droplet can be defined as the size of an equivalent sphere that has the some dimensional properties (*e.g.* volume, superficial area).

It is convenient to represent the size of droplets in a polydisperse emulsion by an average diameter, based on characteristic dimensions (*e.g.* length, superficial area, volume), rather than stipulating the full particle size distribution. Some of the most used average diameters used to describe particle sizes in colloidal systems are presented in Table 2.3.

Table 2.3. Average particle size diameters used to describe colloidal systems.

Name	Symbol	Definition
Average diameter	$d_{(1,0)}$	$D_{(1,0)} = \sum d/n$
Sauter mean diameter	d_{sv} or d_{32}	$d_{sv} = \sum nidi^3/\sum nidi^2$
Volumetric mean diameter	d_{43}	$d_{43} = \sum nidi^4/\sum nidi^3$

*n_i = number of particles with a dimension d_i

The Sauter mean diameter is the most commonly used to analyse droplet size distributions in food emulsions, since it is related to the superficial area (*A*), *i.e.* with the specific area of the droplets exposed to the continuous phase by volume unit:

$$A = \frac{6\Phi}{d_{sv}} \tag{2.1}$$

The polydispersity of an emulsion can be described by the Uniformity (*U)* parameter, which measures the absolute deviation of droplet sizes in relation to the median (monodisperse distributions: *U*~1). Equation 2.2 describes this parameter, where V_i and d_i are the relative volumetric frequency and the average diameter for the dimension *i*, *d*(*v*,0.5) is the median of the distribution:

$$U = \frac{\sum V_i \, |d(v,0.5) - di)|}{d(v,0.5) \sum V_i} \tag{2.2}$$

The experimental determination of the droplet size distribution can be performed through several methods including microscopic ones (*e.g.* optical, electronic, confocal laser, atomic force), measurement of changes in the electric resistance (*e.g.* coulter counter), turbidimetric

techniques (less used) and laser light scattering techniques (adequate for 0.1-1000 μm sizes, suitable for most food emulsions)(Franco, 1995).

The droplet size distribution of an emulsion is affected by several parameters, such as the emulsifier type and concentration, the energy applied to the system, the composition of the aqueous and oil phases or the temperature. The effect of these factors on the droplet size and emulsification process will be discussed in section 2.2.2.4.

2.2.2.3. Emulsion formation

The process of creating an emulsion from two separate immiscible liquids requires the application of high mechanical energy in a process called homogenization, and which is carried out in mechanical devices called homogenizers. An emulsion formed by the homogenization of two separate liquids (primary homogenization), using, for instance, an high speed blender, can be subjected to a secondary homogenization in another homogenizer (*e.g.* high-pressure valve homogenizer) in order to reduce the droplet size and improve the stability of the emulsion (McClemments, 1999). The mechanical conditions involved in the homogenization process have considerable implications in the process efficiency and in the physical characteristics and stability of the emulsions (Franco *et al.*, 1998a).

Creating an emulsion typically comprises three phases (Halling, 1981):

1. Division of the internal phase in droplets (deformation of the interphase);
2. Adsorption of the emulsifier molecules in the recently created interfacial surface;
3. Division of the droplets into smaller ones partly accompanied with re-coalescence.

The two critical steps in the emulsification are the rupture and coalescence of the droplets, both favoured by intense agitation (Walstra, 1993); the size of the resulting droplets depends upon the balance between these two opposed physical processes (Figure 2.17).

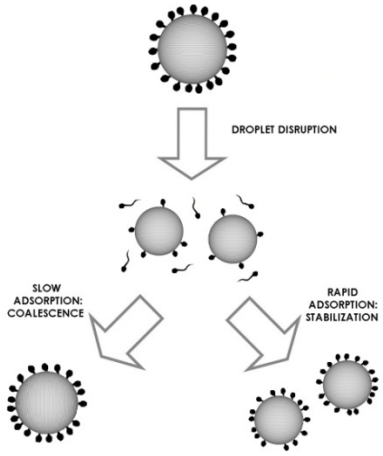


Figure 2.17. Balance between droplets rupture and coalescence during emulsification (Adapted from McClemments, 1999).

This is determined by a balance between interfacial forces that tend to keep droplets together, and rupture forces generated by the homogenizer, that tend to keep droplets separated (Walstra, 1993). The susceptibility of droplets toward rupture is characterized by the Weber number (We) which corresponds to the quotient between rupture and interfacial forces (Walstra, 1993). The droplets are ruptured when the Weber number exceeds a critical value (~ 1) that depends on the physical characteristics of both phases.

2.2.2.4. Main variables influencing the emulsification process

Some of the major factors that influence the emulsification process, and consequently the characteristics and stability of the resulting emulsions are presented below:

- *Concentration and emulsifier type*. The presence of emulsifiers favours the rupture of droplets during the emulsification process and generally leads to a decrease in droplet size, since it decreases the interfacial tension and prevents the re-coalescence of droplets (Walstra, 1993; McClements, 1999).

- *Properties and composition of both phases.* Modifications in the oil type or aqueous phase change the viscosity ratio between the dispersed and continuous phase, from which depends the minimum droplet size achieved in stationary conditions. Moreover, the rheological properties of both phases play a decisive role in the flow mechanisms involved in the emulsification, as well as on the ease of droplet rupture by emulsifier induced deformation (McClements, 1999).

- *Dispersed phase volume fraction.* A large disperse phase fraction requires more energy to reduce the droplet size. The relation between the emulsifier concentration and dispersed phase volume fraction is the most important parameter (Halling, 1981).

- *Applied Energy*. In general, an increase in the energy applied to the system, results in a decrease of the droplet size, resulting in higher stability (Halling, 1981; Franco *et al.*, 1995a, 1995b). However, there are occasions in which an increase in the applied energy over a critical value can produce the opposite effect, due to exposure of the system to an excessive heating and/or high pressures, which is particularly relevant in the case of protein-stabilized emulsions.

- *Type and geometry of the emulsification equipment*. There are different types of homogenizer devices used for the production of food emulsions, such as simple mixers, colloidal mills, high pressure homogenizers, ultrasonic homogenizers and

injectors (Figure 2.18). The type of device used influences the size and distribution of droplets in the emulsions and the efficiency of the droplet rupture during the emulsification process (McClements, 1999; Sánchez *et al.*, 1998). Each device has advantages and disadvantages, being more or less appropriate for a certain kind of material. The selection of a particular homogenizer depends on the emulsion being prepared in an industrial or laboratory scale, the available equipment, the volume of material to homogenize, the nature of the ingredients, the droplet size distribution, the required physicochemical characteristics of the final product and the acquisition and maintenance costs of the equipment.

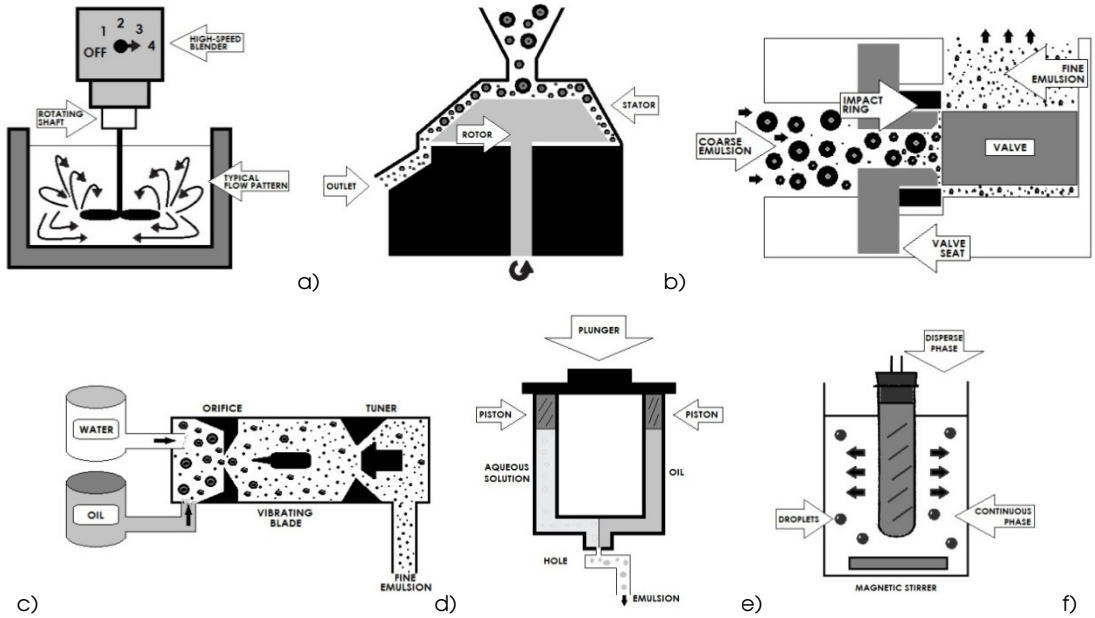


Figure 2.18. Schematic representation of emulsification devices: high-speed blender (a), colloid mills (b), high –pressure valve homogenizers (c), liquid jet generators (d), microfluidizer (e), membrane homogenizer (f) (Adapted from McClemments, 1999).

- *Temperature*. In principle, the use of high temperatures generates lower droplet sizes by increasing the solubility of the emulsifier and promoting a reduction in the interfacial tension, although it can also induce the opposite effect, since it can favour the re-coalescence of already formed droplets (Sánchez *et al.*, 1998), due to a decrease in the viscosity of both phases and an increase in the droplet mobility. On the other hand, in the case of protein stabilized emulsions, an increase in temperature affects their hydrophobicity, inducing a certain denaturation degree, consequently favouring the formation of droplet interactions and improving the emulsion's rheological properties (Franco *et al.*, 1998a).

- *Time*. An increase in the duration of emulsification in discontinuous operations, or the number of recirculations in continuous operations, induces a decrease in droplet size,

as well as in the polidispersity of the distribution (Franco *et al.*, 1995a). However, under certain emulsification conditions there is a minimum droplet size that cannot be reduced. In this situation, to extend emulsification time, besides costly, can result ineffective, since the interfacial layer can deteriorate, causing a decrease in the newly formed emulsion. Moreover, an excessive emulsification time can lead to emulsion heating, causing the effects indicated in the last paragraph.

- *Emulsification procedure*. The method used for adding oil and aqueous phases, as well as the emulsifier, influences the emulsification process; it is possible to emulsify larger oil amounts if mixing is carried out slowly and intermittently rather than continuously.

2.2.2.5. Emulsion Stability

The formation of an emulsion is a thermodynamically unfavourable process that results in a larger interfacial area between oil and water molecules. Consequently, the disperse droplets tend to aggregate spontaneously, in order to reduce the free energy of the system, which eventually leads to complete phase separation. However, it is possible to obtain kinetically stable (metastable) emulsions for considerable periods of time (days, weeks, months and even years) through the creation of a sufficiently high energy barrier (McClemments, 1999).
The stability of an emulsion can be defined as the ability to resist changes in its properties over time, and the main factor to be considered in the processing of this type of food products. An emulsion is physically stable when the changes on the number, size distribution and spatial disposition of the dispersed phase droplets are imperceptible throughout storage time (Franco, 1995; Velev *et al.*, 1993).

2.2.2.5.1. Forces between particles

Many of the emulsions' sensorial and physicochemical properties, as well as long-term stability, are strongly affected by attractive, repulsive or steric interactions between droplets (Rahalkar, 1992), traduced by interparticular forces, such as the following:

- *Brownian motion*. Inherent chaotic translational and rotational particle movement caused by thermal agitation, which leads to a random particle distribution and particle shock.
- *Hydrodynamic forces*. Resistance offered by the continuous media to the movement of two or more droplets, retarding their collision and aggregation.

- *Gravitational forces*. Promote a rising movement of the lower density phase, causing a concentration gradient throughout the sample, possibly leading to a creaming destabilization process (section 2.2.2.5.2).
- *Van der Waals interactions*. Originated from the attraction between oil droplets which have been polarized, electronically or by orientation.
- *Hydrophobic interactions.* Arise when the droplet surface has a non-polar character, either because droplets are totally covered by an emulsifier or because the emulsifier has some non-polar regions exposed in the aqueous phase (McClemments, 1999). Since the interaction between water and non-polar substances is thermodynamically unfavourable, the system tries to minimize the contact area through droplet aggregation.
- *Electrostatic forces*. In many emulsions, droplets have electrically charged surfaces, due to the adsorption of ionic emulsifiers or susceptibility to ionization (*e.g.* proteins, polysaccharides). The electrostatic interactions between droplets (same electric charge) have repulsive nature, playing an important role in the prevention of droplet aggregation and emulsion destabilization. Usually, the surface of droplets is surrounded by oppositely charged ions (counter ions), present in the continuous phase, forming an electrical double layer (Helmholtz, 1879; Gouy, 1909; Stern, 1924).
- *Steric forces*. Take place when the particles possess an adsorbed layer of high molecular weight, usually through the use of polymeric stabilizers (*e.g.* polysaccharide thickeners) and emulsifiers (*e.g.* proteins). The presence of steric stabilizers in the interface usually leads to an increase of the adsorbed double layer, hindering the approach and aggregation of the droplets, minimizing attractive interactions (*e.g.* van der Waals) (Dickinson, 1992; Dickinson and Stainsby, 1988).

The interactions between droplets of an electrically stabilized emulsion are described by the classical DLVO theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). This theory assumes that the global interaction between a pair of droplets in an emulsion is the result of attractive van der Waals interactions and repulsive electrostatic interactions:

$$w(h) = w_{attractive}(h) + w_{repulsive}(h) = w_{VDW}(h) + w_{E}(h) \quad (2.3)$$

Where $w(h)$ is the interdroplet pair potential, *i.e.* the energy required to bring two droplets from an infinite distance apart to a surface-to-surface separation of h; due to van der Waals (w_{VDW}) and electrostatic (w_E) interaction. The overall interaction potential includes all other attractive and repulsive forces that usually have lower influence in food emulsions stabilization (McClemments, 1999).

2.2.2.5.2. Destabilization mechanisms

Food emulsions may become unstable through a variety of physical mechanisms (Figure 2.19). Gravitational separation processes occur due to density differences between the droplets and continuous phase of the emulsion and are usually reversible processes. In oil-in-water emulsions, oil droplets, which have lower density than the aqueous phase, tend to move upward forming an oil layer at the top of the emulsion in a process called creaming. Oppositely, in water-in-oil emulsions, water droplets are denser than the oil phase and tend to move downward forming an aqueous layer at the bottom of the emulsion in a process called sedimentation. Reducing droplet size and increasing the viscosity of the continuous phase (using thickening agents) retards the rate of creaming and sedimentation (Dickinson and Stainsby, 1988; McClemments, 1999).

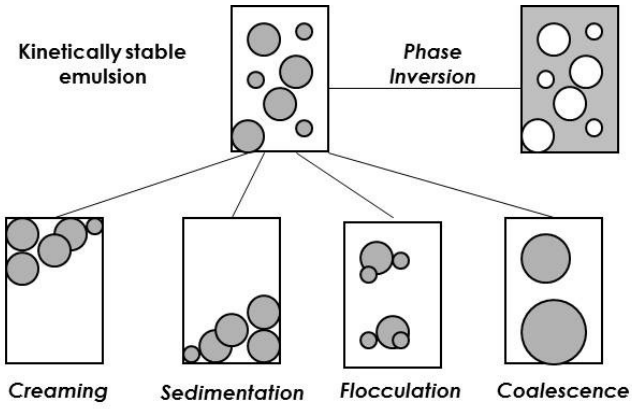


Figure 2.19. Mechanisms of emulsion instability (Adapted from McClements, 1999).

Droplet aggregation processes occur when two or more droplets collide and remain together. In flocculation processes, droplets retain their individual integrity forming aggregates or flocks, whereas in coalescence processes, droplets merge together to form a single larger droplet. Flocculation accelerates the rate of gravitational separation in diluted emulsions, but in concentrated emulsions the flocculated droplets can form a weak gel structure improving the emulsion stability (Dickinson and Stainsby, 1988; McClemments, 1999). Coalescence is an irreversible process involving the disruption of the interfacial membranes, being highly dependent on the thickness and viscoelastic properties of emulsifier films (Phillips *et al.*, 1994). The stability of emulsions to flocculation and coalescence is largely determined by the ability of the interfacial membrane to generate repulsive forces (*e.g.* electrostatic, steric, hydration) between the droplets that are sufficiently strong to overcome the attractive forces (*e.g.* van der Waals, hydrophobic) (Dickinson and Stainsby, 1988; McClemments, 2004).

Phase inversion is the process whereby an emulsion changes from oil-in-water to water-in-oil or vice-versa, and is associated with the occurrence of alterations in the composition or environmental conditions of an emulsion (*e.g.* dispersed-phase volume fraction, emulsifier type, emulsifier concentration, solvent conditions, temperature or mechanical agitation) (Dickinson, 1992).

2.2.2.6. Emulsion composition

Food emulsions are complex materials that can be composed by a variety of different chemical constituents. The constituents in a food emulsion interact with each other, either physically or chemically, to determine the overall physicochemical and organoleptic properties of the final product. The efficient production of high quality food emulsions, therefore depends on knowledge of the contribution that each individual constituent makes to the overall properties and how this contribution is influenced by the presence of the other constituents (McClemments, 1999).

The molecules in an emulsion can be distributed among three regions: the interior of the droplets, the continuous phase, and the interface; according to their concentration and polarity (Wedzicha *et al.*, 1991). The aqueous phase can contain a variety of water soluble polar components, such as sugars, salts, acids, bases, proteins, polysaccharides, minerals, flavours, and preservatives (Dickinson, 1992). Nonpolar molecules tend to be located in the oil phase, including glycerides, free fatty acids, sterols and liposoluble vitamins. Amphiphilic molecules (comprising both polar and nonpolar groups) are usually adsorbed in the interfacial region, being surface active components, such as proteins, small-molecule surfactants (*e.g.* mono- and di-glycerides), phospholipids and alcohols (Dalgleish, 1996; Damodaran, 1996). It should be noted that, even when at equilibrium, there is a continuous exchange of molecules between these three regions. The movement of the molecules may also be promoted by temperature changes, dilution within the mouth or by applying external forces. The rate and type of mass transport process involved have implications in the emulsions texture, flavour and physicochemical stability (Dickinson and Stainsby, 1988).

The nature of the aqueous and interfacial regions, as well as the interactions (competitive or synergic) between their components, has a major impact on emulsion characteristics. This subject will be properly discussed in the next section, particularly the role of proteins and polysaccharides (biopolymers) as emulsion stabilizers (emulsifiers and thickening agents).

The nature of the oil phase influences the nutritional, organoleptic, and physicochemical properties of food emulsions. Attending to the current trend on reducing the fat content of some traditional foods, it is determinant to understand the role played by lipids in the overall physicochemical and organoleptic properties of foods. Usually, low-fat products do not

present the same desirable quality attributes as the original product, and is often difficult to find suitable healthier alternative ingredients to act as fat mimetic.

The flavour profile of food emulsions is intimately related with the nature of lipids present and the chemical changes occurring during processing, storage and handling; which generate products that can be either desirable (*e.g.* butter) or deleterious (*"off-flavours"*, *e.g.* rancidity) (Nawar, 1996).

An important characteristic of lipid components is their ability to crystallize at low temperatures, which may occur during the processing, storage and handling of food emulsions (Walstra, 1987). Therefore, the presence of fat crystals has a marked impact on the texture, mouthfeel, stability and presentation of many food emulsions. The bulk physicochemical properties of edible fats and oils depend on the molecular structure and interactions of the triacylglycerol molecules they contain. The strength of the attractive interactions between molecules and the effectiveness of their packing in a condensed phase determines their melting point, boiling point, density and compressibility (McClemments, 1999).

Some studies (Granger *et al.*, 2003; Rampon *et al.*, 2004) have suggested that the type of fat used in the emulsions formulation participates in the development of the interface characteristics and rheological properties of the emulsions; through interactions of globular proteins hydrophobic domains with the oil phase.

2.2.2.7. Biopolymers as emulsion stabilizing agents

Proteins and polysaccharides are the two most important biopolymers used as ingredients in food emulsions (Damodaran, 1996). Biopolymers provide an important source of energy and essential nutrients in the human diet and have the ability to modify the appearance, texture, stability, and taste of food emulsions due to their unique functional characteristics (*i.e.* their ability to stabilize emulsions and foams, to form gels and to greatly enhance the viscosity of solutions (Damodaran, 1996; Dickinson, 1992; McClemments, 1999).

The main stabilising action of food polysaccharides is via viscosity modification of the aqueous continuous phase. Proteins, on the other hand, have a strong tendency to adsorb at oil-water interfaces and to form stabilizing layers around oil droplets, and so they are able to fulfil both the emulsifying and stabilizing roles (Dickinson, 2003). In addition, both kinds of biopolymers may contribute to the structural and textural characteristics of food products through their aggregation and gelling behaviour (Neirynck *et al.*, 2004).

For a biopolymer to be most effective as an emulsifying agent it must be surface active, *i.e.*, it must have the ability to substantially and rapidly lower the tension at the oil-water interface. The biopolymer must be amphiphilic (*i.e.* with significant amounts of both polar and non-polar groups), strongly adsorbed at the interface, forming a thick steric and charged

stabilising layer and should be present in a concentration that enables full saturation of the interface (Dickinson, 2003).

2.2.2.7.1. Proteins

Proteins are the single most commonly used class of foaming and emulsifying agents used in the food industry. They are natural, non-toxic, cheap and widely available, thus making them ideal ingredients (Wilde *et al.*, 2004). Proteins are heteropolymers with complex structural morphology, displaying the simultaneous occurrence of hydrophilic and hydrophobic groups. They are also amphoteric polyelectrolytes with a large number of positive and negative charges simultaneously present in the protein structure, as a result of the presence of acidic and basic amino acids, as well as polar groups that may be partly dissociated (Piazza, 2004).
Protein emulsifiers stabilize emulsions by forming a viscoelastic adsorbed layer, relatively thin, densely packed and electrically charged (Wilde *et al.*, 2004). These membranes prevent droplet aggregation mainly through steric repulsion and electrostatic repulsive forces. Consequently, protein-stabilized emulsions tend to be more sensitive to environmental conditions (pH, ionic strength, temperature) (McClemments, 2004). The extent of protein adsorption is influenced by surface hydrophobicity and charge (Wilde *et al.*, 2004), hence being possible to slightly modify proteins, chemically or enzymatically, in order to turn them more surface active (Garti, 1999).
Since proteins are large molecules and adsorb to the liquid interface with multiple contact points, the formation of a protein-stabilized emulsion requires that the protein molecules first reach the oil-water interface and then unfold to some extent for optimum contact of nonpolar groups (or domains) with the oil phase (Phillips *et al.*, 1994).
During emulsion formation, protein molecules migrate from bulk solution to the interface by a thermodynamically favourable process, since some conformational and hydration energy of the protein is lost at the interface (Phillips *et al.*, 1994). Once at the interface, most proteins unfold, reorient, rearrange, and spread to form a continuous cohesive film. The hydrophobic loops orient in the non-polar oil phase, while polar charged segments extend into the aqueous phase, but most of the protein segments occupy the interface. Unfolding of proteins at the oil-water interface leads to an increase in the number of contact points per molecule with the interface, making it more difficult for the molecular segments to desorb (Phillips *et al.*, 1994). The high concentration of protein at the surface leads to aggregation and formation of interactions with neighbouring molecules imparting strength and viscosity to the film (Phillips *et al.*, 1994). Hence, the mechanical properties of the adsorbed layer depend on the structure of the adsorbed protein, and the strength of the interactions between them (Wilde *et al.*, 2004).

During or after emulsification some of the individual protein components may be partially or wholly displaced from the interface by other more surface active protein species or by small molecule surfactants (*e.g.* monoglycerides, polysorbates, sucrose esters, lecithin…). In fact, although both proteins and emulsifiers can stabilize foams and emulsions alone, their individual mechanisms of stabilization are incompatible, often resulting in dramatic destabilization when both species are present at the interface (Wilde *et al.*, 2004). Hence, emulsion stability is strongly influenced by the dynamic aspects of protein competitive adsorption and by the nature of the interfacial protein interactions.

The ability of small-molecule surfactants to displace macromolecules from interfaces is related to their higher adsorption energy compared to individual segments of the macromolecules. Provided that adsorption/desorption is an equilibrium process, the surfactants that exert the highest surface pressure will displace all those with lower surface pressures (Garti, 1999). The displacement mechanism of proteins from interfaces can be described by a combination of solubilisation (the water-soluble surfactant binds to the protein to form a soluble protein-surfactant complex) and replacement (the surfactant adsorbs at the interface and displaces the protein because it has a lower interfacial tension) processes (Bos and Vliet, 2001).

Besides their surface activity, proteins that are present in the continuous medium can stabilize emulsions by increasing the viscosity of the continuous phase, possibly through the formation of a network gel, either by themselves or in conjugation with other gelling agents (Franco *et al.*, 1995a).

Food emulsions are traditionally stabilized by milk and egg proteins. Nevertheless a growing interest in alternative protein sources has been evident in the last few years. Several vegetable proteins have been tested successfully in order to stabilize oil-in-water emulsions, namely globular proteins extracted from leguminous seeds, such as soy (Eliazalde *et al.*, 1996; Roesch and Corredig, 2002), lupin (Franco *et al.*, 1998a; Raymundo *et al.*, 2002) and pea (Franco *et al.*, 2000). Vegetable proteins have some additional advantages, since they do not contribute to the cholesterol increase, present superior microbiological quality, and are technologically easier to handle as coagulations occur at higher temperatures. This will be more thoroughly discussed in section 2.2.4.

2.2.2.7.2. Polysaccharides

Polysaccharides make good stabilizing agents due to their hydrophobicity, high molecular weight and gelation behaviour which lead to the formation of macromolecular barriers in the aqueous medium between dispersed droplets (Garti, 1999).

Polysaccharides act as texture modifiers increasing the continuous phase viscosity or even forming a gel in the continuous phase, thereby slowing down droplet movement

(McClemments, 1999). When using gelling agents such as pectin or carrageenan, the droplets become embedded in a polymer gel matrix presenting a solid-like texture. Such a gelled emulsion system will not have easy liquid-like pouring properties, and it may lack the creamy mouthfeel character especially appreciated by consumers of liquid emulsion products. Polysaccharide thickening agents with strongly non-Newtonian rheology are normally preferred for controlling stability of food emulsions (*e.g.* modified starches, cellulose derivatives, guar gum, xanthan gum, etc.) (Dickinson, 2004). These biopolymers are also used in food emulsions as flavour binders, fat replacers and freeze-thaw stabilizers (McClemments, 1999).

Some food hydrocolloids achieve interfacial functionality in emulsions, not so much by adsorbing directly at the oil-water interface during emulsification, but rather by forming an associative interaction after emulsion formation with the stabilising protein layer. Partial unfolding of globular proteins may make them more susceptible to complexation with hydrocolloids in the adsorbed state than in an aqueous solution at the same pH and ionic strength (Garti *et al.*, 1997). Under proper heat and humidity conditions the polysaccharides can *in situ* interact with the proteins via the Maillard reaction, to form a new compound that will be composed of hydrophilic and hydrophobic moieties that will be able to better adsorb on the oil-water interfaces (Garti *et al.*, 1997).

Polysaccharides can influence the state of flocculation of the droplets, especially when the concentrations used are below the values at which a viscoelastic macromolecular network is formed within the bulk aqueous phase. Droplet flocculation may occur either via bridging or depletion mechanism, depending on whether the biopolymer has a net attractive or repulsive interaction with the surface. For emulsion systems with weak attractive protein-polysaccharide interactions, even small changes in pH or ionic strength can have a substantial effect on the bulk rheology and creaming stability, with important consequences for product quality and appearance (Dickinson, 2003; 2004).

Polysaccharides are rigid, water-soluble hydrocolloids and, therefore are not considered as classical emulsifiers. However, at least a few of these biopolymers have been reported to adsorb on oil-water interfaces and to stabilize emulsions (Garti, 1999). The most commonly recognised hydrocolloid emulsifier is gum Arabic which is widely used in the soft drinks industry for emulsifying flavour oils (*e.g.* orange oil) under acidic conditions (Dickinson *et al.*, 1990). The surface activity of polysaccharides is attributed to the presence of hydrophobic groups associated with them, *e.g.* hydrocarbon side groups attached to their backbones (*e.g.* modified starches or celluloses) (McClemments, 2004). In most cases, it seems that the emulsifier ability has its origin in the presence of proteinaceous material covalently bound or physically associated with the carbohydrate polymer (*e.g.* gum arabic, galactomanans) or that it may be present as impurities (Dickinson, 2003). Typically, the emulsions produced are coarser than those which can be made with low-molecular weight surfactants or proteins at

the same emulsifier/oil ratio. However, once strongly adsorbed at the oil-water interface, hydrocolloids can provide a protective layer conferring effective steric stabilization during extended storage and a higher capability to withstand environmental conditions when compared to proteins (Dickinson, 2003).

2.2.3. Food Gels

Gels provide structure and stability to food products, entrapping food ingredients such as flavours, sugar and oil in the gel network; examples of food gels are cheese, (hard) boiled eggs, jelly, custards, sausages, yogurt and tofu. The development of appropriate mechanical, structural and physicochemical properties in most foods is mainly attained by using gelling agents. These agents are essentially hydrocolloids that are usually biopolymers, mainly proteins and polysaccharides (*e.g.* gelatine, pectin, carrageenan, starch) which are easily dissolved or dispersed in water, producing a gelling effect. Besides the biopolymers' crucial contribution to the structure development, water, lipids and flavour components entrapped within the network all contribute to the overall sensory properties of foods with a gel base.

2.2.3.1. Definition

Gels exhibit diverse microstructural and mechanical properties, so it is difficult to define them. The word jelly appeared for the first time in the 14th century and was derived, via the French *gelée*, meaning frost, from the Latin *gelare*, meaning "to freeze". The scientific term *"gel"* was introduced by Thomas Graham (>1869), the founding father of colloid chemistry (Oakenfull *et al.*, 1997). Many definitions of a gel have since been given in the literature: for Flory (1953) a gel consists of polymeric molecules cross-linked is to form a tangled interconnected network immersed in a liquid medium; Hermans (1949) defined it as a two-component system formed by a solid finely dispersed or dissolved in a liquid phase, exhibiting solid-like behaviour under deformation; according to Clark (1991), gel is a material formed by a continuous three-dimensional network that entraps a solvent (water) and immobilizes it; Ziegler and Foegeding (1990) defined a gel as a continuous network of macroscopic dimensions immersed in a liquid medium and exhibiting no steady-state flow, while Wong (1989), defined gelation as aggregation of denatured molecules with a certain degree of order, resulting in the formation of a continuous network. According to Doublier and Thibault (1984) a gel is a biphasic system, constituted by a solid tridimensional network, entrapping a liquid phase.

2.2.3.2. Gel formation

The formation of a gel is a phenomenon that involves biopolymer-biopolymer and biopolymer-solvent interactions and is associated with the following terms (Hermansson, 1978):

- *Aggregation*: general term involving different types of biopolymer interactions, including flocculation, coagulation and gelation.
- *Flocculation:* random aggregation of biopolymers without structural changes. Generally occurs through a decrease in the repulsive electrostatic forces between biopolymer chains.
- *Coagulation:* random aggregation of denatured proteins. Leads to the formation of an heterogeneous gel network or coagulum.
- *Gelation:* ordered aggregation of biopolymers, with important conformational changes. Leads to the formation of an ordered gel network.

The formation of a gel involves a stage called sol-gel transition, which in physical terms corresponds to the transition from the liquid to the solid state. Figure 2.20 schematically represents the stages involved in gel formation:

(*i*) "sol" state, the polymer forms a solution; the macromolecules are not organized.

(*ii*) "gel" state, occurs when the chains are sufficiently organized to form a network.

(*iii*) When the network organization becomes more intense, the gel becomes firmer and usually a sineresis phenomenon occurs, *i.e.*, the gel contracts and exudates part of the liquid phase.

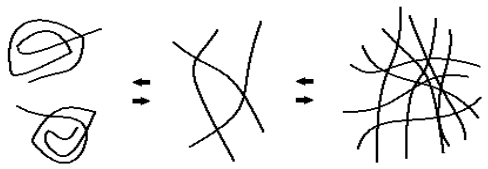


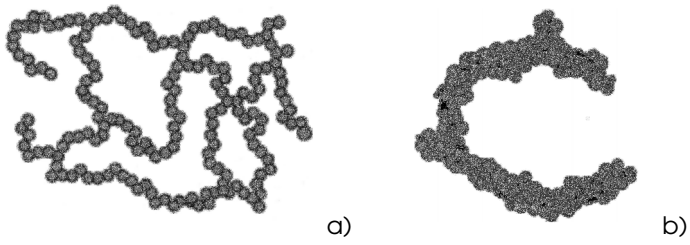
Figure 2.20. Schematic representation of the stages involved in biopolymer gel formation.

The gel state cannot truly be considered a state of equilibrium, because it evolves with time. It represents a compromise between the polymer/polymer and polymer/solvent interaction. Since most of the bonds responsible for the biopolymers gel structure are weak interactions, such as hydrogen, electrostatic or hydrophobic bonds, they can suffer variations within time (Nunes, 2006). The functionality of a gel is determined by the spatial distribution of the macromolecules and by the contribution of the different type of connection forces for the network formation. The contribution of the different type of connections, in addition to the

intrinsic properties of the biopolymer (molecular weight, composition, conformation, hydrophobicity), will depend on the conditions applied along the gel formation process: biopolymer concentration, pH, temperature, ionic strength and type of ions (Kinsella, 1979; Phillips *et al.*, 1994).

2.2.3.2.1. Types of gel structures

Biopolymers generally form two different types of gels, fine stranded and coarse networks (Figure 2.21), but intermediate structures have also been reported (Tombs, 1974). Moreover a biopolymer can form both types of structures according to its charge and the medium's ionic content (Hermansson, 1986).



a) b)

Figure 2.21. Schematic representation of random (a) and ordered (b) gels. (Adapted from Renkema, 2001).

Coarse networks are thought to be formed by random aggregation of macromolecules into clusters, which aggregate to thick strands, originating non-transparent aggregate or particulate gels. This is the case of gels formed by heating and aggregation of denatured globular proteins (*e.g.* whey, soy proteins) at pH values close to the isoelectric point. When the network structure becomes coarser, the ability of the gels to retain water decreases (Hermansson, 1986).

Fine stranded networks are formed by macromolecular ordered association, originating transparent or translucent gels indicating that biopolymer aggregates within the gel are smaller than about 50 nm (Lakemond, 2001). This structure is typical for many polysaccharides, but less common in protein gels, except for gelatine gels. These gels have high water retention capacity and are usually thermoreversible.

Thermoreversible gels are three-dimensional networks cross-linked by physical junction zones that are built up or broke down as the temperature change (thermo-labile interactions) (Flory, 1953; Lopes-da-Silva and Rao, 2007). In fact, temperature plays a crucial role in the formation and disruption of the physical interactions. Normally, gelation occurs with decreasing temperatures, while a gel melts at high temperatures, because the decrease in the number of junction zones with temperature is usually more pronounced than the increase from the entropic contribution (Lopes-da-Silva and Rao, 2007). The thermoreversible gelation of

biopolymer solutions can be considered an equilibrium phenomenon, where a gel can fully recover its initial characteristics after melting (heating) and re-cooling. However, important thermal hysteresis effects, related to the non-equilibrium nature of the gel phase have been identified (*e.g.* Braudo *et al.*, 1991; Haque and Morris, 1993), with melting temperatures far higher than gelling temperatures, due to aggregation of the ordered structures. Polysaccharides such as alginates, carrageenans and agarose form thermoreversible gels, while gels formed by thermal denaturation of globular proteins are irreversible.

Multicomponent gels are formed by polymeric mixtures, in which at least one of the components forms a gel network, and can be classified as complex, mixed or capillary gels (Tolstoguzov, 1998). Complex gels are formed if a polymer connects with another, forming a paired network, while in mixed gels each polymer is associated with itself forming two independent interpenetrated networks. Capillary gels correspond to biphasic gels formed when one of the components forms a continuous network which embeds the dispersed particles (in liquid or gel state)of the other component, that act as filling (Nunes, 2006).

2.2.3.3. Protein gels

The ability of proteins to form gels has been considered of great importance for the structure and properties of many food products (Bacon *et al.,* 1990).

Gelation of a solution of globular proteins can be induced in various ways. Heat-induced gelation is the most commonly studied phenomenon in food science, and responsible for the structure present in many everyday heat-set foods (Totosaus *et al.*, 2002). However there are alternative methods, such as high-pressure processing induced gelation (Aguilar *et al.*, 2007; Alvaréz *et al.*, 2008), salt-induced gelation (Ako *et al.*, 2010), acid-induced gelation (Alting, 2003) or enzyme-induced gelation (Creusot and Gruppen, 2007).

Heat-induced gelation of globular proteins is a process comprising three successive steps:

(i) Unfolding (denaturation) of the protein molecules exposing residues previously buried in the core.

(ii) Interaction of the exposed residues as to form aggregates. Usually, partial denaturation is sufficient to promote aggregation (Clark *et al.*, 2001).

(iii) Arrangement of the aggregates into extended aggregates/precipitates and formation of a continuous network.

The details of the kinetics of both the unfolding and aggregation of protein molecules are important in order to obtain the optimal functionality of globular proteins as thickening or gelling agents in foods (Alting, 2003).

The formation of heat-induced gel networks or aggregates/precipitates arises from a balance of both protein-protein and protein-solvent interactions, through a combination of covalent and non-covalent bonds. The main types of covalent bonds which can be formed

are the disulphide bonds of cysteine residues. These stronger bonds stabilise the structure of many proteins and may prevent the renaturation of the unfolded protein molecule upon cooling, so that the process becomes irreversible (Alting, 2003). The main types of non-covalent bonds include hydrogen bonds, electrostatic bonds and hydrophobic interactions. Electrostatic bonds may be stronger than other non-covalent bonds, but their existence is determined by the pH and salt concentration (Oakenfull *et al.*, 1997).

The type of gel network formed is associated with changes in the balance between these attractive and repulsive forces amid the aggregating proteins (Doi and Kitanabe, 1997). Figure 2.22 depicts how pH and ionic strength influence the final gel properties during heat-induced gelation. At low ionic strength or at pH values far from the isoelectric point (pI) of the active protein, electrostatic repulsive forces hinder the formation of random aggregates and more linear polymers are formed, resulting in transparent and fine-stranded gels. When heat-induced gelation occurs at high ionic strength or at a pH near the pI of the protein, repulsive forces are weaker and denatured proteins aggregate randomly by physical interactions such as hydrophobic and van der Waals interactions into particulate, turbid gels (Alting, 2003).

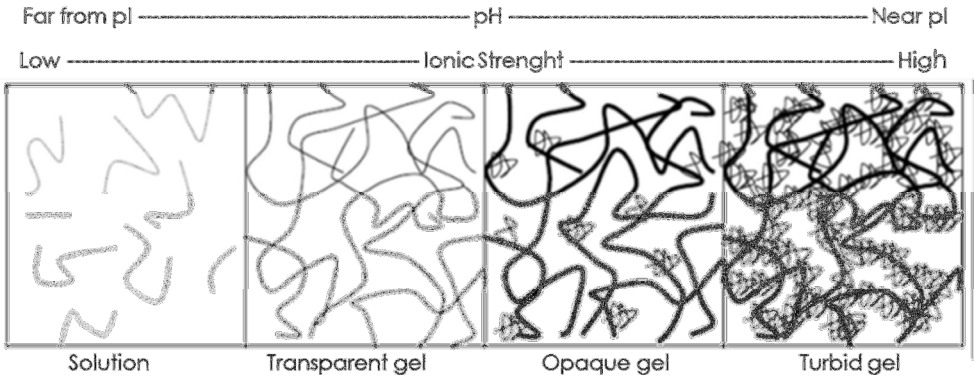


Figure 2.22. Schematic representation of the relation between protein gel appearance and electrostatic repulsion (Adapted from Doi & Kitabatake, 1997).

Further details on the gelation mechanism of pea protein, which has been used in the present work will be discussed in section 2.2.4.1.2.

2.2.3.4. Polysaccharide gels

There are many types of polysaccharides that are able to form gels. In the present work kappa-carrageenan and starch were used, so a detailed description on these biopolymers is given below.

2.2.3.4.1. Kappa-carrageenan

Carrageenans are a family of linear sulphated polysaccharides extracted from certain species of red seaweeds (Rhodophyceae), responsible for the algae flexible structure which enables them to withstand tensions and movements caused by sea currents and waves. These polysaccharides are widely used in the food industry as gelling, thickening and stabilizing agents (Picullel, 1995).

All carrageenans have a common linear chain skeleton, composed of alternating disaccharide repeating units of α-1,3-*D*-galactose-4-sulphate and β-(1,4)-3,6-anhydro-*D*-galactose, as represented in Figure 2.23 (Morris *et al.*, 1980; Rochas and Rinaudo, 1984; Stanley, 1990). Due to the presence of sulphate groups, carrageenan molecules are always ionized and negatively charged, even under acidic conditions.

Depending on the number and position of sulphate groups, three main carrageenan fractions have been identified: iota (ι), kappa (κ) and lambda (λ). The proportions of these different fractions in an alga vary according to the specie and the harvesting process, being the carrageenan type designated by the name of the dominant fraction.

Kappa and iota carrageenan form gelling systems, while solutions of the lambda fraction do not gel, presumably due to its high degree of sulphate substitution (steric hindrance), originating only viscous solutions upon heating (Stanley, 1990).

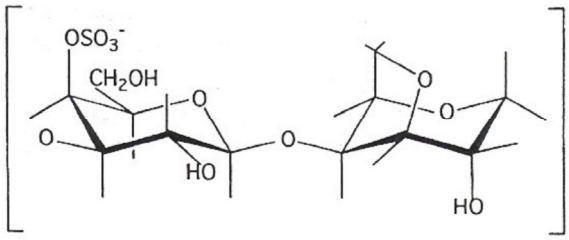


Figure 2.23. κ-Carrageenan molecular structure.

κ-Carrageenan is the mostly used fraction mainly due to its functionality in dairy systems (*e.g.* yoghurts, puddings), since it interacts synergistically with milk proteins, primarily casein micelles (Stanley, 1990). κ-carrageenan sulphate ester groups establish ionic bridges, mediated by bivalent ions such as calcium, with the casein amine groups. This interaction has also been attributed to the incompatibility between κ-carrageenan and casein micelles, leading to phase separation (Schorsch *et al.*, 2000).

The solution of κ-carrageenan may form strong thermally reversible gels upon cooling in the presence of specific cations (counter ions) such as potassium (Rochas and Rinaudo, 1984; Chen *et al.*, 2002).

There are several models in the literature for the gelling mechanism of κ-carrageenan, being generally accepted the model proposed by Anderson *et al.* (1969) and later modified by

Morris *et al.* (1980). According to this "domain model", κ-carrageenan molecules in solution are dispersed as random coils (sol phase) which undergo a conformational transition upon cooling forming helical dimers (ordered phase). Further decrease in temperature, in the presence of counter ions, results in intermolecular association of the ordered double helices (small independent domains acting as junction zones) resulting in helices aggregates and the formation of an infinite network (gel phase), as represented in Figure 2.24.

At higher temperatures or low ionic strengths, the disordered (coil) conformation is favoured due to the high conformational entropy arising from the rotation and fluctuation around the glycosidic bonds along κ-carrageenan backbone (Morris *et al.*, 1977). As temperatures decline or ionic strength increases, conformational entropy decreases allowing chains to overcome thermal motion and adopt an ordered structure (Nickerson *et al.*, 2004).

Since the helical aggregates are more stable than the double helices, the transition temperature of helical aggregates to random coils along heating (gel-sol transition) is superior to the transition temperature of random coils to double helices upon cooling (sol-gel transition), *i.e.* the gels present thermal hysteresis (Stanley, 1990).

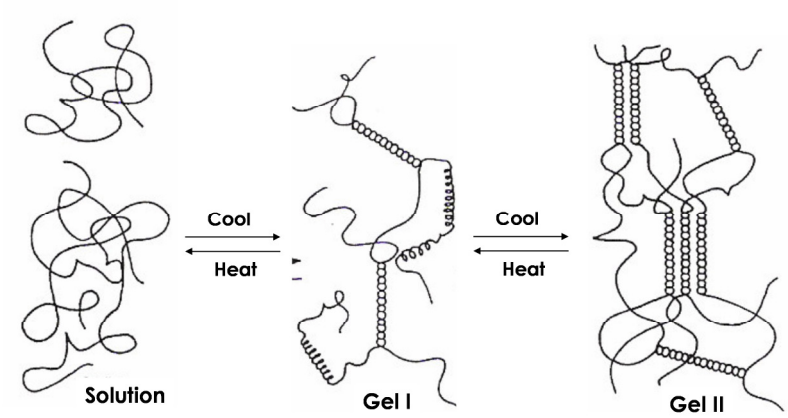


Figure 2.24. Schematic representation of κ-carrageenan gelling mechanism (adapted from de Vries, 2001).

The aggregation phenomenon and physical properties of κ-carrageenan gels are notoriously affected by the presence of even trace amounts of ions (Chen *et al.*, 2002; Morris *et al.*, 1980; Rochas and Rinaudo, 1984; Nickerson *et al.*, 2004). Potassium ions (K^+) have specific affinity to κ-carrageenan by inducing intermolecular association of κ-carrageenan through the formation of an ionic bond between K^+ and sulphate groups of *D*-galactose residues (Thrimawithana *et al.*, 2010), being more effective in reducing the electrostatic repulsions than bivalent ions such as Calcium (Ca^{2+}). Smaller univalent cations, such as Na^+ and Li^+, are usually less efficient in promoting helix formation (Funami *et al.*, 2007; Thrimawithana *et al.*, 2010).

2.2.3.4.2. Starch

Starch is the main storage substance in plants, being the main source of carbohydrates in human diet. It is deposited in vegetable seeds (corn, wheat, sorghum, rice), roots (tapioca, sweet potato) and tubers (potato, cassava), as partially crystalline granules. The chemical composition and functional properties of starch, as well as the size and shape of the granules, are essentially dependent on its botanical origin (Veendam, 1985). Starch granules are optically anisotropic presenting a birefringence pattern (cross) when observed under polarized light, indicating that the molecules are oriented according to a radial direction.

Starch is a polymer of glucose units connected by glicosidic bonds, forming two types of macromolecules: amylose and amylopectin (Whistler and BeMiller, 1997). Amylose is an essentially linear polymer with α-1,4 bonds between *D*-glucose units (Figure 2.25a). Amylopectin is also an α-*D*-glucose polymer, but has higher molecular weight and presents a more branched structure than amylose with α-1,6 bonds in branching sites (Figure 2.25b). Crystalline regions in starch granules are composed by amylopectin molecules while non-crystalline regions are composed by amylose molecules disposed in alternate layers forming a lamellar structure (Lineback, 1986). The proportion of amylose and amylopectin varies according to the botanical origin of starch. Most starches contain 20-30% (w/w) amylose and 70-80% amylopectin (Lineback, 1986; Hoseney, 1998), but there are also waxy-starches (amylose < 20%) and high-amylose starches (amylose > 80%), as for example potato and corn, respectively (Veendam, 1985).

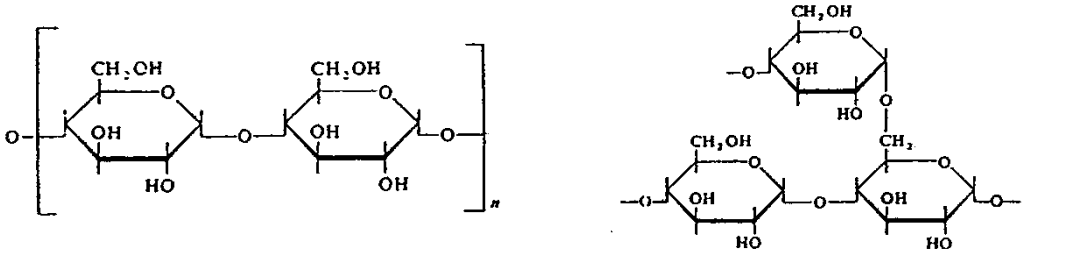


Figure 2.25. Amylose (a) and amylopectin (b) chemical structure.

Starch is widely used in the food industry, due to its unique technological properties and its low cost, as a gelling or thickening agent, in the production of sauces, desserts, beverages and ice creams, among other applications. Starch may be used in its native state or modified by physical, chemical or enzymatic treatment, in order to acquire the properties required for specific applications.

When heating an aqueous solution of starch, one can observe the intumescence (hydration and volume increase) and loss of starch granules structural order, *i.e.*, the loss of birefringence and cristalinity. The group of alterations suffered by starch throughout heating is known as gelatinization, and is schematically represented in Figure 2.26. The critical temperature above

which starch suffers irreversible alterations and gelatinization begins varies between 55 and 80ºC, depending on starch type (Hoseney, 1998; Lineback, 1986). During the heating process, some internal constituents of the granules, mainly amylose, are lixiviated to the exterior, contributing to a viscosity increase of the system, and amylopectin enriched granules. Initially, gelatinization occurs only in amorphous regions, but with temperature increase, the number of granules undergoing gelatinization increases, and with temperatures above 100ºC the hydrogen bonds responsible for granule structural integrity can be broken leading to complete hydration and solubilisation of the granule constituents (Miles *et al.*, 1985; Eliasson, 1986; Lineback, 1986; Morris, 1990; Hoseney, 1998).

If the starch concentration is sufficient (> 6% w/w), the suspension behaves as a viscoelastic paste along cooling (Miles *et al.*, 1985). A starch gel can be considered as a biphasic material in which the starch granules (containing mainly amylopectin) constitute the filling embedded in an amylose/amylopectin gelled matrix (formed mainly by interpenetrated amylose chains). The rheological properties of starch gels depend on the disperse phase (starch granules), the continuous phase (amylose/ amylopectin matrix) and the interactions between phases (Miles *et al.*, 1985; Eliasson, 1986; Morris, 1990). In a longer and more accentuated cooling the gel becomes more rigid forcing water out of the system, *i.e.*, presenting sineresis.

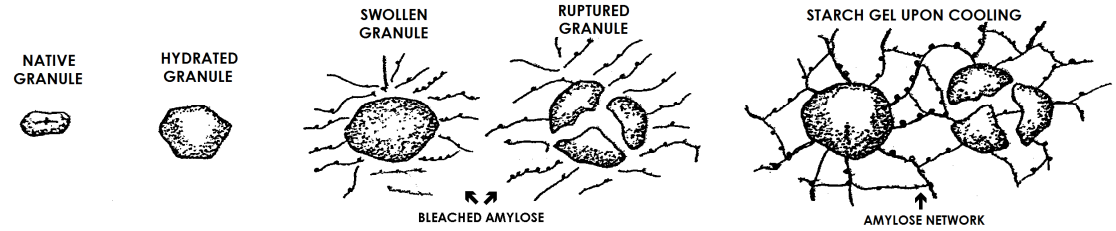


Figure 2.26. Schematic representation of starch gelatinization.

In the course of a prolonged storage, starch gel may suffer a retrogradation phenomenon. In this process, the interactions between starch chains become stronger leading to re-crystallization and the formation of a more opaque and rigid gel. The retrogradation rate depends on several factors, namely the amylose:amylopectin ratio, starch:water ratio, temperature, pH, starch concentration, botanical origin and the presence of other solutes. Retrogradation corresponds to reorganization at the macromolecular level of the starch chains, involving mainly amylose but also amylopectin (Miles *et al.*, 1985). Despite being an inverse process to gelatinization, it does not lead to the initial situation since amylose was lixiviated out of the granules.

Starch gelatinization is a determinant phenomenon in certain foods and can be affected by several factors. The granules molecular integrity can be destroyed by mechanical action, high temperature or extreme pH values. The presence of other ingredients can also have influence; for instance fats have a tendency to interact with starch granules hindering

complete hydration, while soluble hydrophilic solids (sugars, proteins, salts) compete with starch for available water molecules.

2.2.3.5. Mixed protein/polysaccharide gel systems

Proteins and polysaccharides are the macromolecules responsible, in great part, for the structure, mechanical and chemical properties of many food systems, through their gelling, thickening and surface-stabilizing functional properties (Schmitt *et al.*, 1998). Considering that most food products are multicomponent systems, it is important to consider not only the composition, structure and individual properties of each component, but also the properties of the mixture (Tolstoguzov, 1998). The study of protein/polysaccharide mixed systems is important from the fundamental point of view, in order to clarify interaction phenomenon in multicomponent systems, as well as from the technological point of view, in the improvement of the functional properties of conventional products and in the development of novel food products (Nunes, 2006). The structure and individual properties of biopolymers in solution (and concentration), as well as the physico-chemical conditions of the media (*e.g.* pH, ionic strength, temperature), determine the type of interactions that occur between proteins and polysaccharides (Nunes, 2006). Protein/polysaccharide interactions can originate three distinct situations schematized in Figure 2.27: co-solubility, complexation and thermodynamic incompatibility (Tolstoguzov, 1998).

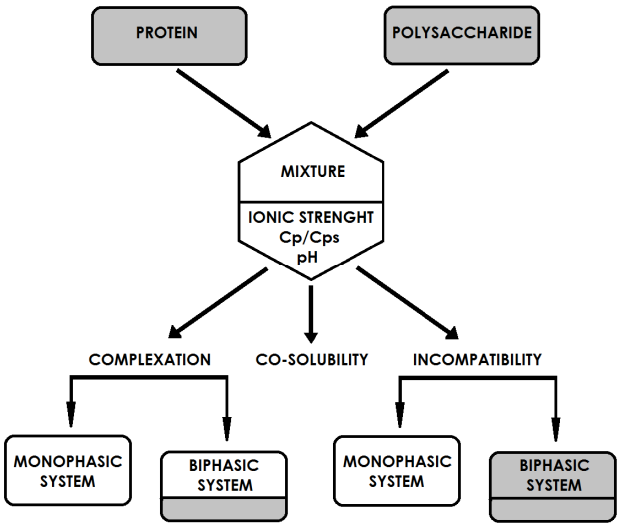


Figure 2.27. Types of protein-polysaccharide interaction. C_p – protein concentration; C_{ps} – polysaccharide concentration (Adapted from Tolstoguzov, 1991).

2.2.3.5.1. Co-solubility

This situation occurs when both macromolecular components form soluble complexes, or do not interact, resulting stable homogeneous solutions. It is the least frequent interaction,

considering the polymeric nature of proteins and polysaccharides and that they both have functional groups.

2.2.3.5.2. Complexation

This phenomenon involves the formation of complexes between proteins and polysaccharides. If these complexes are soluble, monophasic systems are obtained; while if the complexes are insoluble, biphasic systems are formed with both polymers included in one of the phases (Schmitt *et al.*, 1998; Tolstoguzov, 1991; 2003).
Complexation between proteins and anionic polysaccharides generally occurs for pH values lower than the protein isoelectric point and at low ionic force. In these conditions, the protein molecules have an excess of positive charges that are attracted to negative charges in the polysaccharide chains. The neutralization of the biopolymers charges usually results in the precipitation of insoluble complexes. Gel formation may occur through the aggregation of these complexes by hydrophobic, electrostatic and hydrogen bonds (Tolstoguzov, 2003). Soluble complexes are the ones not electrically neutral, as the ones formed in systems with one of the biopolymers in excess (Tolstoguzov, 2003).
It is possible to obtain complexes for pH values above the protein isoelectric point, even with an excess of negative charges in the protein, due to a strong local attraction of positively charged amino acid residues (NH_3^+) with the polysaccharide negative groups (*e.g.* OSO_3^- in sulphated polysaccharides) (Grinberg and Tolstoguzov, 1997).
The functional properties of protein/polysaccharide complexes can be modulated by controlling their formation conditions, *e.g.* pH, ionic strength, biopolymer concentration, biopolymer ratio, heat and mechanical treatments (Schmitt *et al.*, 1998). This enables their industrial application, in a variety of fields (*e.g.* food, biotechnology, medicine, pharmacy and cosmetics), namely in the purification of macromolecules, encapsulation of active molecules, or used as new materials (*e.g.* food ingredients or packagings) (Schmitt *et al.*, 1998).

2.2.3.5.3. Thermodynamic incompatibility

Thermodynamic incompatibility between biopolymers in aqueous media can lead to the formation of either a monophasic or biphasic system whether the total biopolymer concentration is, respectively, lower or higher than the minimum concentration at which phase separation occurs.
This is the most common interaction phenomenon occurring between proteins and polysaccharides due to their different chemical structure and conformation in aqueous media (Grinberg and Tolstoguzov (1997). Above a critical biopolymer concentration, in

certain conditions, the mixed protein/polysaccharide system spontaneously separates in two phases. A biphasic system is formed, each phase containing mainly one of the biopolymers (segregate phase separation), causing an increase on the effective biopolymer concentration in each phase (Grinberg and Tolstoguzov, 1997; Doublier *et al.*, 2000; Tolstoguzov, 1991; 1998; 2003).

This phenomenon is highly dependent on the macromolecule size exclusion volume, which determines the degree of volume occupation in biopolymer mixtures, and accordingly, the minimum biopolymer concentration at which phase separation occurs (Tolstoguzov, 1991; 2003).

Phase separation in protein/polysaccharide systems depends on pH and ionic strength. Incompatibility generally occurs at pH values near the protein isoelectric point and at high ionic strength, conditions that contribute to protein chain auto-association. For anionic polysaccharides, thermodynamic incompatibility also occurs for pH above the protein isoelectric point, since in these conditions both polymers are negatively charged.

Biopolymer phase separation phenomenon has potential technological applications in the food industry, enabling to achieve or enhance certain functional properties in food products, namely through the stabilization of solutions and emulsions (Tolstoguzov, 1991; 2003) and in the attainment of gelled microstructures (Tolstoguzov, 1998). The separated phases act as two complex solvents for the other components in the food system (e.g. sugars, enzymes, flavours, etc.) that are distributed between both phases, affecting the texture, flavour, and other properties of the final food product (Tolstoguzov, 2003).

Phase separation between pea protein and κ-carrageenan, the main biopolymers used in the present study, has been reported in literature (Ipsen, 1997; Musampa *et al.*, 2006; Nunes, 2006; Andrade *et al.*, 2010). Phase diagrams for pea protein/κ-carrageenan systems were determined at 60ºC, pH 7 (above protein isoelectric point) as well as two ionic strengths (0.05 and 0.2 M) by Musampa *et al.* (2006), who observed low thermodynamic compatibility in aqueous media. At pH 7 both polymers are negatively charged, occurring electrostatic repulsion between segments of the polymeric chains. For higher salt concentrations, co-solubility is slightly favoured due to some shielding of these electrostatic repulsions.

2.2.4. Vegetal proteins as emulsifying and gelling agents

In the last few years animal protein consumption has been substantially altered due to animal diseases, cholesterol intake worries and strong demand for healthy food, along with ethic orientations of denying animal intakes of any kind (Bollinger, 2001). The use of vegetable proteins can be an interesting alternative to animal protein based foods. Its high protein level and well-balanced amino-acid composition makes them important sources of protein, with potential to replace meat and dairy proteins, if necessary (van Vliet *et al.*, 2002).

The development of 100% vegetable products based on these proteins present some additional advantages such as no cholesterol, low fat content, low caloric content and expectable superior microbiological quality. Consequently, these products constitute a sustainable alternative to the consumer demand for more natural foods, avoiding animal-related public health concerns (*e.g.* BSE, antibiotics, aphthous fever, avian influenza, and bluetongue) and nutrition-related diseases such as type II diabetes, cardiovascular diseases, digestive problems, allergies and intolerances.

The excessive consumption of red and processed meat has been associated with an increased risk of mortality, namely by cancer and cardiovascular disease (Sinha *et al.*, 2009). According to the World Cancer Research Fund 2007 Report it is recommended to limit the intake of red meat (beef, pork, lamb and goat) and avoid processed meat (*e.g.* smoked, cured or salted, or processed with chemical preservatives). The public health goal is that the average consumption of red meat be no more than 300-500 g a week, very little if any of which to be processed.

According to de Boer *et al.* (2006), European diets include 40 kg of protein per year, of which 62% is of animal origin, being meat the primary dietary protein source (Davis *et al.*, 2010). Several studies have assessed the environmental impact of various food products using life cycle assessment (LCA), which is a methodology that covers the entire *"cradle-to-grave"* impact of products. (*e.g.* Roy *et* al., 2009; Thomassen *et al.*, 2008; Avraamides and Fatta, 2008). These studies generally indicate that vegetable products have lower impact and resource use per kg compared to meat, with dairy products in between; however, differences in agricultural production, transport distances, and transport method can alter the general picture of environmental impact per kg of food for vegetable versus animal products. Moreover, a comparison between products must also encompass differences in nutritional value and preferably other functions of foods, like taste experience and possibly cultural identity (Davis *et al.*, 2010).

Considering all these aspects, globular protein extracted from leguminous seeds, such as soy, pea and lupin protein, have gained additional importance in alternative to animal proteins (Guéguen, 2000; Liu, 2000).

However, for this replacement to be successful, novel protein foods must be acceptable to consumers, namely in terms of flavour and texture, and should nutritionally provide the same amount and quality of protein as meat/dairy products (O'Kane, 2004).

Vegetable proteins are technologically relevant on modern food design, as a consequence of their favourable functional properties (Tolstoguzov, 1998), such as gelling (*e.g.* Batista *et al.*, 2005; Nunes *et al.*, 2003; 2006a; 2006b; O'Kane *et al.*, 2004a; 2005; Sousa, 1993; Kiosseoglou *et al.*, 1999; Lakemond *et al.*, 2003; Renkema and van Vliet, 2002), emulsifying and foaming ability (*e.g.* Raymundo *et al.*, 1998; 2002; Franco *et al.*, 1998a; Gharsallaoui *et al.*, 2009;

Eliazalde *et al.*, 1996; Roesch and Corredig, 2002; Bengoechea *et al.*, 2006; 2010) or flavour retention capacity (Heng *et al.*, 2004).

The term *"functionality"* as applied to food ingredients has been defined as *"any property besides nutritional attributes that influences an ingredient's usefulness in foods"* (Boye *et al.*, 1997) being usually reflected by the sensorial and textural properties of food. This refers to a *"technological functionality"* and should not be confused with the term *"functional foods"* which refers to "*a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease"* (Bellisle *et al.*, 1998, Diplock *et al.*, 1999).

Functional properties of protein ingredients are related to their physical, chemical, and conformational properties (Damadoran, 1996). Therefore, they depend not only on their intrinsic properties but also on their degree of denaturation, or more generally speaking on the changes in native conformation.

Denaturation of globular proteins has been defined as a process in which the spatial arrangement of polypeptide chains within the molecule have changed from that typical of the native protein to a more disordered arrangement (Kauzmann, 1959). More specifically it can be regarded as any modification in conformation (secondary, tertiary or quaternary) not accompanied by the rupture of peptide bonds involved in the primary structure, *i.e.* without alteration of the amino acid sequence (Tanford, 1968). Is a consequence of an altered balance between the different forces, such as electrostatic interactions, hydrogen bonds, disulphide bonds, dipole-dipole interactions, and hydrophobic interactions, which maintain a protein in its native state (Alting, 2003). Various levels of denaturation can be distinguished according to whether the secondary, tertiary or quaternary structure of the protein is involved in the process, so it may be confined to a region of the protein or it may involve the complete molecule (Tanford, 1968).

Heat treatment is the most important food-processing operation which contributes to protein denaturation. The loss of the globular character of proteins during heating can be primarily attributed to the increased entropy of the unfolded state of the protein, leading to rupture of inter- and intramolecular bonds that stabilise the native protein structure (Creighton, 1978). This results in hydrophobic amino acid residues becoming exposed to the solvent and a reorganization of the protein structure (Boye *et al.*, 1997). Thermal denaturation is in principle reversible, since complete unfolding only occurs in the presence of strong denaturants, such as urea or guanidine hydrochloride.

2.2.4.1. Pea Protein

Proteins extracted from leguminous seeds have been subject of particular interest in the last years as functional ingredients for the food industry (Kinsella, 1979; Guéguen, 2000). Soybean is the foremost used, but this crop is not well-suited to the European climate, and approximately 70-80% of all feed protein concentrates used in Europe are imported from South America (Davis *et al.*, 2010). One way of reducing the negative economic and environmental impact of European overseas soy dependence is using grain legumes, *e.g.*, field peas, faba beans, or lupins, grown within Europe.

Dry pea (*Pisum sativum* L.) is a pulse crop of the Leguminaceae Family that is well suited to European temperate climate conditions. It is an interesting alternative, considering that pea protein production in the European Union represented, in 2009, 1.394.339 tons, being France, Germany, Spain and United Kingdom the larger producers (FAOSTAT, 2011). Pea protein isolates are commercially available, in opposition to other leguminous protein isolates such as lupin, and present no labelling constraints, such as no Genetically Modified Organisms (GMO) and gluten free.

Pea seeds (*Pisum sativum*) have 20-30% protein, mainly corresponding to storage proteins. The two principal proteins, legumin (7S) and vicilin (11S), are globulins and represent 65-80% of the total extractable proteins (Schroeder, 1982). They have a regular quaternary structure, hexameric for legumin proteins and trimeric for vicilin proteins, which depends on pH and salt concentration (Gharsallaoui *et al.,* 2009). Legumin is recognized by its sulphur containing amino acid residues (cysteine, methionine). Convicilin is the less representative globular protein, sometimes considered as a contaminant protein (Koyoro and Powers, 1987). O´Kane and co-workers (2004b) concluded that convicilin cannot be considered as a separate fraction, and was designated as a vicilin subunit.

As protein source, pea (*Pisum*) is genetically variable, not only in total protein content, but also in the amino acid sequence and the legumin/vicilin (0.2-1.5) (Casey, 1982), albeit vicilin being the dominant protein in most cultivars (O´Kane *et al.*, 2004b). The convicilin content is also variable (O´Kane *et al.*, 2005). These differences can affect the functionality of the protein isolates obtained from different cultivars.

Legumin and vicilin are similar to 11S (glycin) and 7S (β-convicilin) soy proteins, respectively, presenting similar molecular weights, amino acid profiles and structural subunits (Derbyshire *et al.*, 1976). From the nutritional point of view, pea protein has the advantage of presenting a balanced amino acid composition, especially in lysine, an essential amino acid, despite its low content in tryptophan being a disadvantage (Schneider and Lacampagne, 2000).

Most of the methods used for the production of protein isolates from seeds are based in the extraction of proteins at slightly alkaline pH, followed by precipitation at the isoelectric point (Sumner *et al.*, 1981; Soetrisno and Holmes, 1992a; O´Kane *et al.*, 2005). The separation of the

protein fraction can be carried out by ultracentrifugation (Pompei and Lucisano, 1976), ultrafiltration (Fredrikson *et al.*, 2001), or by using a saline solution, which allows the precipitation of the protein in micelles when the ionic strength is lowered by dilution with water (Guéguen, 1991).

Besides nutritional characteristics, their good functional properties, such as gelling, emulsifying and foaming, have led to a greater interest in this protein source as a promising food ingredient (Bacon *et al.*, 1990; Bora *et al.*, 1994; Guéguen, 1991; 2000; Koyoro and Powers, 1987; Sumner *et al.*, 1981; Tomoskozi *et al.*, 2001). The extraction procedures described above do not cause the protein denaturation as demonstrated by Hermansson (1979) and Sousa *et al.* (1995), allowing the attainment of protein isolates with properties similar to the native proteins, without functionality loss.

2.2.4.1.1. Pea protein as emulsifying agent

Egg yolk is the most commonly used emulsifier in foods like mayonnaise or salad dressings due to its favourable organoleptic and functional properties (Franco, 1995). The production of oil-in- water emulsions using alternative vegetable emulsifiers to fully (or partially) replace egg yolk offers several dietetic advantages (Franco *et al.*, 1998a; Raymundo *et al.*, 2002), such as decreasing cholesterol and fat contents as well as increasing microbiological stability (Riscardo *et al.*, 2003).

Pea protein has good interfacial and emulsifying ability, demonstrated by several authors, as discussed below. Dagorn-Scaviner *et al.* (1986; 1987) have studied the interfacial behaviour of pea (*Pisum sativum L.*) globulins at air/water interface and their emulsifying properties as related to their adsorption behaviours. Pea globulins appeared to be efficient proteins as surface active agents, especially vicilin, which presented generally higher adsorption rate constants. Vicilin also led to better emulsifying properties than legumin, and some inhibiting interactions occurred between the two globulins, legumin adsorption being more disturbed by vicilin than the opposite.

Dissociation of pea legumin results in an improvement in the ability of the protein to diffuse to the interface whereas the charge of the molecule influences the kinetic parameters of anchorage and conformational rearrangement in the occupied interfacial layer (Subirade *et al.*, 1992). Gharsallaoui *et al.* (2009) proposed that at acidic conditions dissociated globulins adsorb slowly to the oil-water interface but form stronger and denser viscoelastic networks, as compared with pHs above the isoelectric point (pI~4.3).

The formation of oil-in-water emulsions stabilized by pea protein isolate has been studied, revealing high emulsion capacities and stability (*e.g.* Soestrisno and Holmes, 1992b). Tomoskozi *et al.* (2001) found a relatively good emulsifying activity and low emulsion stability in comparison to soy protein isolate, while Aluko *et al.* (2009) found that pea had generally

significantly higher emulsion and foam forming properties than soy. When studying different protein fractions, it was observed that ethanol soluble fraction forms emulsion with smaller droplet sizes and higher stability (Adebiyi and Aluko, 2011).

Franco *et al.* (2000) investigated the influence of pH and thermal treatment on the rheology of pea protein-stabilized oil-in-water emulsions, observing higher emulsion viscosity and lower mean droplet sizes, for higher temperature or heating times, and up to pHs close to the protein isoelectric point. This is in accordance to Gharsallaoui *et al.* (2009) which found that pea-protein-stabilized emulsions were more stable to creaming and with more homogeneous particle-size distributions at acidic pH. More recently, attention is being focused on the development of pea protein-based nanoemulsions for delivery of nutraceuticals (Donsi *et al.*, 2010), namely spray dried emulsions for further reconstitution (Gharsallaoui *et al.*, 2010).

2.2.4.1.2. Pea protein as gelling agent

When submitted to controlled thermal denaturation pea globulins can form gels (Bacon *et al.*, 1990; Bora *et al.*, 1994; Guéguen, 2000; O´Kane, 2004; O´Kane *et al.*, 2005).

Bacon *et al.* (1990) studied the properties of gels obtained from legumin and vicilin mixtures. Transparent gels were obtained after thermal treatment under acidic conditions, which enables the use of pea protein as gelatine substitute in vegetable products.

Bora *et al.* (1994) determined that the optimal conditions for the gelation of a pea globulin mixed system corresponds to pH 7.1 and thermal treatment of 87ºC/20 min, while the addition of NaCl at concentrations above 0.05 M reduces gel firmness. These authors also observed that legumin does not form gels and its proportion in the legumin/vicilin mixture is inversely proportional to gel firmness.

O´Kane (2004) extensively studied the gelling capacity of pea legumin and vicilin fractions as well as of protein isolates extracted from different *Pisum sativum* L. cultivars. Pea legumin gelation behaviour was found to be similar to soy glycinine, although gels present distinct properties. For both proteins, it was demonstrated that gel formation is greatly due to hydrophobic and hydrogen bonds, while disulphide bonds are not essential, although they contribute to an increase in the gel structure and stability. Unlike soy glycinin, pea legumin gel networks were susceptible to rearrangements that caused gels to become stronger after reheating/recooling (O´Kane *et al.*, 2004a). Vicilin gelling capacity was reduced by the presence of α-subunit (convicilin). Weaker transparent gels were obtained, at pH 7.6 and ionic strength 0.2 M, in the presence of the α-subunit, which was attributed to electrostatic repulsion between the strongly charged *N*-terminal regions of convicilin (O´Kane *et al.*, 2004c). The minimum gelling concentration of pea protein isolates from five different cultivars in all cases was 16% (m/v). Gel formation did not depend on the formation of disulphide bonds in legumin and the contribution of these bonds to the gels firmness depends on the

cultivar. It was also demonstrated that the isolates from cultivars with high contents of α-subunit form very weak gels at neutral pH, possibly due to electrostatic repulsions between negatively charged *N*-terminal regions (O´Kane *et al.*, 2005).

Batista *et al.* (2005) reported the formation of heat-induced gels at 12.5% (w/w) concentration for a commercial pea protein isolate. These authors also reported higher rheological parameters for gels prepared with 16% (w/w) pea protein isolate in comparison to lupin protein isolate, which was only able to form gels at this concentration.

According to Shand *et al.* (2007) the optimal conditions for formation of strong heat-induced gels from pea protein isolates were 19.6% (w/w) protein content, pH 7.1, 2.0% (w/w) NaCl, and heating at 93ºC.

2.3. RHEOLOGY

2.3.1. Basic Concepts

Rheology was a concept introduced by Professor Eugene C. Bingham (1929), defined as the science which studies the deformation and flow of materials. The word rheology etymologically means the study of flow (from the greek *"rheos"*: flow and *"logos"*: science).
Rheology studies the relation between the external forces that act on a material (body) and the deformation produced. Traditionally, the deformable materials were classified in two groups: elastic solids and viscous fluids (liquids and gases), characterized by classic elasticity and fluid mechanics, respectively. Between these two extremes there is a wide spectrum of materials with different behaviours, which are the object of study in rheology. Rheology, as an independent science reached its greatest development in the second half of the 20th century due to the industrial production at large scale of new materials (plastics, polymers, etc.), which presented special flow characteristics that could not be explained by classical laws.
Most of multiphase and/or formulated materials exhibit complex rheological properties, hence its relevance in many fields of study, such as the processing of polymers, plastics, paints, glues, soil, ceramics, cosmetics, pharmaceuticals, foods (Steffe, 1996; Castro *et al.*, 2001).
Rheology plays an important role in Food Science and Engineering, with numerous application areas in the food industry (Steffe, 1996):

a) Process engineering calculations involving a wide range of equipment such as pipelines, pumps, extruders, mixers, coaters, heat exchangers, homogenizers, and on-line viscometers;
b) Determining ingredient functionality in product development;
c) Intermediate or final product quality control;
d) Shelf life testing;
e) Evaluation of food texture by correlation to sensory data.

The rheological behaviour of food products is directly related to its texture properties and results from their composition and internal structure. The product structure is determined by its composition and the processing applied. The composition of a food product is usually very complex, being the macromolecules, proteins and polysaccharides, responsible for the structural building. Rheological properties explain the behaviour of that building (Sousa, 2001).

2.3.1.1. Stress and deformation in simple shear

The rheological properties of a material are defined through the relation between a force (or a system of forces in equilibrium) that acts on a material (*cause*) and the caused deformation and/or flow (*effect*) (Darby, 1976). The deformation of a material does not depend on the magnitude of the force itself, but on the magnitude of the force per unit area, *i.e.* the stress (or tension). Hence, the applied mechanical solicitation can be mathematically represented by the stress, and the dynamic response of the material is quantified by the deformation (or strain) and/or deformation rate.

Stress (σ) is a magnitude which measures the relation between a force (F) by surface unit (A), and is usually expressed in Pascal (N/m^2). Both force and surface are directional magnitudes that can be represented by three-component vectors, corresponding to the three-dimensional space. So, to completely define stress it is necessary to specify nine components, which result from the application of each component of the force vector upon each of the three components of the surface vector. Stress is a second order tensor, which can be represented in a matrix form (2.3):

$$\overline{\overline{\sigma_{ij}}} = \begin{pmatrix} \sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\ \sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\ \sigma_{zx} & \sigma_{zy} & \sigma_{zz} \end{pmatrix} \tag{2.3}$$

The first sub-index of the stress tensor refers to the plane orientation upon which the force acts, and the second refers to the force orientation. As a consequence of the equilibrium condition between pairs of forces the tensor is symmetric ($\sigma_{ij}=\sigma_{ji}$), so, only six of the nine components are independent.

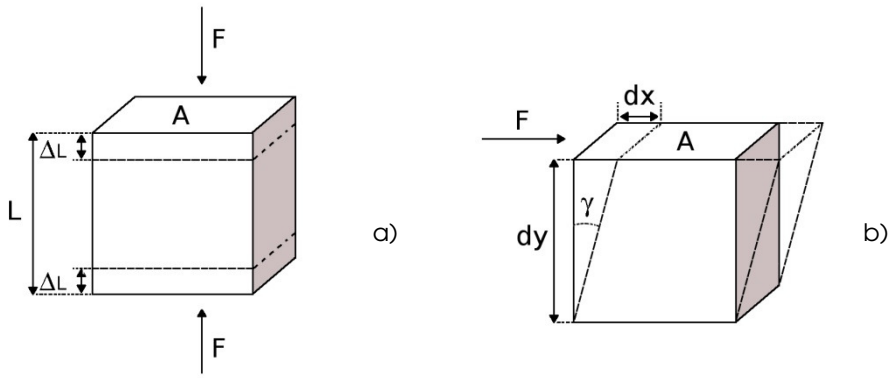


Figure 2.28. Compression deformation caused by uniaxial/normal stress (a); Shear deformation caused by shear/tangential stress (b)

In general, two types of stresses can be externally applied to a material: normal stresses, perpendicularly applied to the surface (compressive or tensile); and shear stresses, tangentially applied to the surface; causing uniaxial and shear deformations, respectively (Figure 2.28).

The total stress tensor can be decomposed as the sum of an isotropic tensor σ, and an anisotropic tensor τ_{ij} (Dealy, 1982).

$$\sigma_{ij} = \sigma\delta_{ij} + \tau_{ij} \tag{2.4}$$

Where δ_{ij} is the unitary tensor or Kronecker delta:

$$\delta_{ij} = 1 \text{ if } i = j \qquad \text{and} \qquad \delta_{ij} = 0 \text{ if } i \neq j \tag{2.5}$$

The isotropic stress, σ, is perpendicular to all the surfaces (with the same magnitude in all directions), causing volume changes but maintaining the material's original shape. The anisotropic stress, τ, can comprise normal (normal stresses) or tangential (shear stresses) components, promoting modifications on the material's shape but keeping its total volume unchanged. The normal component of the anisotropic stress cannot be directly measured but it is possible to measure the components of the total normal stress:

$$\overline{\overline{\sigma_{ij}}} = -P\,\overline{\overline{\delta_{ij}}} + \overline{\overline{\tau_{ij}}} \tag{2.6}$$

By considering differences between normal stresses it is possible to eliminate the contribution of pressure in the total stress components:

$$\sigma_{xx} - \sigma_{yy} = (-P + \tau_{xx}) - (-P + \tau_{yy}) = \tau_{xx} - \tau_{yy} = N_1 \tag{2.7}$$

$$\sigma_{yy} - \sigma_{xx} = (-P + \tau_{yy}) - (-P + \tau_{xx}) = \tau_{yy} - \tau_{xx} = N_2 \tag{2.8}$$

It must be noted that, if the material is incompressible, only the shear stress and the normal stress differences, acting in different directions, have rheological significance. An isotropic stress does not cause changes in an incompressible material, since it can't change its volume. This enables the substitution of the components of the total stress σ_{ij}, by the components of the *"deviation"* stress, τ_{ij}:

$$\sigma_{11} - \sigma_{22} = \tau_{11} - \tau_{22} \tag{2.9}$$

$$\sigma_{22} - \sigma_{33} = \tau_{22} - \tau_{33}$$

$$\sigma_{ij} = \tau_{ij} \text{ if } i \neq j$$

Deformation is a quantitative measure of the extension at which a material element has been deformed. The magnitude of the deformation is determined by the relative displacement of the points, so it is an adimensional magnitude, since it is expressed as the quotient between the distance increment (*dL*) and the initial distance (*L*) between points. Since both the numerator and denominator are three component vectors, deformation and deformation rate are also second order tensors with nine components, as the stress tensor. The deformation ε_{ij}, is a symmetric tensor, so one can define an isotropic deformation ε and an anisotropic deformation γ_{ij}, as:

$$\varepsilon_{ij} = \varepsilon\delta_{ij} + \gamma_{ij} \tag{2.10}$$

The isotropic component indicates a change in volume (dilatation or compression) without shape modification. The γ_{ij}, deviatory deformation tensor, represents the anisotropic deformation and reflects a change in the shape without volume alteration.
The vector deformation rate is obtained by derivation of the deformation tensor in respect to time:

$$\varepsilon_{ij} = d\,(\varepsilon_{ij})\,/dt \tag{2.11}$$

One of the simplest cases of deformation is the so-called *"simple shear"*. This type of deformation is generated when a material is between two parallel plates separated by a gap "*h*" (Figure 2.29) (two plate model). The superior plate is submitted to a force "*F*", which causes it to linearly move a distance "*x*", while the lower plate remains still (Dealy, 1982).

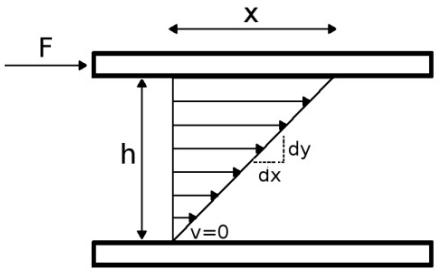


Figure 2.29. Velocity profile in simple shear deformation (two plate model).

If there is no surface slip each element of the material suffers a deformation expressed as:

$$\dot{\gamma} = dx\,/\,dy \tag{2.12}$$

Where γ is the shear deformation; *dx* and *dy* are the displacement of the superior plate (in respect to the inferior plate) in the direction *x* and *y*, respectively; and *h* is the height or

distance between plates. Since the deformation is uniform and independent of the plate size, can be represented as a function of *h* and *x*:

$$\dot{\gamma} = x / h \tag{2.13}$$

In rheological tests, the materials are submitted to a continuous shear by applying a constant force *F*, in the direction *x*, reaching a constant velocity, *v*, which is expressed by:

$$dv = dx / dt \tag{2.14}$$

So, the shear rate or deformation rate is defined as:

$$\dot{\gamma} = \frac{d\gamma}{dt} = \frac{d(dx/dy)}{dt} = \frac{d(dx/dt)}{dy} = \frac{du_x}{dy} \tag{2.15}$$

Or by the following expression:

$$\dot{\gamma} = \frac{d\gamma}{dt} = \frac{d(x/h)}{dt} = \frac{d(dx/dt)}{h} = \frac{v}{h} \tag{2.16}$$

This indicates that the shear rate is constant through the material for simple shear conditions. Since *v* and *h* can be measured without disturbing flow, it is easy to submit a deformation to a material with a known shear rate. However, to produce a totally uniform movement by simple shear it is necessary that both parallel plates have infinite extension.

2.3.1.2. Classification of rheological behaviours

The rheological behaviour of a material can be described by rheological equations of state or constitutive equations, which describe the relation between stress and deformation. In complex materials these equations may include other variables such as time, temperature and pressure (Steffe, 1996). The main types of rheological behaviours are summarized in Table 2.4, considering the relation between applied stress and the deformation or deformation rate in simple shear.

Rigid solids (Euclidean) are not deformable, while elastic solids (Hookean) are deformed when submitted to a tension. In an ideal elastic solid the deformation is totally recovered when the tension is removed, *i.e.* all the energy supplied to the system is stored.

Table 2.4. Types of rheological behaviours.

Solid	Rigid solid (Euclidean)	$\gamma = 0$
	Lineal elastic solid (Hooke)	$\sigma = G\,\gamma$ (G=cte)
	Non lineal elastic solid	$\sigma = G(\gamma).\gamma$
	Viscoelastic	$\sigma = \sigma(\gamma, \dot{\gamma}, t)$
Fluid	Non lineal viscous fluid (non-Newtonian)	$\sigma = \eta(\dot{\gamma}).\dot{\gamma}$
	Lineal viscous fluid (Newtonian)	$\sigma = \eta\,.\,\dot{\gamma}$ (η = cte)
	Non viscous fluid (Pascal)	$\sigma = 0$

In an ideal viscous or Newtonian liquid, the deformation is proportional to the applied tension and remains constant when the tension is removed, *i.e.* all the energy supplied to the system is dissipated in the form of heat.

Most materials simultaneously present both elastic (solid) and viscous (liquid) characteristics, being called viscoelastic materials. These are complex systems in which the relation between the applied tension and the respective deformation is not linear, part of the energy supplied to the system is stored and the other part is dissipated.

A more accurate classification must take in consideration that a material can present different behaviours depending on the magnitude of the stress or the deformation applied, as well as the previous shear history.

2.3.1.2.1 Steady flow

2.3.1.2.1.1. Newtonian flow

Newtonian fluids are ideal liquids, characterized by showing a direct proportionality between shear stress and shear rate, according to the Newton law:

$$\tau = \eta\,\dot{\gamma} \tag{2.17}$$

The proportionality constant, η, is a physical property called dynamic or Newtonian viscosity that depends only of the material's nature (unaltered by shear time or rate), as well as on pressure and temperature conditions. Viscosity reflects the material resistance to flow, resulting from the internal friction between fluid layers, and can be defined as a measure of energy dissipation intensity needed to maintain a fluid continuous deformation, presenting Pa.s units (S.I.). A fluid is considered Newtonian when it continuously flows by applying shear stress. When the stress is removed, the fluid continues to flow until the kinetic energy stored in the material is dissipated as heat due to internal friction. Systems with simple structures, such as water, oil and diluted biopolymer solutions present Newtonian behaviour.

2.3.1.2.1.2. Non-Newtonian flow

For many fluids, the tension is not proportional to the shear rate, *i.e.* their behaviour cannot be described according to equation 2.17, being defined as non-Newtonian fluids. This behaviour is shown by solutions containing macromolecules, emulsions, concentrated suspensions, liquid crystals and micelles, etc.

The ratio between τ and $\dot{\gamma}$ is known as apparent viscosity and varies with shear rate:

$$\eta(\dot{\gamma}) = \tau / \dot{\gamma} \tag{2.18}$$

Non-Newtonian fluids can be classified in terms of the variation of σ with $\dot{\gamma}$ (flow curve) or the variation of apparent viscosity with $\dot{\gamma}$ (viscosity curve) (Darby, 1976). Figure 2.30 presents the flow and viscosity curve characteristics of these fluids. According to the evolution of viscosity with shear rate, fluids are classified into shear-thinning or shear-thickening.

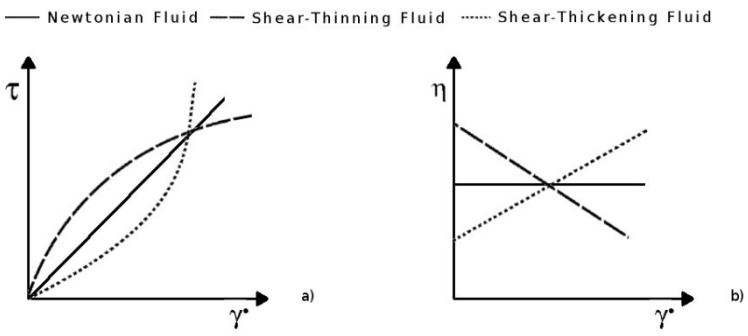


Figure 2.30. Flow (a) and viscosity (b) curves for typical Newtonian and non-Newtonian fluids.

Shear-thinning materials show a decrease in viscosity with increasing shear rate. Most food materials are shear-thinning, namely biopolymer solutions, suspensions, emulsions, etc. A common example is the structural behaviour related to a material structural change with increasing $\dot{\gamma}$, characterized by three differentiated zones (Figure 2.31), corresponding to well differentiated structural behaviours (Mitchell, 1979). At low shear rates (Zone I) the fluid presents a Newtonian behaviour with a constant zero-shear limiting viscosity η_0. In the intermediate region (Zone II) a decrease is observed in the apparent viscosity with shear rate. At higher shear rates (Zone III) the fluid presents again a Newtonian behaviour, characterized by a lower constant limiting viscosity η_∞. These different regions are related to changes in the material structure with increasing shear rates. Many fluids are formed by complex structures randomly distributed under quiet state, maintaining a disordered state which causes a certain flow resistance. By submitting these fluids to a certain stress the microstructural

assemblies are oriented and deformed in the direction of the applied stress and there may be a rupture of the entanglements between molecules, decreasing the flow resistance, and consequently the apparent viscosity. When the applied stress is sufficiently small, there is no preferential orientation of the molecules, and the re-establishment of entanglements is higher than their rupture, which corresponds to the Newtonian behaviour at low shear rates. Likewise, at high shear rates, the different structural units are totally destroyed in respect to their initial state, which explains the achievement of a constant viscosity value η_∞. According to the shape of the curves, there are several mathematical models (*e.g.* Cross, 1965; Carreau, 1972) which can be adjusted to the viscosity or flow curves, depending on the shear rate range where they are valid.

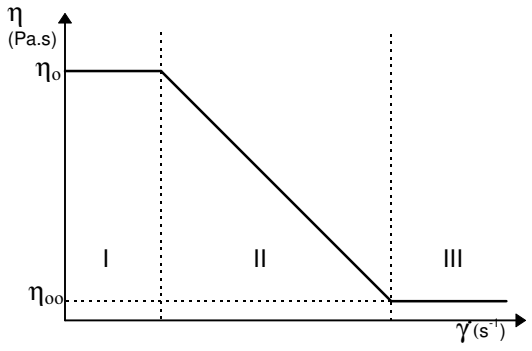


Figure 2.31. Schematic representation of a typical viscosity curve for shear-thinning fluid, with three differentiated zones (I, II, III).

Some shear-thinning materials seem to have a yield value, *i.e.*, do not flow before a certain stress is reached. The stress level required to initiate flow is usually referred to as yield stress and is related to the level of internal structure of the material, which must be destroyed before flow can occur. When these plastic materials begin to flow they can show either a straight line or power-law behaviour, which can be described by Bingham (1916), Herschel-Bulkley (1926) or Casson (1959) models. The existence of a real yield stress as a rheological function of the material was questioned by Barnes and Walters (1985) in their original paper *"The yield stress myth?"*. In fact, with the development of new experimental capabilities (rheometers with shear rates as low as 10^{-6} s^{-1}), it became clear, first for solids and lately for soft solids and structured liquids, that although there is usually a small range of stress over which the mechanical properties change dramatically (an apparent yield stress), these materials nevertheless show slow but continuous steady deformation when stressed for a long time below this level, having shown an initial linear elastic response to the applied stress (Barnes, 1999). However, this empirical parameter is still useful in industrial applications, for predicting product processing conditions (*e.g.* pipeline transportation) or for quality control purposes.

Shear thickening materials show an increase in apparent viscosity with increasing shear rates. This behaviour is far less common than shear-thinning, but it is observable in certain highly concentrated suspensions of solid particles, concentrated polymer solutions or even highly flocculated emulsions. At rest, the space occupied by the liquid between particles is minimal. At low shear rates the friction between particles is relatively low because the liquid acts as lubricant. However, at high shear rates, the movement of the particles upon each other intensifies friction, increasing viscosity (Pal, 1996).

2.3.1.2.2. Time-dependent flow behaviour

In many non-Newtonian fluids, the apparent viscosity measured under simple shear varies not only with shear rate but also with shear time:

$$\eta = \eta(\dot{\gamma}, t) \tag{2.19}$$

Regarding time-dependent rheological behaviour there are different phenomena:

- *Thixotropy*: viscosity decrease with shear time, followed by a gradual recovery when the stress or shear rate is removed;
- *Antithixotropy (Rheopexy)*: viscosity increase with shear time, followed by a gradual recovery when the stress or shear rate is removed;
- *Irreversible thixotropy (Rheodestruction)*: viscosity decrease with time, without recovery when the stress or shear rate is removed.

There are several experimental techniques for evaluating this type of rheological behaviour called transient flow measurements. One of the most used methods is to linearly increase the shear rate from zero to a maximum value and then return at the same rate to zero. If the material presents time-dependent behaviour the upward and downward curves do not match, and an hysteresis loop is generated, being the hysteresis area a measure of the system's thixotropy degree.
Other type of experiment is to study stress variation at a constant shear rate, until a steady-state is attained (stress growth tests). The most important characteristic shown by the fluids exhibiting this rheological behaviour is a maximum stress (*stress overshoot*), followed by a decrease until steady-state is reached (Kokini and Dickie, 1981). This characteristic variation of shear stress with time, at constant shear rate, can be justified by structural changes in the material (thixotropy), but some authors have attributed it to the material viscoelasticity, or both effects (Gallegos and Franco, 1999a).

2.3.1.3. Viscoelasticity

Viscoelastic materials simultaneously present characteristics typical of elastic and viscous behaviour, being possible to discriminate both components in a material. In this type of materials, part of the energy is stored as mechanical energy, and other part is dissipated as heat. For this reason, viscoelastic materials always display a delayed response when a stress load is applied, as well as when it is removed. This way, the relation between stress and deformation cannot be expressed only by material constants, depending also on the deformation history (fluids with memory). The rheological behaviour of viscoelastic materials is characterized by time-dependent material functions.

When both stress and deformation are infinitesimal, the viscoelastic behaviour can also be described by linear differential equations with constant coefficients. This is called linear viscoelastic behaviour and implies that the relation between stress and deformation does not depend on the magnitude of these parameters, being only time-dependent (Darby, 1976). In these conditions the Boltzman superposition principle is valid, *i.e.* it is considered that a series of changes on a cause (*e.g.* deformation) that act upon a linear system, each one occurring sequentially in time, contributes cumulatively to the resulting effect (*e.g.* stress) in a later time moment (Darby, 1976; Ferry, 1980). The total effect will be a linear combination of these separate contributions. Considering $\psi(t-t')$ as a property of the system (influence function), which relates the cause ($\gamma(t)$) and effect ($\sigma(t)$) functions, when a variation on the cause ($\Delta\gamma$) is induced at a time moment t' it produces an effect ($\Delta\sigma$) in a later time moment t. If the change in the cause is produced continuously it can be represented by an integral:

$$\sigma(t)=\int_{\gamma(-\infty)}^{\gamma(t)}\psi(t-t')\,d\gamma(t')=\int_{-\infty}^{t}\psi(t-t')\,\dot{\gamma}(t')\,dt' \qquad (2.20)$$

2.3.1.3.1. Linear viscoelasticity tests

There are mainly three different types of tests used to measure the linear viscoelastic properties of a material: stress relaxation, creep and recovery, and oscillatory tests.

2.3.1.3.1.1. Stress Relaxation

In these tests a constant deformation is applied to the material and the variation of the stress needed to maintain the deformation along time is monitored (Figure 2.32). This method enables to determine the stress relaxation modulus, $G(t)$:

$$G(t) = \sigma(t) / \gamma \qquad (2.21)$$

For an elastic solid (Hooke), an equilibrium situation is rapidly achieved, being defined by the equilibrium rigidity modulus (G_e), whereas for a viscous (Newtonian) liquid the relaxation is instantaneous. Viscoelastic materials present intermediate $G(t)$ *vs.* time evolutions, depending on the internal structure of the materials.

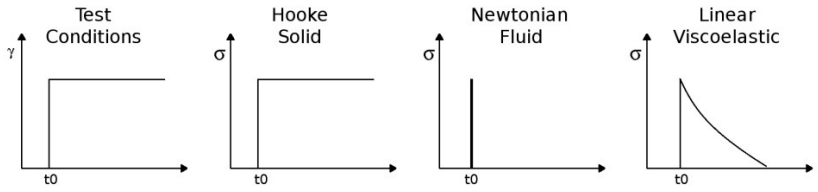


Figure 2.32. Typical stress relaxation test curves.

2.3.1.3.1.2. Creep and recovery

In a creep test, a constant stress is applied to the material, and the resulting deformation is monitored along time (Figure 2.33).

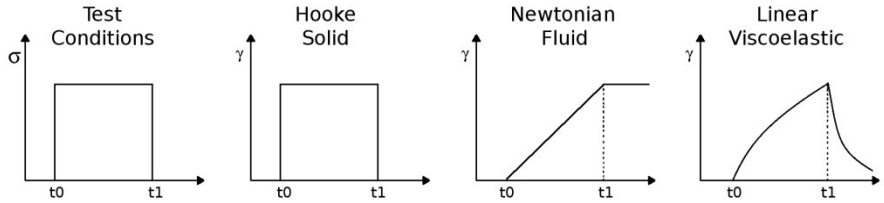


Figure 2.33. Typical creep and recovery test curves.

From the relation between stress and deformation it is possible to determine the viscoelastic compliance, $J(t)$:

$$J(t) = \gamma(t) / \sigma \tag{2.22}$$

For an elastic solid, an equilibrium situation is instantaneously achieved, attaining an equilibrium compliance (J_e). For Newtonian liquids, a linear response is observed:

$$J(t) = t / \eta \tag{2.23}$$

The viscoelastic materials present an intermediate behaviour, power-law for short times and linear for longer times, adjusting the linear part to equation (2.24):

$$J(t) = J_e^0 + t / \eta \tag{2.24}$$

where J_e^0 is the stationary flow compliance, which is a measure of viscoelastic fluids elasticity.

These tests usually include the study of the material behaviour upon stress removal, analysing the recovery of the deformation upon time. The deformation recovery is total for elastic solids, null for Newtonian liquids and partial for viscoelastic materials, defined as γ_f:

$$\gamma_f = \sigma J_e^0 \tag{2.25}$$

2.3.1.3.1.3. Oscillatory tests

Dynamic (oscillatory) rheological tests provide valuable information on the viscoelastic nature of foods (Lopes-da-Silva and Rao, 2007). In comparison to stress relaxation and creep-recovery, oscillatory tests are easier to perform and more reproducible. These are non-destructive tests, characterized by the application of a sinusoidal (harmonic-periodic) stress to a material and measurement of the resulting sinusoidal deformation, or *vice-versa*. For example, the application of a small sinusoidal shear stress to a material can be represented as a cosine (or sine) function:

$$\sigma = \sigma_0 \cos(\omega t) \tag{2.26}$$

The resulting deformation can also be represented by a sinusoidal function, with the same frequency, but with a phase difference δ (rad), also known as phase angle or loss angle, in respect to the applied stress function:

$$\gamma = \gamma_0 \cos(\omega t + \delta) \tag{2.27}$$

where σ_0 and γ_0 are the maximum stress and deformation amplitudes, respectively, ω is the oscillation angular frequency ($\omega = 2\pi f$ rad/s), and t is the time (s).

The value of the phase angle δ varies between 0 and $\pi/2$, depending on the viscoelastic character of the material (Figure 2.34). For an ideal elastic solid, stress is directly proportional to the deformation, so both functions are in phase and δ=0, while for pure viscous liquids, the stress and deformation functions have a phase difference of $\pi/2$.

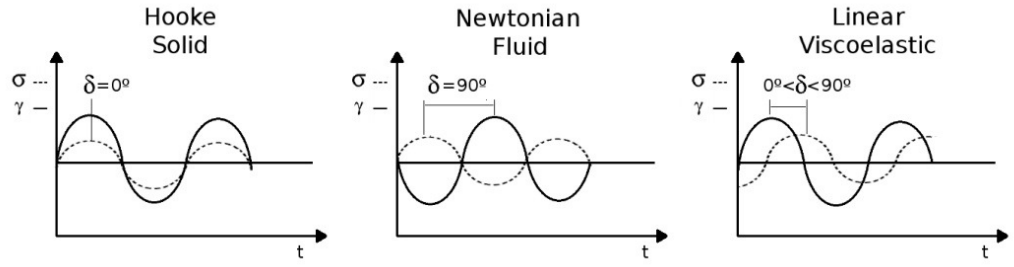


Figure 2.34. Typical dynamic sinusoidal deformation curves in respect to applied sinusoidal stress in small amplitude oscillatory shear tests (SAOS).

Stress and deformation are usually expressed in terms of their complex equivalents (Ferry, 1980), where the prime (') represents the real components and the double prime (") represents the imaginary components:

$$\sigma(\omega t) = \sigma_0 e^{i\omega t} = \sigma_0(\cos(\omega t) + i\,\mathrm{sen}(\omega t)) = \sigma'(\omega) + i\sigma''(\omega) \quad (2.28)$$

$$\gamma(\omega t) = \gamma_0 e^{i(\omega t+\delta)} = \gamma_0(\cos(\omega t+\delta) + i\,\mathrm{sen}(\omega t+\delta)) = \gamma'(\omega) + j\gamma''(\omega) \quad (2.29)$$

The complex shear modulus, G^*, is defined through the relation between the complex oscillatory stress and deformation (Ferry, 1980):

$$G^* = \left(\frac{\sigma_0}{\gamma_0}\right) cos\delta + i\left(\frac{\sigma_0}{\gamma_0}\right) sen\delta = G' + iG'' \quad (2.30)$$

The phase component, G', known as elastic or storage modulus, represents the elastic character of the material being a measure of the deformation energy stored in the material during the shear process and used for deformation recovery after load removal. The viscous or loss modulus, G'', is the out of phase component, representing the viscous character of the material and a measure of the energy lost on the material flowing process. The absolute value of the complex modulus is given by:

$$|G^*| = \sqrt{G'^2 + G''^2} = \frac{\sigma_0}{\gamma_0} \quad (2.31)$$

Considering that both G' and G'' are frequency dependent and can be expressed as:

$$G'(\omega) = \sigma_o/\gamma_o \cos\delta \quad (2.32)$$

$$G''(\omega) = \sigma_o/\gamma_o \sin\delta \quad (2.33)$$

it is possible to calculate the $tan\delta$, or loss tangent, as the ratio between the loss and storage moduli, therefore revealing the ratio of the viscous to the elastic portion of the material:

$$\tan\delta = G'' / G' \quad (2.34)$$

The complex viscosity, η^* (Pa.s) is another commonly used viscoelastic function that can be defined in an analogous way to G^*, through the stress and shear rate ratio:

$$\eta^*(\omega) = \sigma(\omega t)/\dot{\gamma}(\omega t) = \eta'(\omega) - i\eta''(\omega) \quad (2.35)$$

Being the real and imaginary components, respectively:

$$\eta' = G''/\omega \qquad \text{and} \qquad \eta'' = G'/\omega \tag{2.36}$$

It is possible to perform different types of dynamic tests depending on the type of sweep, *i.e.* the function with a variable parameter: amplitude, frequency, time or temperature. Amplitude sweep tests are mostly carried out for the purpose of determining the limit of the linear viscoelastic range. As long as the γ amplitude remain below the critical γ_c value, the G' and G'' are independent of the applied stress or strain value, *i.e.* the structure of the sample is stable; while at $\gamma > \gamma_c$ the structure of the sample has been irreversibly changed or even completely destroyed (Mezger, 2002).

The frequency sweep is probably the most common mode of oscillatory testing because it shows how the viscous and elastic behaviour of the material changes with the rate of application of stress or deformation. The frequency is increased while the amplitude of the applied stress or deformation is maintained constant. The effect of time is examined; short-term behaviour is simulated by rapid movements (at high frequencies) and long-term behaviour by slow movements (at low frequencies) (Mezger, 2002). Mechanical spectra (G', G'' *vs.* ω), are sometimes called *"finger printing"* (Steffé, 1996) due to the microstructural information that can be deduced from these curves.

An isothermal time sweep, where frequency and amplitude are constant, can indicate time-dependent structural changes, such as those associated with the cure or maturation of polymeric materials, namely food gelled systems, in which a chemical or physical network is formed during the test period. The curves obtained are usually called maturation kinetics. Time sweeps may be conducted in conjunction with a controlled change in temperature by applying a temperature/time profile, either by a linear heating (or cooling) rate or by incremental steps. These tests are very useful in monitoring the materials' viscoelastic properties changes occurring during structural transitions, allowing for example the determination of sol-gel and gel-sol transition temperatures (section 2.3.3.2).

2.3.2. Emulsion Rheology

Food emulsions are compositionally and structurally complex materials which can exhibit a wide range of different rheological behaviours, ranging from low-viscosity fluids (such as milk and fruit juice beverages) to fairly hard solids (such as butter) (McClemments, 1999). Many of the sensory attributes of food emulsions (*e.g.* texture, mouthfeel, appearance and flavour) are directly related to their rheological properties (Gunasekaran and Ak, 2000). Ultimately, the rheological behaviour of a food emulsion depends on the type and concentration of the ingredient as well as on the processing and storage conditions.

2.3.2.1. Factors affecting emulsion rheology

The rheological properties of an emulsion are intimately related with the processing conditions and physical stability. Therefore, all the structural parameters that influence the emulsion formation (section 2.2.2.4) and stability (section 2.2.2.5) also affect its rheological behaviour, hampering a systematic understanding of the complex rheological response of concentrated emulsions (Gallegos and Franco, 1999a).

The dispersed phase volume fraction (Φ) is one of the most influencing parameters, since the presence of droplets increases the dissipated energy with the fluid flow, increasing viscosity. In dilute emulsions the droplets are far apart and establish weak interactions, resulting in emulsions with low viscosity and Newtonian behaviour. In concentrated emulsions, the droplets are very near to each other, frequently colliding and strongly interacting, resulting in highly viscous and viscoelastic emulsions (Franco, 1995; Rahalkar, 1992). In fact, above a critical Φ value, the droplets become too compressed, hindering the emulsion flow, which is evidenced by a sharp viscosity increase.

The viscosity of the emulsions is directly proportional to the viscosity of the continuous phase, being directly related with the presence of thickening agents (oil/water emulsions) or fat crystals (water/oil emulsions). The rheology of the dispersed phase has a minor influence because the droplets are covered with a viscoelastic membrane acting as rigid spheres (McClemments, 1999). However, the size and shape of the droplets and droplet size distribution has main influence on the emulsions rheology, depending on both Φ and type of colloidal interactions (*e.g.* van der Waals, electrostatic, steric). In general, emulsions with lower droplet sizes tend to be more viscous and viscoelastic, due to a higher superficial area and higher droplet interaction. This effect is more evident for low Φ in w/o emulsions, and for high Φ in o/w emulsions. Polydisperse emulsions are less viscous (and less stable) than monodisperse emulsions with the same Φ, because the smaller droplets are packed within the available spaces between larger droplets leading to a lower structural resistance to flow.

2.3.2.2. Emulsion flow behaviour

Most of the food emulsions present a non-Newtonian shear-thinning behaviour, which is particularly pronounced in flocculated emulsions (Figure 2.35).

At low shear rates the hydrodynamic forces are not sufficient to disrupt the interactions and entanglements between droplets and the emulsion presents a constant and high viscosity value. As shear rate increases, the hydrodynamic forces become stronger and promote a progressive disruption and deformation of flocs. The deformation of flocs results in their becoming elongated aligned with the shear field resulting in a viscosity decrease. At high shear rates the flocs become completely disrupted and the viscosity reaches a low constant

value (Campanella *et al.*, 1995). Concentrated food emulsions often form long-range three-dimensional network structures, exhibiting constant high viscosity values for a wide range of stress values, that suddenly fall several orders of magnitude, to a much fluid behaviour, as a result of a drastic shear-induced structural breakdown, related to a mechanism of entanglement breakdown and oil droplet de-flocculation (Roberts *et al.*, 2001).

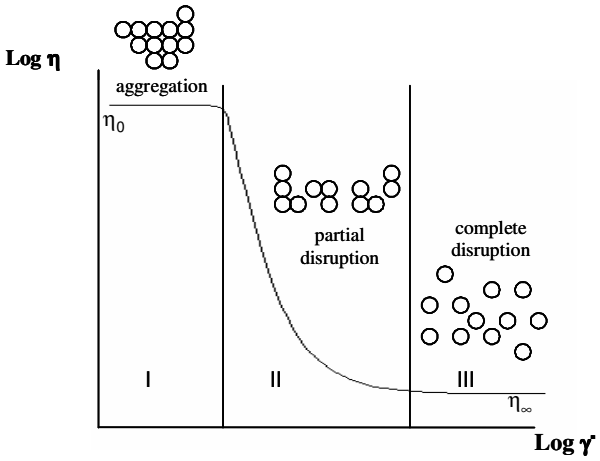


Figure 2.35. Typical flow curve of a shear-thinning emulsion and respective structural changes (Adapted from McClements, 1999).

2.3.2.3. Emulsion linear viscoelasticity

Most food emulsions behave as viscoelastic fluids, and there are numerous studies based on the dynamic response of food emulsions (*e.g.* Gallegos *et al.*, 1992; Franco *et al.*, 1995b; 1998a) It has been observed that the evolution of *G'* and *G"* with frequency is extremely dependent on the emulsion concentration, processing conditions and emulsifier type.

For extensively flocculated emulsions, the disperse phase droplets strongly interact, forming a well-structured tri-dimensional network. The viscoelastic behaviour of this type of emulsion is characterized by *G'* being higher than *G"* for a wide frequency range, with a minimum in *G"* and a plateau region at intermediate frequencies (Figure 2.36a). The Plateau modulus (G^0_N) is a viscoelastic parameter that has been related with the density of the entangled network developed between polymeric molecules (Ferry, 1980). Correspondingly, this parameter has been related with the formation of an entangled network among polymeric molecules (*e.g.* proteins) adsorbed and non-adsorbed at the oil/water interface in an extensively flocculated emulsion (Baumgaertal *et al.*, 1992; Franco *et al.*, 1995a). Hence, G^0_N can be estimated as the value of G' corresponding to the minimum value of the loss tangent (Wu, 1989). Due to the presence of these entanglements in this type of emulsions, there is a deviation of the Cox-Merz rule (Cox and Merz, 1958) which correlates steady shear flow viscosity and dynamic complex viscosity:

$$\eta(\dot{\gamma}) = \eta^*(\omega)|_{\dot{\gamma}=\omega} \quad (2.37)$$

Weakly or non-flocculated oil-in-water emulsions, even in concentrated systems, typically present *G″* values higher than *G′*, in a relatively wide frequency range (Figure 2.36b).

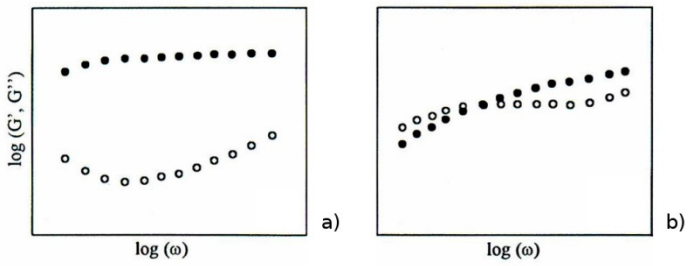


Figure 2.36. Typical dynamic viscoelastic behaviour (mechanical spectra) presented by flocculated (a) and non-flocculated (b) emulsions (● *G′*, ○ *G″*).

2.3.2.4. Wall slip phenomena in emulsion rheological measurements

Colloidal dispersed systems, such as oil-in-water emulsions, often show slip effects under steady-flow conditions or even during oscillatory measurements (Barnes, 1995). Slip occurs due to the displacement of the disperse phase away from solid boundaries, as for instance at the walls of the sensor systems in a rheometer or the walls of pipes or tubes, giving a depleted layer of liquid which then acts as a lubricant. This leads to an apparent decrease in the measured bulk viscosity (Barnes, 1995; Franco *et al.*, 1998b).

In the case of emulsions, the deformability of the droplets and creaming destabilization tendency enhance this effect (Gallegos and Franco, 1999b). Therefore, slip is often more pronounced when an emulsion is formed by large droplets, or when it is weakly flocculated and the flocs behave as individual large droplets under the action of gravity. For this reason, plate-plate or cone-plate geometries (that is, horizontally aligned sensor systems) are more vulnerable to slip (Barnes, 1995; Franco *et al.*, 1998b). The use of roughened plates is the most widely used method for eliminating slip effects in emulsion rheological measurements (Franco *et al.*, 1998b; Sánchez *et al.*, 2001).

2.3.3 Gel Rheology

Rheological studies can provide much useful information on *sol-gel* and *gel-sol* transitions, as well as on gel properties. Generally, the most used tests are frequency, temperature and time sweep dynamic small-amplitude oscillatory shear tests (SAOS) (section 2.3.1.3.1.3), that should be conducted in the linear viscoelastic range.

2.3.3.1. Strong gels and weak gels

Based on the macroscopic behaviour of gel systems, a practical and useful distinction can be made between those systems which are free-standing as a consequence of the development of the three-dimensional network, called *"strong"* or *"true gels"*, and those characterized by a tenuous gel-like network which is easily broken when submitted to a sufficiently high stress, called *"weak gels"* (Doublier *et al.*, 1992). Clark and Ross-Murphy (1987) differentiated gel type based on mechanical spectroscopy measurements, designating as *"strong gels"* networks that have *"finite energy"* and as *"weak gels"* transient systems evidencing hyper-entanglements presence. In the former, the molecular rearrangements within the network over the time scales analysed are very limited, *G'* is higher than *G"* throughout the frequency range and almost independent of frequency (Figure 2.37). In *"weak gels"* there is a higher dependence on frequency for the dynamic moduli, suggesting the existence of relaxation processes occurring even at short time scales, and lower difference between moduli values, indicating that a lower percentage of the stored energy is recovered (Lopes-da-Silva and Rao, 2007).

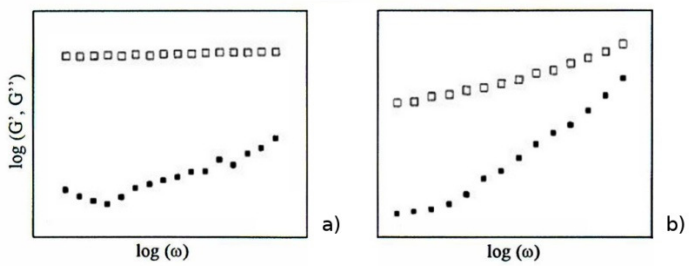


Figure 2.37. Typical dynamic viscoelastic behaviour (mechanical spectra) presented by a *"strong"* (a) and a weak (b) gel (□ *G'*, ■ *G"*).

2.3.3.2. Gel point and sol-gel transition

During gelation, a polymer undergoes a phase transition from a liquid to a gel. The sol-gel transition is not a thermodynamic transition; it is a critical point where one characteristic length scale (namely the size of the largest molecule) diverges. Thus, independently of the system studied or the mechanism involved, gelation is a critical phenomenon where the transition variable will be the connectivity of the physical or chemical bonds linking the basic structural units of the material. Therefore, rheological properties are very sensitive indicators of the critical gel point (Lopes-da-Silva and Rao, 2007).

There are many qualitative definitions of a gel point, which can be related to an instant in time (t_c – gel time) or to a specific temperature (T_{gel} – gel temperature).

From the classic Flory theory (1953), the attributes of the gel point are an infinite steady-shear viscosity and a zero equilibrium modulus at the zero frequency limit. Experimental detection

of the gel point is not always easy since the equilibrium shear modulus is technically zero at the gel point and any applied stress will eventually relax, but only at infinite time. Because continuous shearing affects gel forming, accurate information from viscosity measurement is not possible in the proximity of the gel point. Recently, focus has been placed on the SAOS technique as a method to measure the dynamic moduli during the gelation process in order to identify the gel point. Through this method, the continuous evolution of the viscoelastic properties throughout the gelation process can be monitored. Because the deformation is kept small, modification of molecular structure caused by shear is minimized, which is an important advantage over the classical methods based on the diverging viscosity (Lopes-da-Silva and Rao, 2007).

Several methods have been used to estimate either the gel temperature or the gel time. The time at which G' and G" crossover ($tan\delta$=1), at a given frequency, has been used as criteria for determining the gel-point for some biopolymer systems, such as gelatine, β-lactoglobulin, maltodextrins, and κ-carrageenan gels (Djabourov *et al.*, 1988; Stading and Hermansson, 1990; 1993). However, this parameter was found to be dependent on the oscillatory frequency (Djabourov *et al.*, 1988), and in some cases the viscoelastic moduli may be too low to give a measurable signal by a conventional rheometer. Nevertheless, it is likely that the *G'-G"* crossover time might be close to the sol/gel transition time.

Winter and Chambon (1986; Chambon and Winter, 1987) gave the first experimental demonstration of the power law behaviour of the relaxation modulus, dynamic moduli and dynamic viscosity at gel point in oscillatory frequency. Power law relaxation at gel point seems to be a general property of both chemical gelation and physical gelation involving either synthetic polymers or biopolymers, respectively. It is, however, not a universal parameter for gelation (Lopes-da-Silva and Rao, 2007).

According to the Percolation Theory, $tan\delta$ is independent of frequency at the gel point (Chambon and Winter, 1987). However, because entanglements are neglected, this approach may be of limited application for physical networks that exhibit highly ordered structures before gelation, *i.e.* when the lowest detected value of *G'* can already be greater than *G"* (*e.g.* heat set globular protein gels) (Gosal *et al.*, 2004). Some practical limitations may occur, because for many biopolymer systems gelation begins from a *sol* state characterized by a very low viscosity which is often below the resolution of the rheometer in SAOS experiments; and the lowest detected value of *G'* may already be greater than *G"* (Gosal *et al.*, 2004). Some authors (Stading and Hermansson, 1990; Richardson and Ross-Murphy, 1981) have assessed the gel point as when *G'* has increased to a value greater than the experimental noise level, although this approach is hardly rigorous.

Extrapolation of the rapidly rising values of *G'* to the time or temperature axis, were suggested as an accurate estimate of the gel point, t_c or T_{gel}, respectively (Clark, 1991). The time at which the modulus versus time plot showed a sudden slope increase on a log-log plot, called

the log discontinuity method, was used to determine the gel time (Gosal *et al.*, 2004). The temperature at which a frequency independent phase angle is obtained, is defined as T_{gel}, corresponding to a sharp rise in G' and sharp fall of phase angle upon cooling (Bohlin *et al.*, 1984; Lorén and Hermansson, 2000; Chronakis, 2001; Verbeken *et al.*, 2004).

2.3.3.3. Evaluation of structure development during biopolymer gelation

Aqueous biopolymer gels exhibit a general characteristic behaviour when the structure development is evaluated by dynamic rheological measurements (Clark and Ross-Murphy, 1987). The gradual development of the network structure is reflected by a progressive increase in storage modulus (*G'*). The beginning of the gelation process is dominated by the viscous behaviour of the system (*G"*>*G'*) and the elastic behaviour dominates the final stages of the experiment (*G'*>>*G"*). Both moduli increase as a result of the increasing density of junction zones, but *G'* rises more sharply until it intercepts and then exceeds *G"*. After an initial rapid increase, resulting from the rapid formation of junction zones into the polymer network, G' keeps increasing continuously as a result of the slower formation and rearrangement of junction zones resulting in a net decrease in the length of elastically active chains; values of G' eventually reach a pseudo plateau region. In many biopolymer gelation processes, even after aging for long periods, G' continues to increase steadily as a result of a continuous reorganization of the network in a state close to equilibrium (Ross-Murphy, 1991; Rao and Cooley, 1993).

Most gelation processes involving biopolymers can be seen as thermal effects, because temperature is basically the only thermodynamic parameter changing along the process. Depending on the molecular organization of the polymer network, temperature can have different effects on the viscoelasticity of the gel. The study of the influence of temperature on structure formation in biopolymer systems used as gelling agents in food formulations can be useful in understanding the thermal behaviour of food commodities where they are incorporated and can provide additional insights into the nature of the gelation process of these polymers (Lopes-da-Silva and Rao, 2007).

Structure development rate (SDR), defined as dG'/dt, can be measured under isothermal conditions, at different aging temperatures (maturation cure curves), and also during the gelation process promoted by decreasing the temperature of the biopolymer dispersion (non-isothermal conditions) (Rao and Cooley, 1993). Isothermal tests have the advantage of avoiding the effects of non-equilibrium thermal history of the system.

Kinetic data can also be obtained for the structure development process during cooling of biopolymer dispersions. This kind of analysis is difficult due to the combined effects of time and temperature. Usually, the data are obtained using time as an independent variable and changing the temperature and test sample for each run. Kinetic measurements close to the

gel point can provide important information about the gelation process of biopolymers, since it is under these conditions that the mechanism of gel formation can be reasonably related to its mechanical properties.

2.3.3.3.1. Evaluation of structure loss during melting/softening of biopolymer gels

Many biopolymer gels follow the general behaviour of viscoelastic amorphous polymers: a decrease in viscoelastic parameters with increasing temperature (Ferry, 1980). This behaviour is typical of polysaccharide gels where hydrogen bonding or electrostatic interactions are the only significant interactions that stabilize the polymer network. Consequently, for most biopolymer gels the rupture of junction zones stabilized by thermo-labile interactions may explain the decrease in modulus with increasing temperature, in addition to the increase in flexibility of the polymer chains (Lopes-da-Silva and Rao, 2007).

Application of thermal cycles is one of the methods that has been used to study the thermal behaviour of biopolymer gels. For thermoreversible biopolymer gels, important hysteresis effects, related to the non-equilibrium nature of the gel phase can be identified by different methods, including dynamic rheological methods (Djabourov *et al.*, 1988; Braudo *et al.*, 1991; Haque and Morris, 1993). Thermal hysteresis has its origin in stabilization of the ordered form by further aggregation: when the conformational ordering on cooling is accompanied by aggregation of the ordered structures, the melting of the aggregates usually occurs over a high temperature range. Nishinari *et al.* (1990) described the thermoreversible gel-sol transition by a zipper model approach. The junction zones were assumed to be made up of an association of molecular zippers standing for a rigid ordered molecular structure such as helices or extended molecules. The gel-sol transition for thermoreversible gels was compared to a zipper opening from both ends. In the case of heating, the gel-sol transition caused by the opening of molecular zippers will start as soon as the temperature reaches the point where the segments begin to be released from a zipper. On cooling, in contrast, the pair wise coupling cannot start as easily due to the difficulty in a long molecule to find its partner in appropriate positions for zipper construction. Hence, a state similar to super cooling may occur. This explains why T_m is far higher than T_{gel} and that the rise of G' in cooling is far steeper than the decay of G' on heating, as observed for several thermoreversible biopolymer gels (Lopes-da-Silva and Rao, 2007). Rates of structure disruption or loss rates ($-dG'/dt$) can also be measured when the gels are subjected to an increasing temperature, in order to generate kinetic data for the melting process.

2.3.3.4. Mixed Polymer Gels

The behaviour in systems of two or more polymers is important because it is different than that each would exhibit in the absence of the other, and this knowledge should help to formulate new foods as well as to develop innovative methods to generate attractive food structures and textures (Lopes-da-Silva and Rao, 2007).

Polysaccharides and proteins are the main structure and texture-forming macromolecules in food. Recently, the linear viscoelastic behaviour of pea protein / κ-carrageenan / starch mixed gel systems has been systematically studied by Nunes (2006). The aim of this work was to develop *"dairy desserts"*-like alternative products in which egg and milk proteins are fully replaced by a pea protein isolate.

The effect of temperature and time of thermal treatment in the microstructure and mechanical properties of the pea protein/κ-carrageenan/starch mixed system was investigated (Nunes *et al.*, 2004). It was concluded that temperature favours protein unfolding and biopolymers interaction, resulting in a significant increase on gel texture and viscoelastic parameters. In this work it was also determined that the mechanical properties are significantly affected by gel composition, increasing with pea protein, κ-carrageenan and starch content.

In another work (Nunes *et al.*, 2006a), the influence of cooling conditions on the gel-forming kinetics, final mechanical properties and microstructure of this complex mixture was evaluated. It was shown that slower cooling rates allowed for more complete phase separation with protein molecules structurally organised in larger aggregates, resulting in a decrease of the storage modulus and texture parameters. The texture and rheological parameters of these vegetable gels, with different formulation and thermal processing conditions, were compared to the range of variation of each variable obtained for 12 commercial desserts, analysed as target standard in a previous study (Nunes *et al.*, 2003).

Microscopic observations (Figure 2.38) showed that interaction between pea protein and κ-carrageenan results in phase separation, probably due to a depletion-flocculation mechanism, which increases local polymer concentrations (Nunes *et al.*, 2006b). The water binding effect of the swollen starch granules, promoting the concentration of pea protein and κ-carrageenan in their own phases, reinforces the gel structure. To produce well-structured gels with this selection of polysaccharides, high temperatures, above 95ºC, are necessary. Gels with higher starch content showed higher sineresis which is an undesirable parameter with great importance from a practical point of view, not being well accepted by the consumer.

The gelling ability of each biopolymer *per se* was also studied by dynamic rheological methods, in experimental conditions identical to the ones used in the ternary pea protein/κ-carrageenan/starch mixed gel systems (Nunes, 2006).

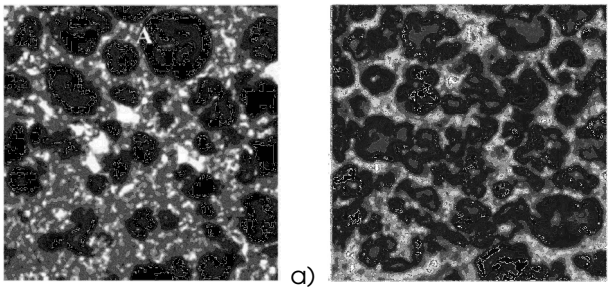


Figure 2.38. CLSM images of pea protein (P) / κ-carrageenan (C) / starch (S) gels with different composition: a) 2.0%P-0.15%C-2.5%S; b) 2.0%P-0.15%C-5%S (Nunes *et al.*, 2006b).

The phase separation between pea protein and κ-carrageenan at neutral pH, by size exclusion volume effect, was explained by the electrostatic repulsion between the two macromolecules, both negatively charged. Phase separation increases the pea protein and κ-carrageenan local concentrations, promoting the aggregation of protein molecules and the formation of junction zones in κ-carrageenan, until gelation hinders the molecule movement forming a structured weak. Confocal laser scanning microscope (CLSM) images revealed that these two biopolymers formed two separate gelled networks, in which κ-carrageenan acts as a continuous matrix dominating system's rheological behaviour. Swelling starch granules hold water, increasing the biopolymers' effective concentration, emphasizing phase separation and reinforcing the gel structure. The authors proposed a model for protein/κ-carrageenan/starch gel systems, schematized in Figure 2.39. SAOS rheological tests evidenced a synergetic effect in the ternary system, considering that it was possible to obtain a well-structured gel using much lower biopolymer concentrations than the ones required for the gelation of each individual biopolymer.

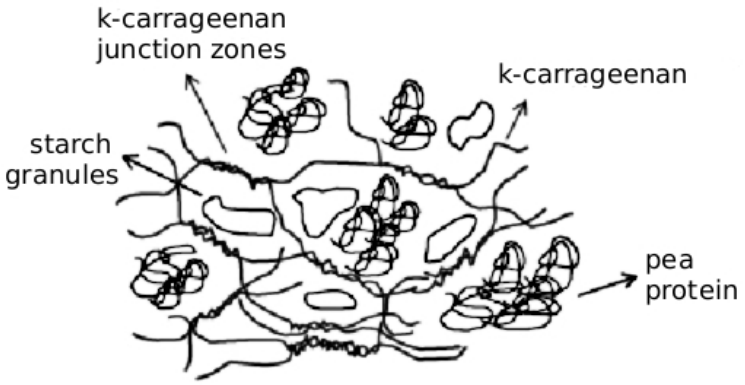
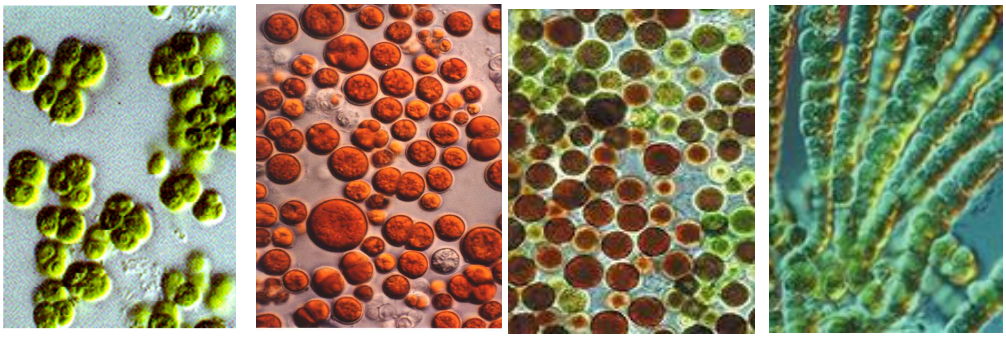


Figure 2.39. Schematic representation of pea protein/κ-carrageenan/starch ternary gel system (Nunes, 2006).

CHAPTER 3

Physicochemical characterization of microalgal biomass



3.1. INTRODUCTION

Microalgae are a potential great source of natural compounds that can be used as functional ingredients. There is a huge number of available microalgae species, and the knowledge of chemical composition is mandatory as a first step in a screening methodology, since it will help to target valuable compounds, antioxidants, PUFAs, etc., in the studied microalga.

As with any higher plant, the chemical composition of algae is not an intrinsic constant factor but varies over a wide range. Environmental factors, such as temperature, salinity, illumination, pH-value, mineral contents or CO_2 supply, affect photosynthesis and productivity of cell biomass, but also influence the pattern, pathway and activity of cellular metabolism and thus dynamic cell chemical composition, with far-reaching biotechnological implications and consequences (Hu, 2004). Most of the environmental parameters vary according to season, and the changes in ecological conditions can stimulate or inhibit the biosynthesis of several nutrients (Plaza *et al.*, 2009), hence the growing conditions can be optimized in order to maximize the production of the biomolecules of interest. Because algae must adapt rapidly to new environmental conditions to survive, they produce a great variety of secondary (biologically active) metabolites, with structures that cannot be found in other organisms (Plaza *et al.*, 2009).

The addition of microalgal biomass to food products is an interesting tool for providing nutritional supplementation with biologically active compounds (*e.g.* antioxidants), besides colouring purposes. Accordingly, the selection of microalgae species with balanced nutritional profiles is fundamental for successful novel foods development. A detailed physicochemical characterization of the microalgae in an essential stage that will allow deciding which algae are best suited for different applications and purposes.

The aim of the present chapter was to determine the proximate composition, *i.e.* fatty acids profile, pigments profile and mineral contents, of five microalgae strains: *Spirulina maxima, Chlorella vulgaris* (green and carotenogenic), *Haematococcus pluvialis* (carotenogenic), *Diacronema vlkianum* and *Isochrysis galbana*. Thermal decomposition of these microalgae was also determined by Thermogravimetric Analysis (TGA). This chemical and physical characterization will provide a basis for understanding the effect of microalgal biomass addition in colloidal food systems presented in chapters 4 (emulsions) and 5 (gels).

The selected microalgae have been extensively cultivated in LNEG (*Spirulina*, *Chlorella*, *Haematococcus*) and IPIMAR (*Diacronema* and *Isochrysis*) for several years, mainly used for animal nutrition purposes regarding their pigment (*e.g.* carotenoids and phycobiliproteins) and omega-3 fatty acids (*e.g.* EPA and DHA) accumulation, respectively.

3.2. MATERIALS AND METHODS

3.2.1. Materials

3.2.1.1. Microalgae production

Spirulina (Arthrospira) maxima (LB 2342), *Chlorella vulgaris* (INETI 58), *Haematococcus pluvialis* (INETI 33), *Diacronema vlkianum* (*IPIMAR*) and *Isochrysis galbana* (*IPIMAR*) microalgae were used in this study. These microalgae were produced in the Biomass Unit of the Department of Renewable Energies – INETI (currently Bioenergy Unit - LNEG) (Lisbon, Portugal), and in the Department of Aquaculture - IPIMAR (Lisbon, Portugal). Throughout the thesis they will be referred simply as: *Spirulina*, *Chlorella* (green or orange), *Haematococcus*, *Diacronema* and *Isochrysis*.

Microalgae were cultivated in appropriate growth media (Vonshak, 1986): *Spirulina* medium, Sorokin and Krauss medium for *Chlorella*, Bold basal modified medium for *Haematococcus* and Wallerstein and Míquel medium (3:1) for *Diacronema and Isochrysis* (Table 3.1).

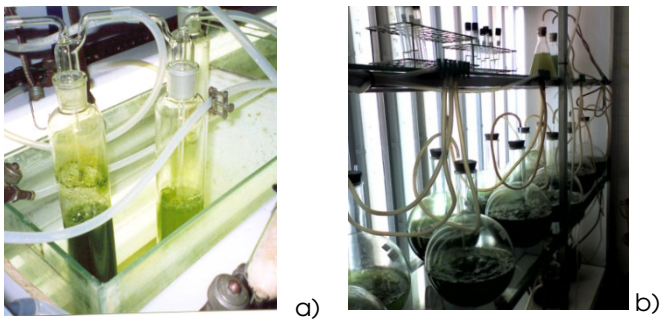
Table 3.1. Growth medium composition for *Spirulina* (S), *Chlorella* (Sorokin and Krauss), *Haematococcus* (Bold Basal Modified), *Isochrysis* and *Diacronema* (Wallerstein and Miquel).

S		**Sorokin and Krauss**		**Bold Basal Modified**		**Wallerstein and Miquel***	
			Nutrients (g/l)				
$NaHCO_3$	(16.8)	KH_2PO_4	(1.25)	KH_2PO_4	(2.73)	$NaNO_3$	(0.15)
$NaNO_3$	(2.50)	KNO_3	(1.25)	Urea	(1.41)	KNO_3	(0.10)
NaCl	(1.00)	$MgSO_4.7H_2O$	(1.00)	K_2HPO_4	(1.17)	$EDTA.Na_2.H_2O$	(0.08)
K_2SO_4	(1.00)	$NaHCO_3$	(0.50)	$MgSO_4.7H_2O$	(1.17)	$MnCl_2.4H_20$	(0.07)
$K_2HPO_4.3H_2O$	(0.50)	$CaCl_2.2H_2O$	(0.11)	EDTA	(0.781)	H_3BO_3	(0.05)
$MgSO_4.7H_2O$	(0.20)	Fe-EDTA	(0.012)	KOH	(0.484)	$NaH_2PO_4.2H_2O$	(0.03)
EDTA	(0.08)			NaCl	(0.39)	$CaCl_2$	(0.01)
$CaCl_2$	(0.04)			$CaCl_2.2H_2O$	(0.39)	Na_2PO_4	(0.01)
$FeSO_4.7H_2O$	(0.01)			H_3BO_3	(0.18)	$ZnSO_4.7H_2O$	(0.01)
				$ZnSO_4.7H_2O$	(0.14)		
			Trace nutrients (mg/l)				
H_3BO_4	(2.860)	H_3BO_3	(0.286)	$FeSO_4.7H_2O$	(78)	$CuSO_4.5H_2O$	(4.000)
$MnSO_4.4H_2O$	(2.040)	$MnSO_4.4H_2O$	(0.203)	Citric acid	(78)	$CoCl_2.6H_20$	(4.000)
$ZnSO_4.7H_2O$	(0.222)	$ZnSO_4$	(0.022)	$MnCl_2.4H_2O$	(23)	Vitamin B_1	(0.150)
$CuSO_4.5H_2O$	(0.074)	$CoSO_4.7H_2O$	(0.009)	$(CuSO_4.5H_2O$	(25)	Vitamin B_{12}	(0.008)
$CoCl_2.6H_2O$	(0.036)	$Na_2MoO_4.2H_2O$	(0.006)	$NH_4)_6Mo_7O_{24}.4H_2O$	(11)	Na_2MoO_4	(0.003)
$Na_2MoO_4.2H_2O$	(0.026)	$CuSO_4$	(0.005)	$CoCl_2.6H_2O$	(8)		
$Na_2WO_4.2H_2O$	(0.024)						
NH_4VO_3	(0.023)						
$Cr(NO_3)_3.9H_2O$	(0.022)						
$Ni(NO_3)_2$	(0.020)						
$TiCl_3$	(0.018)						

* Prepared with filtered seawater.

Microalgae were first grown in 1 litre capacity airlift bioreactors (Figure 3.1), with bubbling air, at low light conditions (150 $\mu E.m^{-2}.s^{-1}$), and the optimal temperature for each microalga: *Spirulina* 34ºC, *Chlorella* and *Haematococcus* 25ºC, *Diacronema* and *Isochrysis* 18ºC (Reis, 2001; Gouveia and Empis, 2003; Donato *et al.*, 2003; Bandarra *et al.*, 2003). Afterwards, they

were transferred to higher capacity polyethylene bags or into outdoors raceway ponds (Figure 3.2). Growth was monitored by optical density measurement at 540 nm (spectrophotometer Hitachi 2000, Japan) and dry weight determination (GF/C glass fibre filter, 1.2 μm pore size).



a) b)

Figure 3.1. *Chlorella vulgaris* a) and *Diacronema vlkianum* b) growing in airlift bioreactors.

Green microalgae (*Spirulina*, *Chlorella* (green), *Diacronema*, *Isochrysis*) were harvested during stationary phase. *Chlorella vulgaris* (orange) and *Haematococcus pluvialis* were further submitted to a carotenogenesis process, through nitrogen starvation and NaCl addition (30% *Chlorella*, 2% *Haematococcus*), at high luminosity favoured by culture dilution (1000 $\mu E.m^{-2}.s^{-1}$) (Figure 3.2b) as it was previously reported by Gouveia *et al.* (1996) and Gouveia and Empis (2003).

Microalgal biomass was harvested without flocculation by simply removing agitation, followed by centrifugation and then frozen and freeze-dried (Figure 3.2).

a) b) c)

Figure 3.2. Polyethylene bags used for *Diacronema vlkianum* growth (a) and *Chlorella vulgaris* carotenogenesis (b); Outdoor raceway ponds for *Chlorella* growth (c).

3.2.2. Methods

3.2.2.1. Moisture

Moisture was determined by drying the sample in an oven at 105ºC for 3-4 hours (until constant weight loss) (AOAC, 2006). The average moisture values were further used in the calculation of the percentages of chemical parameters, expressed in total dry matter.

3.2.2.2. Mineral content

Total ash was determined by incineration at 550ºC in a muffle (AOAC, 2006), after controlled (slow) carbonization in a heating plate.
Phosphorus, potassium, calcium, magnesium, sodium, copper, manganese, zinc and iron contents were determined in an external laboratory (DQAA, ISA, Technical University of Lisbon). The mineral elements were digested by dry via (muffle) followed by acid mineralization and determined by atomic absorption spectrometry except for phosphorus which was determined by molecular absorption spectrometry (reaction with ammonium vanadomolibdate). Total nitrogen was determined by Kjeldhal nitrogen as referred below (section 3.2.2.3).

3.2.2.3. Crude Protein

Total nitrogen was determined by Kjeldhal method after acid digestion, ammonium distillation under steam current, and titration with HCl 0.1N (AOAC, 2006). Crude protein was calculated by multiplying total nitrogen by a conversion factor of 6.25.

3.2.2.4. Lipids

3.2.2.4.1. Total Lipids

Fat matter was extracted by Soxhlet method with petroleum ether for 6 h, after acid digestion with HCl 4N. The lipid extract was concentrated in a rotary evaporator, dried in an oven and weighted (AOAC, 2006).

3.2.2.4.2. Fatty acids profile

Microalgae fatty acids were transesterified in methyl esters by acid catalysis according to the method of Lepage and Roy (1986) modified by Cohen *et al.* (1988). The fatty acid methyl esters preparation was carried out using 300 mg microalgae and 5 ml acetylchloride:methanol solution (5% v/v). The esterification was done at 80 ºC during 1 hour. After cooling, 1 ml of water and 2 ml of *n*-heptane were added to the mixture, which was stirred and centrifuged at 2000 G for 5 min. The organic phase was collected, filtered and dried with anhydrous sodium sulphate. The solvent was evaporated under nitrogen and the methyl esters weighted and solubilized in *n*-heptane (final solution concentration ≈ 10 mg/100 µl). The analysis was performed in a gas chromatograph Varian CP-3800 (USA) equipped with an auto-sampler (CP-8400) and fitted with a flame ionisation detector. The separation was

carried out with Helium as carrier gas (velocity 1 ml/min) in a capillary column DB-WAX of polyethyleneglycol with 30 m length, 0.25 mm internal diameter and 0.25 μm film thickness (J&W Scient., Agilent Tech., USA). Injector (split ratio 1:100) and detector temperatures were kept constant at 250 ºC. The oven temperature program started at 180ºC for 5 min, increased at 4 ºC/min until 220 ºC, and kept constant at this temperature for 25 min.

Fatty acid methyl esters were identified by comparison with the retention time of individual standards (Sigma, St. Louis, USA) (Figure. 3.3.).

Fatty acid quantification was done by using the corrective factor (*F*) proposed by Weihrauch *et al.* (1977) which relates the amount of fatty acids with the total lipid content (*TL*) of the sample.

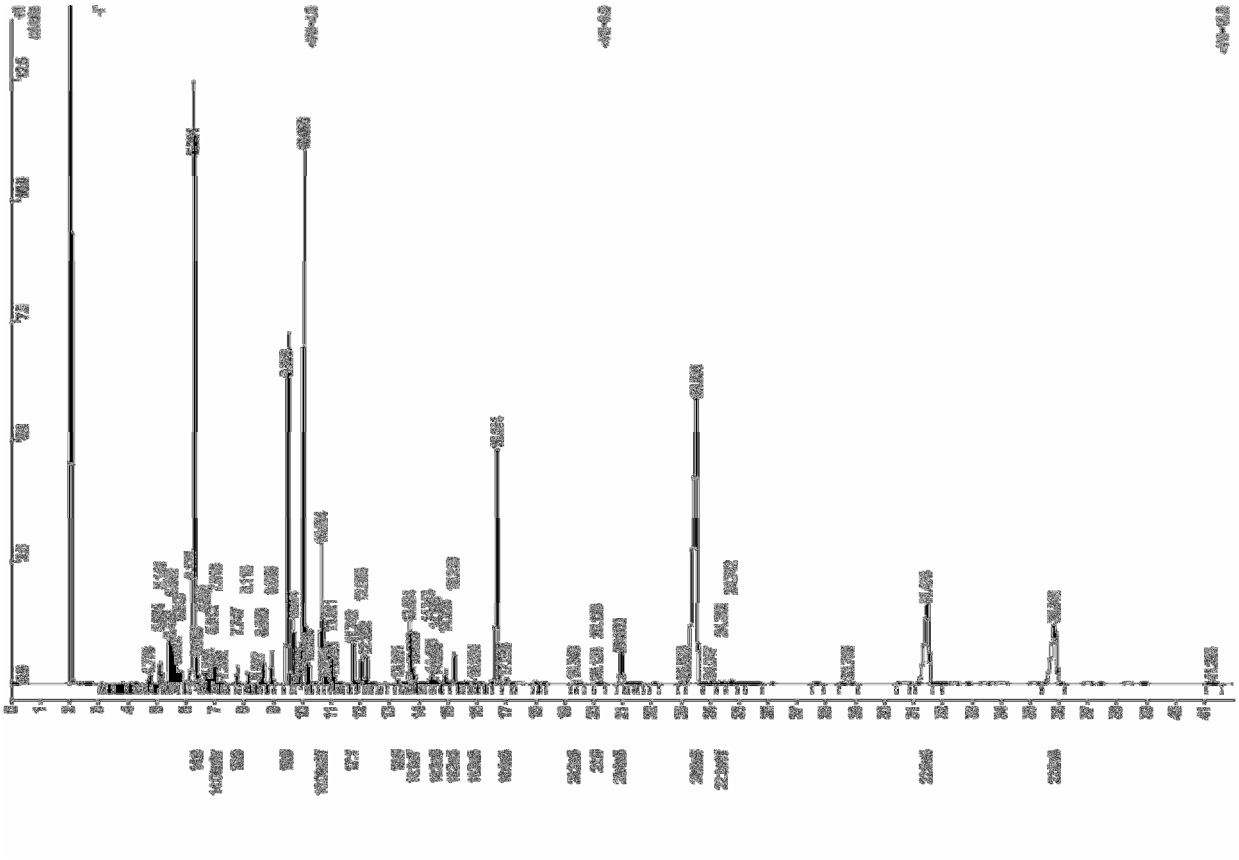


Figure 3.3. Example of a chromatogram obtained for *Diacronema vlkianum* fatty acids methyl esters, determined by GC-FID.

3.2.2.5. Carbohydrates

Total Carbohydrates were estimated by the Anthrone colorimetric method (Dreywood, 1946; Morris, 1948). Carbohydrate materials react with a solution of anthrone in concentrated sulphuric acid, yielding a permanent blue-green colour (Figure 3.4). This method determines both reducing and non-reducing sugars due to the presence of strongly oxidizing sulphuric acid. The specificity of anthrone for carbohydrates is very high and positive reactions with

several mono-, di-, and polysaccharides have been reported (Dreywood, 1946; Morris, 1948). This method has also been used for total or available carbohydrate estimation in microalgae samples (Umamaheswari and Venkateswarlu, 2003; Rebolloso-Fuentes *et al.*, 2001; Tokusoglu and Unal, 2003).

Microalgae samples (1 mg) were suspended in 1 ml distilled water, mixed with 5 ml anthrone reagent (0.2 g/l in 70% H_2SO_4) and then heated in a water bath at 100ºC for 10 min. The solutions were then allowed to cool and its absorbance was measured at 620 nm in a spectrophotometer Ultrospec 4000 (Amersham Pharmacia Biotech, USA). A blank consisting of water (1 ml) and anthrone reagent (5 ml) was included in each batch of analyses in order to eliminate colour effects due to the anthrone.

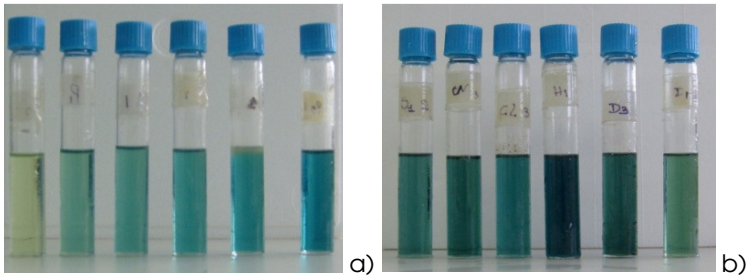


Figure 3.4. Anthrone-Sugar blue-green complex. (a) glucose standards: 0, 40, 80, 120, 160, 200 µg/ml (b) *Spirulina*, *Chlorella* green, *Chlorella* orange, *Haematococcus*, *Diacronema* and *Isochrysis*.

A calibration curve was prepared, using a series of glucose standards of known concentration (0, 40, 80, 120, 160, 200 µg/ml), by reacting 1 ml glucose solution with 5 ml anthrone reagent (incubation at 100ºC/10min) (Figure 3.5). Accordingly, results of total carbohydrates in the microalgae samples are expressed in terms of glucose equivalents.

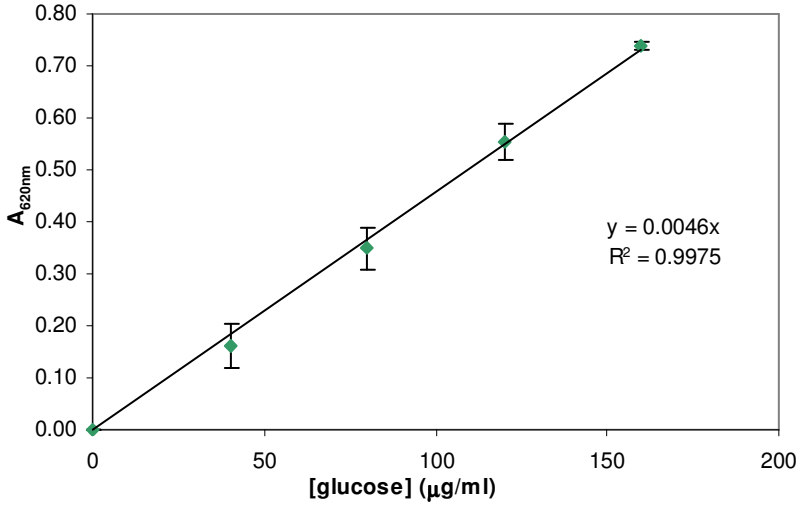


Figure 3.5. Calibration curve applied to the anthrone colorimetric method, using glucose standard solutions.

3.2.2.6. Pigments

3.2.2.6.1. Carotenoids and Chlorophylls

Microalgal carotenoids and chlorophylls were extracted with acetone after breaking the cell walls through mechanical and thermal shocks; about 5 mg of microalgal biomass with glass beads were placed alternately in an ice bath and in a vortex with portions of 5 ml acetone. The extracts were centrifuged at 3500 rpm for 5 min and collected. The procedure was repeated 3-4 times, until both precipitate residue and supernatant became colourless.
Absorption spectra of extracts, from 400 nm to 700 nm, were measured in a spectrophotometer Ultrospec 4000 (Amersham Pharmacia Biotech, USA) (Figure 3.6). Total pigments quantification was made using extinction coefficients ($E^{1\%}_{1cm}$) of 2150 for microalgal pigments, at their absorption maximum in acetone (Gouveia and Empis, 2003), according to equation 3.1:

$$Total\_Pigments(\%) = \frac{A \times V \times f}{E^{1\%}_{1cm} \times m} \qquad (3.1)$$

where *A* is the absorbance (at the wavelength of maximum absorption), *V* is the total volume of the pigment extract (ml), *f* is the dilution factor, $E^{1\%}_{1cm}$ is the extinction coefficient and *m* the weight of the sample (g). The extinction coefficient used was based on an average of the $E^{1\%}_{1cm}$ of the carotenoids mainly found in these microalgae, according to Gouveia and Empis (2003).

Figure 3.6. *Haematococcus pluvialis* and *Chlorella vulgaris* (orange and green) acetone extracts used for total pigment spectrophotometric determination.

The separation of pigments was carried out by thin-layer chromatography (TLC) using plates of silica gel-alumina (Merck) and an eluent mixture of petroleum ether : acetone (72 : 28 v/v). The extracts were also analysed by reversed-phase high performance liquid chromatography (HPLC) on a Hewlett Packard series 1100 (USA) HPLC equipment, with a μ-Boundpak C18 column (250/4.6 mm) and a UV/VIS detector Waters 481 (λ = 460 nm), with methanol :

acetonitrile (75:25 v/v) as eluent. The pigments were eluted over 30 min with a flow rate of 1 ml/min (Figure 3.7.). Peaks were compared with retention times of standard carotenoid solutions (in acetone): astaxanthin (Sigma, 98%), canthaxanthin (Roche, 10%), lutein/zeaxanthin (FloraGlo, Kemin, 5%), echinenone (Roche, 98%) and *trans*-β-carotene (Sigma, 95%).

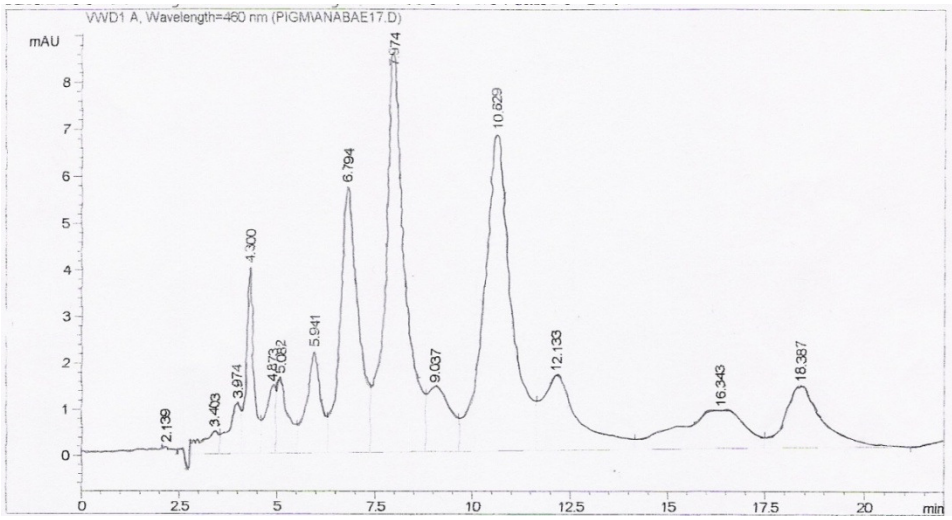


Figure 3.7. Example of a chromatogram obtained for *Haematococcus pluvialis* carotenoid profile determined by RP-HPLC.

3.2.2.6.2. Phycocyanin

Phycocyanin is the major pigment in *Spirulina maxima* and due to its hydrophilic nature it was determined by an alternative method, according to Reis (1998; 2001). About 10 mg of *Spirulina maxima* biomass were digested in the dark in 5 ml of pH 7 phosphate buffer 0.1 M. The extraction occurred in two phases: first at 37ºC with magnetic stirring (100 rpm) during 4 h (to break the cell walls), then 12 h at 5ºC without agitation. Samples were centrifuged at 12000 rpm for 10 min and the supernatant absorbance at 620 nm and 650 nm were measured using a spectrophotometer Ultrospec 4000 (Amersham Pharmacia Biotech, USA) (Figure 3.8).

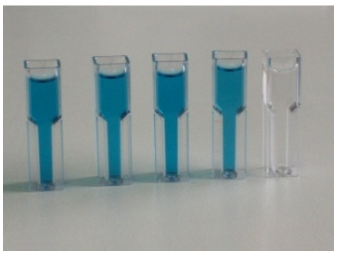


Figure 3.8. Phycocyanin extracts from *Spirulina maxima*.

C-Phycocyanin (*C*- stands for Cyanophyceae) and allophycocyanin quantification was made, according to equations 3.2 and 3.3:

$$Phycocyanin(\%) = \frac{A_{620} \times V \times f}{73 \times m} \quad (3.2)$$

$$Allophycocyanin(\%) = \frac{A_{650} \times V \times f}{58 \times m} \quad (3.3)$$

where A_λ is the absorbance at the given wavelength λ, *V* is the total volume of the pigment extract (ml), *f* is the dilution factor, 73 and 58 are the $E^{1\%}_{1cm}$ of phycocyanin and allophycocyanin, respectively (Boussiba and Richmond, 1979), and *m* the weight of the sample (g).

3.2.2.7. Thermogravimetric Analysis (TGA)

Thermal Analysis (TA) implies the analysis of a change in a sample property, which is related to an imposed temperature alteration. More specifically, in a thermogravimetric analysis (TGA) the change in the sample mass is analysed while the sample is subjected to a temperature alteration (Hemminger and Sarge, 1998).

Microalgal biomass was examined in terms of TGA, using a TGA-Q50 (TA Instruments, UK) equipment, under nitrogen (N_2) flux (inert atmosphere) (Figure 3.9). Samples of 4-5 mg were heated from 35ºC to 900ºC at 20ºC/min rate.

Thermograms were analysed in terms of onset temperature (T_{onset}, ºC), temperature for maximum degradation rate (T_{max}, ºC) and residue at 900ºC, using *Universal Analysis 2000* version 4.2E software (TA Instruments, UK).

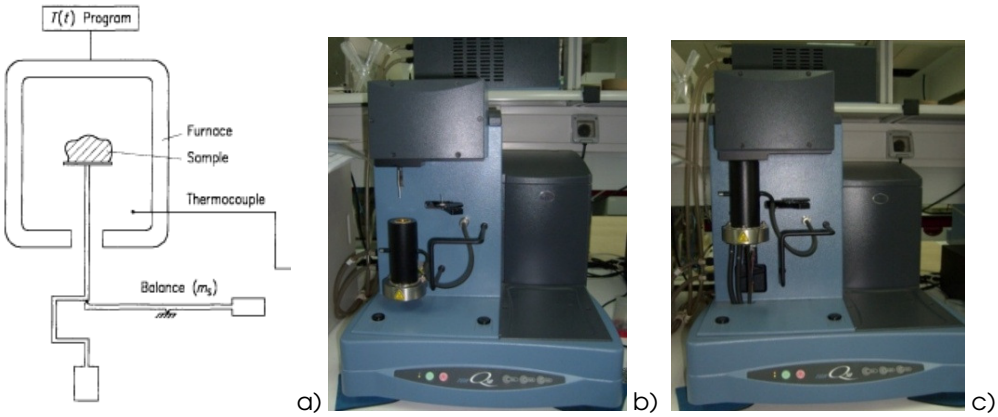


Figure 3.9. Schematic representation of a Thermogravimetric analysis device (a). TGA-Q50 equipment with furnace down (b) and up (c).

3.2.2.8. Statistical analysis

The determinations were repeated at least three times and results are presented as average ± standard deviation. Percentages are expressed as weight/weight in dry matter basis, except when indicated otherwise. The results were evaluated in terms of ANOVA and Factor Analysis, using STATISTICA Software (version 8.0) from StatSoft Inc. (2007), as described below.

3.2.2.8.1. Analysis of Variance - ANOVA

Data were submitted to a variance analysis (ANOVA), in order to compare and evaluate the existence of significant differences between microalgae physicochemical parameters.
Means were compared by *Scheffé Test - Post Hoc Comparisons* to test the hypothesis (3.4):

$$H_0 = \mu_1 = \mu_2 = \ldots \mu_k \qquad \textit{versus} \qquad H_1: \exists\ i,j \text{ such as } \mu_i \neq \mu_j, \qquad (3.4)$$

being μ_i the average population value of the dependent variable (physicochemical parameter) with i-th factor level (microalgae type as independent variable). Statistical significance was tested at 95% probability level (p<0.05).

3.2.2.8.2. Factor Analysis – Principal Components Analysis (PCA)

This statistical method enables to reduce the dimensionality of a data set by transforming n original variables (x_1, x_2, ..., x_n) into p principal component or axis (y_1, y_2, ..., y_p), with $p<n$. Each independent principal component is a linear combination of the original variables that are more or less correlated. The importance of each principal component or factor is indicated by its associated variance, which decreases from y_1 to y_p, expressed as a percentage of the sum of the variances from all components (Kozak and Scaman, 2008). Usually, the first two or three components explain almost all the data variance, so this method can be used to identify combinations of variables that have the largest contribution to variation in the data set. (Powers, 1984). It also enables pattern recognition in a set of multivariate data, evidencing their similarities and differences (Kozak and Scaman, 2008).
This statistical method was applied to the mean values of the analysed parameters of the six microalgae under study, in order to determine which variables better define and differentiate them.

3.3. RESULTS AND DISCUSSION

3.3.1. Proximate composition

Results obtained from the physicochemical characterization of microalgal biomass are collected in Table 3.2. Freeze dried microalgal biomass presented low moisture values (4-11%), and the results of the chemical parameters are expressed as weight in weight percentage, per dry matter (DM).

Table 3.2. Proximate composition of microalgal biomass.

	Spirulina maxima	*Chlorella vulgaris* (green)	*Chlorella vulgaris* (orange)	*Haematococcus pluvialis*	*Diacronema vlkianum*	*Isochrysis galbana*
Dry matter (%)	88.7 ± 0.3 ^{a}	88.8 ± 0.3 ^{a}	94.7 ± 0.3 ^{b}	95.8 ± 0.4 ^{c}	91.3 ± 0.01 ^{d}	96.0 ± 0. 1 ^{c}
Total Ash (% DM)	30.9 ± 1.5 ^{a}	24.2 ± 0.6 ^{b}	34.8 ± 1.8 ^{c}	8.9 ± 0.2 ^{d}	18.4 ± 0.8 ^{bc}	14.5 ± 0.1 ^{cd}
Crude Protein (% DM)	44.9 ± 1.8 ^{a}	38.0 ± 1.5 ^{b}	12.3 ± 0.1 ^{c}	10.2 ± 0.2 ^{d}	38.4 ± 0.2 ^{b}	39.6 ± 0.3 ^{b}
Crude Fat (% DM)	3.6 ± 0.1 ^{a}	5.1 ± 0.01 ^{b}	27.6 ± 1.4 ^{c}	40.7 ± 1.2 ^{d}	17.9 ± 0.5 ^{d}	23.9 ± 0.02 ^{e}
Carbohydrates (% DM)	16.6 ± 1.7 ^{a}	19.9 ± 2.8 ^{a}	22.7 ± 2.5 ^{a}	33.6 ± 2.4 ^{b}	15.1 ± 1.4 ^{a}	13.3 ± 1.1 ^{a}

* Different letters in the same row correspond to significant differences (p<0.05)

Spirulina presents a high mineral content (31%), and is rich in sodium and potassium (Table 3.3.), in accordance with results from other authors (Campanella *et al.*, 1999). *Chlorella vulgaris* green has a total mineral content of 24%, rich in calcium (4.7%) and also in manganese and iron. After carotenogenesis, total minerals increase about 10%, up to 35%, which should be related to the addition of NaCl for inducing carotenoid accumulation. This also occurs with carotenogenic *Haematococcus* with 5.9% sodium, despite its low mineral content (8.9%). This alga has a good profile in terms of microelements such as iron (823 mg/kg), copper (344 mg/kg) and zinc (232 mg/kg). *Diacronema* and *Isochrysis* have intermediate total ash contents (15-18%) rich in phosphorus and manganese.

Table 3.3. Mineral composition of microalgal biomass.

	Spirulina maxima	*Chlorella vulgaris* (green)	*Chlorella vulgaris* (orange)	*Haematococcus pluvialis*	*Diacronema vlkianum*	*Isochrysis galbana*
N (%)	7.19	6.08	2.02	1.64	6.15	6.33
P (%)	1.29	1.53	1.01	1.31	1.49	2.65
K (%)	2.58	0.98	0.45	0.97	0.72	1.19
Ca (%)	0.91	4.73	0.80	0.25	0.91	0.56
Mg (%)	0.35	1.46	0.18	0.22	0.53	0.96
Na (%)	8.53	0.98	4.84	5.87	1.03	1.60
Cu (mg/kg)	1.1	2.2	1.0	344.0	1.9	8.6
Mn (mg/kg)	24.6	471.5	11.7	111.9	2548.7	801.0
Zn (mg/kg)	3.5	17.5	17.8	232.2	91.3	19.2
Fe (mg/kg)	93.6	166.3	17.2	822.7	208.1	14.6

Green microalgae have high protein contents, around 40%, with the highest value for *Spirulina* (44.9%), while carotenogenic microalgae showed low protein values (10-12%) (Table 3.1). Estimations of crude protein include other nitrogenous constituents, *e.g.* nucleic acids, amines, glucosamides, and cell wall materials, which in general is expected to account for about 10% of the total nitrogen found in microalgae (Becker, 2004; 2007). According to Becker (2007), the amino acid pattern of almost every algae compares favourably with that of the reference (WHO/FAO) and other food proteins (*e.g.* egg, soybean).

Spirulina and *Chlorella* green, presented relatively low fat values (4% and 5%, respectively) while *Diacronema* and *Isochrysis* have significantly high lipid contents (18-24%) with an interesting composition in terms of PUFA-ω3. Carotenogenic algae, showed very high lipid contents (28% and 41%, for *Chlorella* orange and *Haematococcus*, respectively), in opposition to the low protein content referred above. This is due to the fact that the biosynthesis and accumulation of lipids is enhanced in nitrogen-limited cultures of microalgae (Hu, 2004). Since there is no nitrogen available for cellular division, carbon is accumulated in the form of lipids within the cells.

Triacylglycerols are the most common storage lipids in microalgae constituting up to 80% of the total lipid fraction. Free fatty acids, phospholipids, glycolipids, sterols and tocopherols are present in smaller amounts (Becker, 2004).

Microalgae lipid fraction was analysed in terms of fatty acid profile, the proportion of total saturated (SFA), monounsaturated (MFA), and polyunsaturated (PUFA) –ω_3 and -ω_6 fatty acids are presented in Figure 3.10 and the main fatty acids are identified in Table 3.4.

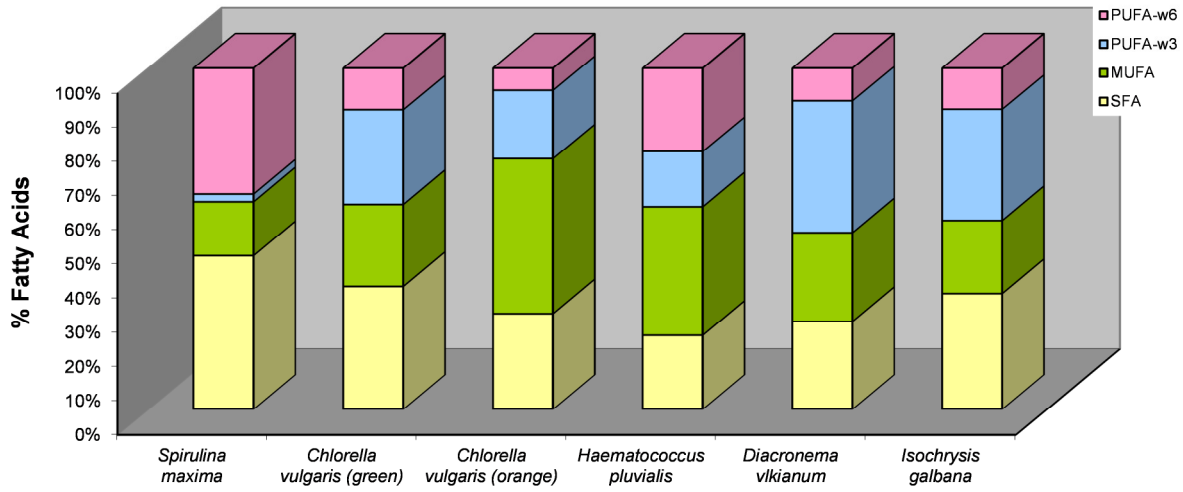


Figure 3.10. Proportion of total saturated (SFA), monounsaturated (MFA), and polyunsaturated (PUFA) ω_3 and ω_6 fatty acids in microalgae lipids.

Spirulina contains 40% SFA, mainly palmitic acid (16:0), and 35% PUFA, with a much larger proportion of ω_6 in relation to ω_3 acids (16:1). Besides linoleic acid (18:2ω_6), *Spirulina* is rich (452 mg/100g) in γ-linolenic acid (GLA 18:3ω_6). GLA has been associated with several beneficial

health effects, such as LDL reduction, precursor of C_{20} eicosanoids (prostaglandins, leukotriens and thromboxanes), anti-inflammatory effects, transepidermal water loss regulation (helps healing eczema), apoptosis of cancer cells stimulation, reduction of pain and inflammation in rheumatoid arthritis, among others (Van Hoorn *et al.*, 2008). *Spirulina* is a well-known source of GLA, since in cyanobacteria this fatty acids plays an analogue role to the α-linolenic acid (ALA 18:3ω_3) in algae and superior plants (Reis, 2001).

Chlorella green has 31% SFA (mainly palmitic acid), 21% MUFA (mainly oleic acid 18:1), and 35% PUFA, with higher proportion of ω_3 acids. In fact, except for *Spirulina* and *Haematococcus*, all the other microalgae studied present a ω_3/ω_6 ratio higher than 2, as can be observed in Table 3.4. In fact, in cyanobacteria (*e.g. Spirulina*) the unsaturated double bindings are preferably in ω_6 position while in Chlorophyceae are mainly in ω_3 position.

When submitted to the carotenogenesis process *Chlorella* fatty acids profile suffers large variations, particularly through the accumulation of oleic acid (10.0 g/100g), resulting in a higher proportion of MUFA (44%). There is also a significant increase in palmitic (5.6 g/100g), α-linolenic (3.7 g/100g) and linoleic (1.5 g/100g) acids, which is also related to the increase in total lipids along carotenogenesis (from 5 to 28%). The levels of EPA+DHA are very low, either in *Chlorella* green or orange, and absent in *Spirulina*.

Table 3.4. Main fatty acids present in the microalgae biomass (mg/100 g).

	Spirulina Maxima	*Chlorella vulgaris* (green)	*Chlorella vulgaris* (orange)	*Haematococcus pluvialis*	*Diacronema vlkianum*	*Isochrysis galbana*
14:0	9 ± 0.2	124 ± 13	210 ± 1	154 ± 1	2081 ± 38	3272 ± 3
16:0	1078 ± 26	1016 ± 20	5606 ± 1	5977 ± 12	1413 ± 10	2711 ± 6
18:0	32 ± 1	25 ± 1	406 ± 3	603 ± 10	14 ± 1	50 ± 38
Other SFA	26 ± 2	88 ± 12	408 ± 61	988 ± 13	78 ± 5	648 ± 51
Σ SFA	1146 ± 24	1254 ± 45	6630 ± 61	7722 ± 1	3586 ± 23	6681 ± 60
16:1	189 ± 5	78 ± 2	38 ± 1	102 ± 2	2425 ± 41	3275 ± 3
18:1	115 ± 4	449 ± 3	9965 ± 133	11125 ± 51	253 ± 5	584 ± 1
Other MUFA	10 ± 1	110 ± 21	329 ± 6	1065 ± 3	193 ± 82	354 ± 18
Σ MUFA	402 ± 10	836 ± 23	10733 ± 141	13387 ± 46	3620 ± 128	4213 ± 21
16:4 ω_3	4 ± 0.2	165 ± 1	688 ± 2	1160 ± 6	112 ± 20	-
18:3 ω_3 (ALA)	40 ± 0.1	661 ± 12	3665 ± 1	3981 ± *2*	14 ± 1	421 ± 5
18:4 ω_3	2 ± 0.6	-	-	-	1121	-
20:5 ω_3 (EPA)	-	19 ± 1	39 ± 1	579 ± 6	3212 ± 57	4875 ± 108
22:6 ω_3 (DHA)	-	16 ± 1	80 ± 1	-	836 ± 41	1156 ± 40
Other PUFA-ω_3	11 ± 6	111 ± 1	308 ± 3	52 ± 10	113 ± 47	10 ± 1
Σ PUFA-ω_3	58 ± 35	971 ± 14	4781 ± 2	5770 ± 14	5407 ± 146	6461 ± 153
18:2 ω_6	481 ± 31	292 ± 16	1502 ± 1	7844 ± 20	49 ± 5	123 ± 1
18:3 ω_6 (GLA)	452 ± 28	112 ± 11	23 ± 1	472 ± 8	112 ± 3	-
20:4 ω_6	-	-	12 ± 0.2	292 ± 1	191 ± 1	162 ± 3
22:5 ω_6	-	4 ± 1	-	-	976 ± 33	-
Other PUFA-ω_6	12 ± 0.2	20 ± 1	10 ± 6	159 ± 20	15 ± 23	2116 ± 75
Σ PUFA-ω_6	945 ± 59	428 ± 28	1547 ± 7	8767 ± 230	1343 ± 21	2401 ± 76
ω_3/ω_6	0.1	2.3	3.1	0.7	4.1	3.6

Haematococcus also presents large amounts of oleic acid (11.1 g/100g). It has been reported by Zhekisheva *et al.* (2002) that under nitrogen depletion conditions (carotenogenesis), *Haematococcus* produced, for each picogram of astaxanthin, five picograms of fatty acids, particularly oleic acid-rich triacylglycerols, suggesting that these two processes are interrelated and they enable oil globules to maintain the high content of astaxanthin esters. As for *Chlorella* orange, *Haematococcus* also has high contents of palmitic and α-linolenic acids (6.0 and 4.0 g/100g, respectively). Although it presents a considerable amount of EPA (0.6 g/100g), the proportion of ω_6 fatty acids in this alga overcomes the ω_3 fraction (24 *vs.* 16%), which is mainly due to its high linolenic acid content (7.8 g/100g).

Diacronema and *Isochrysis* Haptophyte microalgae have 40% PUFA with a favourable ω_3/ω_6 ratio around 4, rich in EPA and DHA. These results are in accordance with findings of other authors (Bandarra *et al.*, 2003; Fidalgo *et* al., 1998; Donato *et al.*, 2003; Ponis *et al.*, 2006). *Diacronema* has 3.2 g EPA and 0.8 g DHA per 100 g microalgal biomass, while *Isochrysis* has even higher values, with 4.9 g EPA and 1.2 g DHA per 100 g microalgal biomass. The main PUFA-ω_6 in *Diacronema* is 22:5ω_6 (DPAω_6), representing 6.5% of total fatty acids (1.0 g/100g). Recently, Nauroth *et al.* (2010) proved the potent anti-inflammatory activity of DPAω_6 from algal source. DPAω_6 can be converted into oxylipins, resolvin-like molecules, with potent anti-inflammatory activity which could contribute to the reduction of inflammatory response in vivo. Was also referred the synergistic effect of DPAω_6 with DHA, suggesting that algal biomass may be a novel anti-inflammatory supplement.

For these microalgae the main SFA is myristic acid (14:0) and the main MUFA is palmitoleic acid (16:1). Considering the health benefits associated to the consumption of EPA and DHA, particularly in the prevention of cardiovascular diseases and cognitive development (*e.g.* Thies *et al.*, 2003; Ghys *et al.*, 2002), these microalgae have an enormous potential for application in healthy food products development. Typical Western diets provide dramatically skewed ω_3/ω_6 ratios toward omega-6, promoting the pathogenesis of many diseases (Simopoulos, 2002). This is mainly due to the disproportionate consumption of ω_6-rich vegetable oils (*e.g.* sunflower, peanut, corn) in detriment to seafood, nuts and other sources of ω_3.

The unsaturation index (*UI*) for each fatty acid within the total lipid was calculated by multiplying the number of double bonds in each fatty acid by the percentage of that fatty acid and dividing it by 100 (Huynh and Kitts, 2009). The unsaturation index for the total lipid, generated by summing the individual fatty acid unsaturation indexes, was compared to the unsaturation index provided by EPA+DHA (Figure 3.11). Using this measure, either a small percent number of a highly unsaturated fatty acid or a large number of a monounsaturated will produce an equal UI number (Huynh and Kitts, 2009). It is clear from Figure 3.11 that *Diacronema* and *Isochrysis* present a significantly higher UI (2.0-2.4) than the other

microalgae. In fact, the contribution of EPA+DHA alone (1.4) to the UI is larger than the total UI of the other microalgae (1.0-1.4), with the highest values for *Haematococcus.*

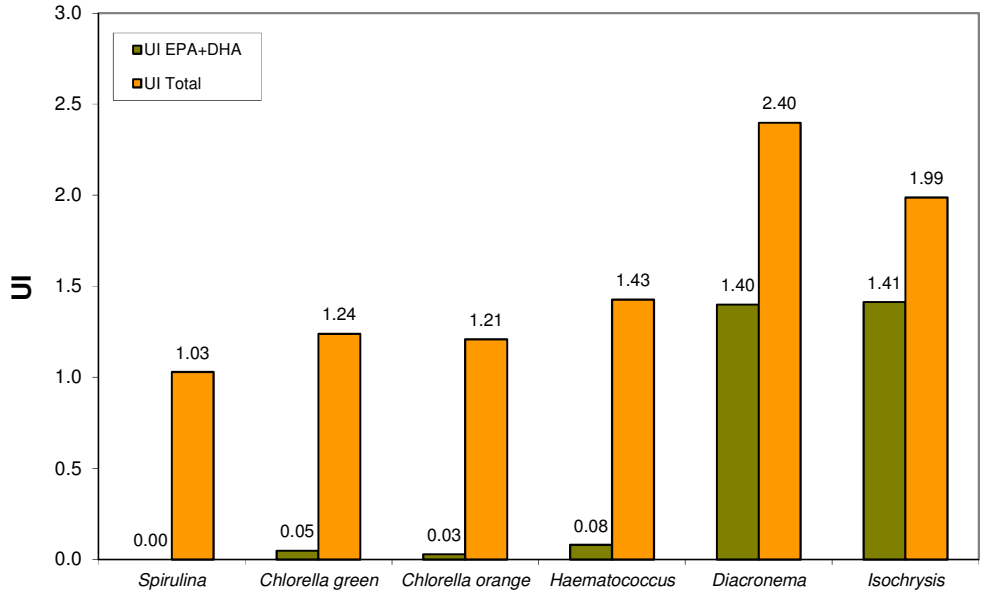


Figure 3.11. Comparative unsaturation indexes (UI) of total and EPA+DHA fatty acids of different microalgae.

Finally, looking back at Table 3.2 we can observe that the carbohydrates in the microalgae studied range between 13-15% for *Diacronema* and *Isochrysis*, with slightly higher values for *Spirulina* (17%) and *Chlorella,* with a value of 20% which increased up to 23% upon carotenogenesis. The highest carbohydrate content was found for *Haematococcus* (34%). The increase in carbohydrates in carotenogenic algae is well recognised, since under nitrogen limiting conditions and high light intensities algae tend to accumulate sugars (Hu, 2004). There were many experimental difficulties in determining the fibre fraction, as well as the profile of sugars and polysaccharides present in the algae, so the results are not shown. Carbohydrates of algae can be found in the form of starch, cellulose, and other polysaccharides, as storage products or cell wall components. While eukaryotic green algae, such as *Chlorella* and *Haematococcus*, accumulate starch and have rigid cell walls (cellulose and hemicellulose), *Spirulina* uses glycogen as storage product and has thin and fragile peptidoglycan cell walls. For *Diacronema* e *Isochrysis* haptophytes, the storage product is chrysolaminarin, a β-1,3-linked glucan (Becker, 2004).

3.3.2. Pigment analysis

Microalgae acetone extracts UV-VIS absorption spectra from 400 to 700 nm are presented in Figure 3.1.2. Green algae typically show an absorption maximum at 430-432 nm which

corresponds to lutein (and/or its isomer zeaxanthin), which should be the main carotenoid present in these algae. A distinctive peak at 663 nm is also observable in all the green algae analysed, corresponding to chlorophylls.

Carotenogenic microalgae present absorption maximum at higher wavelengths (460-480 nm) and no prominent peaks at 660 nm, indicating an accumulation of secondary carotenoids accompanied by a decrease in the chlorophyll content of the cells. In the case of *Chlorella* orange, the absorption maxima is situated at 460 nm corresponding to canthaxanthin, while for *Haematococcus* the maximum is observed at 474 nm, corresponding to astaxanthin. Unlike primary carotenoids (*e.g.* β-carotene, lutein, violaxanthin) which are distributed within the chloroplasts of green algae (tilacoid membranes), secondary carotenoids, such as canthaxanthin and astaxanthin, are accumulated in lipid globules outside the chloroplast plastids.

Carotenogenesis process occurs in response to nutrient starvation or other stressed conditions *i.e.* environmental conditions that result in a metabolic imbalance which requires biochemical and metabolic adjustments before a new steady state of growth can be established (Vonshak and Torzillo, 2004). In response to high light intensity, chlorophyll *a* and other pigments directly involved in photosynthesis decrease while the secondary carotenoids, which serve as photoprotective agents, increase (Hu, 2004). This process is similar in green microalgae, but the relative proportion of each pigment and ester, as well as the number of different mono- and di-esters, varies (Orosa *et al.*, 2000). The esterified form of secondary pigments seems to be the optimal and the most commonly found accumulation form in these microalgae (Orosa *et al.*, 2000; Gouveia *et al.*, 1996).

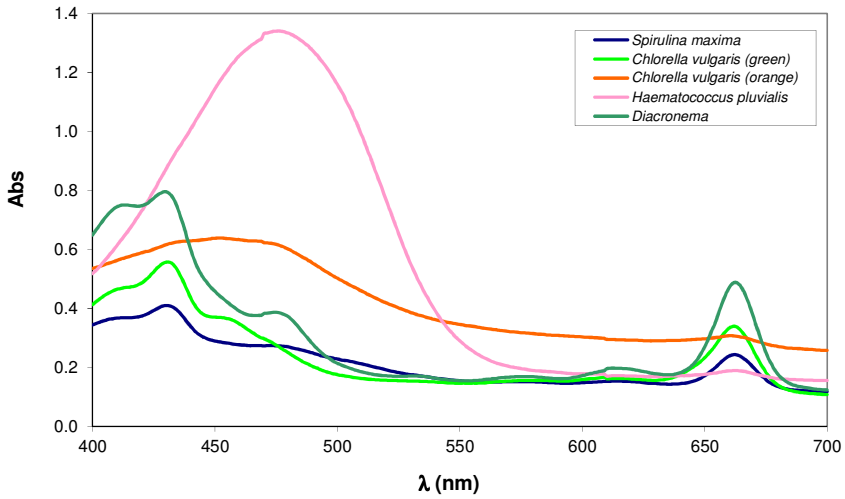


Figure 3.12. UV-VIS absorption spectra of microalgae acetone extracts.

Microalgae pigment extracts were analysed by TLC and HPLC, and the main pigments separated and identified (as compared to chromatography standards) are presented in Table 3.5.

Chlorella green had a total pigment content of 1.2%, with Lutein/Zeaxanthin as main carotenoids and presence of both Chlorophyll *a* and *b*, typical of Chloropyceae.

Generally, Chlorophycean algae contain the same set of major carotenoids as higher plants, namely β-carotene, α-carotene, lutein, violaxanthin and neoxanthin (Goodwin and Britton, 1988).

After carotenogenesis, *Chlorella* total pigment content slightly increases (1.3%) but the carotenoid profile is deeply modified, being observed a dominance of secondary keto-carotenoids, mainly canthaxanthin, either free or esterified (mainly with oleic acid).

Table 3.5. Pigment composition of microalgae acetone extracts.

	Total pigments (% DW)	**Main Pigments**
Spirulina	0.9 ± 0.1	Lutein/Zeaxanthin β-carotene Chlorophyll *a*
Chlorella green	1.2 ± 0.1	Lutein/Zeaxanthin Chlorophyll *a* Chlorophyll *b*
Chlorella orange	1.3 ± 0.03	Astaxanthin Lutein/Zeaxanthin Canthaxanthin mono-, di- esters of canthaxanthin and/or astaxanthin Chlorophyll *a*/*b*
Haematococcus	3.0 ± 0.2	Astaxanthin Lutein/Zeaxanthin Canthaxanthin mono-, di- esters of canthaxanthin and/or astaxanthin Chlorophyll *a*/*b*
Diacronema	2.4 ± 0.3	Fucoxanthin Lutein/Zeaxanthin β-carotene Chlorophyll *a* Chlorophyll c
Isochrysis	3.2 ± 0.4	Fucoxanthin Lutein/Zeaxanthin β-Carotene Chlorophyll *a* Chlorophyll c

It is evident that *Haematococcus* has an extremely high pigment yield of 3.0% (Table 3.5), mainly astaxanthin (free and esterified). This microalga is one of the few microorganisms that are able to synthesize astaxanthin, including *Brevibacterium, Agrobacterium auratim, Phaffia* and *rhodozyma*, being able to accumulate the highest level of astaxanthin in nature (Todd-Lorenz and Cysewski, 2000).

Astaxanthin accumulates inside *Haematococcus* during the morphogenetic transformation of green vegetative cells to red cyst cells under unfavourable environmental conditions, such as nitrogen source exhaustion, excess acetate addition, strong light intensity, phosphate deficiency or salt stress (Boussiba and Vonshak, 1991; Kobayashi *et al.*, 1997; Harker *et al.*, 1996; Fábregas *et al.*, 2003). Mature red cysts form a thick amorphous layer as a secondary wall inside the extracellular matrix (primary wall) and a large interspace between the

plasmalemma and the secondary wall developed (Hejazi and Wijffels, 2004; Kang and Sim, 2008).

Haematococcus primarily contains astaxanthin in the monoester form (~70%), while the remaining fraction is in diester form (25%) and free form (5%) (Nobre *et al.*, 2006; Mendes-Pinto *et al.*; 2001; Todd-Lorenz and Cysewski, 2000). Fatty acids (mainly 16:0, 18:1 and 18:2) are esterified onto the 3' hydroxyl group(s) of astaxanthin after biosynthesis of the carotenoid thereby increasing its solubility and stability in the cellular lipid environment (Todd-Lorenz and Cysewski, 2000).

Diacronema and *Isochrysis* also show high pigment yields (2.4 and 3.2%, respectively), with presence of chlorophyll *a* and *c* (c_1 and c_2), as typical for Haptophyceae. Besides Lutein/Zeaxanthin and β-carotene these algae show characteristic high levels of fucoxanthin, a carotenoid responsible for the brownish colour of *Isochrysis*.

Spirulina showed the lowest pigment yield (0.9%), being detected Lutein/Zeaxanthin, β-carotene and chlorophyll *a*. It is reported in the literature that Cyanobacteria also produce echinenone and an unusual glycosylated carotenoid named myxoxantophyll (Vermaas, 2004).

Despite its low content in lipophilic pigments *Spirulina* is very rich in blue phycobiliprotein aqueous pigments, with 7.0% *c*-Phycocyanin and 4.4% allophycocyanin per dry matter (Figure 3.13), corresponding to 15.6% of *Spirulina's* crude protein (44%), which is in agreement with results from other authors (Campanella *et al.*, 1999).

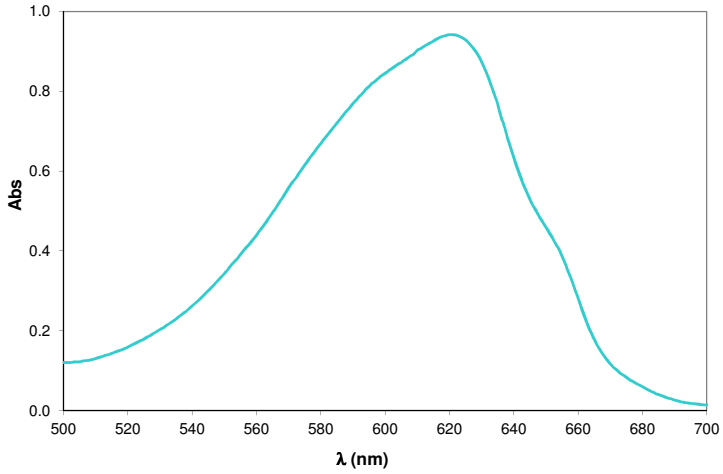


Figure 3.13. UV-absorption spectra of phycocyanin extract from *Spirulina*.
C-PC: *c*-phycocyanin; APC: allophycocyanin.

3.3.3. Thermal analysis

Thermograms resulting from thermogravimetric analysis (TGA) of microalgae samples, expressing weight loss (%) in respect to temperature, are presented in Figure 3.14, including the derivative thermogravimetric (DTG) curves. It can be observed that microalgae undergo

a series of alterations upon heating that can be described by different stages, which are related to the complex mixture of biomolecules in algal cells, as previously discussed, each group with different thermal stabilities (Pane *et al.*, 2001).

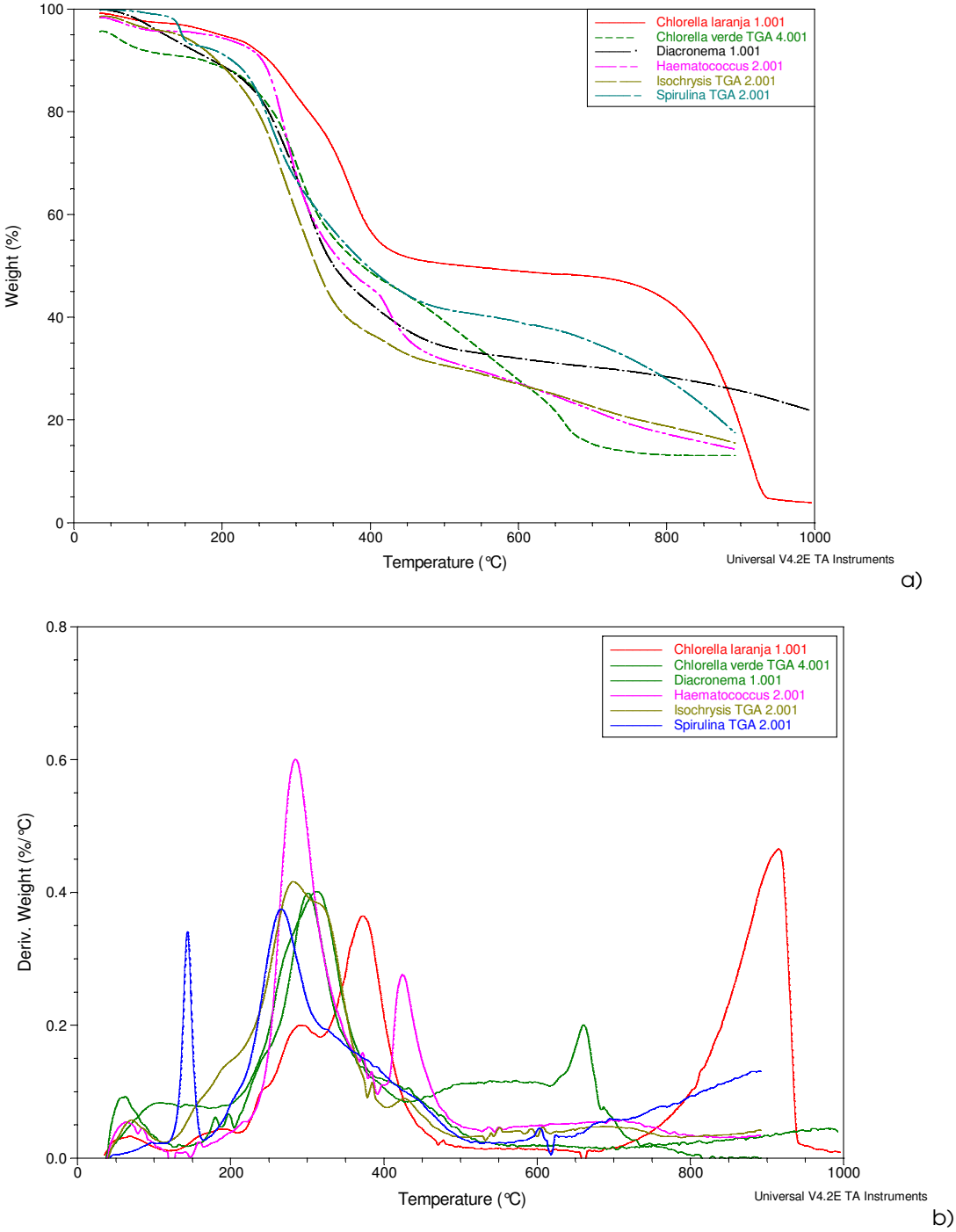


Figure 3.14. TGA (a) and DTG (b) curves of microalgae samples.

The first stage of weight loss occurs between 40 and 200-250°C range and corresponds to the loss of free water and water loosely bound to biomolecules, with values similar to the moisture content (<10%). In this process, the cell structure is progressively destroyed, and phenomena such as alteration of lipid structures and protein thermal unfolding occur (Pane *et al.*, 2001).

For *Spirulina* this initial weight loss is more pronounced than for the other algae, which can be related to the degradation of more thermal-sensitive compounds, such as phycobilliprotein hydrophilic pigments.

This is followed by a wider stage where major weight loss occurs, at onset temperatures above 221ºC (Table 3.6). In general, higher onset temperatures are observed for carotenogenic algae (254-258ºC) as related to green algae (221-243ºC), indicating higher thermal stability for the former.

Table 3.6. Parameters from TGA measurements on microalgal samples.

	T_{onset1} (ºC)	T_{max1} (ºC)	T_{max2} (ºC)	T_{max3} (ºC)	Residue $_{T=900}$ (%)
Spirulina	228.9 ± 8.1	264.1 ± 0.5	-	> 900ºC	14.8 ± 4.0
Chlorella green	224.6 ± 1.2	301.9 ± 1.8	-	666.8 ± 8.4	13.1 ± 0.1
Chlorella orange	254.3 ± 3.9	290.4 ± 1.7	370.1 ± 0.5	906.5 ± 14.4	3.5 ± 0.6*
Haematococcus	258.1 ± 2.9	288.2 ± 5.9	417.2 ±10.1	-	13.2 ± 10.1
Diacronema	243.3 ± 5.0	309.7 ± 2.9	-	-	25.9 ± 0.1
Isochrysis	221.3 ± 8.2	282.8 ± 0.4	-	-	16.5 ± 1.3

**residue calculated at 993ºC*

From Figure 3.14 it is possible to observe that this major degradation stage can be divided into three zones, with different maximum degradation rates, as evidenced by peak temperatures in DTG curves (Figure 3.14b), indicated as T_{max1}, T_{max2} *and* T_{max3} in Table 3.6.

The first degradation zone occurs from 221 to around 350ºC for all algae, with T_{max} ranging from 264ºC (*Spirulina*) to 310ºC (*Diacronema*). This major weight loss in the 200-400ºC range is generally associated with the decomposition of proteins and carbohydrates (Pane *et al.*, 2001; Ross *et al.*, 2008), as well as other volatile components, which are gradually released and/or converted to other products. Second and/or third main peaks in DTG curves can be observed for some algae, indicating the presence of heat resistant compounds that are degraded at higher temperatures.

A second zone of degradation, at 350-500ºC, can be observed for carotenogenic microalgae, with T_{max2} at 370ºC and 417ºC, for *Chlorella* orange and *Haematococcus*, respectively (Table 3.6). This could be related to the high lipid content characteristic of these microalgae, which is in agreement with the study of Na *et al.* (2011) that reported the degradation and volatilization of triacylglycerols at a temperature around 390ºC. According to TGA previous studies on *Nannochloropsis* sp. (Marcilla *et al.* 2009), the devolatilization stage (180-540ºC), where the main degradation processes take place, is very complex, involving overlapped steps according to a complex biochemical composition. These authors have concluded that the lipid compounds are decomposed at a higher temperature compared to other cell components.

A third degradation zone, above 500-550ºC, can be observed for some algae, which can be related to the presence of other components in the microalgal biomass (*e.g.* ashes). For *Chlorella* green a T_{max3} observed at 667ºC. For *Chlorella* orange, almost no weight variations

(plateau) are observed from 400 to 800ºC, with a residue around 46% of the sample's initial weight. Above 800ºC *Chlorella* orange suffers a significant weight loss, with maximum degradation rate (T_{max3}) at 906.5ºC. This could be related to the high mineral content of this alga (34.8%). In the case of *Spirulina*, a new degradation peak seems to occur at temperatures above 900ºC, so it is considered a T_{max3} > 900ºC.

After the initial weight loss and major degradation stages, in a final step the residue is slowly decomposed at a very slow rate until complete oxidation of the organic matter, resulting in the formation of a loose porous non-degradable residue (Pane *et al.*, 2001; Li *et al.*, 2011). The percentage of non-degradable residue at 900ºC is presented in Table 3.6, which in general accounts for 13-17% of the sample's initial weight, with a higher value for *Diacronema* (25.9%). In the case of *Chlorella* orange the sample was further heated, since at 900ºC the weight was not stabilized, so the 3.5% residue corresponds to a temperature of 993ºC.

The ability of the application of thermogravimetric analysis (TGA) for the study of microalgal species has been proved by several authors. As an example, Pane *et al.* (2001) applied TGA to study the effects of temperature on a marine planktonic alga, reporting the existence of marked differences between the different phases of growth, related to the presence of different molecules produced during the algal growth and to the differences in the thermal properties of these intracellular molecules. Some authors have also studied the thermogravimetric properties of algal samples for pyrolysis purposes, *i.e.*, using microalgal biomass as feedstock for liquid biofuel production (Peng *et al.*, 2001; Ross *et al.*, 2008; Li *et al.*, 2011).

3.3.4. Principal Component Analysis

The first step in a Principal Component Analysis is the elaboration of the correlation matrix for the studied variables (physicochemical parameters) which is presented in Table 3.7. This matrix assesses the linear association between variables through the Pearson correlation coefficient (r), being symmetric in relation to the main diagonal line. The stronger correlations, with r>0.75, are highlighted in red on Table 3.7. As an example of positive correlations, it can be observed that higher total PUFA-ω_3 are associated to higher 14:0, 16:1, EPA and DHA levels, in the samples (microalgae) studied. Positive correlations between total PUFA-ω_6 and 18:2ω_6, as well as with Cu, Zn and Fe mineral content, were also observed. Negative correlations were observed between total fat and protein or moisture contents, for example. In respect to the thermogravimetric analysis parameters, a strong negative correlation of T_{onset} with protein content is observed, as well as strong positive correlation of T_{max2} and T_{max3} with total fat and ashes, respectively, which is in accordance with the TGA results discussion in section 3.3.3.

Table 3.7. Correlation Matrix of Variables.

	Moisture	Ashes	Protein	Fat	Carb.	P	K	Ca	Mg	Na	Cu	Mn	Zn	Fe	SFA	MUFA	PUFA-ω3	PUFA-ω6	14:0	16:0	18:0	16:1	18:1	16:4ω3	18:3ω3	20:5ω3	22:6ω3	18:2ω6	18:3ω6	20:4ω6	UI	Pigment	C-PC	T_{onset}	$T_{max\,1}$	$T_{max\,2}$	$T_{max\,3}$	$Res_{900°C}$
Moisture	**1.00**	0.39	0.59	-0.92	-0.33	-0.42	0.43	0.62	0.30	-0.03	-0.48	0.10	-0.45	-0.41	-0.96	-0.90	-0.86	-0.72	-0.39	-0.81	-0.65	-0.31	-0.64	-0.56	-0.63	-0.41	-0.35	-0.51	0.20	-0.62	-0.27	-0.75	0.56	-0.46	-0.00	-0.62	0.51	0.26
Ashes	0.39	**1.00**	0.01	-0.47	-0.57	-0.52	0.08	0.18	-0.20	0.28	-0.65	-0.33	-0.72	-0.77	-0.43	-0.29	-0.52	-0.73	-0.43	-0.10	-0.17	-0.43	-0.02	-0.23	-0.01	-0.51	-0.39	-0.51	-0.89	-0.53	-0.86	0.39	-0.03	-0.24	-0.01	0.92	-0.55
Protein	0.59	0.01	**1.00**	-0.79	-0.69	0.44	0.54	0.28	0.56	-0.36	-0.65	0.40	-0.60	-0.53	0.45	-0.58	-0.14	-0.58	0.45	-0.94	-0.96	0.51	-0.99	-0.96	-0.99	-0.99	0.47	-0.77	-0.15	-0.31	0.28	-0.14	0.43	-0.89	-0.13	-0.99	-0.01	0.61
Fat	-0.92	-0.47	-0.79	**1.00**	0.61	0.12	-0.48	-0.61	-0.47	-0.76	-0.38	-0.31	-0.74	-0.68	-0.78	0.98	0.67	0.85	0.13	0.90	0.85	0.07	0.82	0.64	0.79	0.09	0.17	0.78	0.06	0.74	0.20	0.70	-0.55	0.72	0.11	0.81	-0.53	-0.23
Carb.	-0.33	-0.57	-0.69	0.61	**1.00**	-0.21	-0.40	0.10	-0.06	0.04	-0.13	0.27	-0.13	-0.05	0.66	0.01	0.05	0.73	-0.37	0.56	0.72	-0.40	0.64	0.82	-0.40	-0.30	-0.40	0.56	0.36	0.56	-0.12	0.36	-0.50	0.55	0.34	0.64	-0.36	-0.16
P	-0.42	-0.52	0.44	0.12	-0.21	**1.00**	0.03	-0.13	0.53	-0.51	-0.13	0.27	-0.13	-0.05	0.66	0.01	0.05	0.76	0.19	0.88	-0.17	-0.45	-0.84	-0.40	-0.40	-0.38	0.88	0.84	-0.43	0.34	-0.43	0.68	-0.29	-0.57	-0.03	-0.42	-0.61	0.35
K	0.43	0.08	0.54	-0.48	-0.40	0.03	**1.00**	-0.14	-0.08	0.55	-0.08	-0.31	-0.23	-0.09	-0.31	-0.49	-0.39	-0.09	-0.14	-0.45	-0.35	-0.08	-0.44	-0.41	-0.47	-0.29	-0.12	-0.15	0.61	-0.23	-0.36	-0.27	0.92	-0.49	-0.83	-0.42	0.26	0.15
Ca	0.62	0.18	0.28	-0.61	0.10	-0.13	-0.14	**1.00**	0.77	-0.49	-0.34	-0.05	-0.37	-0.28	-0.57	-0.57	-0.52	-0.51	-0.29	-0.50	-0.41	-0.30	-0.38	-0.30	-0.29	-0.33	-0.29	-0.36	-0.25	-0.53	-0.26	-0.49	-0.15	-0.46	0.40	-0.39	0.35	-0.07
Mg	0.30	-0.20	0.56	-0.47	-0.06	0.53	-0.08	0.77	**1.00**	-0.76	-0.38	0.18	-0.39	-0.26	-0.10	-0.51	0.03	-0.33	0.34	-0.58	-0.61	0.31	-0.62	-0.54	-0.54	0.30	0.31	-0.47	-0.47	-0.22	0.17	0.03	-0.29	-0.77	0.33	-0.64	-0.12	0.23
Na	-0.03	0.28	-0.36	0.16	0.04	-0.51	0.55	-0.49	-0.76	**1.00**	0.38	-0.66	0.19	0.23	-0.10	0.25	-0.36	0.29	-0.60	0.39	0.49	-0.59	0.46	0.41	0.41	-0.57	-0.61	0.47	0.75	-0.09	-0.30	0.68	0.39	-0.78	0.48	0.64	0.43	-0.44
Cu	-0.48	-0.65	-0.65	0.74	0.87	-0.13	-0.08	-0.34	-0.38	0.38	**1.00**	-0.27	0.94	0.98	-0.32	0.68	0.18	0.93	-0.28	0.65	0.80	-0.29	0.68	0.83	0.66	-0.19	-0.32	0.98	0.63	0.73	-0.10	0.51	-0.21	0.60	-0.04	0.68	-0.45	-0.08
Mn	0.10	-0.33	0.40	-0.10	-0.21	0.27	-0.31	-0.05	0.18	-0.66	-0.27	**1.00**	0.06	-0.10	-0.05	-0.26	0.30	-0.21	0.63	-0.42	-0.47	0.70	-0.47	-0.39	-0.51	0.64	0.69	-0.37	0.91	0.37	0.91	0.36	-0.32	-0.07	0.65	-0.47	-0.58	0.85
Zn	-0.45	-0.72	-0.60	0.74	0.84	-0.13	-0.23	-0.37	-0.39	0.19	0.94	0.06	**1.00**	0.97	0.28	0.64	0.24	0.87	-0.13	0.56	0.71	-0.12	0.60	0.77	0.56	-0.04	-0.15	0.90	0.53	0.86	0.18	0.60	-0.33	0.68	0.21	0.60	-0.62	0.17
Fe	-0.41	-0.77	-0.53	0.68	0.87	-0.05	-0.09	-0.28	-0.26	0.23	0.98	-0.10	0.97	**1.00**	0.28	0.59	0.20	0.91	-0.17	0.52	-0.18	0.54	0.74	0.53	0.53	-0.08	-0.21	0.93	0.61	0.81	0.05	0.58	-0.25	0.53	0.08	0.55	-0.68	0.11
SFA	-0.96	-0.43	-0.34	0.78	0.16	0.66	-0.31	-0.57	-0.10	-0.10	0.32	-0.05	0.28	0.28	**1.00**	0.74	0.94	0.63	0.59	0.62	0.42	0.51	0.41	0.32	0.40	0.60	0.54	0.32	0.31	0.57	0.36	0.79	-0.51	0.18	-0.06	0.39	-0.56	-0.15
MUFA	-0.90	-0.29	-0.87	0.98	0.58	0.01	-0.49	-0.57	-0.51	0.25	0.68	-0.26	0.64	0.59	0.74	**1.00**	0.58	0.78	-0.01	0.97	0.90	-0.08	0.90	0.86	0.89	0.03	-0.04	0.75	0.04	0.59	0.04	0.55	-0.51	0.76	0.04	0.89	-0.34	-0.42
PUFA-ω3	-0.86	-0.52	-0.14	0.67	0.05	0.76	-0.39	-0.52	0.03	-0.36	0.18	0.30	0.24	0.20	0.94	0.58	**1.00**	0.49	0.81	0.40	0.19	0.75	0.17	0.12	0.16	0.82	0.78	0.13	0.45	0.63	0.48	0.88	-0.59	0.08	0.16	0.15	-0.73	0.16
PUFA-ω6	-0.72	-0.73	-0.58	0.85	0.73	0.19	-0.09	-0.51	-0.33	0.29	0.93	-0.21	0.87	0.91	0.78	0.03	0.49	**1.00**	0.03	0.69	0.75	-0.00	0.63	0.74	0.46	0.99	0.90	0.02	0.73	0.83	0.08	0.73	-0.29	0.49	-0.11	0.63	-0.61	-0.05
14:0	-0.39	-0.43	0.45	0.13	-0.37	0.88	-0.14	-0.29	0.34	-0.60	0.38	-0.27	-0.13	-0.17	0.59	-0.01	0.81	0.03	**1.00**	-0.21	-0.42	0.99	-0.43	-0.47	-0.45	0.99	1.00	-0.02	0.84	0.39	0.68	0.34	-0.55	0.68	0.63	0.74	-0.68	0.68
16:0	-0.81	-0.10	-0.94	0.90	0.56	-0.17	-0.45	-0.50	-0.58	0.39	0.65	-0.42	0.56	0.52	0.62	0.97	0.40	0.69	-0.21	**1.00**	0.95	-0.29	0.97	0.90	0.96	-0.19	-0.24	0.75	0.10	0.41	0.34	-0.41	-0.30	0.80	-0.04	0.96	-0.12	-0.60
18:0	-0.65	-0.17	-0.96	0.85	0.72	-0.35	-0.35	-0.41	-0.61	0.49	0.80	-0.47	0.71	0.68	0.42	0.90	0.19	0.75	-0.42	0.95	**1.00**	-0.47	0.98	0.98	0.97	-0.37	-0.45	0.89	0.34	0.44	-0.30	0.26	-0.30	0.85	-0.05	0.98	-0.09	-0.55
16:1	-0.31	-0.43	0.51	0.07	-0.40	0.84	-0.08	-0.30	0.31	-0.59	-0.29	0.70	-0.12	-0.18	0.51	0.41	0.90	-0.00	0.75	-0.29	-0.47	**1.00**	-0.49	-0.51	-0.52	0.99	1.00	-0.24	0.87	0.40	0.87	0.66	-0.28	-0.39	0.20	-0.51	-0.68	0.65
18:1	-0.64	-0.02	-0.99	0.82	0.64	-0.40	-0.44	-0.38	-0.62	0.46	0.68	-0.47	0.60	0.54	0.41	0.90	0.17	0.63	-0.43	0.97	0.98	-0.49	**1.00**	0.96	0.99	-0.40	-0.45	0.79	0.22	0.32	-0.32	0.17	-0.34	0.87	-0.00	1.00	0.01	-0.64
16:4ω3	-0.56	-0.23	-0.96	0.81	0.82	-0.40	-0.41	-0.30	-0.54	0.41	0.83	-0.39	0.77	0.74	0.32	0.86	0.12	0.74	-0.47	0.90	0.98	-0.51	0.96	**1.00**	0.95	-0.42	-0.49	0.91	0.36	0.47	-0.27	0.25	-0.37	0.87	0.09	0.96	-0.13	-0.48
18:3ω3	-0.63	-0.01	-0.99	0.79	0.67	-0.38	-0.47	-0.29	-0.54	0.41	0.66	-0.51	0.56	0.53	0.40	0.89	0.16	0.61	-0.45	0.96	0.97	-0.52	0.99	0.95	**1.00**	-0.43	-0.48	0.78	0.18	0.28	-0.36	0.15	-0.38	0.83	0.02	0.99	0.04	-0.68
20:5ω3	-0.41	-0.51	0.42	0.17	-0.30	0.88	-0.12	-0.33	0.30	-0.57	-0.19	0.64	-0.04	-0.08	0.60	0.03	0.82	0.11	0.99	-0.19	-0.37	0.99	-0.40	-0.42	-0.43	**1.00**	0.99	-0.30	0.48	0.48	0.86	0.74	-0.34	-0.34	0.19	-0.42	-0.74	0.60
22:6ω3	-0.35	-0.39	0.47	0.09	-0.40	0.84	-0.15	-0.29	0.31	-0.61	-0.32	0.69	-0.15	-0.21	0.54	-0.04	0.78	-0.02	1.00	-0.24	-0.45	1.00	-0.45	-0.49	-0.48	0.99	**1.00**	-0.42	0.57	0.38	0.87	0.65	-0.33	-0.36	0.23	-0.47	-0.66	0.59
18:2ω6	-0.51	-0.51	-0.77	0.77	0.86	-0.24	-0.13	-0.36	-0.47	0.47	0.98	-0.37	0.90	0.93	0.32	0.75	0.13	0.90	-0.38	0.75	0.89	-0.41	0.79	0.91	0.78	-0.30	-0.42	**1.00**	0.61	0.64	0.41	0.41	-0.20	0.70	-0.07	0.80	-0.32	-0.24
18:3ω6	0.20	-0.23	-0.15	0.06	0.36	-0.43	0.61	-0.25	-0.47	0.75	0.63	-0.36	0.53	0.61	-0.31	0.04	-0.45	0.45	-0.56	0.10	0.34	-0.51	0.22	0.36	0.18	-0.48	-0.57	0.61	**1.00**	0.24	-0.43	-0.08	0.59	0.26	-0.50	0.24	0.04	0.05
20:4ω6	-0.62	-0.89	-0.31	0.74	0.56	0.34	-0.23	-0.53	-0.22	-0.09	0.73	0.37	0.86	0.81	0.57	0.59	0.63	0.83	0.39	0.41	0.44	0.40	0.32	0.47	0.28	0.48	0.38	0.64	0.24	**1.00**	0.59	0.90	-0.43	0.42	0.42	0.32	-0.92	0.45
UI	-0.27	-0.53	0.28	0.20	-0.12	0.56	-0.36	-0.26	0.17	-0.66	-0.10	0.91	0.18	0.05	0.36	0.03	0.66	0.08	0.84	-0.18	-0.30	0.87	-0.32	-0.27	-0.36	0.86	0.87	-0.21	-0.43	0.59	**1.00**	0.68	-0.48	-0.05	0.56	-0.33	-0.80	0.77
Pigment	-0.75	-0.85	-0.14	0.70	0.36	0.68	-0.27	-0.49	0.03	-0.30	0.51	0.36	0.60	0.58	0.79	0.55	0.88	0.73	0.68	0.34	0.26	0.66	0.17	0.25	0.15	0.86	0.65	0.41	-0.08	0.90	0.68	**1.00**	-0.54	0.15	0.20	0.16	-0.94	0.40
C-PC	0.56	0.39	0.43	-0.55	-0.50	-0.29	0.92	-0.15	-0.29	0.68	-0.21	-0.32	-0.33	-0.25	-0.51	-0.51	-0.59	-0.29	-0.34	-0.41	-0.30	-0.28	-0.34	-0.37	-0.38	-0.34	-0.33	-0.20	-0.59	-0.43	-0.48	-0.54	**1.00**	-0.30	-0.79	-0.32	0.52	0.02
T_{onset}	-0.46	-0.03	-0.89	0.72	0.55	-0.57	-0.49	-0.46	-0.77	0.39	0.60	-0.07	0.68	0.53	0.18	0.76	0.08	0.49	-0.38	0.80	0.85	-0.39	0.87	0.87	0.83	-0.34	-0.36	0.70	0.26	0.42	-0.05	0.15	-0.30	**1.00**	0.22	0.88	-0.09	-0.29
$T_{max\,1}$	-0.00	-0.24	-0.13	0.11	0.34	0.03	-0.83	0.40	0.33	-0.78	-0.04	0.65	0.21	0.08	-0.06	0.04	0.16	-0.11	0.20	-0.04	-0.05	0.20	0.09	0.09	0.02	0.19	0.23	-0.07	-0.50	0.24	0.56	0.20	-0.79	0.22	**1.00**	-0.01	-0.38	0.32
$T_{max\,2}$	-0.62	-0.01	-0.99	0.81	0.64	-0.42	-0.42	-0.39	-0.64	0.48	0.68	-0.47	0.60	0.55	0.39	0.89	0.15	0.63	-0.45	0.96	0.98	-0.51	1.00	0.96	0.99	-0.42	-0.47	0.80	0.24	0.32	-0.33	0.16	-0.32	0.88	-0.01	**1.00**	0.02	-0.63
$T_{max\,3}$	0.51	0.92	-0.01	-0.53	-0.36	-0.61	0.26	0.35	-0.12	0.43	-0.45	-0.58	-0.62	-0.58	-0.56	-0.34	-0.73	-0.61	-0.68	-0.12	-0.09	-0.68	0.01	-0.13	0.04	-0.74	-0.66	-0.32	0.04	-0.92	-0.80	-0.94	0.52	-0.09	-0.38	0.02	**1.00**	-0.64
$Res_{900°C}$	0.26	-0.55	0.61	-0.23	-0.16	0.35	0.15	-0.07	0.23	-0.44	-0.08	0.85	0.17	0.11	-0.15	-0.42	0.16	-0.05	0.56	-0.60	-0.55	0.65	-0.64	-0.48	-0.68	0.60	0.59	-0.24	0.05	0.45	0.77	0.40	0.02	-0.29	0.32	-0.63	-0.64	**1.00**

Subsequently, the principal components are then extracted by calculating the eigenvalues of the correlation matrix, which indicate the importance of each component in explaining the results variability. Table 3.8 presents the eigenvalues in a descending order being its sum equal to the number of variables (38). The first component explains 42.7% of total variance, the second 30.6%, the third 11.3%, the fourth 9.8% and the fifth 5.5%. Thus, the first three components explain 85% of the total variance.

Table 3.8. Eigenvalues – Extraction of Principal Components.

Factor	Eigenvalue	% Total Variance	Cumulative - Eigenvalue	Cumulative - % Total Variance
1	16,24050	42,73816	16,24050	42,7382
2	11,63215	30,61092	27,87265	73,3491
3	4,29167	11,29386	32,16431	84,6429
4	3,73132	9,81926	35,89563	94,4622
5	2,10437	5,53781	38,00000	100,0000

The correlation of each variable to the different factors is presented in Table 3.9, reflecting the weight carried by each variable on the factors' total variance (*factor loadings*).

Table 3.9. Factor Loadings.

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Moisture	0,71574	0,50534	0,238830	0,404738	-0,107195
Ashes	0,36815	0,68559	-0,484401	-0,316508	-0,244144
Protein	0,91187	-0,22817	0,329726	-0,043501	0,076266
Fat	-0,91693	-0,35296	-0,112463	-0,144860	-0,032037
Carbohydrates	-0,77661	0,00729	0,083147	0,561808	0,272560
P	0,20829	-0,81512	0,049964	-0,300146	0,446780
K	0,36864	0,31985	0,766426	-0,394420	0,137211
Ca	0,47029	0,27764	-0,279544	0,645875	0,454360
Mg	0,56405	-0,30604	-0,163192	0,385314	0,642722
Na	-0,39516	0,64901	0,423475	-0,473082	-0,139636
Cu	-0,87573	-0,03545	0,410937	0,202675	0,147956
Mn	0,33051	-0,67826	0,006664	0,360759	-0,548211
Zn	-0,82138	-0,21426	0,379570	0,352372	-0,105737
Fe	-0,79003	-0,16192	0,473806	0,330336	0,126575
SFA	-0,50998	-0,63581	-0,195405	-0,490522	0,238474
MUFA	-0,93061	-0,19004	-0,227794	-0,214392	-0,000587
PUFA-ω_3	-0,32076	-0,85519	-0,190245	-0,349349	0,086721
PUFA-ω_6	-0,85720	-0,29538	0,358709	-0,038376	0,218672
14:0	0,26764	-0,92671	-0,049531	-0,258805	-0,012286
16:0	-0,92907	0,04184	-0,280861	-0,237038	-0,002171
18:0	-0,97603	0,18965	-0,093669	-0,051178	0,002724
16:1	0,31733	-0,91710	0,022240	-0,223156	-0,089052
18:1	-0,93330	0,23387	-0,253437	-0,084612	-0,053487
16:4ω_3	-0,96915	0,18852	-0,081692	0,135866	-0,008802
18:3ω_3	-0,91693	0,25009	-0,304665	-0,051317	0,035327
20:5ω_3	0,20921	-0,94905	0,028199	-0,232829	-0,023236
22:6ω_3	0,29922	-0,91742	-0,061573	-0,237888	-0,091763
18:2ω_6	-0,93183	0,08355	0,302879	0,145409	0,108746
18:3ω_6	-0,34904	0,44296	0,822119	0,060595	-0,049003
20:4ω_6	-0,61939	-0,66628	0,369018	0,154177	-0,111716
UI	0,12290	-0,91051	-0,007269	0,147646	-0,366083
Pigments	-0,45111	-0,86455	0,185063	-0,049699	0,111074
C-PC	0,38192	0,56233	0,603072	-0,395119	-0,134583
T_{onset}	-0,82981	0,15889	-0,157520	0,103974	-0,500538
$T_{max\ 1}$	-0,02131	-0,39936	-0,474912	0,751634	-0,222622
$T_{max\ 2}$	-0,93210	0,25041	-0,237042	-0,083194	-0,073334
$T_{max\ 3}$	0,30241	0,88710	-0,284871	-0,200552	0,014914
Residue$_{900°C}$	0,37912	-0,60997	0,506826	0,335586	-0,338701
Expl.Var	16,24050	11,63215	4,291667	3,731319	2,104367
Prp.Totl	0,42738	0,30611	0,112939	0,098193	0,055378

Twenty-six of the 38 initial variables are strongly correlated with the first two factors, while two variables (K, 18:3ω_6) are more correlated to factor 3 and one (T_{max1}) to factor 4. Accordingly, it was decided to represent the distribution of the variables in the space defined by factors 1 and 2 (Figure 3.15), without considerable loss of information.

The variables are widely distributed in the factorial plan (Figure 3.15) being possible to detect some groups of variables in the different quadrants of the plan. Moisture, ashes, phycocyanin (C-PC), Ca, K and $Residue_{900ºC}$ are located on the upper-right quadrant, although these variables are poorly correlated with factors 1 and 2. On the lower-right quadrant, protein is strongly associated to factor 1 positive values, while a cluster of 14:0, 16:1; 20:5ω_3, 22:6ω_3, UI and P are associated to negative factor 2 values. Manganese, Mg and T_{max3} are also located on this quadrant. Total pigments and PUFA-ω_3 are also correlated with negative factor 2 values, but are located on the lower-left quadrant (factor 1 negative), as well as 20:4ω_6 and SFA. T_{max1} does not correlate to either factor 1 or 2, so it is located in the axis. Fat, carbohydrates, Cu, Zn, Fe, MUFA, PUFA-ω_6, 16:0, 18:0, 18:1, 16:4ω_3, 18:3ω_3, 18:2ω_6, T_{onset}, and T_{max2} variables are strongly, negatively correlated to factor 1. On the upper-left quadrant are located the variables 18:3ω_6, and Na, which are poorly correlated to factors 1 and 2.

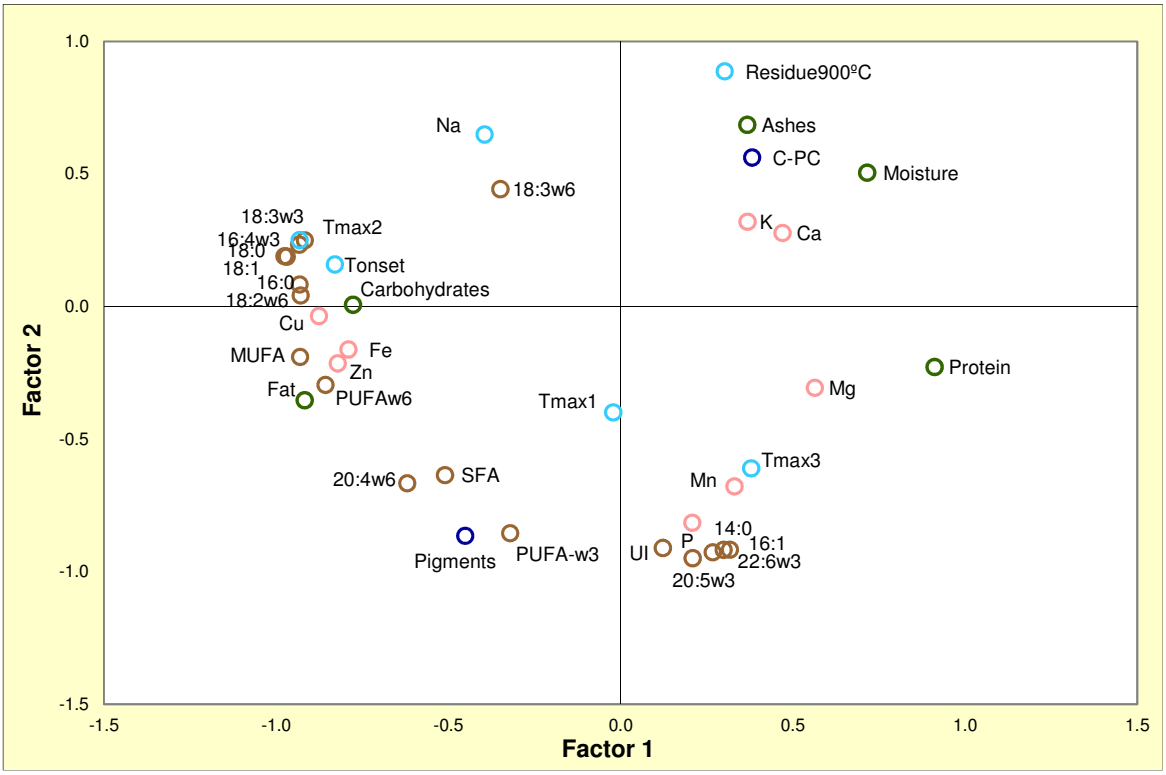


Figure 3.15. Projection of the variables in the factorial plan defined by factor 1 and factor 2.

Figure 3.16 shows the projection of the microalgae samples on the plan defined by factor 1 and 2. It is clear that the microalgae are distributed on the plan according to its physicochemical composition.

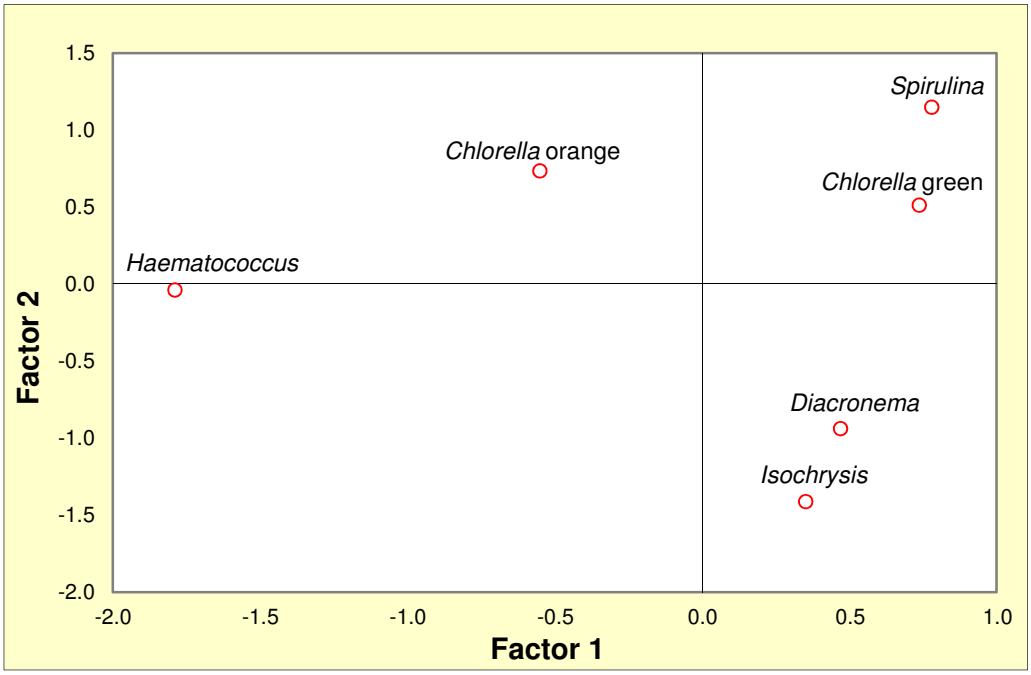


Figure 3.16. Projection of the microalgae samples in the factorial plan defined by factor 1 and factor 2.

Green algae are located on the positive factor 1 axis while carotenogenic algae are located on the negative factor 1 axis, reflecting protein rich *versus* fat/carbohydrate rich biomasses, among other differences in chemical composition variables. Among green algae, *Chlorella* and *Spirulina* are located in the positive factor 2 axis, while *Isochrysis* and *Diacronema* are on the negative factor 2 axis, confirming their physicochemical similarity. In fact, *Chlorella* and *Spirulina* are characterized by high protein (38-45%), moisture (11%) and mineral (24-31%) contents, namely K (*Spirulina*: 2.6%) and Ca (*Chlorella*: 4.7%). Obviously, C-PC positioning is associated to *Spirulina* since this pigment is only present in cyanobacteria. *Isochrysis* and *Diacronema* have similar chemical composition being clustered mainly in association to their characteristic fatty acid profile (14:0, 16:1, 20:5ω_3, 22:6ω_3, UI) and also to its high phosphorus (1.5-2.7%) and Mn (0.8-2.5%) levels. Regarding carotenogenic microalgae, *Chlorella* orange and *Haematococcus* are also located in different regions on the left side of the principal factors plan. *Haematococcus* is situated in the negative end of factor 1, close to factor 2 axis origin, associated to higher fat (41%) and carbohydrate (34%) contents, in opposition to a lower protein (10%), and to its fatty acid profile, rich is 16:0, 18:1, 18:3ω_3 and 18:2ω_6, as well as high contents in Zn, Cu and Fe (232, 344 and 823 mg/kg, respectively). *Chlorella* orange is positioned in the upper-left quadrant, less correlated to factor 1 than *Haematococcus*, but positively correlated to factor 2. An association of carotenogenic algae with some parameters from TGA analysis (T_{onset}, T_{max2}) can also be observed. It should be noted that these algae present a characteristic second zone of thermal degradation (350-500ºC), with T_{max2} closely related to their high lipid content, as confirmed in the variables correlation matrix (Table 3.7).

3.4. CONCLUSIONS

Microalgae can show different biomass profiles according to their origin, but mainly due to their environmental culture conditions (*e.g.* light, temperature, nutrient status, salinity). In fact, when grown under optimal conditions, most microalgae present similar cell compositions in terms of relative amounts of crude proteins, lipids, and carbohydrates. However, when submitted to unfavourable environmental conditions the same alga suffers a dramatic adaptive change in cell composition, inducing the accumulation of certain protective biochemical compounds such as carotenoids.

Of the six microalgae studied, the major differences in physicochemical composition were in fact related to the induction of carotenogenesis process in *Chlorella* (orange) and *Haematococcus*. Nevertheless, each microalga presented a typical biomass nutrient profile, allowing the selection of desired physicochemical characteristics for specific food technology applications.

Spirulina maxima is a Cyanobacteria, characterized by high protein (44%) and low fat content (4%), although it presents an interesting proportion of γ-linolenic acid (0.5 g/100g). It also presents high mineral content (31%), rich in sodium and potassium (8.5 and 2.6%, respectively). In terms of pigment content, its main feature is the production of blue-coloured phycocyanin (7%) an water-soluble phycobiliprotein associated with several health benefits.

Chlorella vulgaris green also presented high protein (38%) and low fat content (5%), being rich in Calcium (4.7%). However, after carotenogenesis process, *Chlorella* becomes orange due to the accumulation of carotenoid pigments, mainly cantaxanthin, in association to a chlorophyll decrease. As a result of this process it is also observed an increase in fat (28%) and a decrease in protein (12%) contents. This should be related to the fact that, when deprived of nitrogen, the cells are unable to divide themselves and accumulate fat within them. Higher total ash content is also observed, mainly due to NaCl addition. Although *Chlorella* green is widely used as food ingredient/supplement, particularly in Asian countries, the carotenogenic alga is not authorized for consumption yet. Recent toxicity assays revealed no mortalities nor relevant clinical signs or behavioural changes in mices, as well as in rats fed with the carotenogenic *Chlorella* studied in the present work (Gouveia *et al.*, submitted).

Haematococcus pluvialis after carotenogenesis process accumulated up to 3.0% carotenoids, mainly astaxanthin esterified with oleic acid (18:1ω9). This alga presented very high fat content (41%), as well as high carbohydrate (34%) and low protein (10%) levels, along with interesting iron (823 mg/kg), copper (344 mg/kg) and zinc (232mg/100kg) microelements concentrations.

Diacronema vlkianum and *Isochrysis galbana* are marine microalgae from the Haptophyceae Family, less studied in terms of human food application but widely used for aquaculture purposes. Like the other green algae studied, they have high protein contents

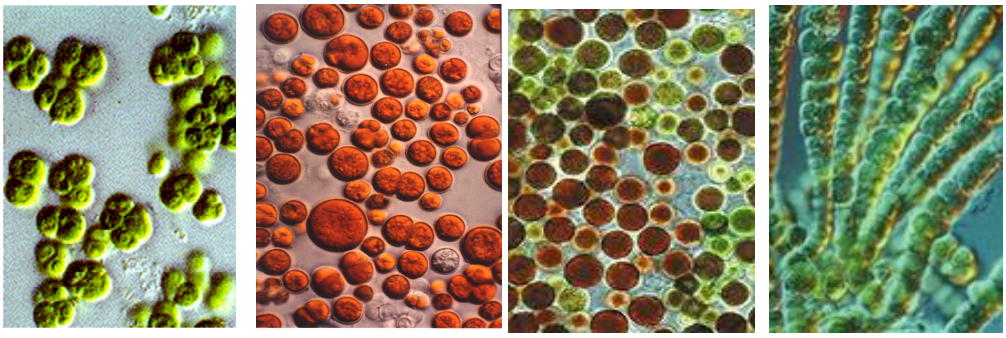
(38-40%) but also high fat contents (18-24%), rich in long chain PUFA-ω_3, particularly EPA (3.2-49 g/100g) and DHA (0.8-1.2 g/100g). These algae are also rich in carotenoids (2-3%), mainly fucoxanthin which gives them a brownish character. This interesting chemical composition should be associated to a higher antioxidant action (*Diacronema*), when compared to *Chlorella vulgaris* in a parallel study (Batista *et al.*, 2010).

The algae were also analysed in terms of their thermal properties through Thermogravimetric Analysis which enables to assess their thermal resistance upon heating, which may be useful considering some technologies used in food processes. Higher temperatures (254-258ºC) for the onset of biomass degradation are observed for carotenogenic microalgae, as well as a characteristic second zone of thermal degradation (350-500ºC) with T_{max2} closely related to their high lipid content. This indicates that carotenogenic microalgae, which have higher fat contents, seem to be able to resist better to thermal treatment.

A factor analysis statistical method (PCA) was applied to 38 analysed physicochemical parameters of the six microalgae under study, in order to determine which variables better define and differentiate them. It is possible to group and correlate several variables into five principal components (factors), of which the first three describe 85% of the results variability. By displaying the variables and the microalgae in the bidimensional plan defined by factors 1 and 2 it is possible to visualize that each microalgae is strongly correlated to a specific group of variables. Nevertheless, it is possible to distinguish the main differences arising from green and carotenogenic algae (upon factor 1), and that *Diacronema* and *Isochrysis* are clearly differentiated from the other green algae.

CHAPTER 4

Microalgal biomass and natural pigments in oil-in-water pea protein-stabilised emulsions



4.1. INTRODUCTION

Oil-in-water emulsions form the basis of many kinds of foods, as for example mayonnaises, which are one of the oldest and most widely consumed sauces in the world today. They can be an efficient vehicle for functional ingredients (*e.g.* carotenoids), which can be encapsulated either in the oil or aqueous phase.

Natural pigments can be used as an alternative to synthetic colourings in the food industry, which are perceived as undesirable and harmful and some are considered to be responsible for allergenic and intolerance reactions. On the other hand, most natural pigments are phytochemicals which have been associated to a nutraceutical effect (*e.g.* antioxidant action), thus providing additional health benefits besides colouring purposes.

Lutein (3,3'-dihydroxy-α-carotene) is a xanthophyll carotenoid with two hydroxyl groups in the conjugated polyene chain (Alves-Rodrigues and Shao, 2004). Lutein can be found in green leafy vegetables like broccoli and spinach and is commercially extracted from the Marigold Flower (*Tagetes erecta*) for the production of orange colourings for the food and beverages industry and production of dietary supplements (Sowbhagya *et al.*, 2004). Epidemiological studies provide evidence to suggest that lutein may protect against Age-related Macular Degeneration (AMD), a leading cause of blindness in people over 65 (Chong *et al.*, 2007) and other eye diseases such as cataracts. This is related to the fact that lutein (and its stereo-isomer zeaxanthin) is deposited in the lens and the macula lutea of the retina acting as blue light filter protecting the underlying tissues from phototoxic damage (Alves-Rodrigues and Shao, 2004). There is also evidence to suggest that lutein may protect against cardiovascular heart diseases and certain types of cancer (*e.g.* breast), through its role as an antioxidant (Johnson, 2000).

Recent studies have investigated the stability of emulsions and microemulsions enriched with carotenoids, such as lycopene (Ribeiro *et al.*, 2003; Spernath *et al.*, 2002), astaxanthin (Ribeiro *et al.*, 2005; Wackerbarth *et al.*, 2009), β-carotene (Neves *et al.*, 2008; Yuan *et al.*, 2008) and lutein (Amar *et al.*, 2004; Losso *et al.*, 2005; Santipanichwong and Suphantarika, 2007). These systems can be used as vehicles for nutraceutical carotenoids in food applications. All carotenoid molecules present a hydrophobic character, and are consequently dissolved in the non-polar oil phase of the emulsion. Nevertheless, it should be considered that the presence of polar hydroxyl groups at the end of some carotenoid molecules, such as lutein and astaxanthin, can provide a certain polar character (Khachatryan, 2003; Shibata *et al.*, 2001) and interfacial activity, as well as the ability to bind with proteins through intermolecular hydrogen bonds (Bassi *et al.*, 1993; Moros *et al.*, 2002).

Phycocyanin is a blue photosynthetic pigment extracted from cyanobacteria with reported antioxidant activity *in vitro* (Romay *et al.*, 2003). It is a hydrophilic phycobiliprotein composed by an apoprotein linked to coloured prosthetic groups (tetrapyrrolic chromophores).

Biliproteins are highly soluble in water, which renders their application in non-polar systems difficult. To overcome this limitation, some experimental studies have tested the incorporation of these compounds in multiphase systems, such as reverse micelles and microemulsions (Bermejo *et al.*, 2000; 2003). However, due to its protein nature, it is possible that phycocyanin presents some surface activity (Chronakis *et al.*, 2000).

The use of microalgal biomass as a colouring source in food products presents additional advantages. Not only pigment extraction and purification costs are eliminated, an improved colour stability is expected, since the pigments are encapsulated inside the microalgal cell. Besides colouring action microalgae may also provide additional benefits through the enrichment of foods with other biologically active compounds such as polyunsaturated fatty acids ω_3.

The introduction of natural colourings (pigments and microalgal biomass) in oil-in-water emulsions can also change the microstructure and perceived texture, since the emulsions' behaviour during its production, processing and storage is highly dependent on composition (Granger *et al.*, 2003). The physical properties of the aqueous and fat phases may be modified according to the nature and polar/non-polar affinities of the colourings, which may possess surface-active properties. The interactions of colourings with emulsifier and stabilising agents at the surface of the emulsion droplets as well as in the aqueous medium among the droplets (Dickinson, 2003; Klinkerson *et al.*, 2004) can contribute to a reinforcement of the emulsion structure through the formation of physical entanglements (Clark *et al.*, 1992; Riscardo *et al.*, 2003). However, if these constituents are incompatible, the emulsion may become less stable, as in the case of surface viscoelasticity loss induced by the displacement of the protein from the interface (Dickinson *and* Hong, 1995).

The aim of the present chapter is to study the effect of adding natural pigments and microalgal biomass to oil-in-water pea protein-stabilized emulsions.

Initially, lutein and phycocyanin were added to the oil and aqueous phases of the emulsion, respectively, at various concentrations, as well as mixtures of both pigments (section 4.3.1). Besides colouring purposes it was intended to develop a comprehensive study on the full rheological characterisation of these systems, involving texture analysis, linear viscoelasticity, steady-state flow and transient stress growth experiments, as well as monitoring some flow tests with an optical analysis system in order to interpret the shear-induced structural modifications when these natural pigments are present in the formulation. These results were related with droplet size distribution measurements of the emulsions.

Afterwards, the ability of microalgal biomass to act as a pigment in food emulsions was evaluated (section 4.3.2). *Spirulina maxima*, *Chlorella vulgaris* (green and carotenogenic) and *Haematococcus pluvialis* (carotenogenic) biomass was added, at various concentrations, to the aqueous phase of the emulsion and their colour and texture stability monitored during 5 weeks (section 4.3.2.1). Subsequently, microalgal biomass *"fat mimetic"*

capacity was investigated (section 4.3.2.2). This deals with the cooperative effect between the protein emulsifier and the microalgal components, resulting in a reinforced emulsion structure, which allows reducing the total oil content, yielding emulsions with the same rheological and sensory properties. The study was conducted by lowering the oil content from 65% to 50% at constant microalgal biomass concentration (2%). Emulsions were analysed in terms of colour, texture analysis, linear viscoelasticity, flow behaviour and droplet size distribution.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Pea protein isolate Pisane HD®, kindly provided by Cosucra Groupe Warcoing (Belgium), has the following characteristics (Pisane® technical information): protein content 90 ± 2%, ash 5 ± 1% (sodium 0.70%, calcium 0.15%, phosphorus 0.52%, magnesium 0.20%, potassium 0.40%), carbohydrates maximum 4.5%, fats (E.P. extract) maximum 0.5%, isoelectric point 4.5. Vegetable oil of commercial grade was used.

Kemin Foods (USA) kindly offered FloraGlo® which is crystalline lutein, derived from marigold flowers (*Tagetes erecta*), suspended in safflower oil. This product has the following quality specifications (FloraGlo® technical information): Lutein ≥ 20.0%, Zeaxanthin ≥ 0.86%, Moisture ≤ 1%, Ash ≤ 1%.

Phycocyanin was produced, extracted and purified from the cyanobacteria *Spirulina* (*Arthrospira*) *maxima*, by the Research Group of the Renewable Energy Department - INETI (Portugal), according to the method described by Reis (2001).

For microalgae biomass production see description in section 3.2.1.1.

4.2.2. Methods

4.2.2.1. Preparation of oil-in-water emulsions

Oil-in-water emulsions were prepared with 65% (w/w) vegetable oil, 32% (w/w) deionised water and 3% (w/w) pea protein isolate. The protein isolate was dispersed in water under magnetic stirring (30 min, at room temperature) and emulsification was carried out by gradual oil addition, using an Ultra Turrax T-25 homogenizer (IKA, Germany) at 13000 rpm, 3 min, as reported previously by Franco *et al.* (1998a).

Lutein and phycocyanin were dissolved in the aqueous and oil phases respectively. Emulsions containing various concentrations of lutein and phycocyanin (0.25-1.25% w/w) were prepared, as well as emulsions with mixtures of both pigments, in different proportions (0.50% w/w total pigment).

Emulsions with microalgal biomass were prepared with 0.25-2.00% (w/w) *Spirulina maxima* and *Chlorella vulgaris* (green and orange) and 0.05-2.00% (w/w) *Haematococcus pluvialis*. For the evaluation of microalgal fat mimetic effect, the emulsions were prepared with 3% pea protein isolate, 2% microalgal biomass and oil concentrations ranging from 50% up to 65%.

The emulsions were placed in cylindrical glass containers (60 mm diameter, 45 mm height) and stored at 4ºC for 24 h, before performing the tests.

4.2.2.2. Colour measurement

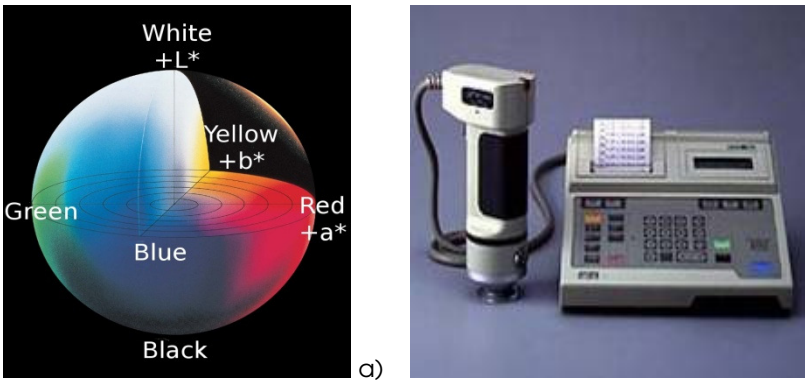
The tristimulus theory of colour vision states that retina cones are grouped into 3 types, each responding to a portion of the light spectrum, with peak responses corresponding to blue, green, and red light. The interaction of these groups is then responsible for the stimulus which is interpreted by the brain as colour, resulting that all colours are seen as mixtures of the three primary colours. Colorimeters are simple and easy to handle equipments based on reflectance measurements, which express colour numerically according to international standards. The Colorimeter includes a set of three photocells ("*Observer*") filtered to closely match the same colour sensitivity as the human eye (corresponding to the three retina cones). Colorimeters use a constant illumination method and constant light source, according to standardized illuminants, being D_{65} the most commonly-used corresponding to the average of noon daylight all over the world (CIE, 1978).

The emulsion colour was measured instrumentally using a Minolta CR-300 (Japan) tristimulus colorimeter with standard illuminant D_{65} and a visual angle of 2º (Figure 4.1.). The results were expressed in terms of the CIELAB uniform colour system (CIE, 1978). L^* corresponds to the lightness, ranging from dark/black (L^*=0%) to bright/white (L^*=100%). The chromaticity coordinates a^* and b^* indicate the colour direction, increasing the saturation from the dull/achromatic centre (a^*=0, b^*=0) to $+a^*$ (red), $-a^*$ (green), $+b^*$ (yellow), or $-b^*$ (blue).

The total colour difference between samples was calculated according to:

$$\Delta E = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2} \quad (4.3)$$

The measurements were conducted under the same light conditions, using a white standard (Y^*92.7, a^*-0.02, b^*1.72), at 20ºC, replicated 5 times (50 mm^2 measuring area per measurement) at weekly intervals until 6th week. The emulsions were kept refrigerated at 5ºC.

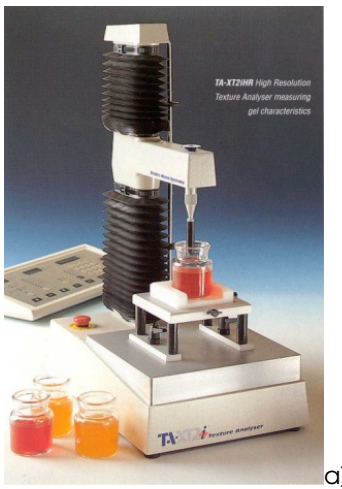


a) b)

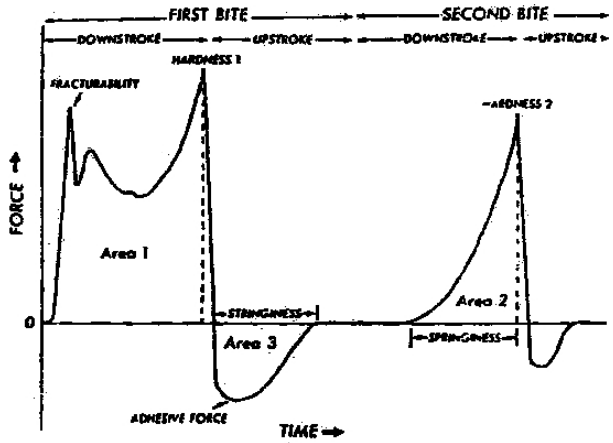
Figure 4.1. Representation of colour solid for CIELAB colour space (a); Colorimeter CR-300 Minolta (b).

4.2.2.3. Texture Profile Analysis

Food texture is an important quality attribute which can be defined as a group of physical characteristics deriving from the structural elements that constitute the food product (Bourne, 2002). These properties are sensorially detected (tact, vision, audition) and are related to the deformation, disintegration and flow of the food material when submitted to an acting force.
Some instrumental methods enable to determine food texture properties, with good correlation with sensory analysis tests. This can be economically advantageous, at an industrial level, considering the high costs associated to training and maintaining a sensory analysis panel.
The texturometer is composed by a dynamometer which provides mechanical energy to a probe (at a constant rate) that will contact or penetrate the sample causing a deformation. The response of the material is recorded in force *vs.* time or force *vs.* distance curves. These are empirical tests that depend upon the conditions used, namely probe type, velocity, distance, sample geometry.
In the present study, *"texture profile analysis"* (TPA) or *"two-bite"* tests were performed in a texturometer TA-XT2 (Stable Micro System, UK). In this type of test the probe penetrates twice in the material (with a waiting time between penetration cycles) to imitate the mouth action of two bites in the food (Szczesniak, 1963). From the resulting texturogram curves it is possible to determine a series of texture attributes, which are strongly correlated with sensory analysis (Figure 4.2.).



a)



b)

Figure 4.2. Texturometer (a). Typical force *vs.* time curves from TPA tests, with texture attributes (b).

The emulsions, placed in cylindrical glasses (60 mm diameter, 45 mm height), were punctured with a cylindrical probe of 38 mm diameter in a load cell of 5000 g (5 mm penetration, 2 mm/s crosshead speed, 3 s waiting time). Firmness was considered as the height of the force peak during the first compression cycle of the force *vs.* time curve (Figure 4.2), and was the

parameter with the best discriminating ability to compare emulsions (data not presented in this work). Other TPA parameters such adhesiveness, cohesiveness and springiness were also calculated, as indicated in Figure 4.2b.

4.2.2.4. Droplet Size Distribution

Droplet size distribution measurements were carried out by laser light scattering using a Malvern Mastersizer-X analyser (Malvern, UK) in the Fourier conformation. This technique is based in the measurement of the pattern of laser light scattering by the particles dispersed in a media, using a system of lenses. In this case, 45 mm lens was used, in order to detect oil droplets with 0.1-80 μm characteristic average sizes.

Aliquots of emulsions were carefully dispersed in deionized water, step by step, applying gentle agitation in order to disrupt droplet flocs. Values of the Sauter mean diameter, d_{32}, which is inversely proportional to the specific surface area of droplets, were obtained as follows (Sprow, 1967):

$$d_{3,2} = \frac{\sum n_i d_i^{\,3}}{\sum n_i d_i^{\,2}} \qquad (4.4)$$

where n_i is the number of droplets with a diameter d_i.

4.2.2.5. Rheological measurements

4.2.2.5.1. Rheometers

Rheometers are sophisticated equipments capable of measuring rheological properties, through controlled stress (torque) or controlled shear-rate devices which can act in stationary and oscillatory mode. The possibility of applying very low torque (or shear-rate), triggered by compressed air-bearing systems, enables to perform non-destructive tests under very low deformation levels (Mezger, 2002).

In the present work, oscillatory and steady-state flow measurements were performed in a controlled-stress rheometer (RS-75, Haake, Germany) while transient tests were carried out in a controlled-rate rheometer (ARES, Rheometrics Scientific, Germany).

Several cone-plate and plate-plate geometries were used in this study. The sample is placed between a fixed lower plate (coupled to a temperature control system) and a moveable cone or plate, associated to an axis and motor, which allows the rotational movement. The samples were covered with a layer of paraffin oil to prevent moisture loss.

Some rheological experiments were repeated, under the same conditions, in a controlled-stress rheometer coupled with an optical analysis system and video camera (RheoScope 1, Haake, Germany), in order to monitor the structural modifications that occur during the tests. For these measurements, a mirror cone-glass plate (70 mm diameter, 1º angle) geometry was used.

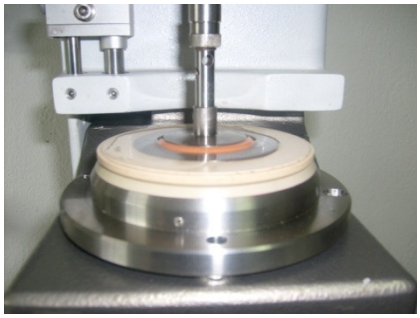

a)

b)

Figure 4.3. Controlled-stress rheometer from Haake (a); and respective cone-plane geometry system measuring an emulsion sample (b).

4.2.2.5.2. SAOS tests

Dynamic tests were performed using a cone and plate sensor system (35 mm diameter, 2º angle). The linear viscoelastic region was previously determined by stress sweep tests (0.45 – 2000 Pa) at 1 Hz oscillation frequency. Frequency sweep tests were ranged from 0.00628 to 628 rad/s and performed at constant stress values, comprised in the linear viscoelastic region for each emulsion (4-20 Pa). The *"plateau"* modulus, G^0_N, was estimated as the value of G' for the minimum value of the loss tangent ($tan\delta = G''/G'$) (Wu, 1989):

$$G_N^o = [G']_{tan\,\delta \to minimum} \tag{4.5}$$

4.2.2.5.3. Steady-state flow tests

For steady-state flow measurements, a serrated parallel plate sensor system was used (20 mm diameter), in order to overcome slip effects (Franco *et al.*, 1998b). Viscosity versus shear stress curves were performed using a logarithmic stepped ramp of stresses increasing in 30 min from 0.1 to 2000 Pa. The Carreau model (1972) was adjusted to the flow curves:

$$\eta = \frac{\eta_0}{\left[1 + \left(\frac{\dot{\gamma}}{\dot{\gamma}_c}\right)^2\right]^s} \tag{4.6}$$

where η_0 is the limiting viscosity for the first Newtonian region, $\dot{\gamma}_c$ is the critical shear rate for the onset of the shear-thinning behaviour and *s* is a parameter related to the slope of this region.

The relative deviation of the Cox-Merz rule (1958), correlating steady shear flow viscosity and dynamic complex viscosity data, was also calculated as follows:

$$\eta_{rel} = \left. \frac{\eta^* - \eta}{\eta^*} \right|_{\dot{\gamma}=\omega} \tag{4.7}$$

4.2.2.5.4. Transient shear flow tests

Transient shear flow (stress growth) measurements were performed, using serrated plate-plate geometry (25 mm diameter), applying constant shear rates (0.01, 0.1, 1 and 10 s^{-1}) for 1800 s.

Stress growth curves were fitted with the Leider-Bird model (1974) in its generalized form (Navarro *et al.*, 1999) with three exponential terms:

$$\tau = \tau_\infty \left[1 + \left(b\dot{\gamma}t - 1 \right) \sum_{j=1}^{3} w_j e^{\left(-t/\lambda_j\right)} \right] \tag{4.8}$$

being τ_∞ the equilibrium shear stress, *b* a fitting parameter related to the absolute values of transient stress, w_j fitting parameters which assign relative weights to the exponential terms and λ_j time constants.

The time for the overshoot (t_{max}) was deduced by deriving equation (4.8) with respect to time and making this derivative equal to zero. The value of the stress overshoot was then obtained by substituting t_{max} in equation (4.8). The amount of overshoot (relative peak height) is a parameter related to the viscoelastic response and the degree of structural rupture occurring in the system, defined as (Navarro *et al.*, 1999):

$$S^+ = \frac{\tau_{\max} - \tau_\infty}{\tau_\infty} \tag{4.9}$$

4.2.2.6. Fluorescence microscopy

Emulsions with 2% microalgal biomass addition were analysed by fluoresce microscopy using an Olympus BX61 optical microscope in epifluorescence mode with 10x (UPlanApo)

objectives. A Mercury arc lamp was used as excitation source and DAPI, TRITC, FITC fluorescence filter sets were used to select different excitation and emission wavelengths. Both microalgae and pea protein isolate presented autofluorescence.

4.2.2.7. Statistical analysis

All tests were replicated at least three times and performed at 20ºC. Statistical analysis, ANOVA Post-Hoc Comparisons – Scheffé Test, was performed using the Software STATISTICA (Version 6.0, Statsoft Inc., USA). The significance level was set at 95%.

4.3. RESULTS AND DISCUSSION

4.3.1. Emulsions coloured with lutein and phycocyanin

4.3.1.1. Colour stability

Lutein and phycocyanin imparted appealing and innovative colourations to food emulsions, as can be observed in Figures 4.4-4.6.

Lutein emulsions presented orange tonalities, as a result of positive *a** and *b** values, *i.e.* in the red and yellow domain, respectively (Figure 4.4). A linear increase in *a** values with lutein concentration was observed (R^2>0.97). Emulsions with 0.25% showed slightly negative *a** values (-2.3), increasing up to 8.1. at 1.25% lutein. Regarding the yellow tonality, adding only 0.25% lutein yields a high *b** value of 46.9. This corresponds to a high colour saturation, so upon further increasing lutein concentration from 0.5% to 1.25% no significant differences (p<0.05) were observed in *b** (49.0-49.9).

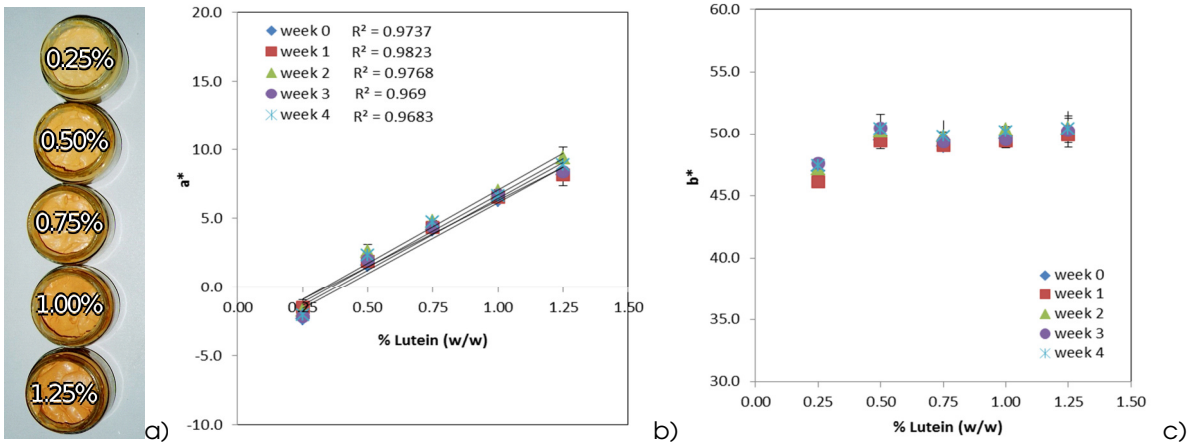
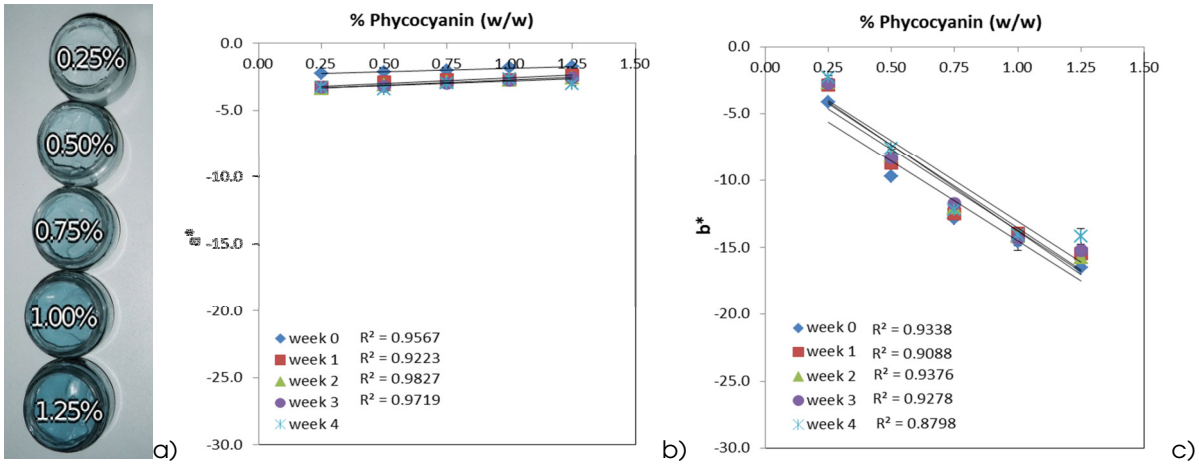


Figure 4.4. Emulsions coloured with different lutein concentration (a); evolution of chromatic coordinates *a** (b), and *b** (c) along time.

Regarding phycocyanin emulsions (Figure 4.5), intense blue colourations were obtained, expressed by dominant negative b* values and small contribution of *a** component to overall tonality. In fact, *a** ranged from -2.3 to -1.8, slightly increasing to a maximum value of -3.4 upon time. The component *b** linearly increased (in modulus) from -4.1 to -16.5 (week 0) with phycocyanin concentration, although R^2 decreased from 0.93 to 0.88 with time.

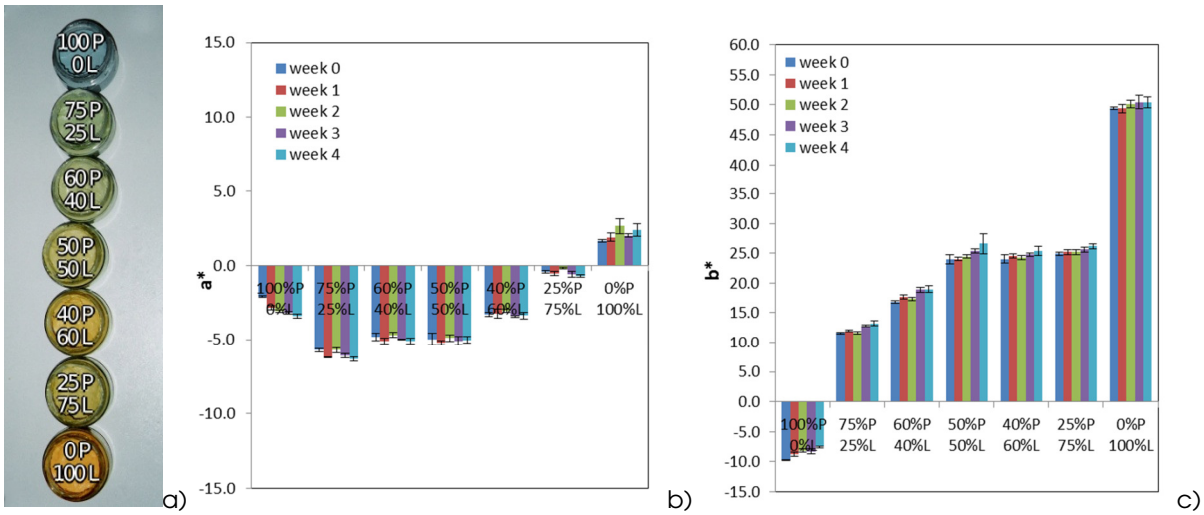
When using both lutein and phycocyanin, in different proportions, a range of greenish tonalities were obtained, as can be observed in Figure 4.6. Consequently, all emulsions showed negative *a** values (except 100% lutein emulsion), which increased from -0.4 to -5.8 with increasing lutein proportion. Regarding *b** values, all emulsions (except 100% phycocyanin emulsion) showed positive values, *i.e.* in the yellow domain. Lutein proportions

of 25% and 40% yielded *b** values around 12 and 17, respectively, whereas lutein proportions between 50% and 75% resulted in similar yellow tonalities (*b**: 24.0-24.9), which is in accordance with *b** results for the lutein emulsion (Figure 4.4b).



a) b) c)

Figure 4.5. Emulsions coloured with different phycocyanin concentration (a); evolution of chromatic coordinates *a** (b), and *b** (c) along time.



a) b) c)

Figure 4.6. Emulsions coloured with different proportions of lutein (L) and phycocyanin (P), with 0.5% total pigment concentration (a); evolution of chromatic coordinates *a** (b), and *b** (c) along time.

In terms of luminosity (results not shown), a linear decrease (R^2>0.94) was observed on *L** with pigment concentration, *i.e.* the emulsions became darker, with values ranging from 85.1 (25% lutein) to 68.2 (1.25% phycocyanin). The emulsions tended to become slightly darker with time (*L** decrease).

The stability of the emulsion colour along time can be measured through the total colour difference parameter (*ΔE**) presented in Table 4.1. For all the emulsions studied, *ΔE**<3, when comparing the colour presented during time with the initial colour for each emulsion. Although the visual perception of colour difference changes with the location of colour within the CIELAB space and with the direction of the difference, a mean value of *ΔE** can be assumed as a sensible basis for perceptible colour differences between two samples. Some

authors defend that this threshold value corresponds to $\Delta E^*<1$ (Gonnet, 1998) while others consider that the human eye is only able to distinguish among colours for $\Delta E^*>5$ (Castellar *et al.*, 2006). Francis and Clydesdale (1975) stated that total colour differences bellow 1 are not obvious for the human eye, but for $1<\Delta E^*<3$ values colour differences are not appreciative, being $\Delta E^*>3$ the threshold value for obvious colour differences.

Table 4.1. Total colour difference (ΔE^*) of emulsions with lutein (L), phycocyanin (P), and both pigments in different proportions (total pigment concentration: 0.50% w/w), in respect to the initial colour (week 0)

	ΔE^*			
	week 1	**week 2**	**week 3**	**week 4**
0.25% L	1.77	0.67	1.50	1.73
0.50% L	0.92	1.24	2.25	2.30
0.75% L	0.69	1.08	1.69	2.97
1.00% L	0.87	1.20	2.01	1.93
1.25% L	0.37	1.40	1.84	2.33
0.25% P	2.39	2.58	2.27	2.45
0.50% P	1.82	2.33	2.46	2.83
0.75% P	1.06	1.75	1.92	2.17
1.00% P	1.65	1.65	1.72	1.65
1.25% P	1.56	1.72	1.75	2.75
100P:0L	1.82	2.33	2.46	2.83
75P:25L	0.70	0.14	1.29	1.91
60P:40L	0.97	0.49	2.15	2.40
50P:50L	0.37	0.59	1.52	2.77
40P:60L	0.75	0.41	1.18	1.68
25P:75L	0.84	0.46	1.01	1.68
0P:100L	0.92	1.23	2.25	2.30

4.3.1.2. Texture analysis

Texture profile analysis results are presented in terms of firmness, since this parameter showed better discriminating ability between samples. Other TPA parameters such adhesiveness, cohesiveness and springiness were also calculated, as indicated in Figure 4.2b.

For each texture parameter determined, a significant ($p<0.05$) alteration upon lutein addition was observed, while there were no differences between emulsions with varying lutein concentrations. Firmness decreased from 0.652 N down to 0.354-0.380 N (Figure 4.7a), adhesiveness from 1.225 –N.s down to 0.369-0.538 –N.s, cohesiveness from 0.887 down to 0.729-0.753, while springiness increased from 0.904 up to 0.959-0.971.

Phycocyanin emulsions presented a significant ($p<0.05$) linear ($R^2=0.9021$) firmness increase with pigment concentration (Figure 4.7b). For the remaining texture parameters studied (adhesiveness, cohesiveness and springiness), no significant differences were found between the results of the emulsions at different phycocyanin concentrations.

Firmness also increased with phycocyanin proportion in emulsions containing both pigments (Figure 4.7c). However, no significant ($p<0.05$) differences were found between the firmness values of the lutein-free emulsion and the 75P:25L and 60P:40L emulsions. For Adhesiveness there were only significant differences ($p<0.05$) between the 100P:0L (2.228 –N.s) and 0P:100L (0.538 –N.s). No significant differences ($p<0.05$) were found between cohesiveness results and springiness decreased with phycocyanin proportion.

Texture results are in agreement with the rheological results further discussed.

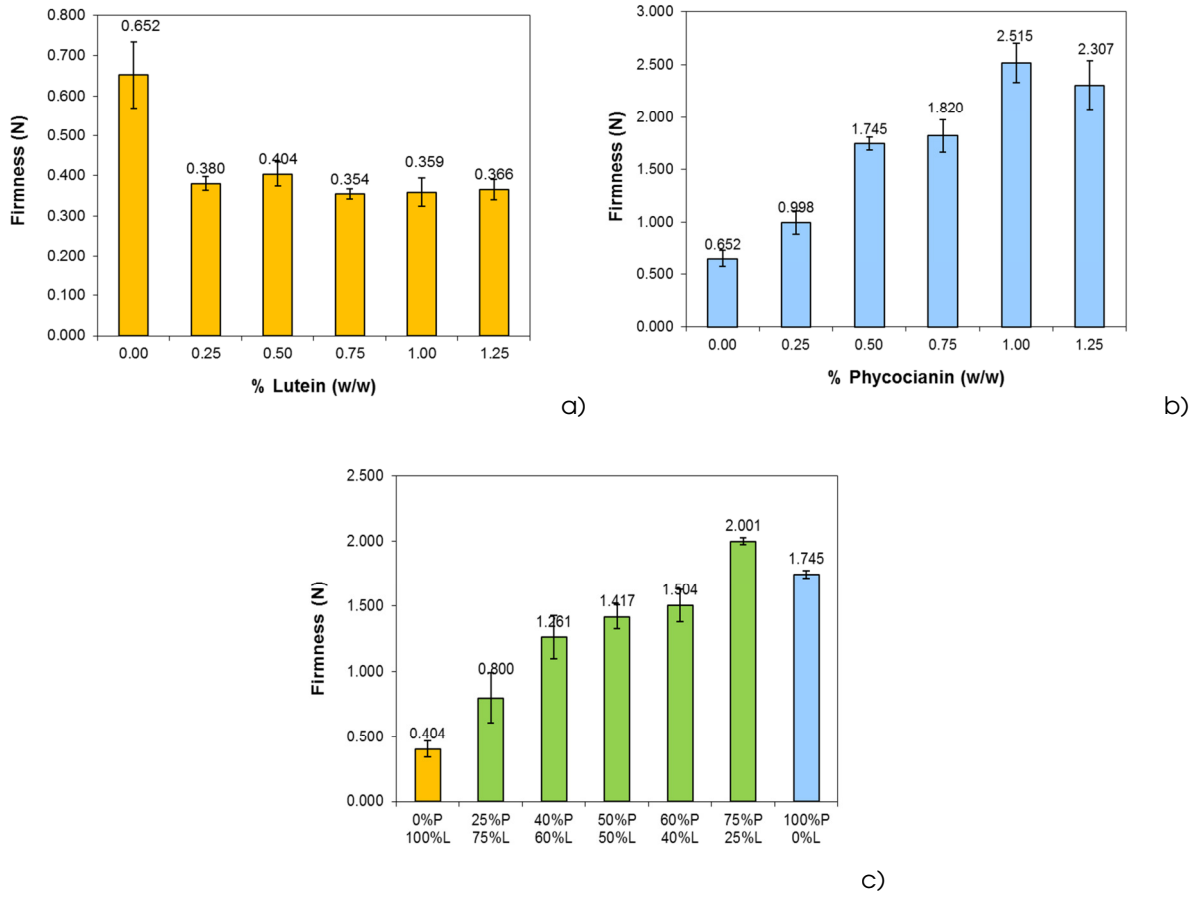


Figure 4.7. Firmness of pea protein stabilized o/w emulsions, at different lutein (a) and phycocyanin (b) concentrations; as well as with different proportions of both pigments (total pigment concentration: 0.50% w/w) (c).

4.3.1.3. Droplet size distribution

Droplet size distribution curves for selected emulsions are presented in Figure 4.8a. A similar behaviour was observed for all samples, denoting a bimodal distribution of droplet sizes.

For the control emulsion (without pigment addition) a Sauter mean diameter (d_{32}) of 2.23 μm was determined. The addition of lutein resulted in slightly higher droplet sizes, except for the emulsion with 1.25% lutein (Figure 4.8.b).

The addition of phycocyanin promoted a significant (p<0.05) increase on the d_{32} values, except for the emulsion containing 0.75% pigment. Figure 4.8c shows the evolution of d_{32} values with phycocyanin concentration, which can be described by a polynomial equation (d_{32}=9.0(phyc)^2+11.5(phyc)+5.8; R^2=0.9817) with a minimum for 0.75% pigment. Increasing the phycocyanin content from 0.25 up to 0.75% caused a reduction in d_{32} values, while for higher phycocyanin levels (1.00-1.25%) an increase on d_{32} values was observed.

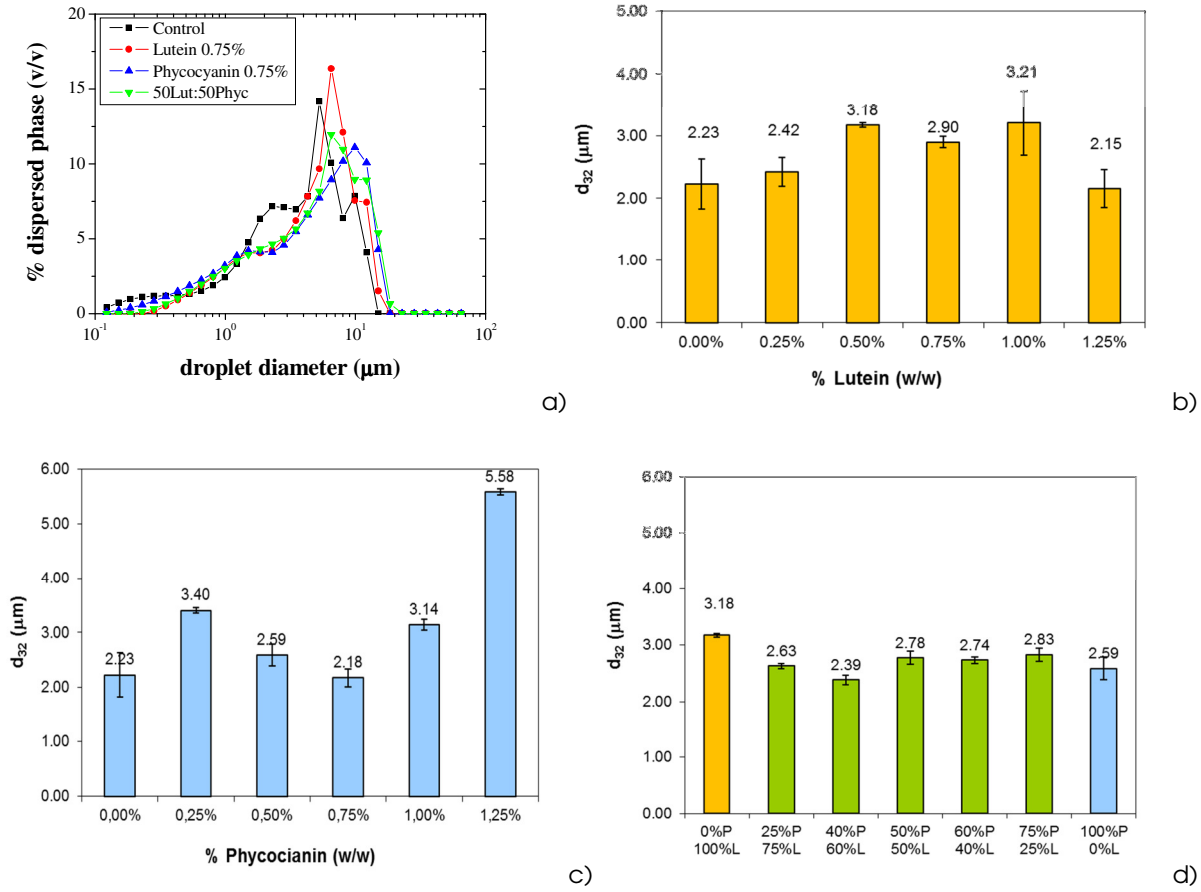


Figure 4.8. Droplet size distribution curves (a) and respective Sauter mean diameter of o/w emulsions with different lutein (b) or phycocyanin (c) concentrations, and different proportions of both pigments (total pigment concentration: 0.50% w/w) (d).

In the case of emulsions containing both pigments, the phycocyanin-free emulsion, which showed much lower texture properties, presented significantly (p<0.05) higher d_{32} values, while the other emulsions presented similar but smaller d_{32} values (Figure 4.8d). This fact evidences an important role played by phycocyanin on stabilising these o/w emulsions.

4.3.1.4. Linear viscoelastic behaviour

The linear viscoelastic regions of some selected emulsions obtained from stress sweep tests are presented in Figures 4.9 and 4.10. Microscope photographs, retrieved by the RheoScope® equipment, represent emulsion microstructure changes occurring at different strain values during the test.

The lutein and control emulsions (Figure 4.9) presented a similar behaviour: a gradual decrease on the storage modulus (G') values after a certain critical strain was exceeded.

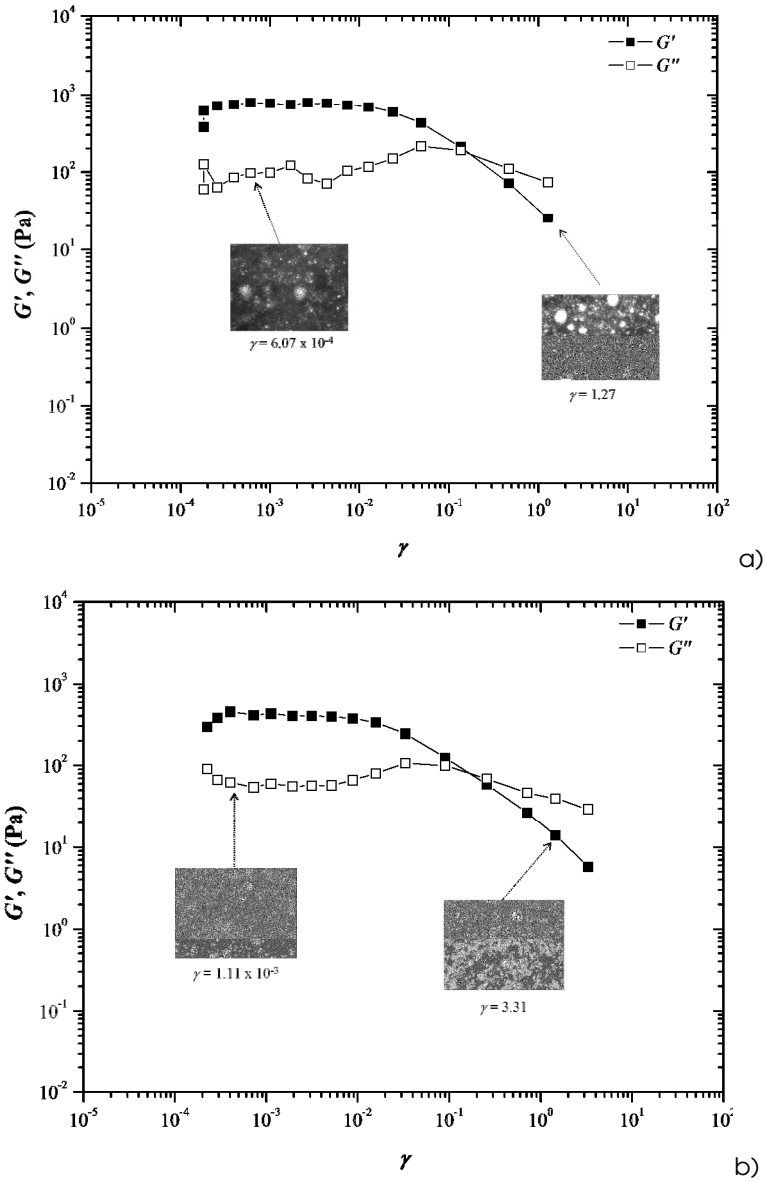
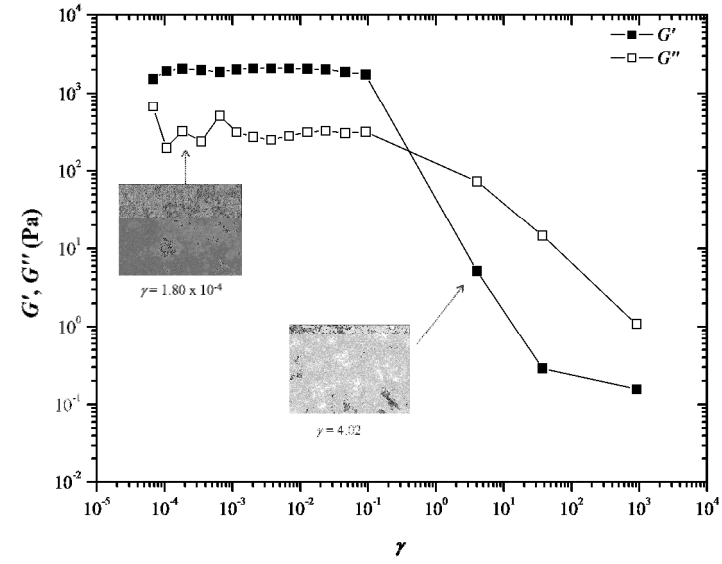
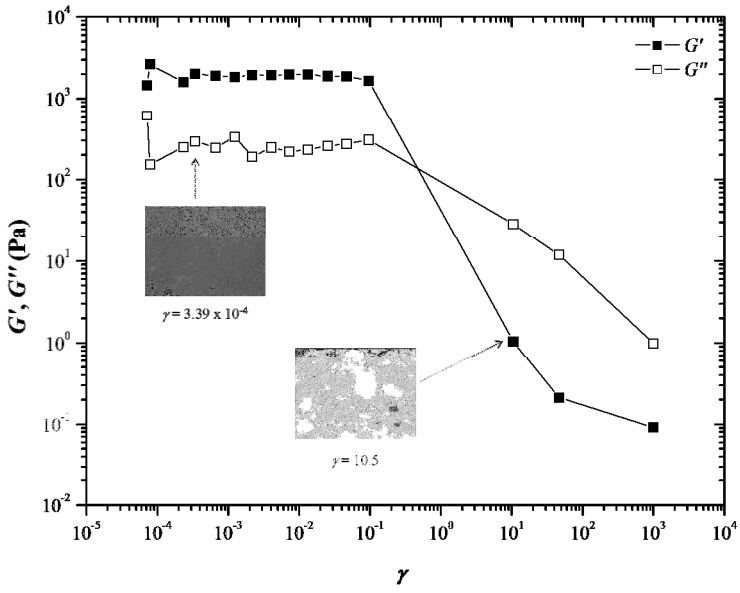


Figure 4.9. Linear viscoelastic range of o/w pea protein emulsions without pigment addition (a), and with 0.75% lutein (b). The microscope photographs retrieved by the RheoScope® equipment represent the emulsions' microstructure changes occurring at different shear values during the test.

For the emulsion containing 0.75% phycocyanin and both types of pigments in the same proportion (0.5% total content) (Figure 4.10), the linear viscoelastic range is more extended ($\gamma_c \approx 0.10$) and values of both the storage and loss moduli (G', G'') are higher. However, starting from this critical strain value, a steep decrease of these functions is observed, more sharply for emulsions containing phycocyanin. Moreover, from the comparison of the microscope images corresponding to strain values of the same order of magnitude ($\gamma \approx 1\text{-}10$), it is possible to observe a higher structural breakdown for both phycocyanin and blended emulsions, in which an alteration of the flocculated state is rather apparent outside the linear viscoelastic regime. On the contrary, for both the lutein and control emulsions, shear-induced structural modifications are less pronounced by applying the same stress programme.



a)



b)

Figure 4.10. Linear viscoelastic range of o/w pea protein emulsions with 0.75% phycocyanin (a) and 0.50% total pigment (lutein and phycocyanin in the same proportion) (b). The microscope photographs retrieved by the RheoScope® equipment represent the emulsions' microstructure changes occurring at different shear values during the test.

Figures 4.11-4.13 represent the evolution of storage (*G'*) and loss (G") moduli with frequency, inside the linear viscoelastic region, for these emulsions (mechanical spectra). For each sample *G'* was always higher than G" within the experimental frequency range. Hence the emulsions presented a predominantly elastic response, showing a plateau region with a minimum in G" and a slight frequency dependence of *G'*. This behaviour is typical of protein-stabilised emulsions in which an elastic network develops due to the occurrence of an extensive bridging flocculation process (Pal, 1995; Franco *et* al., 1998a; Raymundo *et al.*, 2002). The development of the plateau region has been previously related to the formation of physical entanglements among macromolecules adsorbed and non-adsorbed at the oil/water interface of the oil droplets, yielding a structural network (Franco *et al.*, 1995a). The

plateau modulus (G^0_N) is a viscoelastic parameter defined for polymers as the extrapolation of the entanglement contribution to the viscoelastic functions at high frequencies (Baumgaertel *et al.*, 1992). This parameter can be considered as a characteristic parameter of this region and may be easily estimated from the minimum in the loss tangent ($tan\ \delta = G''/G'$) as indicated in eq. 4.5.

The viscoelastic properties of emulsions with lutein were significantly (p<0.05) lower than those evidenced by the control emulsion, *i.e.*, without lutein addition (Figure 4.11). However, there were no significant differences (p<0.05) in the G^0_N values of the emulsions with different contents of added lutein. This means that the addition of lutein weakens the emulsions network but this effect is independent on pigment concentration, within the range tested.

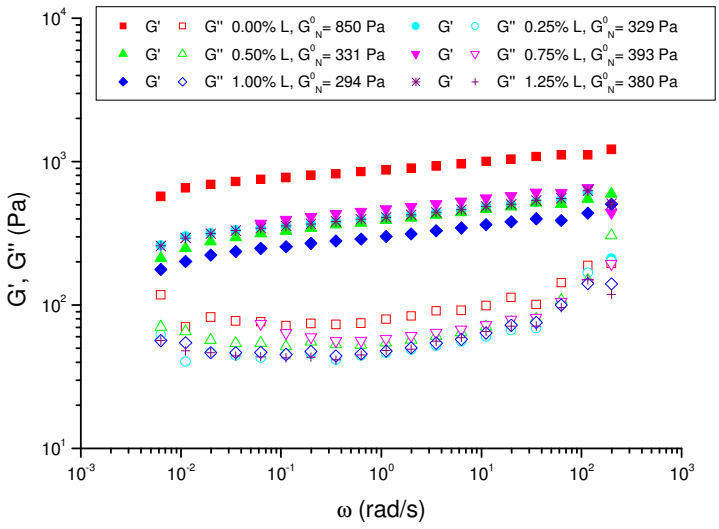


Figure 4.11. Mechanical spectra for o/w emulsions with different lutein (L) concentrations, and respective plateau modulus (G^0_N).

Phycocyanin emulsions presented significantly (p<0.05) higher values of viscoelastic functions (G', G'' and G^0_N) than those found for the control emulsion, indicating a more developed three-dimensional structure. The plateau modulus (G^0_N) increased continuously and significantly (p<0.05) with phycocyanin concentration (Figure 4.12).

For the emulsions containing both pigments, a significant (p<0.05) increase on G^0_N with phycocyanin proportion can be observed (Figure 4.13). However, it was also observed that emulsions with higher phycocyanin and lower lutein concentrations (75P:25L and 60P:40L) presented higher G^0_N values than the lutein-free emulsion (100P:0L). This fact evidences a surprising synergistic effect between both pigments, resulting in a reinforcement of the emulsion network. Finally, the phycocyanin-free emulsion (0P:100L) presented a much smaller G^0_N value than the rest of emulsions containing this pigment.

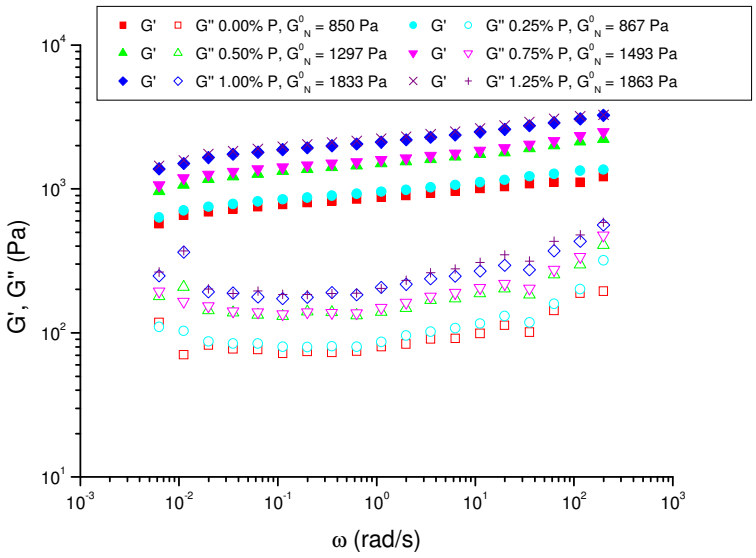


Figure 4.12. Mechanical spectra for o/w emulsions at different phycocyanin (P) concentrations, and respective plateau modulus (G^0_N).

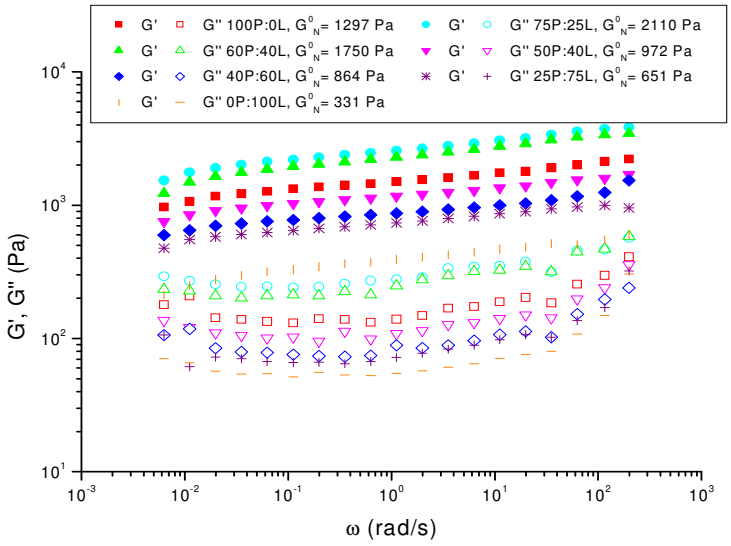


Figure 4.13. Mechanical spectra for o/w emulsions with different phycocyanin (P) and lutein (L) proportions (total pigment concentration: 0.50% w/w) and respective plateau modulus (G^0_N).

The microscopic images of the emulsions, taken during SAOS measurements, did not present significant changes because these tests were performed within the linear viscoelastic range of stresses, corresponding to the *quasi*-undisturbed emulsion microstructure. Thus, these photographs, taken along the whole frequency range studied, are very similar to the photographs presented on Figures 4.9 and 4.10 at low strain values before the onset of the non-linear viscoelastic region.

4.3.1.5. Steady-state flow behaviour

All the emulsions studied showed similar flow curves under steady-state shear conditions presenting a strong shear-thinning behaviour (Figures 4.14-4.16). Shear-thinning is a consequence of a dramatic shear-induced structural breakdown, related to a mechanism of oil droplet deflocculation, as previously reported for other protein-stabilized concentrated food emulsions (Franco *et al.*, 1998a; 2000).

Lutein emulsion steady-state flow curves (Figure 4.14) presented a shear-thinning behaviour with a zero-shear rate limiting-viscosity (η_0) at very low shear rates ($\dot{\gamma}$). The lutein-free emulsion (control) presented η_0 values almost one order of magnitude higher than those found in lutein emulsions, evidencing a negative effect of this pigment addition in the emulsion microstructure, which is in agreement with the results obtained from SAOS measurements. A significant decrease in the Carreau parameter *"s"* for lutein emulsions (0.38-0.40) was also observed, when compared to the control (0.47), which is related to the viscous nature of these emulsions, since the s parameter is a measure of the shear-thinning character of the material.

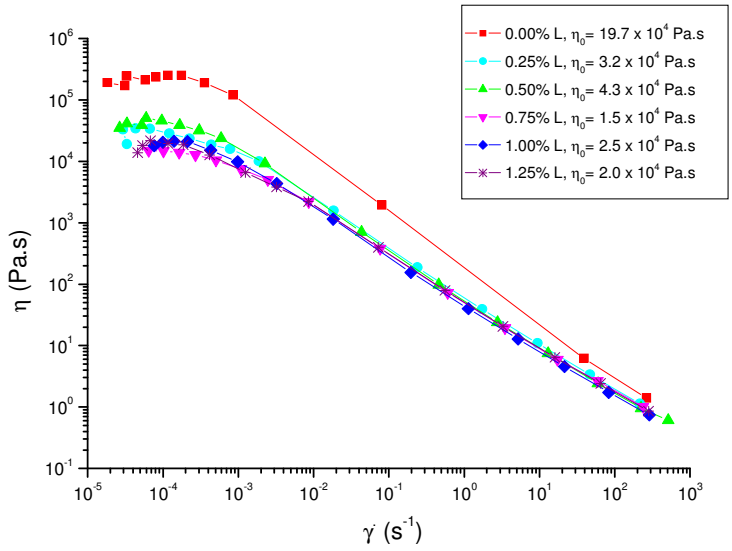


Figure 4.14. Steady-state flow curves for o/w emulsions with different lutein (L) concentrations, and respective limiting viscosity (η_0).

For phycocyanin emulsions (Figure 4.15) a significant (p<0.05) linear increase of the η_0 values with phycocyanin content was observed, while for the *"s"* and $\dot{\gamma}_c$ parameters of the Carreau equation, no significant differences were found.

For emulsions containing both pigments (Figure 4.16), the η_0 values increased with phycocyanin proportion and yielded higher values for the mixtures with low lutein proportions (under 50%) than for the lutein-free emulsion. This tendency was also observed for the *s*

parameter, which increased from 0.43 up to 0.48 with phycocyanin proportion (P25:L75 to P75:L25), and presenting 0.40 and 0.46 for the phycocyanin- and lutein-free emulsions, respectively.

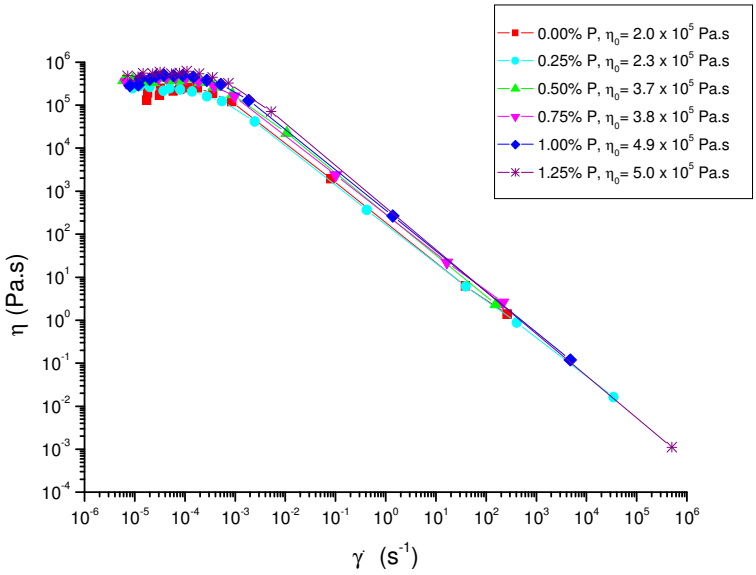


Figure 4.15. Steady-state flow curves for o/w emulsions at different phycocyanin (P) concentrations, and respective limiting viscosity (η_0).

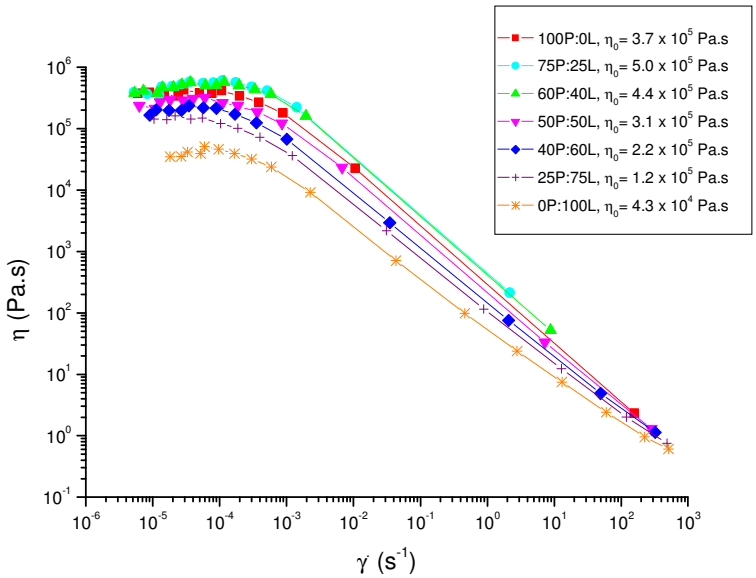


Figure 4.16. Steady-state flow curves for o/w emulsions at different phycocyanin (P) and lutein (L) proportions (total pigment concentration: 0.50% w/w) and respective limiting viscosity (η_0).

The evolution of emulsions microstructure along the Newtonian and shear-thinning region can be observed by the microscope photographs shown in Figures 4.17 and 4.18 as a function of the stress applied. At low stress values these emulsions exhibit a constant high viscosity value ($\eta_0 \approx 10^5$ Pa.s) that suddenly falls several orders of magnitude (Roberts *et al.*, 2001).

From Figure 4.17 a progressive shear-induced deflocculating process can be observed, for both the control and 0.75% lutein emulsions, by visualising different concentric flow regions, especially for high shear rates.

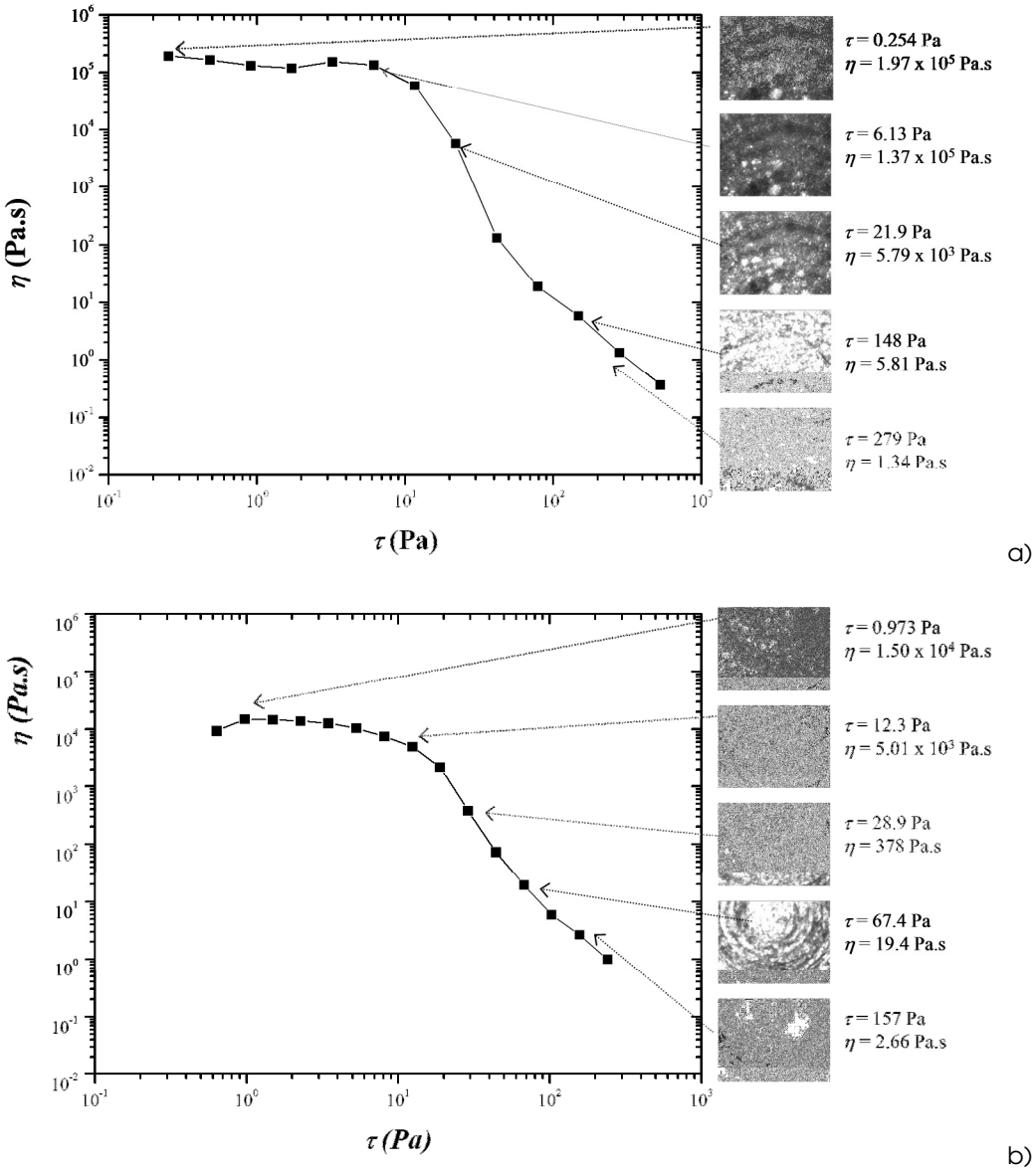


Figure 4.17. Steady-state flow curves of o/w pea protein emulsions without pigment addition (a), and with 0.75% lutein (b). The microscope photographs retrieved by the RheoScope® equipment, represent the emulsions' microstructure changes occurring at different stress values during the test.

The emulsions containing phycocyanin presented a wider plateau region (Figure 4.18). The viscosity decrease at high stress values is much sharper, as a consequence of a dramatic structural breakdown after a critical stress. The same qualitative behaviour was observed for the emulsion containing both types of pigments. In spite of this structural breakdown, phycocyanin-based emulsions presented higher viscosity values than the control emulsion for the shear stress values applied. As may be seen in the microscope photographs (Figure 4.18), the microstructure of these two emulsions is not significantly altered previously to the viscosity

decrease. However, a structural modification can be observed afterwards. In addition to this, some zones which are not occupied by the sample, appear at very high shear stresses, which are attributed to the beginning of the expulsion of the sample from the gap. These last points and the corresponding micrographs have been maintained in Figure 4.18, in order to illustrate the phenomenon of fracture and subsequent expulsion of the sample from the gap although it is evident that no physical interpretation may be deduced from that situation.

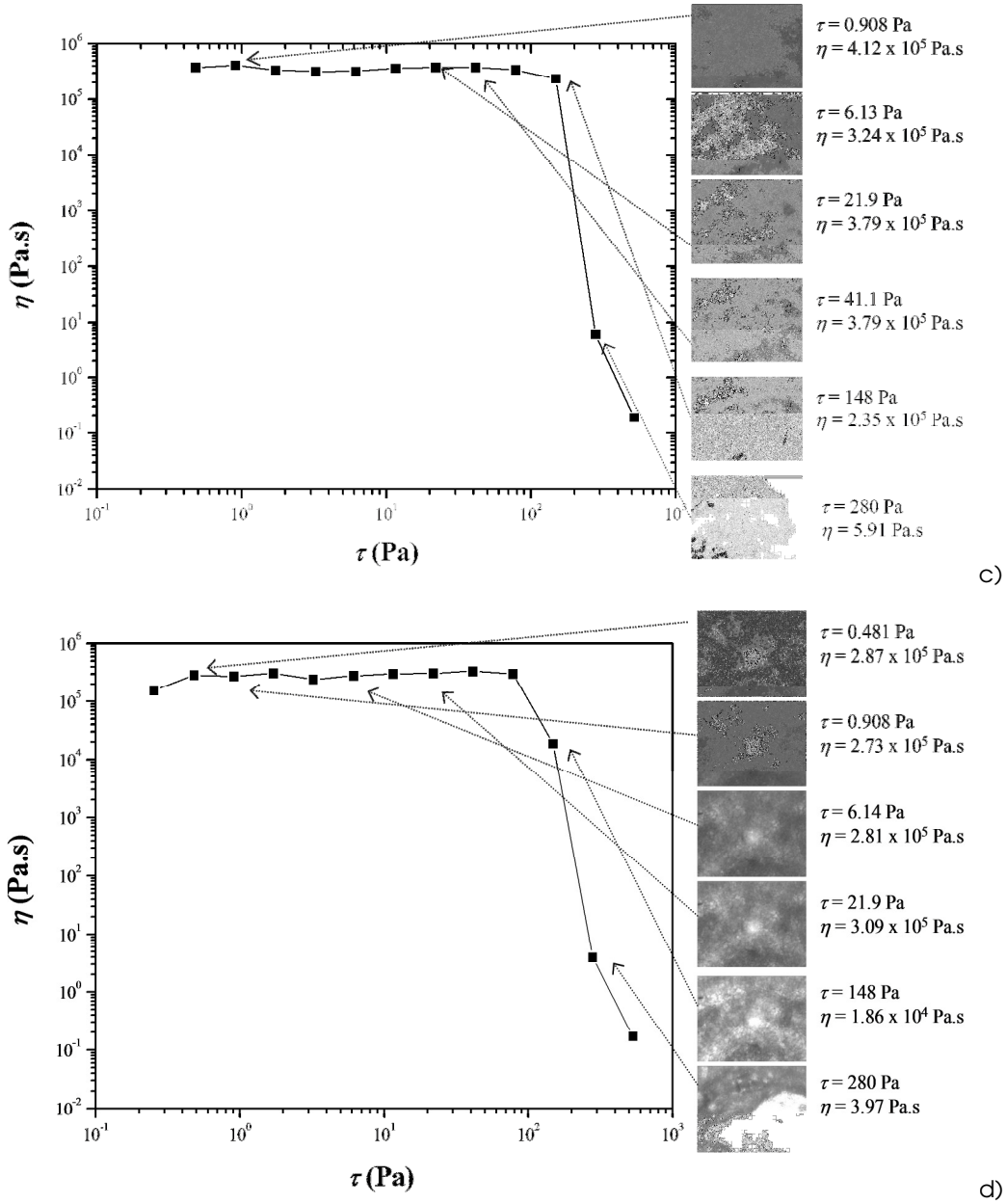


Figure 4.18. Steady-state flow curves of o/w pea protein emulsions with 0.75% phycocyanin (a) and 0.50% total pigment (lutein and phycocyanin in the same proportion) (b). The microscope photographs retrieved by the RheoScope® equipment, represent the emulsions' microstructure changes occurring at different stress values during the test.

Steady shear flow viscosity data can be related to the dynamic complex viscosity (η^*), through the empirical Cox-Merz rule (1958) which states that the complex viscosity is equal to

the steady shear viscosity when the shear rate and frequency are equal. This correlation has been confirmed experimentally for several synthetic polymers (Ferry, 1980) and polysaccharide solutions (Lopes-da-Silva and Rao, 1992), but a deviation of the Cox-Merz rule has been usually observed in food products (Bistany and Kokini, 1983; Rao and Cooley, 1992; Riscardo *et al.*, 2005).

Figure 4.19 presents both steady-state and dynamic viscosity results for the emulsions under study. It was observed that complex viscosity values were always higher than steady shear viscosity, with a very similar power-law decrease in both types of measurements, as previously reported for other emulsions (Riscardo *et al.*, 2005), excepting for the lutein emulsion. Considering this fact, the relative deviation of the Cox-Merz rule (η_{rel}) was obtained, independently of the frequency or shear rate, according to eq. 4.7.

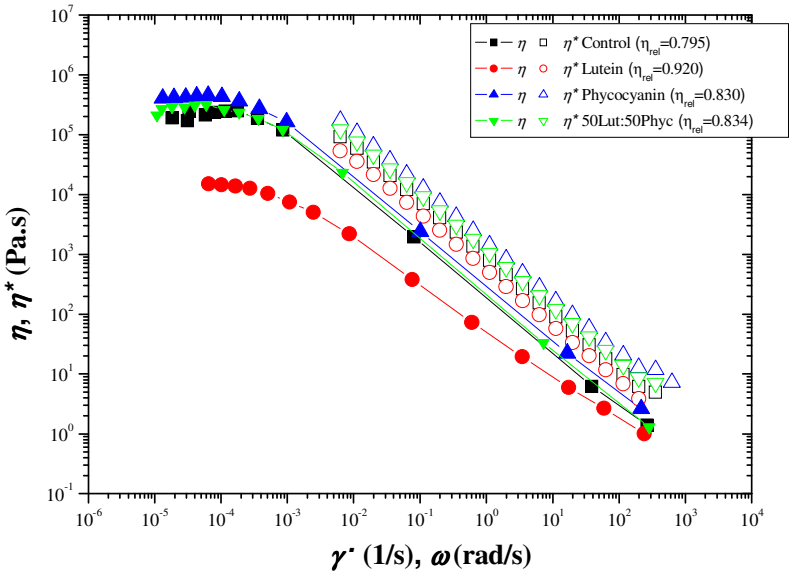


Figure 4.19. Comparison between steady-state and complex viscosities and relative deviation of the Cox-Merz rule for o/w pea protein emulsions without pigment addition (control), with 0.75% lutein, 0.75% phycocyanin and 0.50% total pigment (lutein and phycocyanin in the same proportion) addition.

The emulsions containing phycocyanin and pigment blend showed higher (p<0.05) η_{rel} values (0.83) than the control emulsion (0.795). As above mentioned, for the lutein emulsion, the potential decrease on the $\eta = f(\dot{\gamma})$ and $\eta^* = f(\omega)$ curves was different, so the η_{rel} values varied between 0.87 and 0.95, being presented on Figure 4.19 an average value. In any case, the Cox-Merz deviation was always higher than those observed for the rest of emulsions.

The deviation from Cox-Merz rule is attributed to a shear-induced structural breakdown. By definition, applied strain is much lower in SAOS measurements, and high enough in shear flow test to break structured inter- and intramolecular associations (Mills and Kokini, 1984; Franco *et al.*, 1995a; Gunasekaran and Ak, 2000). According to this, we can consider that the shear

induced structural breakdown in pea protein-stabilized emulsions increased with pigment incorporation, particularly for lutein.

4.3.1.6. Transient flow

Emulsion transient flow behaviour was studied by stress growth experiments, in which a constant shear rate was suddenly imposed on a viscoelastic fluid held previously at rest (*"start-up flow"*) (Steffe, 1996). The evolution of the emulsion transient stress during these tests, at different shear rate values (0.01, 0.1, 1 and 10 s^{-1}), can be observed in Figure 4.20.

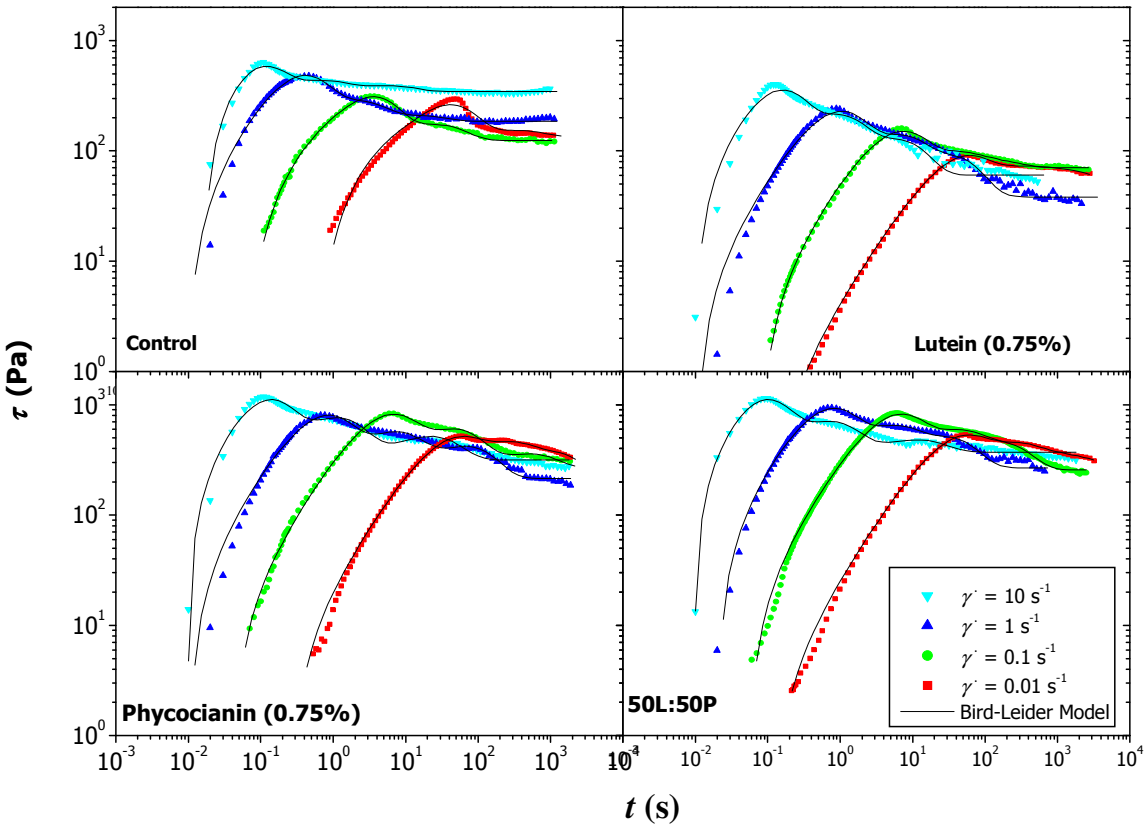


Figure 4.20. Stress growth experiments at constant shear rates (0.01, 0.1, 1 and 10 s-1) and Leider-Bird model fitting for o/w pea protein emulsions without pigment addition (control), with 0.75% lutein, with 0.75% phycocyanin and 0.50% total pigment (lutein and phycocyanin in the same proportion) addition.

All the emulsions studied presented a characteristic viscoelastic behaviour with two distinct regions in the transient flow curves, as it has been observed in other food emulsions (Franco *et* al., 1995a; Campanella and Peleg, 1987; Partal *et al.*, 1999). At relatively short times, the onset of shear originates a sudden stress overshoot ($\tau_{overshoot}$) that corresponds to the viscoelastic response of the material. After reaching this maximum, the shear stress decays to an almost constant (steady-state) equilibrium value (τ_∞) at long times, as a result of time-dependent shear-induced structural modifications (Navarro *et al.*, 1999). From the curves on

Figure 4.20 it can be clearly observed that characteristic times and stresses are dependent on the applied shear rate and emulsion composition.

The Leider-Bird model (1974) has shown to be adequate in describing the transient behaviour of several food products (Kokini and Dickie, 1981; Dickie and Kokini, 1982), and it was used to fit the emulsion stress growth curves in its generalised form (Navarro *et al.*, 1999) with three exponential terms (eq. 4.8).

The Leider-Bird equation fitted the experimental results quite well (Figure 4.20), with determination coefficients (R^2) greater than 0.97 and reduced qui-square values (χ^2) smaller than 10 for all the curves analysed.

Figure 4.21 collects the most relevant parameters of stress-growth curves deduced from equation (4.8). τ_∞ represents emulsion equilibrium shear stress values, obtained at different shear rates. Emulsions containing phycocyanin (isolated and blended) showed higher values than the control emulsion; contrary to that found in lutein-based emulsions, which is in concordance with the results previously shown. As may be observed, almost constant values of τ_∞ were attained in all cases, since the shear rate range corresponds to the drastic viscosity decrease seen in Figures 4.17 and 4.18.

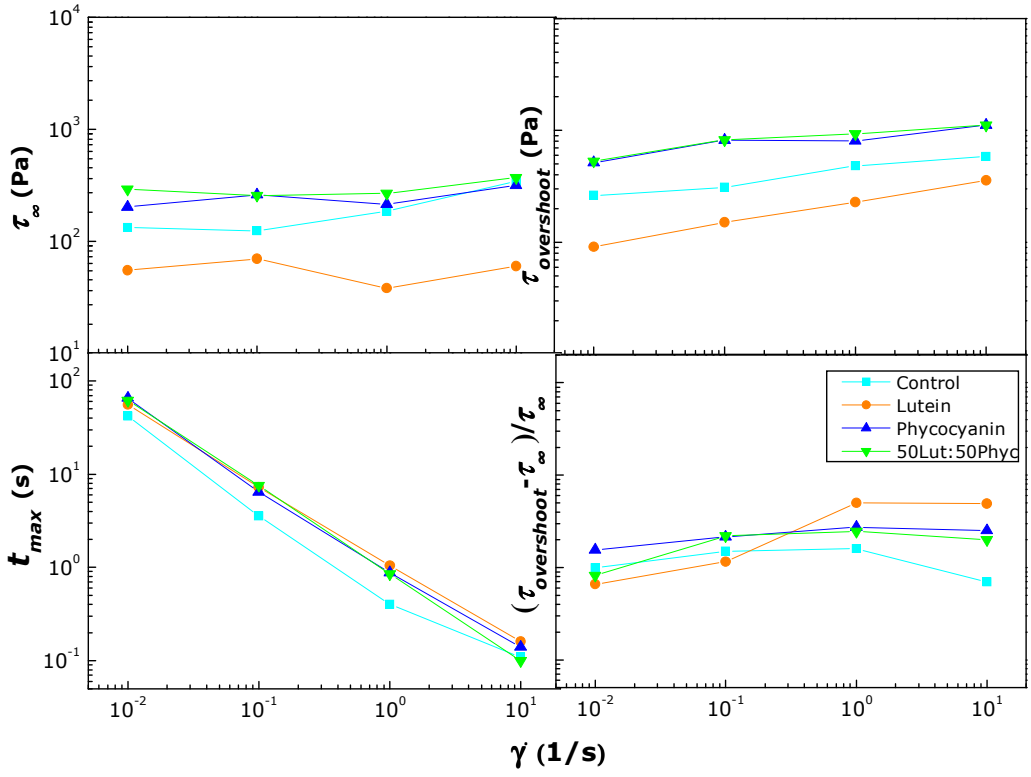


Figure 4.21. Equilibrium shear stress τ_∞ , stress overshoot τ_{max}, time for the overshoot t_{max} and amount of overshoot S^+ for o/w pea protein emulsions without pigment addition (control), with 0.75% lutein, 0.75% phycocyanin and 0.50% total pigment (lutein and phycocyanin in the same proportion) addition.

Stress overshoot values follow the same tendency with pigment addition as those described by τ_{∞}. The structural breakdown was faster for the control emulsion, which presented the stress overshoot at shorter times, indicating that the addition of pigments induces the formation of structural networks which are able to resist higher deformations. For all the emulsions, a slight linear increase on stress overshoot and a linear decrease on time overshoot with shear rate were observed.

The amount of overshoot (S^+) is a parameter related to the viscoelastic response and the degree of structural breakdown occurring in the system at constant shear rate. In general, the introduction of phycocyanin (isolate or blended) in pea protein-stabilised emulsions resulted in a higher amount of overshoot, as compared to the control emulsion, which confirms the results shown in Figure 4.19. In respect to lutein addition, at low shear rates (0.01-0.1 s^{-1}) the relative overshoot peak height was lower than the control emulsion, while at higher shear rates (1-10 s^{-1}) it suddenly increased to considerably higher values than for other emulsions, as also observed for η_{rel} (Figure 4.19).

The transient tests were repeated on the RheoScope®, in order to monitor the resulting time-dependent structural changes of the emulsions with the optical system. Figure 4.22 shows the images obtained for the lutein emulsion, at 1 s^{-1} shear rate, taken before, during and after the overshoot (which occurred at different times and stresses). We can clearly observe the structural breakdown taking place once the stress overshoot is reached, which is much more apparent in the equilibrium state. Once again, this microphotograph evidences that a significant structural modification takes place when the viscous response is predominant over the elastic one.

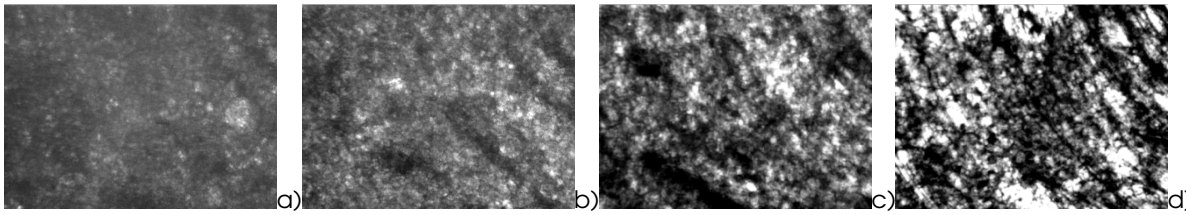


Figure 4.22. Microscope photographs of an o/w pea protein emulsion with 0.75% lutein, retrieved by the RheoScope® equipment during a stress growth experiment at constant shear rate (1 s^{-1}), before stress overshoot (a), during (b) and immediately after (c) stress overshoot, and at equilibrium time (d).

4.3.1.7. Structural considerations

The incorporation of lutein and phycocyanin imparted relevant modifications on the structural and rheological properties of oil-in-water pea protein emulsions, which may be related with different droplet size distributions, changes on the rheology of the bulk continuous phase or to specific molecular interactions at the interface.

In general, lutein emulsions presented lower texture and rheological properties than the control emulsion, inclusively a shear induced structural breakdown higher than the other emulsions studied.

Adding lutein to the oil fraction could have modified the nature of the emulsions' dispersed phase. Fats and oils influence the nutritional, organoleptic, and physicochemical properties of food emulsions in a variety of ways. The bulk physicochemical properties of edible fats and oils depend on the molecular structure and interactions of the triacylglycerol molecules they contain. The strength of the attractive interactions between molecules and the effectiveness of their packing in a condensed phase determines their melting point, boiling point, density and compressibility (McClements, 1999).

Considering that lutein possesses two terminal hydroxyl groups, some interfacial activity might be expectable from its presence, indeed some competition with pea protein molecules for interface binding locations. However, at constant emulsifier (pea protein) concentration, only small variations on the droplet size distribution occurred with the addition of lutein, which indicates that the major role at the interface is played by pea protein, present at higher concentrations (3% w/w) than lutein (0,25-1,25% of a 20% oil suspension).

The interaction between proteins and lipid emulsifiers in mixed adsorbed layers at liquid and air-water interfaces has been widely studied in the last years (Wilde, 2000; Wilde *et al.*, 2004; Patino *et al.*, 2003; Bos and Vliet, 2001). Although both proteins and lipid emulsifiers can stabilise foams and emulsions alone, their individual mechanisms of stabilisation are incompatible, often resulting in a dramatic destabilisation when both species are present at the interface – competitive destabilisation (Wilde *et al.*, 2004).

Although carotenoids are lipid molecules, they present significant structural differences from common used low molecular weight lipid emulsifiers, like mono- and diacylglycerols, and consequently should present different interfacial behaviour. In fact, terpenic compounds are not even considered as emulsifiers (Garti, 1999).

However, a recent trend is to use lipid fractions (oleoresins) extracted from fruits, flowers, spices, leaves, etc., naturally rich in carotenoids (*e.g.* lycopene in tomato, or lutein in Marigold flower) to produce emulsions. These oleoresins consist of various triacylglycerols, non-saponifiable fatty components (*e.g.* waxes) and monoacylglycerol derivatives, and sometimes are *"self emulsifiable"* and can form *"in situ"* water-in-oil emulsions. These are called *"natural occurring self-emulsifying oils"* for w/o emulsions, and can provide other functional properties due to the presence of carotenoids with potential antioxidant activity (Garti, 1999).

Some authors (Granger *et al.*, 2003; Rampon *et al.*, 2004) have suggested that not only the surfactant molecules, *i.e.* emulsifiers and proteins, but also the fat used in the emulsions formulation participates in the development of the interface characteristics and rheological properties. Moreover, it is well known that globular proteins exhibit hydrophobic domains that

may also interact with the oil phase. This can be particularly relevant if we consider that lutein molecules are mainly lipophilic molecules but present polar hydroxyl groups in both ends of the conjugated polyisoprenoid chain. According to this, we can suppose that lutein molecules, located inside oil droplets could penetrate in the interface, interacting with pea protein, creating weaker and disordered layers and poor rheological properties.

The addition of phycocyanin resulted in a significant improvement of the emulsions texture and rheological properties. The emulsion structure was clearly reinforced, being observed a higher resistance to structural breakdown, occurring at higher stress or shear rate values. Hence, a dramatic decrease on rheological characteristics is observed upon the onset non-linear viscoelastic stresses and on steady-state shear flow curves (confirmed by RheoScope® images); as well as by the deviation of Cox-Merz rule and transient shear growth test results.

The presence of phycocyanin protein molecules may have contributed to a marked increase in the viscosity of the aqueous continuous phase, thus retarding the oil droplet association movements and consequently enhancing emulsion stability. The formation of an entangled three-dimensional network, favoured by the association of phycocyanin molecules and pea protein could explain the difficulty in obtaining accurate RheoScope® images, which present major differences from the control and lutein emulsions.

It is also possible that phycocyanin protein molecules, due to their amphiphilic nature, interact in the interfacial protein adsorbed layer at the surface of oil droplets, lowering the tension at the oil-water interface, reinforcing the pea protein emulsifier film and imparting stability to emulsions. In fact, previous studies have demonstrated that a protein isolate from blue-green algae (*Spirulina platensis* strain *Pacifica*) was capable of reducing the interfacial tension at the aqueous/air interface at relatively lower bulk concentrations compared to common food proteins. The surface active components were likely to be protein and/or protein-pigment complexes rather than individual protein molecules (Chronakis *et al.*, 2000).

The presence of different types of proteins in oil-in-water emulsions might lead to a synergistic interaction, providing an improvement in the quality of the final product (Clark *et al.*, 1992; Carrera, 2000) by reinforcing the protein layer. On the other hand, a decrease in the emulsion stability, due to the formation of weaker and more disordered mixed interfacial layers has also been reported (Imm *and* Regenstein, 1999), due to thermodynamic incompatibility (Polyakov *et al.*, 1997) or to competitive adsorption of protein molecules at the interface (Dickinson *et al.*, 1990; Mine and Keeratiurai, 2000). In fact, during and after emulsification, some of the individual protein components may be partially or wholly displaced from the interface by other more surface-active protein species or by small surfactant molecules (Dickinson, 1998). Hence, emulsion stability is strongly influenced by the dynamic aspects of protein competitive adsorption and by the nature of the interfacial protein interactions (Dickinson, 1998); and will be determined by the balance between these competitive and cooperative effects.

The addition of phycocyanin produced a decrease in the Sauter mean diameter of the emulsions, probably due to the formation of a disordered, mixed-protein layer. At increasing phycocyanin concentrations (0.25-0.75% w/w), the pigment either becomes more competitive with pea protein for the interface layer, or could instead be more involved in supramolecular entities with the pea protein, enhancing the local packing density at the adsorbed layer (Raymundo *et al.*, 1999). As a result, the emulsions' structure was reinforced, yielding higher values for the rheological and textural parameters as well as smaller droplet diameters.

For higher phycocyanin concentrations (1.00-1.25%) a drastic increase of the droplet size diameter occurred. This can be due to the competition mechanism referred to above. Phycocyanin proteins, in larger amounts, could have been substantially adsorbed at the oil-water interface during the emulsification process, faster than the pea protein molecules, and the latter become statistically unable to displace them. Accordingly, the rheological and textural parameters of the emulsions continued to increase with phycocyanin concentration. This should be related to an increase on the viscosity of the continuous phase due to the presence of important amounts of unadsorbed proteins and to the formation of supramolecular entanglements among protein molecules adsorbed and non-adsorbed at the interface (Riscardo *et al.*, 2003).

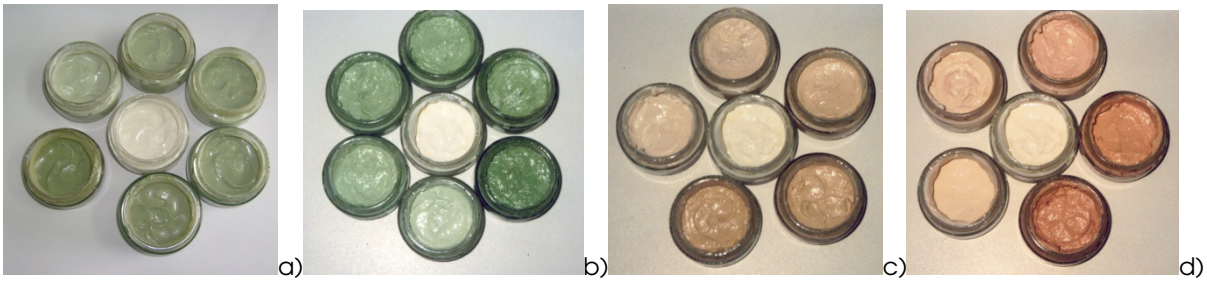
The overall characteristics of the emulsion containing lutein and phycocyanin result from a combination of the effects observed on emulsions containing each pigment individually. The rheological properties are intermediate between lutein and phycocyanin emulsions. The phycocyanin proportion imparted an improved structural resistance to shear stress, evidenced by the stress growth, steady-state flow and stress sweep results, as well as by the RheoScope® microscope images, which are similar to the phycocyanin emulsion.

The microstructural evolution of the emulsions monitored by the RheoScope® equipment did not evidence any clear trends, but it was possible to obtain a qualitative evaluation of the main differences between emulsions and the structural modifications induced by rheological tests. In order to allow the capture of microscopic images by the optical system it was necessary to use a cone and glass plate system. Therefore, in some photos at high shear rates partial sample exclusion from the gap or wall slip of the sample seemed to occur, which corresponded to erroneous rheological data. Considering this effect, the rheological parameters presented correspond to the results obtained with serrated plate-plate geometries that are able to avoid these slip effects (Franco *et al.*, 1998b).

4.3.2. Emulsions coloured with microalgal biomass

4.3.2.1. Effect of microalgal biomass concentration on the colour and texture of emulsions

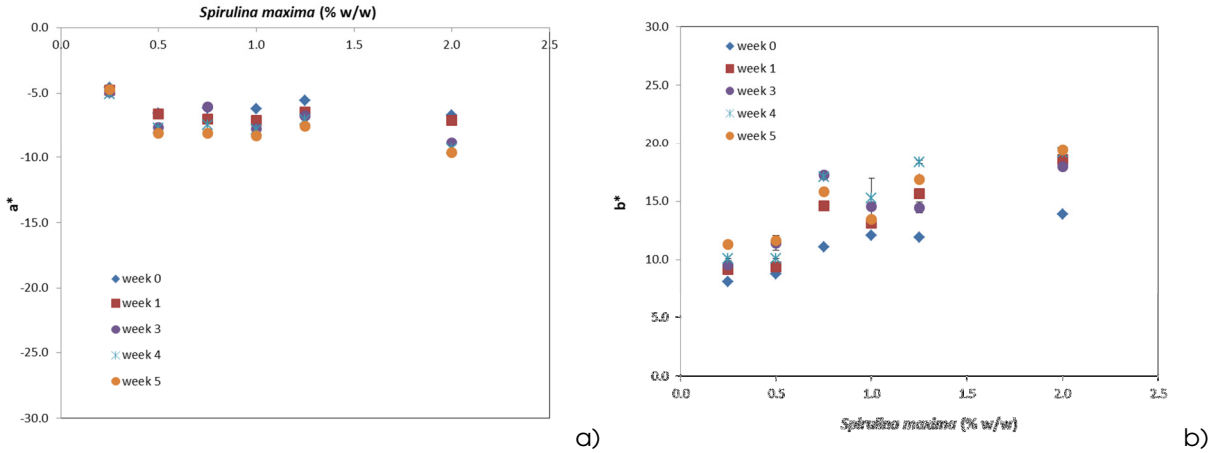
The use of microalgal biomass from *Spirulina maxima*, *Chlorella vulgaris* (green and carotenogenic – orange) and *Haematococcus pluvialis* resulted in oil-in-water emulsion with a wide variety of appealing colours (Figure 4.23).



a) b) c) d)

Figure 4.23. Oil-in-water pea protein stabilized emulsions coloured with different concentrations of *Spirulina maxima* (a), *Chlorella vulgaris* green (b), *Chlorella vulgaris* orange (c) and *Haematococcus pluvialis* (d) biomass.

Spirulina and *Chlorella* green promoted green tonalities due to their dominant chlorophyll content, in relation to other pigments (section 3.3.2). Consequently, these emulsions presented negative *a** values, *i.e.* in the green domain (Figures 4.24a and 4.25a).



a) b)

Figure 4.24. Evolution of *a** (a) and *b** (b) values of o/w emulsions coloured with different concentrations of *Spirulina maxima* biomass, over time.

Spirulina colouration was duller, with *a** values initially ranging from -4.6 to -6.9 as opposed to -8.5 to -15.2 for *Chlorella* green (week 0). Moreover, for *Chlorella* there is a tendency to increase *a** values with biomass concentration (not linearly), while for *Spirulina* there are no significant (p<0.05) variations on *a** when increasing biomass concentration from 0.5% to 1.25%. In terms of *b** parameter, both *Spirulina* and *Chlorella* microalgae showed positive

values, *i.e.*, in the yellow domain (Figures 4.24b and 4.25b). Both type of emulsions showed a similar behaviour, with *b** values increasing with microalgae biomass concentration. As well as for the *a** chromatic coordinate, *Spirulina* emulsions presented *b** values (8.2-13.9) lower than *Chlorella* (11.8-20.7), resulting less saturated overall colourations when using the same microalgal biomass concentration.

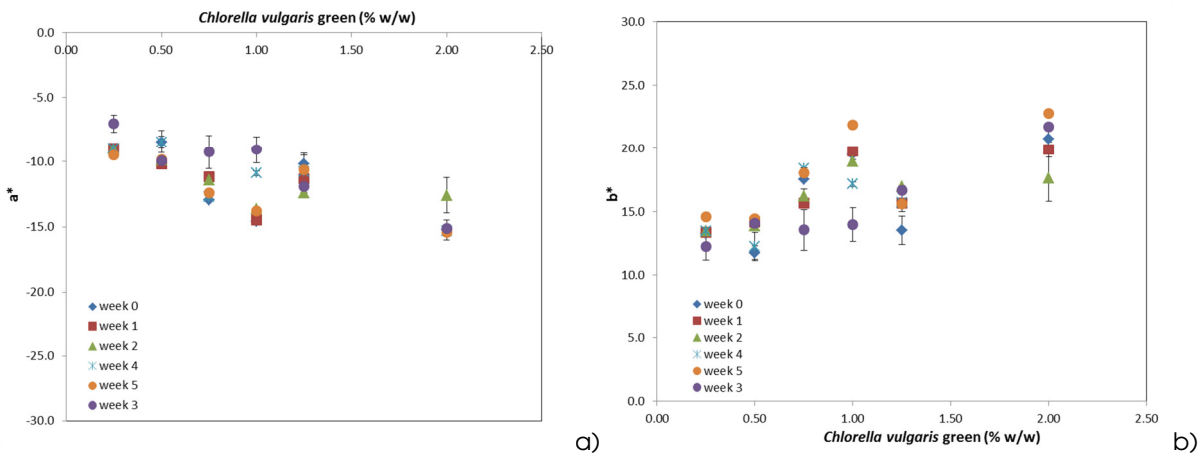


Figure 4.25. Evolution of *a** (a) and *b** (b) values of o/w emulsions coloured with different concentrations of *Chlorella vulgaris* green biomass, over time.

Carotenogenic microalgae, *Chlorella* orange and *Haematococcus*, provided orange to pink tonalities to the emulsions, according to their high content in carotenoid pigments (section 3.3.2). Accordingly, these emulsions presented positive *a** and *b** values corresponding to red and yellow tonalities, respectively (Figures 4.26 and 4.27). For both microalgae, an increase in emulsion *a** and *b** values was observed, reflecting higher colour saturation, with increasing biomass concentration. However, while for *Chlorella* orange this increment is linear (R^2>0.82), for *Haematococcus* there is a logarithmic increment (R^2>0.84). In fact, an emulsion with only 0.05% *Haematococcus* presents a colour (*a**2.40, *b**12.7) similar to an emulsion with 0.25% *Chlorella* (*a**2.15, *b**12.7). This higher pigmentation capacity is related to a much higher total pigment content of *Haematococcus* (3.0%) in relation to *Chlorella* (1.3%) (Table 3.5). The main difference is related to the red (*a**) component, with *Haematococcus* showing maximum values of 17.3 (week 0) and *Chlorella* only 7.4; while for the yellow (*b**) component the differences are not so evident (maximum 28.7 and 22.3, for *Haematococcus* and *Chlorella*, respectively). This should be related to the main carotenoid pigments accumulated in *Haematococcus*, being astaxanthin (red) resulting in pink emulsions; and canthaxanthin (orange) for *Chlorella*, resulting orange emulsions.

In terms of lightness (results not shown), *L** values were lower (darker) for the emulsions containing higher biomass concentrations, for all the microalgae studied, with values ranging from 85.8 (0.05% *Haematococcus*) to 57.8 (2.00% *Chlorella* green). This decrease was not linear for *Chlorella* green, and for the remaining algae the linear correlation coefficients (R^2)

ranged between 0.83 and 0.99. There was not a clear tendency for emulsions to become darker or lighter with time.

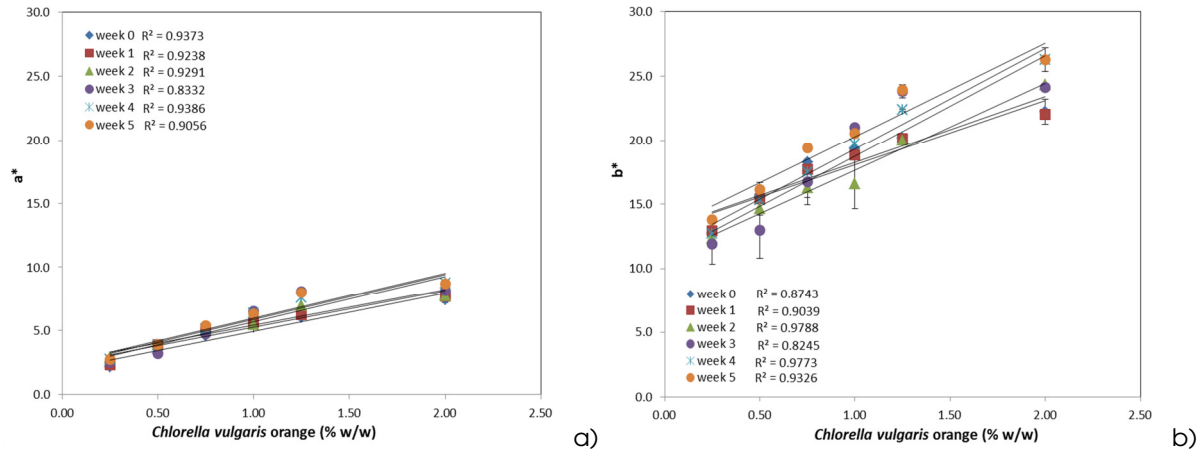


Figure 4.26. Evolution of *a** (a) and *b** (b) values of o/w emulsions coloured with different concentrations of *Chlorella vulgaris* orange biomass, over time.

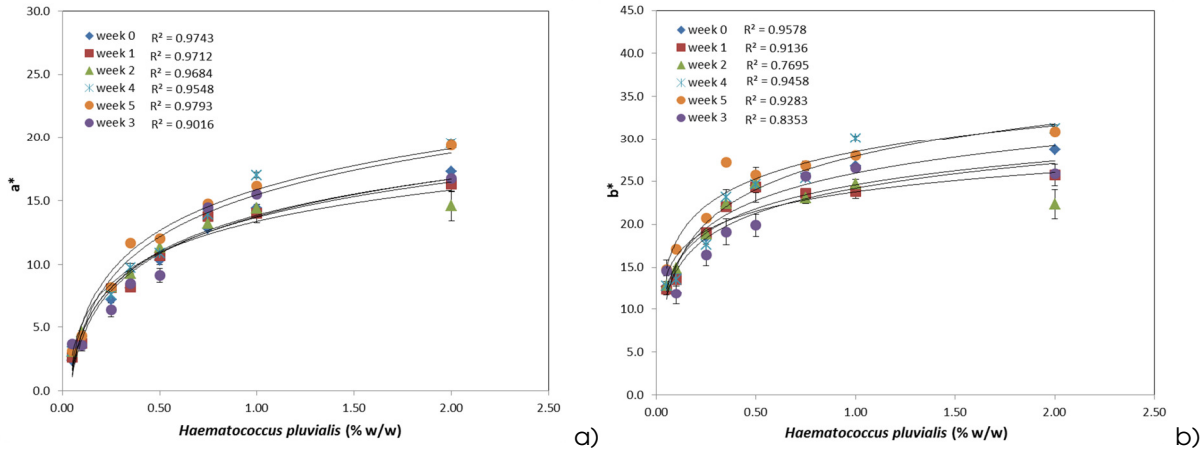


Figure 4.27. Evolution of *a** (a) and *b** (b) values of o/w emulsions coloured with different concentrations of *Haematococcus pluvialis* biomass, over time.

The stability of the emulsions colour along time was measured through the total colour difference (ΔE^*) by comparing the colour presented during time with the initial colour for each emulsion. This parameter is presented in Table 4.2, highlighting in red the cases in which $\Delta E^*>5$, the threshold for perceptive colour difference for human eye according to Castellar *et al.* (2006). In general, perceptive colour differences arise upon 3 weeks storage, more evident for higher microalgal biomass concentrations. However, it seems that some experimental problems may have occurred on the 3rd week because significantly higher ΔE^* values were observed (up to 18.6). Ultimately, it should be noted that after 5 weeks, total colour differences were lower than 9.8 for all the emulsions studied. . The overall highest stability was observed for *Chlorella* orange emulsion, which is an important result considering the issues associated with natural pigment stability and colour loss in food products upon storage.

Table 4.2. Total colour difference (ΔE^*) of emulsions with microalgal biomass addition, in respect to the initial colour (week 0).

		ΔE^*				
		week 1	**week 2**	**week 3**	**week 4**	**week 5**
Spirulina maxima	0.25%	2.0	-	2.5	2.1	5.7
	0.50%	1.4	-	3.6	1.8	3.4
	0.75%	4.4	-	6.2	6.4	5.3
	1.00%	2.3	-	2.9	3.6	2.6
	1.25%	4.0	-	2.8	6.7	5.5
	2.00%	6.4	-	6.7	6.5	7.3
Chlorella vulgaris **green**	0.25%	2.9	1.7	16.2	1.4	2.4
	0.50%	11.0	12.8	13.2	6.3	9.8
	0.75%	5.0	2.6	8.8	1.9	0.7
	1.00%	1.5	2.2	18.6	9.1	2.7
	1.25%	10.2	11.5	11.7	6.7	5.9
	2.00%	2.1	5.1	2.3	1.8	2.9
Chlorella vulgaris **orange**	0.25%	1.4	1.3	4.0	1.5	1.7
	0.50%	1.4	1.6	8.8	1.0	1.0
	0.75%	2.5	2.2	2.1	1.0	2.4
	1.00%	0.5	3.8	2.4	2.7	1.9
	1.25%	1.2	2.6	5.7	3.0	4.7
	2.00%	1.6	6.0	6.2	5.8	4.7
Haematococcus pluvialis	0.05%	0.9	1.3	14.5	15.4	2.4
	0.10%	0.8	1.3	15.4	7.9	3.4
	0.25%	2.6	1.1	9.4	1.6	3.5
	0.35%	0.0	1.3	15.7	7.2	7.4
	0.50%	0.7	1.0	17.3	3.5	7.5
	0.75%	1.1	1.0	3.6	3.1	6.7
	1.00%	7.1	2.0	2.1	6.4	4.6
	2.00%	4.3	10.1	5.5	6.8	8.1

The firmness of emulsions coloured with different microalgae, is presented in Figure 4.28, for different biomass concentration. This texture parameter showed the better discriminating ability between samples, relatively to other TPA parameters.

The addition of microalgal biomass originated emulsions with higher firmness than the control emulsion (without biomass addition). For all microalgae types studied, a linear positive relation (R^2>0.84) between biomass concentration and firmness was observed, although this dependence assumes distinct features. The addition of 0.25-0-75% *Spirulina* does not promote significant (p<0.05) firmness differences in relation to the control emulsion. Even at 2% incorporation level, the average emulsion firmness attained was 1.8 N, a much lower value than for the rest of algae. In fact, for *Chlorella* vulgaris green, maximum firmness values of 6.8 N were attained for the emulsion with 2% biomass incorporation, followed by 6.0 N for 2.00% *Haematococcus* and 4.2 N for 2% *Chlorella* orange. However, below 1% biomass concentration the emulsion with *Chlorella* green showed firmness values in the same order of magnitude (in same cases even lower) than the emulsions with carotenogenic algae.

The increase in emulsion texture properties, through microalgal biomass addition, can be due to an increase of the viscosity of the emulsion continuous phase, by a stabilizing mechanism similar to the effect of polysaccharide addition such as xanthan gum or starch (Xie and Hettiarachchy, 1997; Raymundo *et al.*, 2002). The possibility of establishing interactions between pea protein and microalgal biomass, should also be considered, since it can contribute to a structural reinforcement through the formation of physical entanglements

(Clark *et al.*, 1992; Riscardo *et al.*, 2003). The lower firmness values obtained for *Spirulina* emulsions are quite unexpected, considering the favourable texturizing effect shown by phycocyanin (main pigment in *Spirulina*) in the previous section (4.3.1). Still, these results are in agreement with the texture and rheological behaviour of gelled systems with *Spirulina* addition, which shall be discussed in chapter 5.

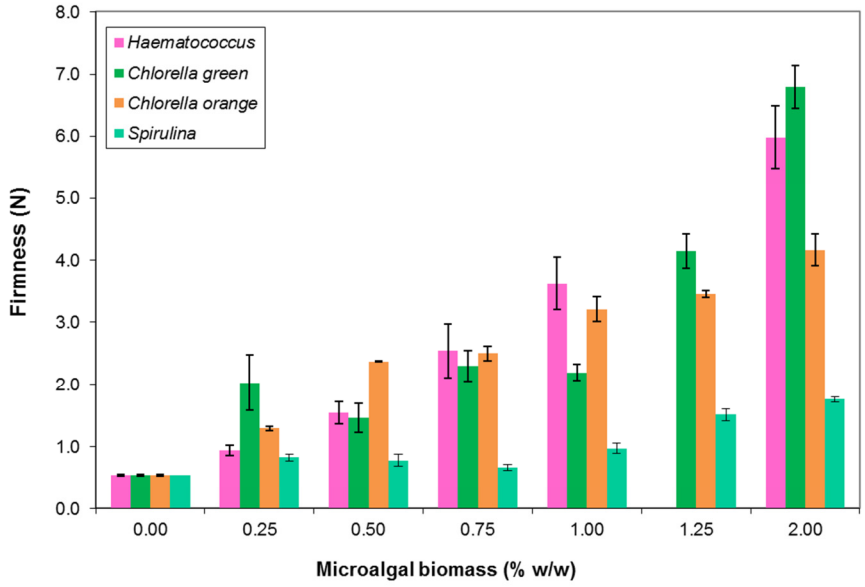


Figure 4.28. Firmness of o/w emulsions coloured with different concentrations of *Spirulina maxima*, *Chlorella vulgaris* green, *Chlorella vulgaris* orange and *Haematococcus pluvialis* biomass.

4.3.2.2. Fat mimetic capacity of microalgal biomass in oil-in-water emulsions

The structural reinforcement arising from microalgae biomass can be very advantageous, considering technological and nutritional applications. One of the main issues regarding emulsion technology is the reduction of fat content, since for example traditionally mayonnaises are oil-in-water emulsions with 70-80% fat (Depree and Savage, 2001). High fat intake is associated to an increased risk for obesity, high blood cholesterol, coronary heart disease and some types of cancer, so in the last years food industry has been pressed to develop alternative low-fat products. In addition to nutritional aspects, fat influences the rheological properties and sensory characteristics of foods, particularly colloidal systems in which an oil phase is dispersed. Therefore, food technologists have focused on investigating fat replacers, such as fat mimetics *i.e.* substances that imitate triglycerides' organoleptic or physical properties (Akoh, 1998). Some fat mimetics such as modified starch, cellulose, pectin, inulin or whey protein have been used to stabilize emulsions and to increase the viscosity of light mayonnaise (*e.g.* Wendin *et al.*, 1997; Chouard, 2005; Liu *et al.*, 2007; Murphy, 1999).

In the present work, microalgal biomass was studied as fat mimetic in oil-in-water pea protein-stabilized emulsions. The emulsifier (pea protein) was maintained constant (3%), as well as the microalgal biomass (2%), and the oil content was reduced from 65% to 50%. The emulsions were analysed in terms of rheological behaviour, and compared with a control system (3% pea protein, 65% oil, 0% microalga).

In terms of colour, the reduction of the oil content did not cause significant (p<0.05) changes, with total colour differences ΔE^*<3.5 between samples with different oil contents (except for *Chlorella* orange 50% oil, ΔE^*=5.2).

The effect of oil addition on the viscoelastic properties of the emulsions can be observed on Figure 4.29, in terms of the evolution of the viscoelastic functions (G' and G'') with frequency.

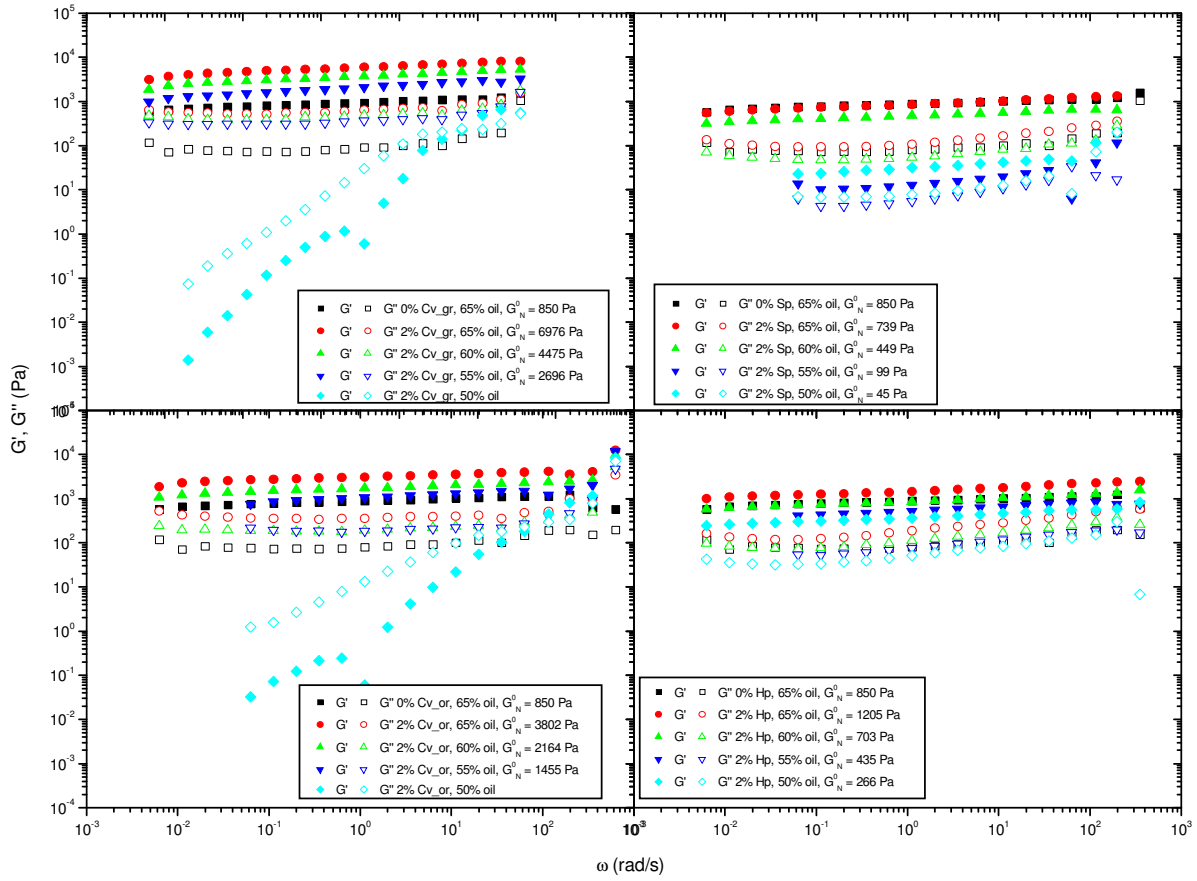


Figure 4.29. Mechanical spectra of o/w emulsions with 3% pea protein and 2% of *Spirulina maxima* (Sp), *Haematococcus pluvialis* (Hp), *Chlorella vulgaris* green (Cv_gr) and orange (Cv_or) microalgal biomass, for different oil content and respective values of G^0_N.

These emulsions present mechanical spectra typical of protein-stabilized emulsions in which an elastic network develops due to the occurrence of an extensive bridging flocculation process (*e.g.* Franco *et al.*, 1998a). Except for the *Chlorella* (green and orange) emulsions with 50% oil, the values of the storage modulus (G') were always higher than those found for the loss modulus (G''), in the whole frequency range studied, showing a clear tendency to

the development of the plateau region previously described. The plateau modulus (G^0_N) was analysed as a characteristic parameter of this region.

From the dynamic measurements, it was observed that, for a certain protein and biomass concentration, higher oil content induced a reinforcement of the emulsion structure, for all the microalgae studied. This was demonstrated by the increase of the plateau modulus (G^0_N). For the emulsion systems with addition of *Chlorella* (green and orange) microalgal biomass, the G^0_N value was significantly higher than for the standard. It was possible to produce emulsions with 55% oil that were more structured than the emulsions with 65% oil and no microalgal biomass addition. This effect was more significant for *Chlorella* green, which is in agreement with texture results (section 4.3.2.1), and could be related to a much higher protein content in *Chlorella* green (38%) than in orange (12%) (Table 3.2). The preparations with 50% oil showed no phase separation, but a much weaker structure was obtained as can be deduced from the mechanical spectrum (Figure 4.29). In fact, G' is lower than G'', showing both functions power-law evolutions with frequency and a crossover, typical of the flow (terminal) region of the spectrum.

Regarding *Spirulina* and *Haematococcus*, lower G^0_N values were obtained by reducing the emulsions oil content, when compared to the control. In the case of *Spirulina,* this is in agreement with the texture results but not for *Haematococcus* (section 4.3.2.1). However, for both microalgae, when reducing the emulsions oil content to 50% an incipient microstructure was detected, as reflected by G' values always higher than G'' for the whole frequency range.

Flow curves for the microalgae emulsions prepared with different oil contents are presented in Figure 4.30, together with zero shear rate-limiting viscosity values obtained from the fitting to the Carreau equation (eq. 4.6). The flow behaviour of these emulsions is in agreement with dynamic measurements, as η_0 increases with the increase of the oil content. Determinations under steady shear involve internal structure destruction as shear rate increases. The zero shear limiting viscosity represents the viscosity where the internal structure was not damaged by shear rate and thereby, measures the highest resistance to flow of the system. In this sense, the flow behaviour could provide information more directly related to the technological ability of using microalgal biomass as fat mimetic.

For *Chlorella* (green and orange), the structuring effect of microalgal biomass is most obvious, with η_0 values of 3.7 and 4.7x10^5 Pa.s (respectively) with only 55% oil, as opposed to 2.0x10^5 Pa.s for the control (65% oil, 0% algae). In the *Spirulina* emulsion, flow behaviour is consistent with dynamic measurements, without effective reinforcement of the emulsion microstructure upon biomass addition evidenced. *Haematococcus* emulsions with 65% and 60% oil presented η_0 values higher than the control emulsion, comparable to *Chlorella* green (at 65% oil) values, which is more approximate to the texture results (Figure 4.28). It seems that *Haematococcus* structuring effect is closely related to the emulsions oil content, which could

be related to the fact that this carotenogenic microalga accumulates large amounts of lipids (41%) as fat droplets within the cells.

Systems with 50% oil presented η_0 values ranging from 0.9x10^4 (*Spirulina*) to 3.0x10^5 Pa.s (*Chlorella* green). In these cases, even if only an incipient microstructure was achieved, the flow was still difficult due to relatively high apparent viscosities. Some attempts to prepare emulsions without any protein addition were undertaken, *i.e.*, using microalga as the sole emulsifier, but only a very unstable suspension was obtained. The development of the emulsion structure did not occur when microalgal biomass fully replaced the vegetable protein as an emulsifier, and phase separation was instantaneous. This was not the case of the systems with 50% oil, 3% protein and 2% microalga discussed previously, which were very stable and did not present phase separation.

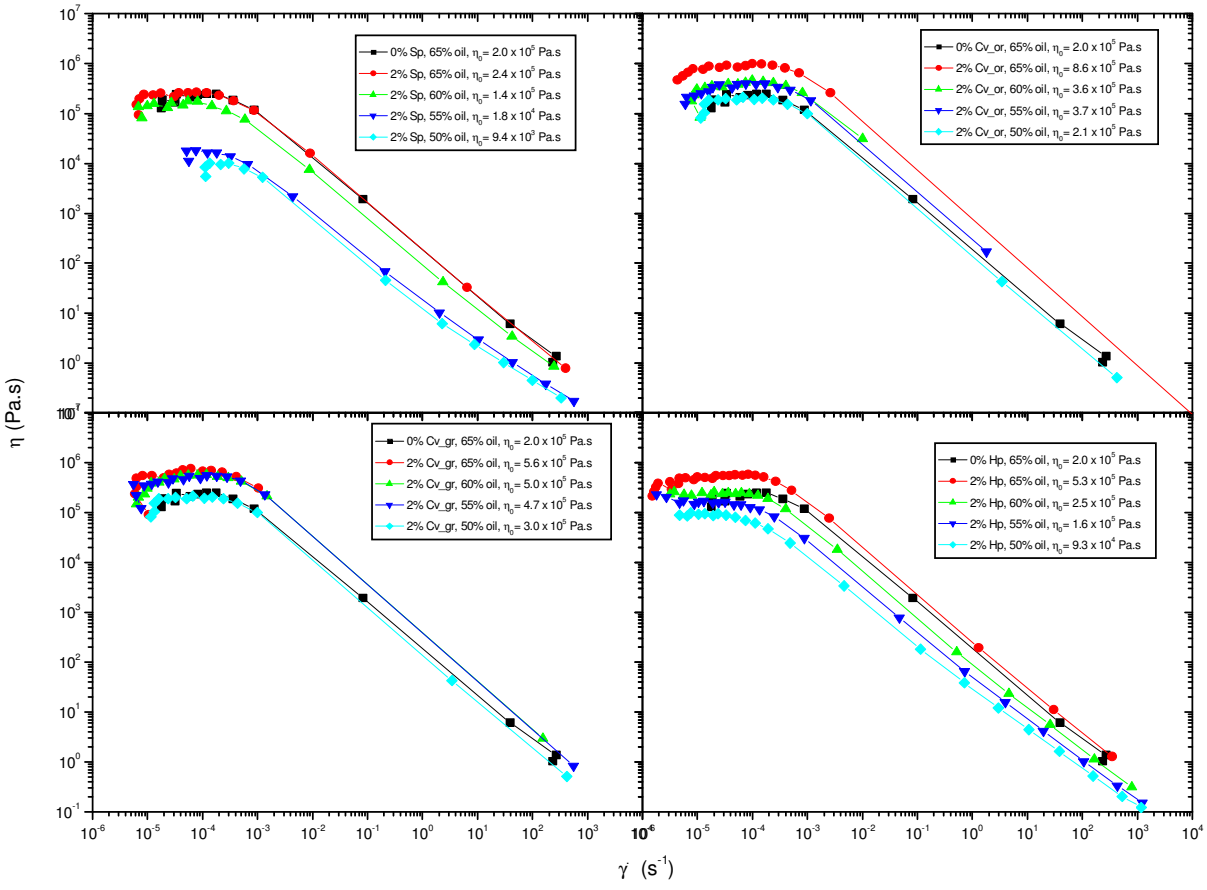


Figure 4.30. Flow curves of o/w emulsions with 3% pea protein and 2% of *Spirulina maxima* (Sp), *Haematococcus pluvialis* (Hp), *Chlorella vulgaris* green (Cv_gr) and orange (Cv_or) microalgal biomass, for different oil content and respective values of η_0.

Fluorescence microscope images of pea protein oil-in-water emulsions with 65% oil and 2% microalgal biomass addition are presented in Figure 4.31. Pea protein auto-fluorescent molecules can be perceived as white areas, while dark areas should correspond to the lipid fraction. Carotenoid pigments, clearly detected as red/pink dots, appear widely distributed

in emulsion with *Chlorella* orange and *Haematococcus* carotenogenic microalgae, being present in smaller amounts in *Chlorella* green emulsion. *Spirulina* emulsion photograph shows a different type of fluorescence which should be related to the presence of phycobiliprotein pigments.

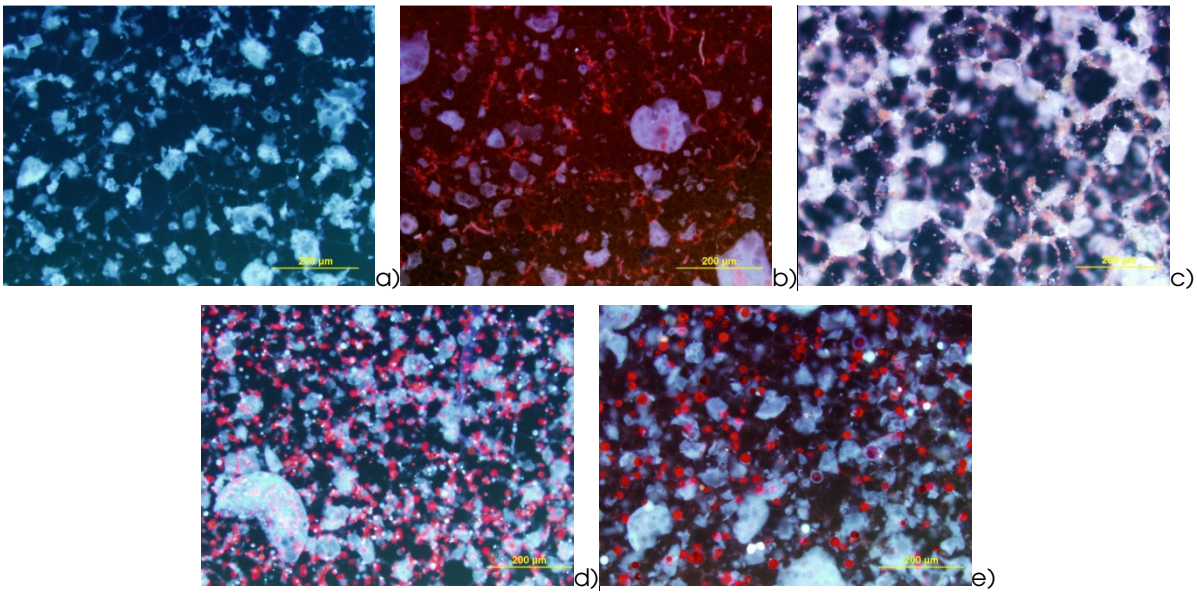


Figure 4.31. Fluorescence microscopy images of o/w emulsions with 3% pea protein and 65% oil (a), and 2% *Spirulina maxima* (b), *Chlorella vulgaris* green (c), *Chlorella vulgaris* orange (d), *Haematococcus pluvialis* (e). (DAPI filter, 10x).

4.4. CONCLUSIONS

Addition of microalgal biomass, and natural pigments, in oil-in-water pea protein-stabilized emulsions proved to be an efficient and innovative procedure, which simultaneously enables original and stable colourations, via health-beneficent natural pigments, and microstructural reinforcement, as observed through emulsions rheological response.

Lutein and phycocyanin pigments imparted markedly different effects, according to the pigment nature, particularly the affinity to the aqueous (continuous) or to the oil (disperse) phase, and its interactions with the emulsifier molecules at the interface seems to be of major importance.

Lutein addition modified the nature of the oil phase originating emulsions with much smaller rheological and textural properties but with similar d_{sv} results, comparing with the control emulsion (0.00% lutein). The addition of phycocyanin had a significant influence on the rheology of the continuous phase and through interactions with pea protein at the interfacial layer. As a result, the rheological and textural parameters increased linearly with phycocyanin concentration, while d_{sv} results presented a polynomial behaviour with a minimum for 0.75% (w/w) pigment concentration. When using combinations of both pigments the rheological and textural characteristics of the emulsions increased with phycocyanin proportion while d_{sv} values remained similar. A synergistic effect was observed when using small amounts (under 50% proportion) of lutein.

Microscope photographs taken during the rheological tests (RheoScope®) also provided useful information, especially for the control and lutein emulsions which are more fluid, while for the emulsions containing phycocyanin and blend of pigments which were very consistent and opaque, it was more difficult to obtain accurate microscopic measurements.

Phycocyanin provided an intense blue colouration, which although unexpected in a food product, is valuable considering that stable natural blue colourings are hard to find. So, this study opens perspective to phycocyanin use in colloidal emulsion systems,

Chlorella vulgaris (green and orange, after carotenogenesis) and *Haematococcus pluvialis* (red, after carotenogenesis) microalgal biomass, showed to be appropriate purveyors of pigments in oil-in-water food emulsions, imparting stable and appealing colours to food emulsions, with additional advantages in terms of providing other bioactive molecules, e.g. antioxidants, PUFA-ω_3. *Haematococcus* proved to be the more effective colouring agent, which should be related to its high pigment content (3.0%). Higher colour stabilities were attained for biomass concentrations below 1.0%, for all microalgae studied.

The addition of these microalgae also presented a positive effect on the emulsion's texture and rheological characteristics. The addition of this biomass material proved to be beneficial in terms of enabling lesser oil contents for the emulsions, without disturbing their structural and

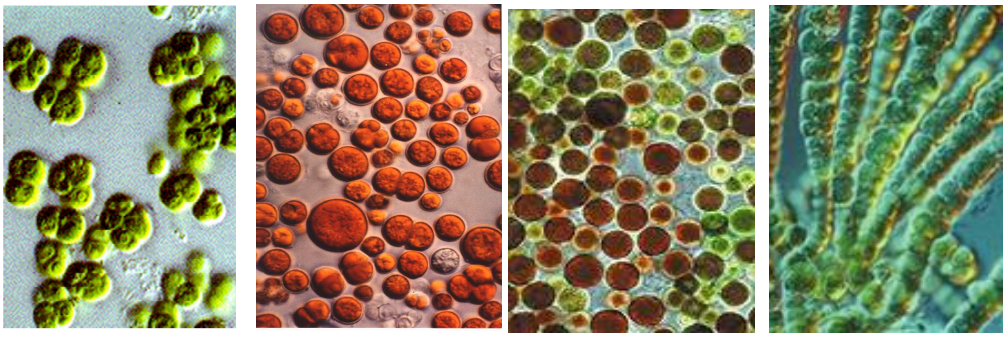
texture properties. These results support the potential use of microalgae material to act as a fat mimetic, besides the possible advantages as colouring agent.

Spirulina seemed to be the less effective microalgae, either as colouring and texturizing agent. Duller green colourations were attained, even at high biomass concentrations, and less significant effects on firmness and viscosity improvement were observed.

The results presented in this chapter have been published in 4 articles in peer-review international journals (*Food Hydrocolloids*, *Food Biophysics*, *European Food Research & Technology* and *Food Research International*) (Appendix A) and presented in several national and international conferences, including Rheology meetings (IBEREO2004, AERC2006) and the *International Congress of Pigments in Food* (Appendix B).

CHAPTER 5

Microalgal biomass and natural pigments in pea protein mixed gel systems



5.1. INTRODUCTION

Natural biopolymers, such as globular proteins and polysaccharides, have been widely used in the formulation of structured gelled food products (Doublier *et al.*, 2000). Most food products have a complex composition, comprising at least three biopolymer types whose interaction determines the products' structural and mechanical properties (Tolstoguzov, 2003). Recently, pea protein isolate has been successfully used in combination with κ-carrageenan and starch polysaccharides, to develop strictly vegetable gelled desserts, as a vegan alternative to dairy-desserts (Nunes *et al.*, 2003; 2006a; 2006b). The gelation process of these mixed systems is thermally induced, involving extensive denaturation and/or conformation changes of the biopolymers and subsequently the development of a gel network upon cooling. The thermal profile used on gel-setting is determinant for the development of the gel structure which is reflected by its rheological properties (Nunes *et al.*, 2006a).

These biopolymer gels, prepared with pea protein, served as model systems to study the addition of microalgal biomass in gelled matrixes, in the present chapter. The impact of microalgae addition in different food matrixes reflects their interactions with other food components such as biopolymers (*e.g.* proteins and polysaccharides). Besides colouring purposes, from a novel product development point of view, it is essential to define the texture/sensorial and rheological characteristics of these systems, since they reflect the microstructural modifications that might arise as a consequence of microalgal biomass addition to gel matrixes, which may distress the product stability. So, the aim of the present chapter was to study the impact of adding different microalgae biomass addition on the rheological properties of protein-polysaccharide mixed food gels.

Some preliminary studies were carried out with the purpose of developing a formulation of vegetable based gelled-desserts (similar to "dairy-desserts") with different microalgal biomass types and concentrations (section 5.3.1). The microalgae gels were analysed in terms of colour and texture, and compared to gels with commercial pigments addition. For *Spirulina* and *Diacronema*, fatty acid profile and pigment analysis of gels prepared at different temperatures were also carried out.

Section 5.3.2 covers the rheological characterization of the different microalgae gels, in terms of maturation kinetic curves, monitored through small-amplitude oscillatory shear (SAOS) time sweep tests. The mechanical spectra (frequency sweep) and thermoreversible character (temperature sweeps) of the formed gels was also analysed.

Spirulina and *Haematococcus* gels presented a markedly different rheological behaviour compared to the control mixed biopolymer gelled system, so their rheological behaviour was more thorough studied. Subsequently, the effect of different gel setting conditions (time/temperature treatment, heating/cooling rates) on the linear viscoelastic properties of

Spirulina and *Haematococcus* gels was investigated (section 5.3.3). The interaction of these microalgae with pea protein, kappa-carrageenan and starch biopolymers, in model binary (biopolymer/microalga) and ternary (pea protein/κ-carrageenan/microalga; protein/starch/ microalga) gel systems was also studied in terms of linear viscoelastic behaviour and fluorescence microscopy (Section 5.3.4). The aim of these studies was to clarify how these microalgae are arranged during the formation of gel structure and how they interact with each biopolymer present in the complex mixed gel system.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Pea protein isolate (Pisane HD) used for emulsion studies (chapter 4) was not commercially available at the time gel studies were conducted. Cosucra Warcoeing Group had developed four new pea protein isolates (C9, F9, H9, M9), which were tested in terms of the produced gel firmness (determined as indicated in section 5.2.2.4). Pisane F9 yielded the highest value, comparable to a commercial gelled dessert (Figure 5.1), and was used for gel studies in the present chapter.

Other biopolymers used were κ-Carrageenan Satiagel AMP45 (Degussa, France) and native maize starch Vitena A (Copam, Portugal). All these biopolymers were kindly provided by the respective manufacturers.

About microalgae biomass production see description in section 3.2.1.1.

Commercial carotenoid pigments were used: astaxanthin (Carophyll Pink 8%, Roche, Switzerland), canthaxanthin (Carophyll Red 10%, Roche, Switzerland), β-carotene (10% Roche, Switzerland), lutein (5% Kemin, USA). Phycocyanin, described in section 4.2.1, was also used.

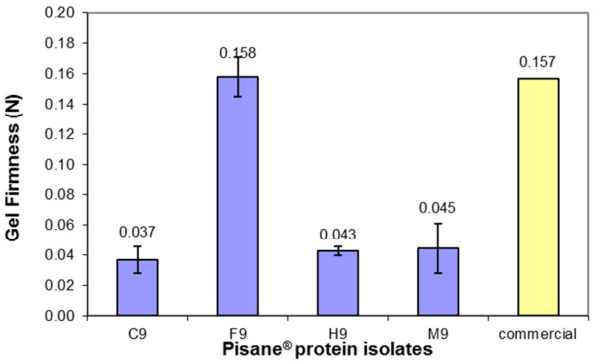


Figure 5.1. Firmness of gels prepared with different Pisane® protein isolates, compared to a commercial gelled dessert.

5.2.2. Methods

5.2.2.1. Gel preparation

The gels were prepared based on a formulation previously optimised by Nunes *et al.* (2006a; 2006b): 4% (w/w) pea protein isolate, 0.15% (w/w) κ-carrageenan, 2.5% (w/w) native maize starch and 15% (w/w) sugar. Microalgal biomass and pigments were added at 0% (control), 0.25% and 0.75% concentration. For the binary systems studies (section 5.3.4) microalgal biomass was added to these systems in the same proportion to each biopolymer as in the mixed gel formulation. Accordingly, the systems studied were: pea protein 12% + microalga

2.25%; κ-carrageenan 0.75% + microalga 3.75%; starch 5% + microalga 1.5%; pea protein 4% + κ-carrageenan 0.15% + microalga 0.75%; pea protein 6% + starch 3.75% + microalga 1.125%. No adjustments were made to the natural pH of the systems and no salts were added to keep in solution solely the salts carried along with the biopolymers and microalgae.

The ingredients were dispersed in demineralized water, by mechanical agitation (300 rpm, 1 h) at room temperature (Figure 5.2a).

Thermal treatment of the dispersions was conducted in a thermally controlled water bath at 75-90ºC for 5 min (Figures 5.2b-c). The samples were placed in cylindrical glass containers (33 mm diameter and 33 mm height) and allowed to set at 5ºC in a refrigerator, being colour, pigments, fatty acids and texture measured after 24 h maturation. For the rheological studies presented in section 5.3.2, samples were transferred to the rheometer-measuring device, which was preheated to 40ºC, after thermal treatment at 90ºC/5min. Regarding sections 5.3.3 and 5.3.4, thermal treatment (heating/cooling) was controlled in the rheometer plate-plate geometry by a Peltier system and the gelification process was monitored *in situ*.

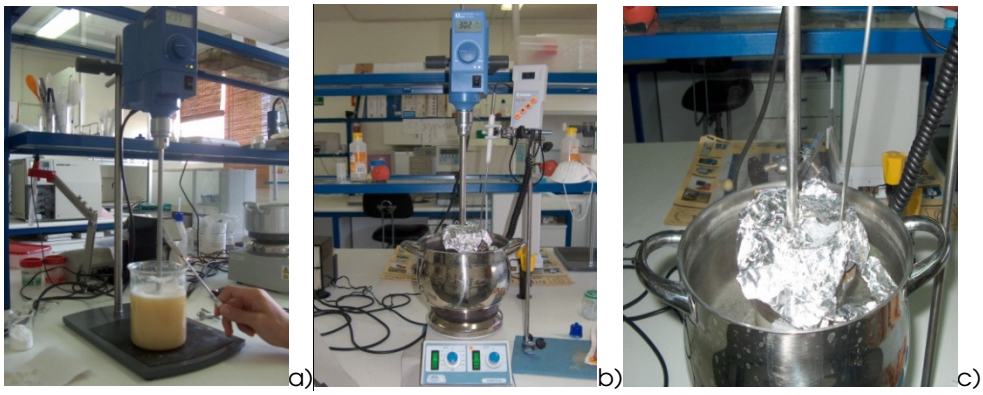


Figure 5.2. Gel preparation: ingredient dispersion at 300 rpm (a); thermal treatment in thermally controlled water bath (b). Samples were covered with aluminium foil during thermal treatment to prevent water loss due to evaporation (c).

5.2.2.2. Colour and pigments analysis

Food gels are translucent materials that are seen partially by reflected light and partially by transmitted light that passes through them. These samples require special handling since any variations in their thickness (path length) or background will affect their colour (Good, 2003). To overcome this, a fixed amount of sample (10 mm height) was poured into a measuring cup, which was then surrounded with a black paper strip, against a white backing (Figure 5.3).

The gels' colour was measured instrumentally using a Minolta CR-300 (Japan) tristimulus colorimeter (D_{65}, 2º), which was calibrated with a white standard porcelain plate (Y=92.7, x=0.3161, y=0.3322), with a measuring area per measurement of 50 mm^2. The results were

expressed in accordance to the CIE L*a*b* uniform colour system. Saturation Chroma (C^*_{ab}) and hue angle (h_{ab}) were also calculated, as defined by the following equations:

$$C^*_{ab} = (a^{*2} + b^{*2})^{1/2} \quad (5.1)$$

$$h_{ab} = \arctan(b^*/a^*) \quad (5.2)$$

where h_{ab} 0º = red (+a^*), h_{ab} 90º = yellow (+b^*), h_{ab} 180º = green (-a^*) and h_{ab} 270º = blue (-b^*). The total colour difference (ΔE^*) between samples with different gelling temperatures and different microalgal biomass concentrations were calculated according to equation 4.3.

Figure 5.3. Gel sample (control) preparation for colour measurement.

Total pigment content of the gels was determined as indicated in section 3.2.2.6.

5.2.2.3. Fatty acid profile

Spirulina and *Diacronema* gels were freeze-dried, and analysed in terms of fatty acids profile, according to the methodology described in section 3.2.2.4.2.

5.2.2.4. Texture profile analysis

The gels texture was measured objectively using a texturometer TA.XTplus (Stable MicroSystems, UK) in the penetration mode with a cylinder probe of 10 mm diameter, plunged 7 mm at 0.5 mm/s, with 5 s waiting time between penetration cycles. Firmness was considered as the height of the force peak during the first compression cycle of the force *versus* time curve, and was the parameter with better discriminating ability to compare this type of gels. Measurements were done in triplicate.

5.2.2.5. Rheological tests

SAOS measurements were performed in a controlled-stress rheometer (RS-300, Haake, Germany), using a cone-plate geometry system (35 mm, 2º). A constant shear stress within

the linear viscoelastic region (previously determined for each sample) was applied in all measurements. Samples were covered with a layer of paraffin oil to prevent moisture loss.
In the first rheological tests (section 5.3.2), the samples were transferred to the plate-plate geometry, preheated at 40ºC. Thereafter, SAOS tests (τ=0.2 Pa, ω=6.28 rad/s) were used to monitor the viscoelastic functions, storage modulus *G'* (elastic function) and loss modulus G" (viscous function), while decreasing temperature from 40ºC to 5ºC at 0.5ºC/min. In the subsequent tests a Universal Temperature Control – Peltier system was available, so it was possible to monitor the gelation process *in-situ*. Different thermal profile conditions were studied in section 5.3.3. The samples were heated from 20 to 70-90ºC, maintained at this temperature for 5-30 min, and then cooled down to 5ºC, at varying heating/cooling rates (0.5-10.0ºC/min) (0.2 Pa, 6.28 rad/s). For binary gels studies (section 5.3.4), the samples were heated from 20ºC to 90ºC, kept at 90ºC for 5 min, and then cooled down to 5ºC, at 1ºC/min (ω=6.28 rad/s).
Subsequently, time sweep tests were conducted at 5ºC during 24 h (0.5 Pa, 6.28 rad/s) followed by frequency sweep tests (0.5 Pa, 0.01-111.7 rad/s). In section 5.3.2 the maturated gels were also submitted to heating (5 to 80ºC) and cooling (80 to 5ºC) temperature sweep ramps (0.2 Pa, 6.28 rad/s) to access their thermoreversible character.

5.2.2.6. Fluorescence microscopy

The gels were analysed by fluoresce microscopy using an Olympus BX61 optical microscope in epifluorescence mode with 4x and 10x (UPlanApo) objectives. A Mercury arc lamp was used as excitation source and DAPI, TRITC, FITC fluorescence filter sets were used to select different excitation and emission wavelengths. After thermal treatment (on the Peltier - rheometer plate), in the same conditions used for the rheological measurements, samples were poured between a microscope slide and a coverslip, sealed with varnish to prevent dehydration, and kept in a refrigerator at 5-7ºC for 24 h to allow gel maturation. *Spirulina*, *Haematococcus* and pea protein presented autofluorescence. Starch was non-covalently stained with Rhodamine B (Sigma), in the absence of pea protein. Rhodamine was added during biopolymers mechanical dispersion at 0.002% concentration level.

5.2.2.7. Statistical analysis

Results are presented as average ± standard deviation of tests that were replicated at least three times. Percentages are expressed as weight/weight in dry matter basis, except when indicated otherwise. Statistical analysis, ANOVA Post-Hoc Comparisons – Scheffé Test, was performed using the Software STATISTICA (Version 6.0, Statsoft Inc., USA). The significance level was set at 95%.

5.3. RESULTS AND DISCUSSION

5.3.1. Physical and chemical characterization of microalgal and natural pigment gels

5.3.1.1. Colour and pigments analysis

The incorporation of microalgal biomass (0.25% and 0.75% w/w) resulted in gels with a wide range of appealing colours, presenting less intense tonalities than gels obtained with pigment addition (Figure 5.4), since the effective colouring content is much lower.

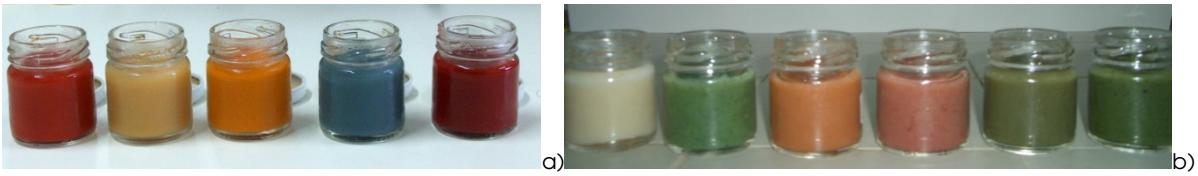

a) b)

Figure 5.4. Vegetable gelled puddings coloured with commercial pigments (a): canthaxanthin, lutein, β-carotene, phycocyanin, astaxanthin (left to right); and microalgal biomass (b): Control, *Chlorella vulgaris* (green and carotenogenic), *Haematococcus pluvialis* (carotenogenic), *Spirulina maxima, Diacronema vlkianum* (left to right); at 0.75% (w/w) incorporation level.

Results of colour parameters of vegetable gels with pigments and microalgal biomass addition, prepared at 90ºC/5min, are presented in Tables 5.1 and 5.2, respectively.

The addition of pigments resulted in gels darker than the control (L^* 60.05) especially for astaxanthin and canthaxanthin addition (L^* 30 to 37), except for phycocyanin (L^* 77.38 and 79.18). Gels with astaxanthin and canthaxanthin presented very intense red tonalities with positive and high a^* (red) and b^*(yellow) values, reflecting high saturation (C_{ab}* 36-39 and 44-47 for astaxanthin and canthaxanthin, respectively). Although less saturated, the astaxanthin gels presented a hue value closer to pure red (h_{ab}º~20) than canthaxanthin. β-carotene imparts a highly saturated (C^*_{ab}>50) and predominantly orange hue (h_{ab}º~60), the contribution of the b^* chromatic coordinate being much more relevant than that of a^*. The lutein gels evidenced a paler yellow tonality which is reflected by a negligible contribution of the a^* parameter, the colour saturation (C^*_{ab} 17-25) being mainly determined by b^*. Phycocyanin induced a blue coloration to the gels (h_{ab}º 276-291), expressed by negative b^* values (-15 to -38). Increasing pigment concentration from 0.25% to 0.75%, an increase in a^* and b^* in the colour saturation was observed. However, for astaxanthin, canthaxanthin and β-carotene gels the total colour differences were very small (ΔE^*<5) (Castellar *et al.*, 2006) resulting from non-significant differences in b^* value, while for lutein and phycocyanin the colour differences were clearly detected (ΔE^* 10.22 and 23.62, respectively).

The *Spirulina* gels presented an unappealing tonality which is reflected by small a^* and b^* values yielding a low chroma (C^*_{ab}<10). The addition of *Chlorella* (green) and *Diacronema* resulted in gels with negative a^* and positive b^* values, originating green hues (h_{ab}º 113-140)

which are more saturated for *Diacronema* (C^*_{ab}~20). The algae submitted to carotenogenesis originated tonalities in the red (positive a^*) and yellow (positive b^*) ranges. In *Chlorella* orange gels there is a dominance of the yellow tonality over the red one ($b^*>a^*$), resulting an orange hue (h_{ab}º 52-81); on the other hand, for *Haematococcus* gels the red tonality is predominant over the yellow ($a^*>b^*$) yielding a pink coloration (h_{ab}º 29-58). Increasing microalgae biomass concentration from 0.25% to 0.75% resulted in appreciable total colour differences (ΔE^*> 5) (Castellar *et al.*, 2006), particularly for *Haematococcus* and *Chlorella* orange (ΔE^* 13.89 and 12.94, respectively).

Table 5.1. Colour parameter values of gels coloured with commercial pigments.

		L^*	a^*	b^*	C_{ab}*	$h°_{ab}$	ΔE^*
Control	0.00%	60.05 ± 1.18 ^{a}	-2.29 ± 0.15 ^{a}	5.87 ± 0.23 ^{a}	6.31 ± 0.20 ^{a}	111.37 ± 1.79 ^{a}	
Astaxanthin	0.25%	30.81 ± 0.17 ^{b}	33.96 ± 0.21 ^{b}	12.49 ± 0.31 ^{b}	36.18 ± 0.27 ^{b}	20.20 ± 0.40 ^{b}	
	0.75%	29.89 ± 0.08 ^{b}	36.93 ± 0.38 ^{bc}	13.14 ± 0.26 ^{b}	39.20 ± 0.33 ^{b}	19.59 ± 0.48 ^{b}	3.18
β-Carotene	0.25%	55.46 ± 1.72 ^{c}	23.09 ± 1.13 ^{d}	48.26 ± 3.28 ^{c}	53.55 ± 2.59 ^{c}	64.33 ± 2.50 ^{c}	
	0.75%	56.23 ± 2.26 ^{cd}	28.36 ± 1.84 ^{e}	47.51 ± 1.94 ^{c}	55.37 ± 1.51 ^{c}	59.15 ± 2.27 ^{c}	5.38
Canthaxanthin	0.25%	37.18 ± 0.08 ^{e}	38.61 ± 0.03 ^{c}	22.04 ± 0.08 ^{de}	44.46 ± 0.05 ^{d}	29.71 ± 0.08 ^{d}	
	0.75%	35.31 ± 0.11 ^{e}	42.74 ± 0.16 ^{f}	21.01 ± 0.06 ^{d}	47.63 ± 0.16 ^{d}	26.18 ± 0.06 ^{bd}	4.65
Lutein	0.25%	57.97 ± 0.57 ^{d}	-0.47 ± 1.87 ^{ag}	16.99 ± 0.94 ^{f}	17.09 ± 0.96 ^{e}	91.34 ± 6.26 ^{e}	
	0.75%	54.50 ± 0.66 ^{c}	5.53 ± 1.93 ^{h}	24.51 ± 1.72 ^{e}	25.20 ± 1.57 ^{f}	77.21 ± 4.66 ^{f}	10.22
Phycocyanin	0.25%	77.38 ± 0.53 ^{f}	1.50 ± 0.39 ^{g}	-15.14 ± 0.66 ^{g}	15.22 ± 0.68 ^{e}	275.65 ± 1.30 ^{g}	
	0.75%	79.18 ± 0.45 ^{f}	13.69 ± 0.37 ^{i}	-35.29 ± 0.92 ^{g}	37.85 ± 0.97 ^{b}	291.21 ± 0.29 ^{g}	23.62

*Different letters in the same column correspond to significant differences (p<0.05)

Table 5.2. Colour parameter values of gels coloured with microalgae biomass.

		L^*	a^*	b^*	C_{ab}*	$h°_{ab}$	ΔE^*
Control	0.00%	60.05 ± 1.18 ^{a}	-2.29 ± 0.15 ^{a}	5.87 ± 0.23 ^{a}	6.31 ± 0.20 ^{a}	111.37 ± 1.79 ^{a}	
Chlorella	0.25%	47.60 ± 0.79 ^{bc}	-8.66 ± 0.11 ^{b}	10.48 ± 0.48 ^{b}	13.60 ± 0.38 ^{b}	140.40 ± 1.26 ^{b}	
vulgaris green	0.75%	39.46 ± 0.41 ^{d}	-9.86 ± 0.62 ^{b}	9.20 ± 0.88 ^{bc}	13.49 ± 1.05 ^{b}	137.06 ± 0.95 ^{b}	8.33
Chlorella	0.25%	50.65 ± 0.17 ^{e}	3.06 ± 0.11 ^{c}	19.52 ± 0.45 ^{d}	19.76 ± 0.46 ^{cd}	81.09 ± 0.14 ^{c}	
vulgaris orange	0.75%	43.74 ± 0.47 ^{fg}	13.91 ± 0.48 ^{d}	18.06 ± 2.69 ^{de}	22.86 ± 1.86 ^{e}	52.09 ± 5.23 ^{d}	12.94
Haematococcus	0.25%	47.88 ± 0.51 ^{b}	5.30 ± 0.13 ^{e}	8.34 ± 0.13 ^{c}	9.88 ± 0.12 ^{f}	57.60 ± 0.82 ^{d}	
pluvialis	0.75%	39.11 ± 0.63 ^{d}	16.05 ± 0.23 ^{f}	9.02 ± 0.39 ^{bc}	18.41 ± 0.29 ^{c}	29.34 ± 1.05 ^{e}	13.89
Spirulina	0.25%	45.77 ± 0.22 ^{cf}	-3.00 ± 0.09 ^{ag}	8.31 ± 0.02 ^{c}	8.83 ± 0.03 ^{fg}	109.86 ± 0.58 ^{a}	
maxima	0.75%	36.49 ± 0.96 ^{h}	-3.22 ± 1.08 ^{g}	7.20 ± 0.91 ^{ac}	7.91 ± 1.22 ^{g}	113.62 ± 4.99 ^{af}	9.35
Diacronema	0.25%	42.90 ± 0.54 ^{g}	-9.13 ± 0.25 ^{b}	18.82 ± 0.47 ^{d}	20.92 ± 0.48 ^{d}	115.87 ± 0.66 ^{f}	
vlkianum	0.75%	34.58 ± 0.32 ^{i}	-7.18 ± 0.25 ^{h}	16.64 ± 0.68 ^{e}	18.13 ± 0.72 ^{c}	113.36 ± 0.25 ^{af}	8.82

*Different letters in the same column correspond to significant differences (p<0.05)

For *Spirulina* and *Diacronema*, gels were prepared at different temperatures ranging from 75ºC to 90ºC. The total colour difference between samples with different temperatures was not detectable by visual observation (ΔE^*<5) (Castellar *et al.*, 2006) (Table 5.3). These results are supported by the gels total pigments content, which remained relatively constant for the different gelling temperatures used. Considering the amount of pigments from microalgae (*Spirulina:* 0.9%; *Diacronema*: 2.4%; Table 3.5) that were added to the gels, the pigment losses were less than 20%, as it can be seen for *Spirulina* gels in Figure 5.5. Therefore, it can be

concluded that the gels colour and pigment content presented a good thermal stability when increasing the processing temperature from 75ºC to 90ºC, revealing an efficient pigment protection inside the *Spirulina* and *Diacronema* cells. A similar effect was previously observed for microalgae incorporation in biscuits systems (Gouveia *et al.*, 2008). The resistance of the microalgae bioactive molecules to different heat transfer processes, through "dry" and "wet" food matrices, evidences the potential of microalgae as food ingredients and/or nutraceutical delivery systems.

Table 5.3. Total colour difference (*ΔE**) between gels with 0.75% *Spirulina* and *Diacronema* microalgal biomass prepared at different temperatures.

	*ΔE**	
Temperature (ºC)	***Spirulina* gels**	***Diacronema* gels**
75	-	-
80	0.8	3.5
85	1.1	5.1
90	2.5	2.2

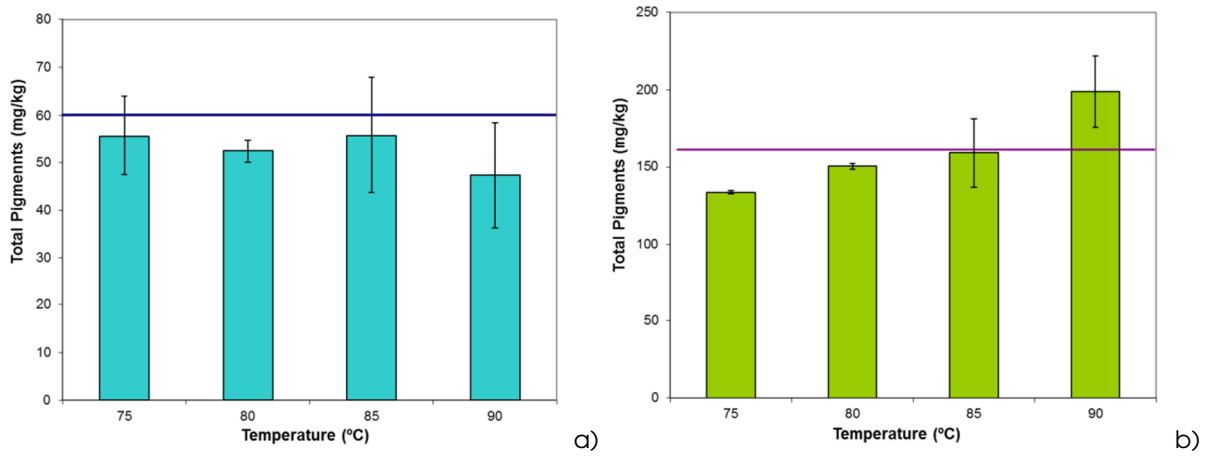


Figure 5.5. Temperature effect on total pigments content of gelled desserts with 0.75% *Spirulina* (a) and *Diacronema* (b) microalgal biomass. Bold line represents the amount of pigments added to the gel, considering the microalga pigment content (*Spirulina*: 0.9%; *Diacronema*: 2.4%).

5.3.1.2. Fatty acids profile

Both *Spirulina* and *Diacronema* gels were analysed in terms of fatty acids profile (Table 5.4). These microalgae have shown an interesting fatty acid profile (Table 3.4), particularly *Diacronema* which is rich in EPA (~21%) and DHA; while *Spirulina* is rich in γ-linolenic acid (GLA, 18:3ω_6) (17%).

Accordingly, *Diacronema* gels showed the presence of EPA (3-4%) and DHA (0.7-0.9%), and *Spirulina* gels the presence of GLA (1.3%), which are absent in the control gel (without alga addition. Linoleic (42-50%), oleic (16-20%) and palmitic (12-16%) are the main fatty acids for all the gels studied. In fact, a high level of linoleic acid was registered in the final product gels (42-50%), even in the samples prepared with *Diacronema*, where linoleic acid contribution is very low (0.3%). This result is probably due to the addition of residual lipids from maize starch and pea protein isolate used as ingredients in the preparation step, because linoleic acid is

the main fatty acid present in maize and pea oil (He *et al.* 2007; Chavan *et al.*, 1999; Yoshida *et al.*, 2007). The range of temperatures used in the preparation of these gels seems not to affect GLA, EPA and DHA percentage, suggesting that these microalgae cells are resistant to thermal treatments, as was referred by Gouveia *et al.* (2008) in a previous work with *Isochrysis galbana* submitted to a temperature of 121 ºC in a biscuit matrix. However this type of food (vegetable gelled desserts) products presents very low lipid content (0.4%) contributing to a limited level of these components.

Table 5.4. Fatty acid composition (percentage of total fatty acids) of gels prepared with *Spirulina* (*Sp*) and *Diacronema* (*Di*), at 75ºC and 90ºC; and control gel (without alga addition).

	Control Gel	*Sp* Gel 75ºC	*Sp* Gel 90ºC	*Di* Gel 75ºC	*Di* Gel 90ºC
SFA					
14:0 – miristic	0.2	-	0.2	2.7	3.2
16:0 – palmitic	14.3	15.8	15.8	13.3	12.2
18:0 – stearic	3.1	3.5	3.5	2.8	2.3
∑ SFA	17.7	19.7	19.2	19.4	18.2
MUFA					
16:1 ω_7 palmitoleic	-	0.5	0.5	3.2	3.8
18:1 ω_9 – oleic	18.7	19.7	19.2	16.7	15.6
∑ MUFA	19.1	20.8	20.6	20.7	20.3
PUFA-ω3					
18:3 ω_3 - α-linolenic	5.8	6.9	6.7	5.8	6.2
18:4 ω_3	-	-	-	1.2	1.5
20:5 ω_3 – EPA	-	-	-	3.1	4.1
22:6 ω_3 - DHA	-	-	-	0.7	0.9
EPA+DHA	-	-	-	3.9	5.0
∑ PUFA-ω_3	6.6	6.9	6.7	11.1	12.6
PUFA-ω6					
18:2 ω_6 – linoleic	47.1	50.4	49.1	41.9	43.0
18:3 ω_6 - γ-linolenic	-	1.3	1.3	0.2	0.2
22:5 ω_6	-	-	-	0.9	1.1
∑ PUFA-ω_6	47.1	51.8	50.4	42.1	43.3
∑ PUFA total	53.7	58.7	57.1	53.2	55.9

SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids

5.3.1.3. Texture profile

The addition of carotenoid pigments resulted in a significant increase of the gels firmness (Figure 5.6a), particularly for the highest concentration tested (0.75%). However, gels with 0.75% phycocyanin showed lower firmness values than the control gel. This is an interesting result, considering that for oil-in-water pea protein emulsions (section 4.3.1), an inverse tendency was observed *i.e.* lower firmness values for the lutein emulsions and higher for phycocyanin emulsions (Figure 4.7). This substantiates that using the same natural pigment to colour food products can produce different texturizing or de-structuring effects depending on the type of colloidal system

Generally, the addition of microalgal biomass promoted much smaller variations on the control gel firmness, as compared to the addition of pigments, and there were no significant differences on the gels firmness with increasing biomass concentration (Figure 5.6b). As

observed for phycocyanin, *Spirulina* gels also presented lower firmness values than the control, which decreased with microalgal biomass concentration.

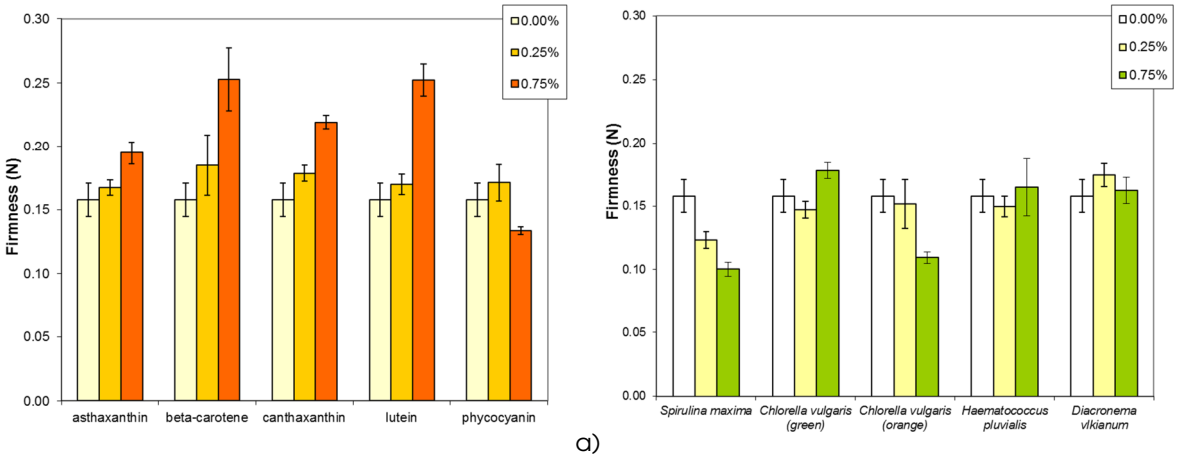


Figure 5.6. Firmness of gels with, different concentrations of natural pigments (a) and microalgal biomass (b), prepared at 90ºC/5min.

Diacronema gels presented a tendency of linearly increasing firmness with processing temperature (Figure 5.7), as it was observed for the control gel (0% microalga). This behaviour is typical of biopolymer systems, in which the gelation process requires denaturation and subsequent interaction of the exposed residues to form aggregates (Clark *et al*, 2001). High temperatures favour biopolymer interactions by inducing protein unfolding, κ-Carrageenan maximal hydration and total starch gelatinization, as demonstrated by Nunes *et al*. (2006a; 2006b).

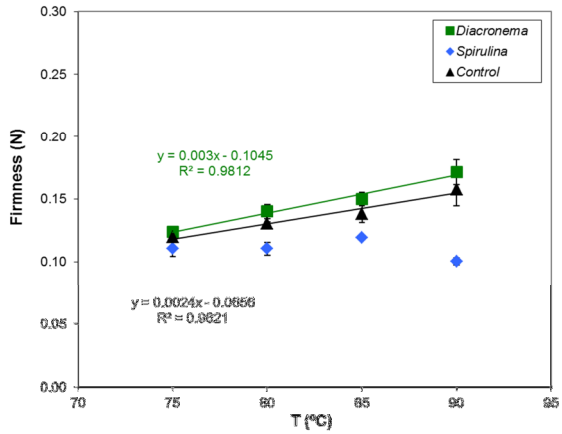


Figure 5.7. Effect of temperature on the firmness of gels with *Spirulina* and *Diacronema* (0.75% microalgal biomass), and the control gel.

However, *Spirulina* gels showed a different behaviour with a steep decrease on firmness for gelling temperatures above 85ºC (Figure 5.7). This destabilization process could be associated to a competitive interaction, or thermodynamic incompatibility, between the microalgal protein (44.9% of the dry biomass) and pea protein isolate. In fact, proteins isolated from

Spirulina have been reported (Chronakis, 2001) as being quite intricate biomaterials, likely to be protein and/or protein-pigment (phycocyanin) complexes rather than individual protein molecules. Therefore, *Spirulina* denaturation and gel formation is a complex phenomenon *per se*, which can probably interfere with the gelling process of the biopolymers present in the mixed gel system.

5.3.2. Rheological characterization of microalgae gels

The addition of different microalgae to pea protein/κ-carrageenan/starch gels had significant physical implications, as reflected by colour and texture changes studied in the previous section (5.3.1). The rheological behaviour of these gels (0.75% microalgal biomass) was studied, after thermal treatment at 90ºC/5 min in a controlled water bath. The samples were cooled in the rheometer plate-plate geometry from 40ºC to 5ºC at 0.6ºC/min, and the evolution of the viscoelastic functions (*G´, G"*) was monitored through dynamic temperature sweep tests.

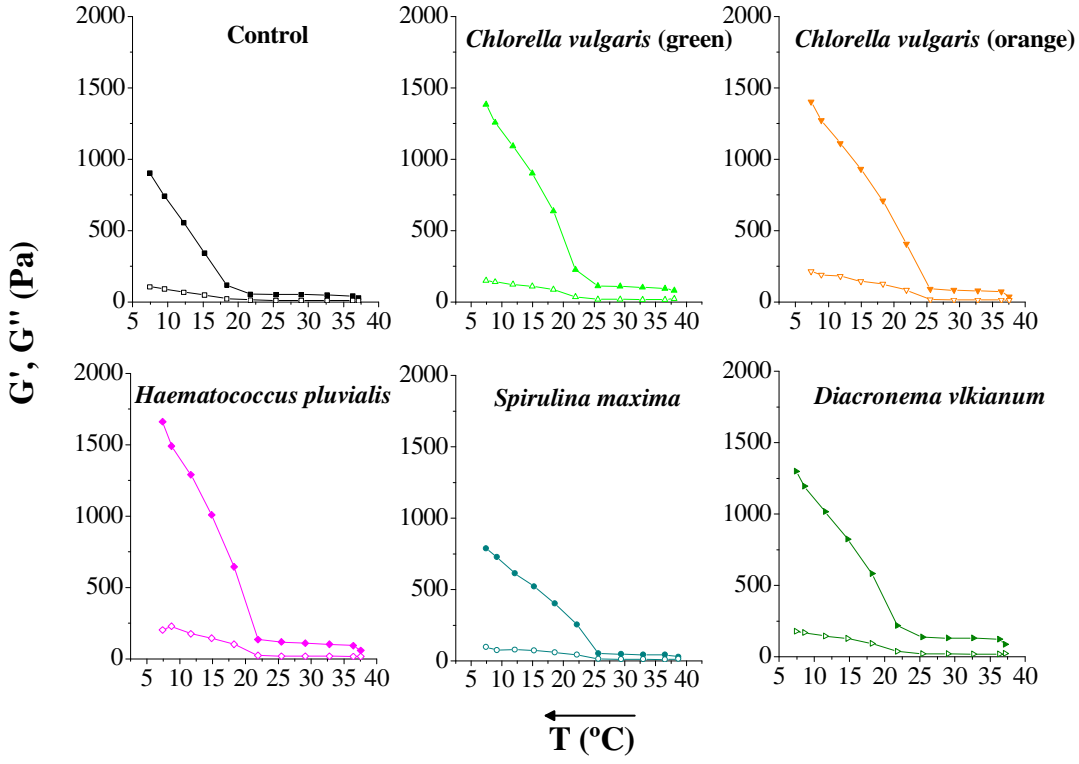


Figure 5.8. Evolution of SAOS functions in cooling curves, from 40ºC to 5ºC at 0.6ºC/min, for pea/κ-carrageenan/starch gels with 0.75% microalgal biomass addition. *G´* (filled symbol), G" (open symbol).

For all the gels studied, at 40ºC, the respective *G´* values were higher than *G"* values (Figure 5.8). However, a sharp rise in *G´*, associated with a decrease in the phase angle (δ), was observed upon cooling, revealing a structural reinforcement (Figure 5.9). The temperature at which this occurs may be regarded as the *"gelation temperature"* which reflects the

changes in the rheological properties of the gel network formation (Chronakis, 2001; Verbeken *et al.*, 2004). The addition of microalgal biomass resulted in an increase of the gelation temperature from 18.4ºC (control gel) to 21.8-22.0ºC (*Chlorella*, *Haematococcus* and *Diacronema*). The *Spirulina* gel presented even higher gelation temperatures of 25.8ºC, meaning that gel formation occurs earlier during the cooling process, although lower final values of SAOS functions were observed.

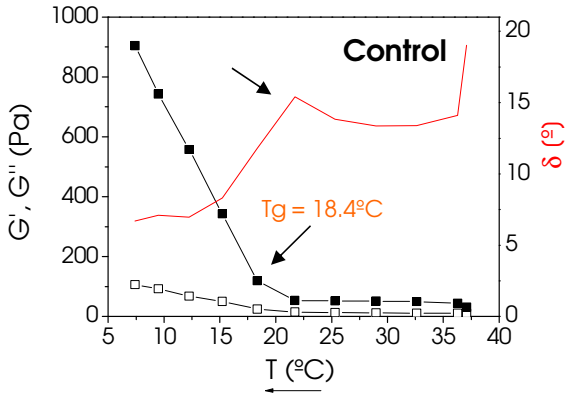


Figure 5.9. Method for determination of gelation temperature. *G´* (filled symbol), G" (open symbol).

The samples were further allowed to maturate at 5ºC during 24 h, monitoring the viscoelastic parameters by oscillatory time sweep tests. The maturation behaviour is typical of biopolymer gelation (Nunes *et al.*, 2003), with *G´* increasing rapidly at first and then more slowly (Figure 5.10).

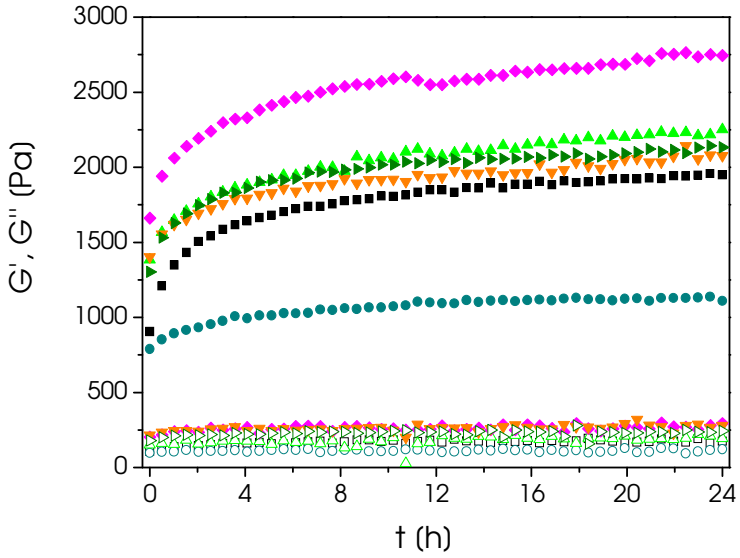


Figure 5.10. Maturation kinetic curves at 5ºC, of pea/κ-carrageenan/starch gels (■) with 0.75% microalgal biomass addition: *Chlorella vulgaris* green (▲) and orange (▼), *Haematococcus pluvialis* (♦), *Spirulina maxima* (●), and *Diacronema vlkianum* (►). *G´* (filled symbol), G" (open symbol).

The evolution found for gel maturation kinetic curves (Figure 5.10) allows a simple definition of an equilibrium G' value (G'_{eq}) *i.e.* the value of G' at infinite time (Nunes *et al.*, 2003; Clark *et al.*, 2001), where the gel reaches a stable and fully developed structure:

$$G'_{eq} = \lim_{t \to \infty} G'(t) \tag{5.3}$$

which can be rewritten as:

$$G'_{eq} = \lim_{\frac{1}{t} \to 0} G'(t) \tag{5.4}$$

being k the reciprocal time, *i.e.* $1/t$. Results can be fitted to a second order exponential decay of the form:

$$G'(k) = y_0 + A_1 e^{\frac{-k}{b_1}} + A_2 e^{\frac{-k}{b_2}} \tag{5.5}$$

where y_0, A_1, A_2, b_1 and b_2 are fitting parameters.
Estimation of G'_{eq} can be easily achieved by fitting experimental data to Equation 5.5, as exemplified in Figure 5.11.

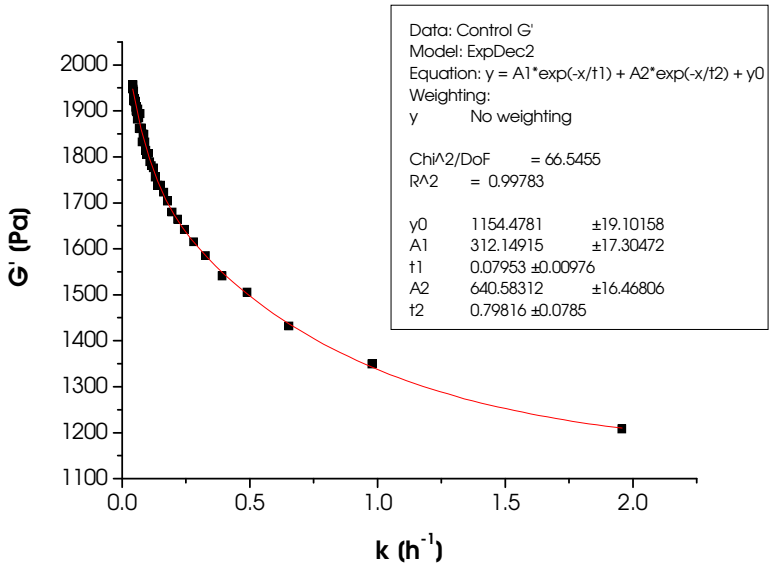


Figure 5.11. Fitting of eq. 5.5 to experimental maturation kinetic curves, represented as G' *vs.* k (1/t), for G'_{eq} determination.

G'_{eq} values are listed in Table 5.5 along with the exponential decay fitting parameters. By comparing the calculated G'_{eq} values with the experimental G' attained after 24 h maturation (G'_{24h}) it is possible to define a maturation index, given by the ratio $(G'_{24h}/G'_{eq})\times 100$. Full gel maturation was not achieved during the experimental procedure as

a result of continuous reorganization of the polymeric molecules in the gel network. However, for all gels the maturation index was above 90%.

The addition of *Chlorella* (green and orange) and *Diacronema* biomass resulted in gels with slightly higher viscoelastic properties (G'_{eq}=2219-2473 Pa) but a similar maturation pattern than the control gel (G'_{eq}=2107 Pa). *Haematococcus* gel presented much higher viscoelastic properties (G'_{eq}=2972 Pa) than the other gels while the addition of *Spirulina* promoted a drastic reduction on gel strength (G'_{eq}=1186 Pa).

Table 5.5. Parameters of exponential decay and calculated G'_{eq} (Eq. 5.5), experimental G' values after 24 h maturation (G'_{24h}), and maturation index (G'_{24h}/G'_{eq})×100 for pea/κ-carrageenan/starch gels with 0.75% microalgal biomass addition.

Gel	y_0 (Pa)	A_1 (Pa)	b_1 (h^{-1})	A_2 (Pa)	b_2 (h^{-1})	χ^2	R^2	G'_{eq} (Pa)	G'_{24h} (Pa)	(G'_{24h}/G'_{eq})×100 (%)
Control	1154	312.1	0.079	640.6	0.798	66.5	0.9978	2107	1957	92.5
Chlorella vulgaris (green)	1553	427.0	0.064	492.9	0.565	274	0.9910	2473	2249	90.9
Chlorella vulgaris (orange)	1558	405.2	0.451	256.5	0.056	557	0.9646	2219	2142	96.5
Haematococcus pluvialis	1973	711.6	0.396	286.8	0.047	572	0.9850	2972	2762	93.0
Spirulina maxima	838.5	177.3	0.783	170.7	0.138	75.9	0.9858	1186	1137	95.9
Diacronema vlkianum	1513	521.8	0.609	200.5	0.075	166	0.9919	2236	2142	95.8

The structural reinforcement action of *Haematococcus* may be related to its significantly higher fat content (40.7%) in relation to other microalgae. The influence of fat content on gelling behaviour has been studied in milk gelled systems, such as acid milk gels (Houzé *et al.*, 2005; Lucey *et al.*, 1998) and dairy custard model systems (Vélez-Ruiz *et al.*, 2005), from which it was concluded that using high fat milk rather than skim milk results in stronger gels, despite the low fat level used. The modification on the rheological properties of fat-containing gels are usually attributed to fat droplets acting as active filler particles embedded in the protein matrix (Houze *et al.*, 2005; Lucey *et al.*, 1998).

The destabilization process induced by *Spirulina* biomass addition in terms of rheological behaviour, had already been observed for texture results (Figures 5.6-5.7). As referred before, this effect could be associated to interferences in the gelling process of the biopolymers present in the mixed gel system, considering that *Spirulina* proteins denaturation and gel formation is a complex phenomenon (Chronakis, 2001).

After the maturation period, without disturbing the sample, frequency sweeps were conducted at 5ºC. From the resulting mechanical spectra (Figure 5.12) can be observed that G' values are always around one decade higher than those found for G" in the frequency range studied, showing both SAOS functions only a slight dependence with frequency. G' values obtained at different frequencies follow the same tendency than that previously mentioned for G'_{eq}, *i.e.* values similar to the control for *Chlorella* (green and orange) and

Diacronema gels, much higher values for *Haematococcus* addition and much lower for *Spirulina* gels (about 40% variation in both cases).

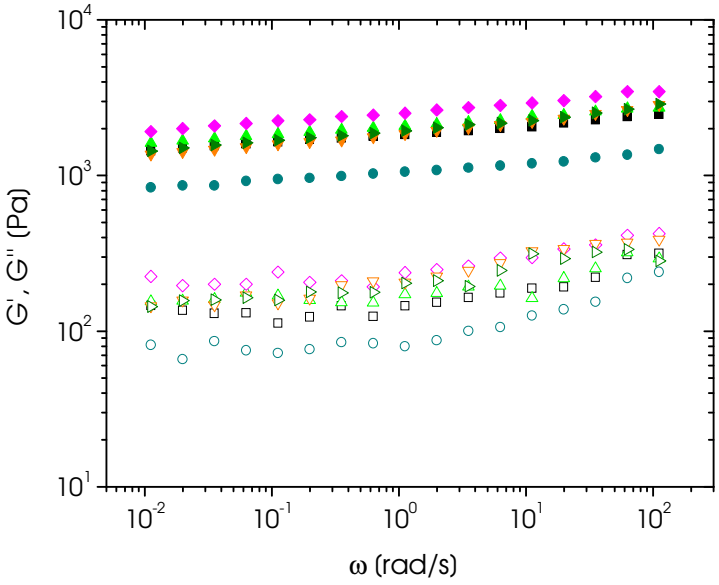


Figure 5.12. Mechanical spectra of pea/κ-carrageenan/starch gels (■) with 0.75% microalgal biomass addition: *Chlorella vulgaris* green (▲) and orange (▼), *Haematococcus pluvialis* (♦), *Spirulina maxima* (●), and *Diacronema vlkianum* (▶). *G'* (filled symbol), G" (open symbol).

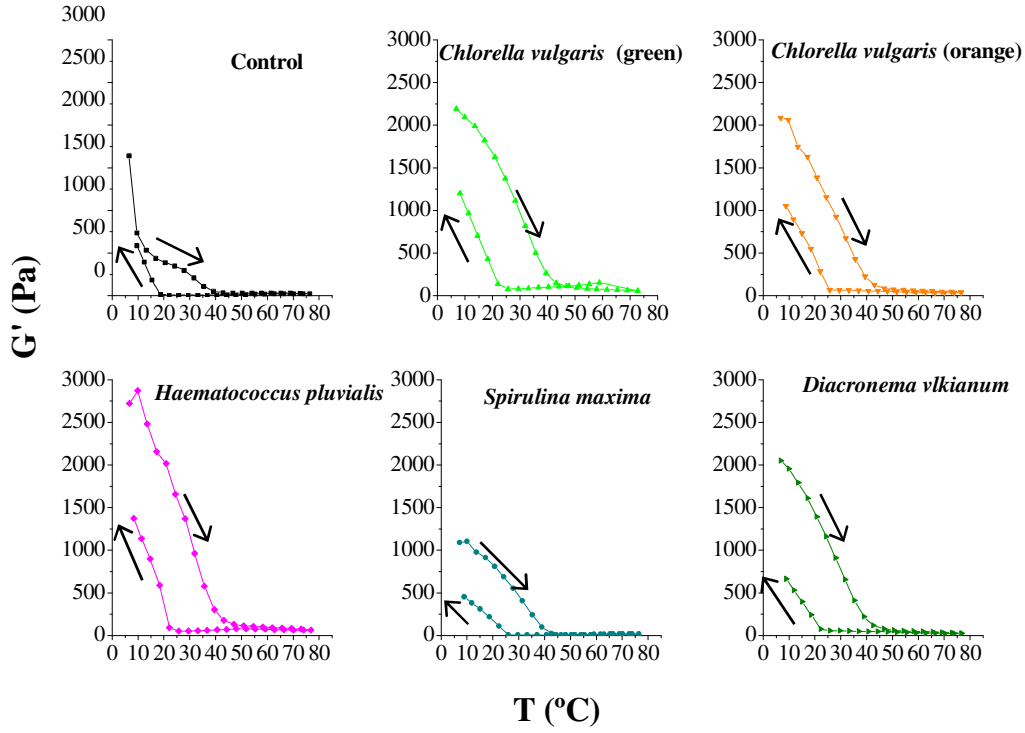


Figure 5.13. Evolution of G' in heating (from 5º to 80ºC) and cooling (from 80º to 5ºC) curves, at 0.5ºC/min, for pea/κ-carrageenan/starch gels with 0.75% microalgal biomass addition.

In order to access the gels thermoreversible character, the samples were afterwards continuously heated up from 5º to 80ºC and subsequently cooled down from 80º to 5ºC at 0.5ºC/min by a temperature sweep test. From Figure 5.13, it can be observed that the storage modulus values decrease sharply during the heating up process until 40-50ºC (much higher than the gelation temperature) and then remain relatively constant until 80ºC ($G'>G''$, results not shown). Upon subsequent cooling it was observed that all gels were only partially thermo-reversible displaying a thermal hysteresis.

5.3.3. Effect of gel setting conditions on the linear viscoelasticity of *Spirulina* and *Haematococcus* gels

Considering the distinct rheological behaviour presented by *Spirulina* and *Haematococcus* microalgae in pea protein/κ-carrageenan/starch gelled systems, it was decided to extend the rheological studies on these particular systems. So, in the present section, the effect of gel setting conditions on the linear viscoelasticity is discussed.

5.3.3.1. Effect of processing temperature and time

The effect of thermal treatment on the gels' rheological behaviour was studied by setting different maximum temperature and time combinations – 70ºC/5min, 80ºC/5min, 90ºC/5 min, 90ºC/15min and 90ºC/30 min. Figure 5.14 presents G' and G'' evolution upon thermal treatment, as well as through heating (up from 20ºC) and cooling (down to 5ºC) ramps (±5ºC/min).

Pea/κ-carrageenan/starch suspensions at 20ºC presented very low G' and G'' values, which were of the same order of magnitude (around 1 Pa). Heating the samples induced a decrease on the viscoelastic functions, until a slight increase occurs around 84ºC corresponding to starch gelatinization. This phenomenon is related to the swelling of the starch granules, and subsequent amylose solubilisation, which causes an increase on the systems viscosity (Morris, 1990). Therefore, in the samples heated only at 70ºC and 80ºC starch gelatinization did not occur, at least at its full length, causing a negative impact on the gels mechanical properties. In all the samples with *Spirulina* addition, this transition was not detected indicating that this microalga interferes with the starch gelatinization process, perhaps by competing for water binding sites during the granules hydration process, as proposed in section 5.3.4. *Haematococcus* suspensions always presented $G'>G''$ during gel setting, with initial G' values around 60 Pa, suggesting the existence of an incipient internal microstructure in this state.

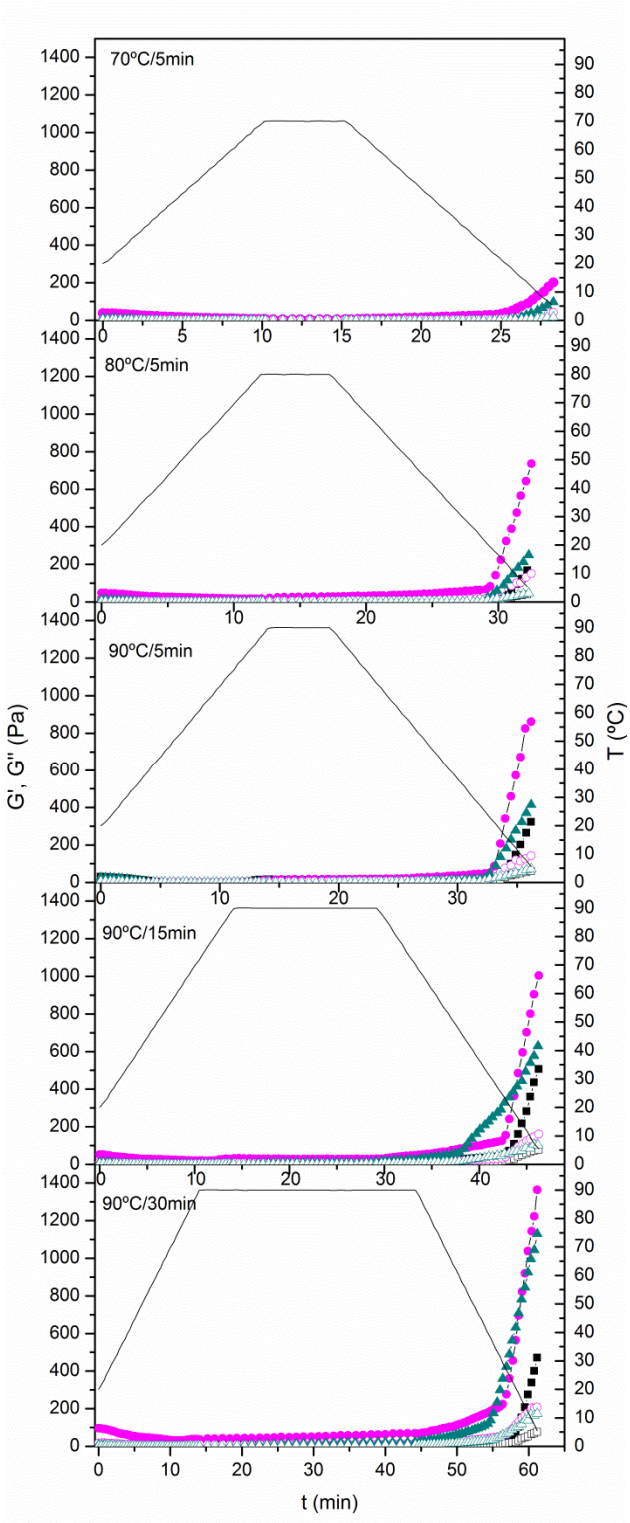


Figure 5.14. Evolution of *G´* and *G´´*, of pea/κ-carrageenan/starch suspensions (■) with *Haematococcus* (●) and *Spirulina* (▲), during thermal treatment, performed at different temperature/time conditions (70ºC/5min, 80ºC/5min, 90ºC/min, 90ºC/15min and 90ºC/30min). (Heating/cooling rates: ± 5.0ºC/min). *G´* (closed symbol), *G"* (open symbol), *T* (line).

After thermal treatment, and upon cooling to 5ºC, it is observed a small increase in the viscoelastic functions, until a sharp rise in *G´* arises (Figure 5.14), which is coincident with a dramatic decrease in phase angle (δ), revealing an important structural reinforcement (as in

Figure 5.9). The temperature at which this occurs may be regarded as a "*gelation temperature*" which reflects the changes in the rheological properties of the gel network formation (Chronakis, 2001; Verbeken *et al.*, 2004). These "*gelation temperatures*" (T_{gel}) should not be regarded as true "*gel points*", meaning the appearance/disappearance of an infinite network, as defined by other authors (Winter and Chambon, 1986). These gelation temperatures are presented in Table 5.6. T_{gel} values of control gel increased from 10.3 to 16.4ºC with increasing temperatures (70-90ºC/5min); and up to 25.0ºC, by extending thermal treatment duration to 90ºC/30min.

Table 5.6. Gelation temperature (T_{gel}), parameters of exponential decay, and calculated G'_{eq} (Eq. 5.5), experimental G' values after 24 h maturation (G'_{24h}), and maturation index (G'_{24h}/G'_{eq})×100 of pea/κ-carrageenan/starch gels with *Haematococcus* and *Spirulina*, after thermal treatment performed at different temperature/time conditions. (Heating/cooling rates: ± 5.0ºC/min).

T/t (ºC/min)	T_{gel} (ºC)	y_0 (Pa)	A_1 (Pa)	b_1 (h^{-1})	A_2 (Pa)	b_2 (h^{-1})	χ^2	R^2	G'_{24h} (Pa)	G'_{eq} (Pa)	$(G'_{24h}/G'_{eq})\times 100$ (%)
Haematococcus											
70/5	18.6	674	585	0.045	750	0.546	151	0.997	1642	2010	81.7
80/5	18.8	1504	389	0.044	827	0.605	284	0.994	2428	2720	89.3
90/5	20.7	1551	1694	0.019	871	0.423	448	0.992	2510	4116	61.0
90/15	22.9	1767	786	0.053	787	0.744	402	0.994	2890	3340	86.5
90/30	27.2	2084	1531	0.033	920	0.622	707	0.993	3430	4535	75.6
Spirulina											
70/5	21.6	-	-	-	-	-	-	-	414	-	-
80/5	21.8	495	186	1.089	299	0.028	9	0.996	720	980	73.5
90/5	22.9	702	339	0	194	1	4	0.997	917	1235	74.3
90/15	37.4	726	214	0.114	264	1.022	18	0.998	1128	1204	93.7
90/30	41.6	1597	457	0.070	213	0.252	100	0.994	2033	2268	89.7
Control											
70/5	10.3	231	309	0.071	497	0.791	9	0.999	878	1037	84.7
80/5	14.9	651	317	0.081	515	1.153	10	0.999	1338	1482	90.3
90/5	16.4	881	463	0.048	669	0.731	35	0.999	1706	2013	84.8
90/15	20.6	1127	657	0.039	809	0.732	96	0.998	2122	2593	81.8
90/30	25.0	1118	660	0.049	721	0.725	100	0.998	2094	2498	83.8

Samples with microalgal biomass addition presented the same behaviour with a higher T_{gel} with increasing temperature and time, which for *Haematococcus* ranged from 18.6 to 27.2ºC and for *Spirulina* ranged from 21.6 to 41.6ºC. The achievement of a higher T_{gel} means that gel formation occurs earlier during the cooling process, being possible to obtain gels at room temperature by increasing the intensity of the thermal treatment. From the results obtained, it can be assumed that by using microalgae biomass it is possible to attain higher T_{gel} while using milder thermal processing conditions, which enable the preservation of natural compounds such as natural pigments (*e.g.* astaxanthin and phycocyanin).

As previously discussed, the maturation behaviour of these gels is typical of biopolymer gelation (Clark *et al.*, 2001), with G' increasing rapidly at first and then more slowly, as a result of continuous reorganization of the polymeric molecules in the gel network (Figure 5.15). The evolution found for gel maturation kinetic curves allowed the calculation a G' value at the pseudo-equilibrium-state (G'_{eq}) according to eq. 5.5, which are presented in Table 5.6 along

with the experimental G' attained after 24 h maturation (G'_{24h}), and the maturation index, given by the ratio (G'_{24h}/G'_{eq})×100.

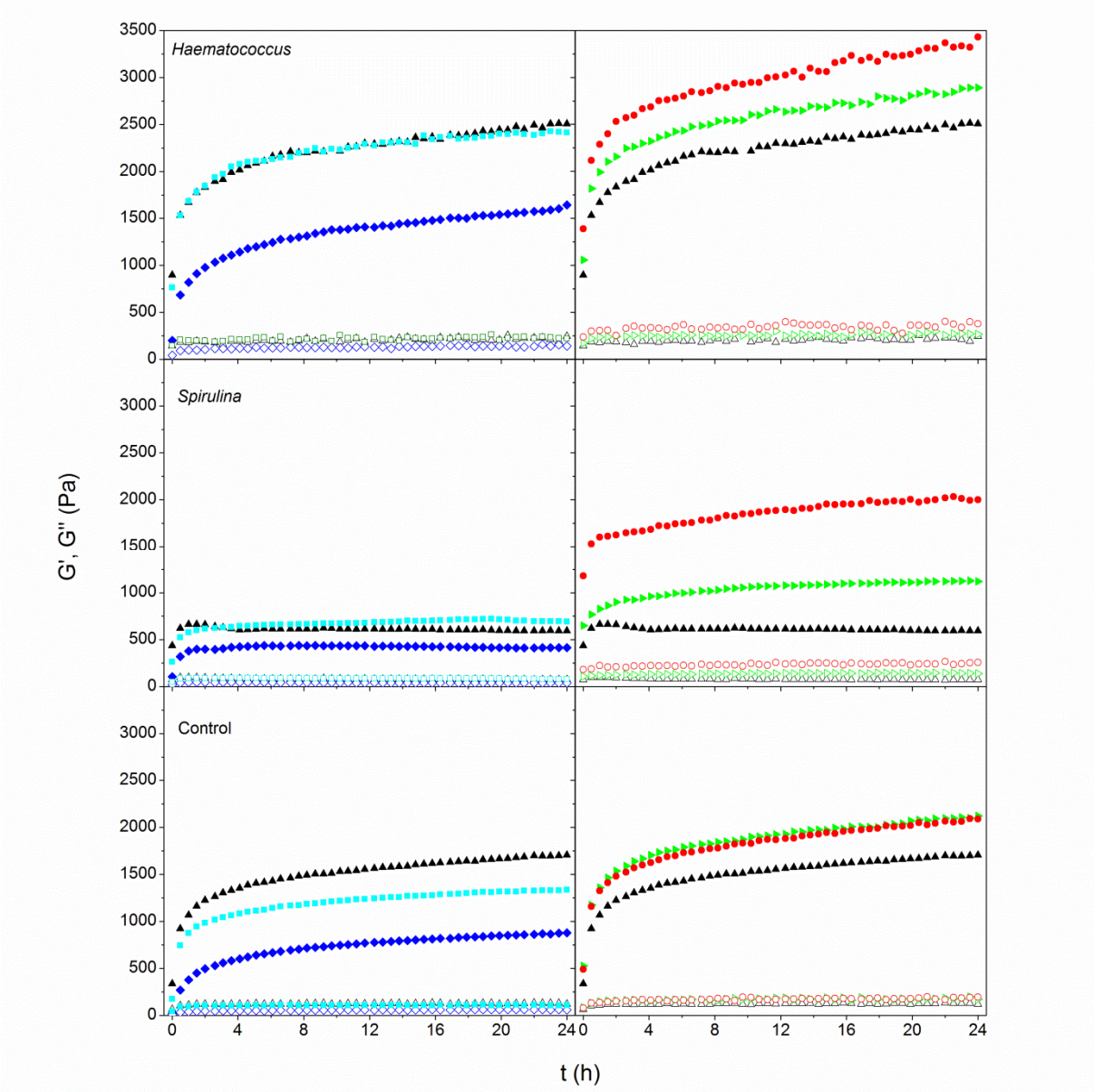


Figure 5.15. Maturation kinetic curves at 5ºC, of pea/κ-carrageenan/starch gels with *Haematococcus* and *Spirulina*, after thermal treatment at 70ºC/5min (♦), 80ºC/5min (■), 90ºC/5min (▲), 90ºC/15min (►) and 90ºC/30min (●). (Heating/cooling rates: ± 5.0ºC/min). G' (closed symbol), G'' (open symbol).

Pea/κ-carrageenan/starch control gels presented increasing G'_{24h} and G'_{eq} when increasing processing temperature from 70 to 90ºC. The effect of processing time was less important (small variations between 15 and 30 min), which is in agreement with previous studies (Nunes *et al.*, 2004). High temperature induces globular protein unfolding and exposure of higher number of linking sites with subsequent reinforcement of gel structure (van Vliet *et al.*, 2002). κ-Carrageenan also requires high temperatures to achieve maximal hydration (Schmidt and Smith, 1992) which will support subsequent gelation involving conformational transition (single

chain to double helix) and aggregation when the system is cooled (Morris *et al.*, 1980). Starch gelatinization process is also temperature dependent, as previously discussed.

Haematococcus gels presented much higher G'_{24h} and G'_{eq} values than the control gel (Table 5.6). As referred before, this structural reinforcement action may be related to its high fat content (41%), since fat droplets can act as active filler particles embedded in the protein matrix (Houzé *et al.*, 2005). In fact, even when submitted to a milder thermal treatment (70ºC/5min) *Haematococcus* gels presented a G'_{eq} of 2010 Pa, similar to the control gel heated at 90ºC/5min (2013 Pa). This enables the use of lower temperatures to reach a similar product, avoiding thermal degradation of natural biomolecules (*e.g.* astaxanthin). All gels presented higher viscoelastic properties with increasing temperature, as a result of improved protein unfolding and biopolymer interaction. The effect of heating time was less pronounced on the gel properties.

In the case of *Spirulina* gels, they always presented lower G'_{24h} and G'_{eq} values than the control gel (Table 5.6), which is in agreement with previous results (section 5.3.2). In fact, for the different thermal treatments applied *Spirulina* gels provide G'_{24h} values about half of those found for the control, except in the case of 90ºC/30min, where the values are of the same order of magnitude (2033 Pa and 2094, respectively). This means, that in order to obtain a *Spirulina* gel with mechanical properties similar to the control a more extensive thermal treatment, involving higher temperatures and times of exposure, should be applied. It is suggested that *Spirulina* protein molecules compete for water binding sites, hindering the hydration of starch granules, since this microalga (Cyanobacteria) lacks a rigid cell-wall, according to results presented in the following section (5.3.4). It is thus possible to provide conditions for more extensive starch granules swelling and amylose release by increasing the intensity of the thermal treatment.

5.3.3.2. Effect of heating and cooling rates

Gel setting conditions play a major role on the development of gel structure and resulting rheological properties, and are determined not only by the temperature/time applied but also by the heating and cooling rates implemented (*e.g.* Turgeon and Beaulieu, 2004; Nunes *et al.*, 2006a). To study the influence of these factors on pea/κ-carrageenan/starch systems with microalgal biomass, tests were conducted at 90ºC/5min, applying different heating and cooling rates. Figure 5.16 presents G' and G'' evolution of the samples that were submitted to the same heating and cooling rates: ±0.5ºC/min, ±1.0ºC/min and ±5.0ºC/min. For the slower heating/cooling rates (±0.5ºC/min, ±1.0ºCmin) an increase in T_{gel} was observed (Table 5.7) compared to the faster heating/cooling rate (±5.0ºC/min): from 16.4 to 23.6ºC in the control; from 20.7 to 25ºC in *Haematococcus*, and from 22.9 to 35ºC in *Spirulina*. Although an increase

of gel setting temperature is an advantageous feature, it should be kept in mind that the time length of the processing (5.3 h and 2.7 h against 36 min) is a major drawback.

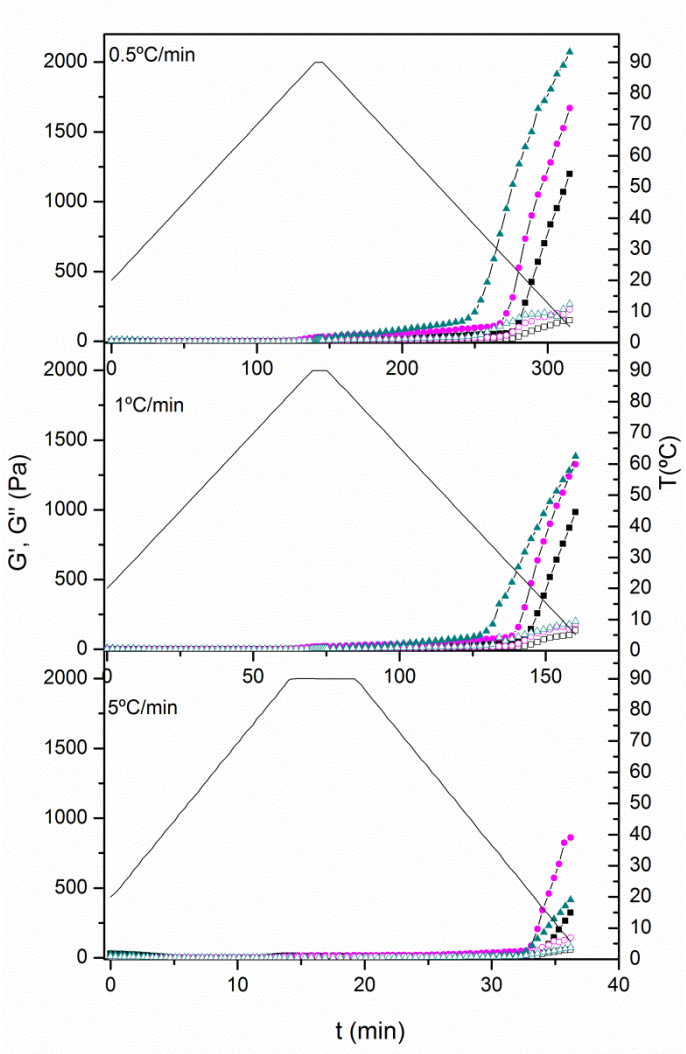


Figure 5.16. Evolution of *G´* and *G´´*, of pea/κ-carrageenan/starch suspensions (■) with *Haematococcus* (●) and *Spirulina* (▲), during thermal treatment (90ºC/5min), performed at the same heating and cooling rates (± 0.5, 1.0 and 5.0ºC/min). *G´* (closed symbol), *G´´* (open symbol), *T* (line).

Maturation kinetic curves are presented in Figure 5.17 and the respective fitting parameters derived from eq. 5.5 are presented in Table 5.7. For the Control gel, higher G'_{24h} and G'_{eq} (~2300 and 2700 Pa) are observed for lower heating/cooling rates with only small differences between them. In the case of *Haematococcus* gels, the differences in G'_{24h} are less pronounced for the various rates (2510-2971 Pa) and in terms of calculated G'_{eq} these differences are even smaller, being the highest value attained for the faster rates (4116 Pa). Oppositely, *Spirulina* gels show major differences between each of the three rates, indicating that for these systems it is highly advantageous to use lower heating/cooling rates upon gel setting, in order to attain a higher structuring degree. In fact, at ±0.5ºC/min *Spirulina* gel attained higher G'_{24h} and G'_{eq} values than the control system (2871-3329 Pa against 2346-2763 Pa).

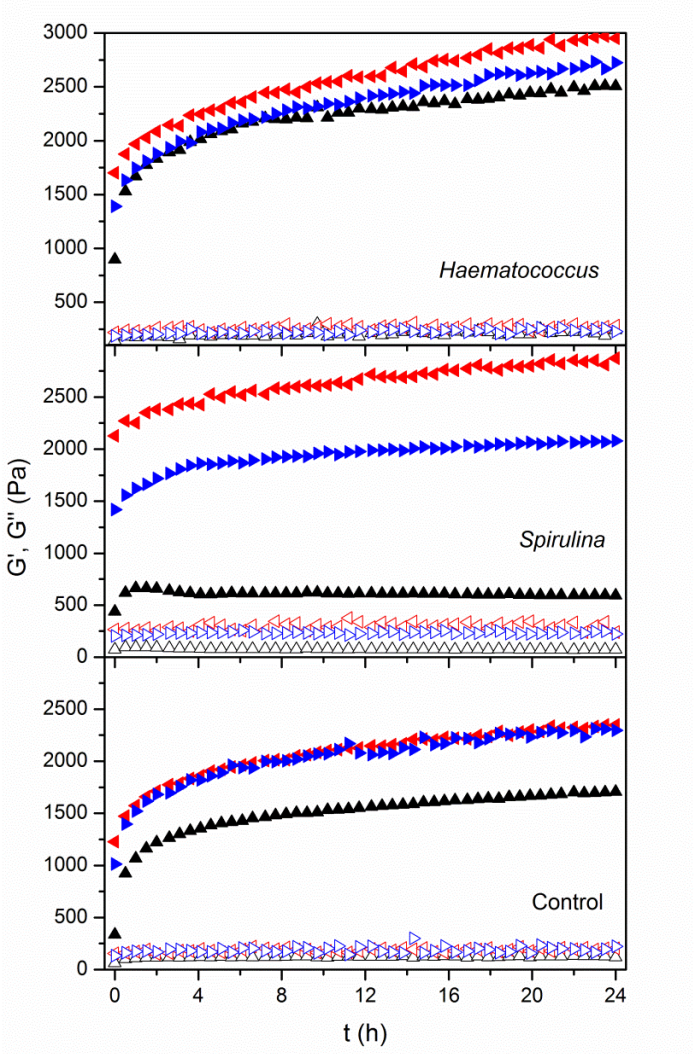


Figure 5.17. Maturation kinetic curves at 5ºC, of pea/κ-carrageenan/starch gels with *Haematococcus* and *Spirulina*, after thermal treatment (90ºC/5min) performed at the same heating and cooling rates, ±0.5ºC/min (◄), ±1.0ºC/min (►) and ±5.0ºC/min (▲). *G´* (closed symbol), *G"* (open symbol).

Table 5.7. Gelation temperature (T_{gel}), parameters of exponential decay, and calculated ***G'eq*** (Eq. 5.5), experimental *G´* values after 24 h maturation (G'_{24h}), and maturation index (G'_{24h}/G'_{eq})×100 of pea/κ-carrageenan/starch gels with *Haematococcus* and *Spirulina*, after thermal treatment (90ºC/5min) performed at the same heating and cooling rates.

Heating/cooling rate (ºC/min)	T_{gel} (ºC)	y_0 (Pa)	A_1 (Pa)	b_1 (h^{-1})	A_2 (Pa)	b_2 (h^{-1})	χ^2	R^2	G'_{24h} (Pa)	G'_{eq} (Pa)	(G'_{24h}/G'_{eq})× 100 (%)
Haematococcus											
+0.5/-0.5	25.6	1894	1387	0.037	708	0.367	452	0.995	2971	3989	74.5
+1.0/-1.0	24.7	1648	1149	0.037	774	0.401	421	0.995	2733	3571	76.5
+5.0/-5.0	20.7	1551	1694	0.019	871	0.423	448	0.992	2510	4116	61.0
Spirulina											
+0.5/-0.5	34.8	2257	663	0.041	408	0.366	462	0.985	2871	3329	86.2
+1.0/-1.0	35.6	1544	328	0.036	471	0.523	103	0.994	2079	2343	88.7
+5.0/-5.0	22.9	702	339	0	194	1	4	0.997	917	1235	74.3
Control											
+0.5/-0.5	23.6	1464	664	0.051	636	0.533	158	0.997	2346	2763	84.9
+1.0/-1.0	23.6	1393	615	0.047	719	0.521	928	0.983	2312	2726	84.8
+5.0/-5.0	16.4	881	463	0.048	669	0.731	35	0.999	1706	2013	84.8

Many studies on biopolymers evidence that cooling rate influences the mechanical properties of simple gels (*e.g.* Lopes-da-Silva and Rao, 1995) as well as the phase separation process and physical properties of mixed systems (Lorén *et al.*, 1999; Turgeon and Beaulieu, 2004). Considering that cooling rate plays a major role on the development of gel structure, larger than the heating rate does, it was decided to maintain the thermal treatment at 90ºC/5min and using a constant heating rate of 5ºC/min while studying the effect of different cooling rates: -0.5º, -1.0, -5.0 and -10.0ºC/min.

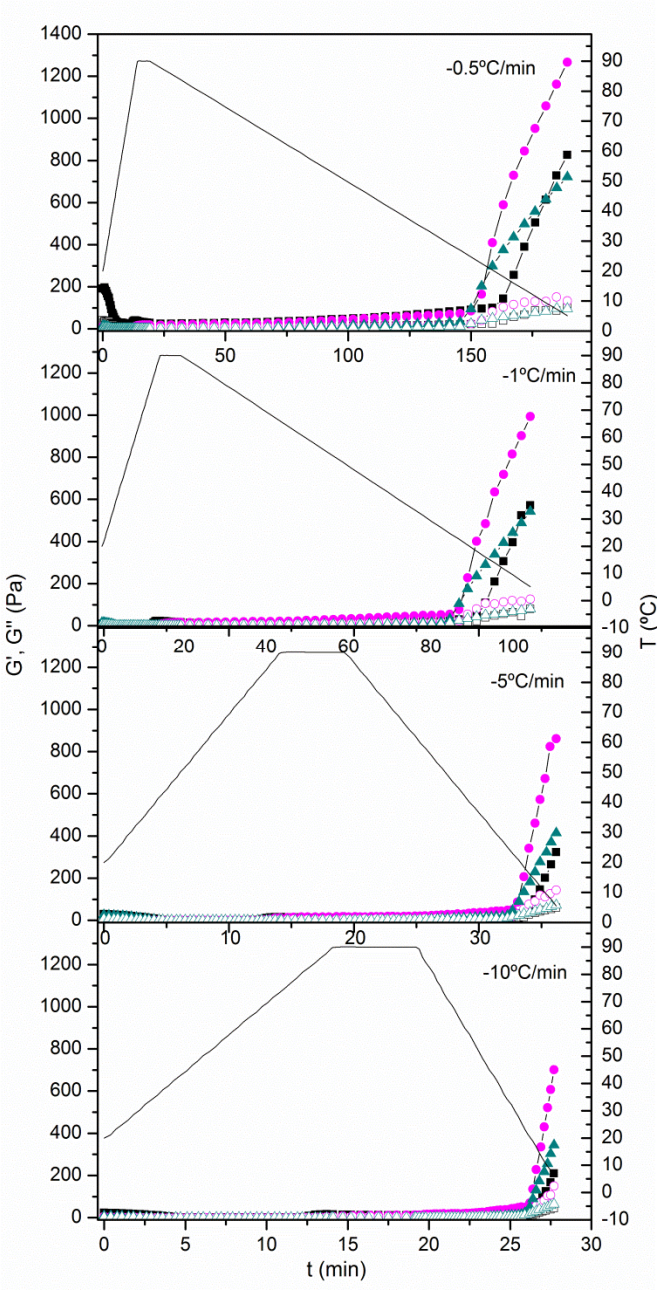


Figure 5.18. Evolution of *G´* and *G´´*, of pea/κ-carrageenan/starch suspensions (■) with *Haematococcus* (●) and *Spirulina* (▲), during thermal treatment (90ºC/5min), performed at different cooling rates (-0.5, -1.0, -5.0 and -10.0ºC/min). *G´* (closed symbol), *G"* (open symbol), *T* (line).

From Figure 5.18 and Table 5.8 it can be observed that control gels presented a T_{gel} of 18.1-18.2ºC for lower cooling rates (-0.5 and -1ºC/min), and 16.4-16.6ºC for higher cooling rates (-5.0 and -10ºC/min). As previously discussed, samples with microalgae addition presented higher T_{gel} values which increased with decreasing cooling rates. T_{gel} ranged from 18.7 to 22.5ºC for *Haematococcus* gels, and from 21.0 to 24.7ºC, for *Spirulina* gels. At higher cooling rates the temperature decreases too rapidly (8.5 and 17 min for -5 and -10ºC/min, respectively), not allowing the solution to reach equilibrium at each temperature step, requiring lower temperatures for gel setting, since gelation time increases exponentially with temperature (Tosh and Marangoni, 2004).

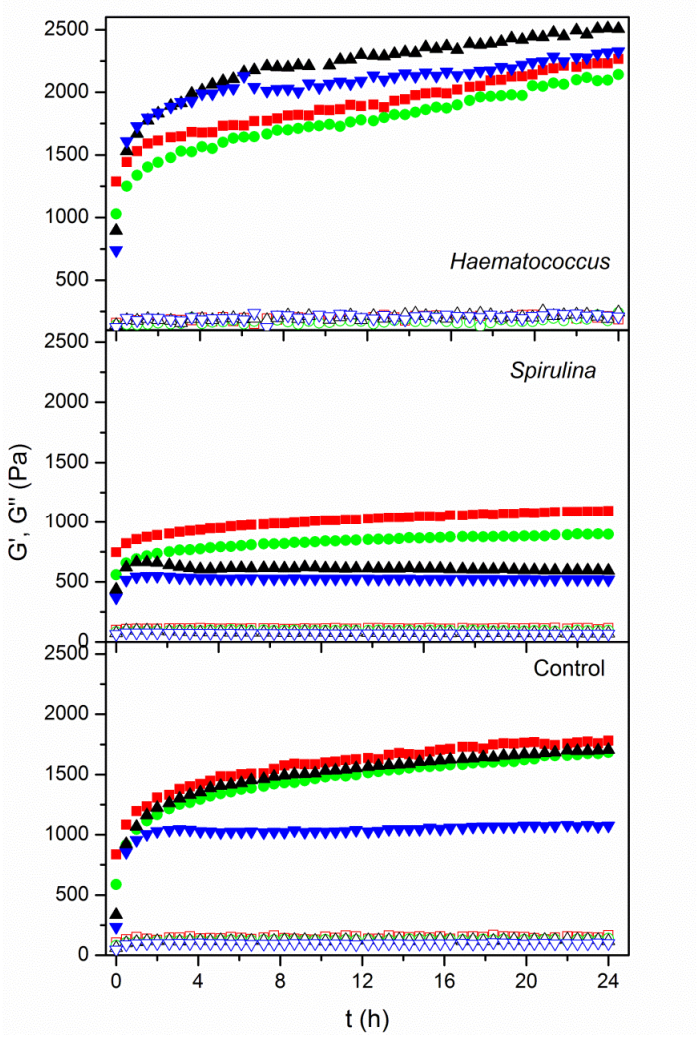


Figure 5.19. Maturation kinetic curves at 5ºC, of pea/κ-carrageenan/starch gels with *Haematococcus* and *Spirulina*, after thermal treatment (90ºC/5min) performed at different cooling rates, -0.5ºC/min (■), -1.0ºC/min (●), -5.0ºC/min (▲) and -10.0ºC/min (▼). *G´* (closed symbol), *G"* (open symbol).

Maturation kinetic curves are presented in Figure 5.19, and the respective fitting parameters derived from eq. 5.5 are listed in Table 5.8. *Spirulina* gels presented significantly smaller G'_{24h} and G'_{eq} (<1200 Pa) values, than the control gels (>1600 Pa, excepting for cooling rates of 10ºC/min). This is in accordance with previous results (section 5.3.3.1) and could be related to

a competitive interaction between the microalga and the other biopolymers present in the mixed gel system, namely starch, which is supported by the present heating curves results (Figure 5.18).

Table 5.8. Gelation temperature (T_{gel}), parameters of exponential decay, and calculated G'_{eq} (Eq. 5.5), experimental G' values after 24 h maturation (G'_{24h}), and maturation index (G'_{24h}/G'_{eq})×100 of pea/κ-carrageenan/starch gels with *Haematococcus* and *Spirulina*, after thermal treatment (90ºC/5min) performed at different cooling rates.

Heating/cooling rate (ºC/min)	T_{gel} (ºC)	y_0 (Pa)	A_1 (Pa)	b_1 (h^{-1})	A_2 (Pa)	b_2 (h^{-1})	χ^2	R^2	G'_{24h} (Pa)	G'_{eq} (Pa)	(G'_{24h}/G'_{eq})× 100 (%)
Haematococcus											
+5.0/-0.5	22.5	1444	1901	0.029	397	0.566	258	0.995	2266	3742	60.6
+5.0/-1.0	21.1	1260	543	0.441	2166	0.224	269	0.995	2141	3969	53.9
+5.0/-5.0	20.7	1551	1694	0.019	871	0.423	448	0.992	2510	4116	61.0
+5.0/-10.0	18.7	1582	553	0.690	2540	0.017	350	0.987	2328	4675	49.8
Spirulina											
+5.0/-0.5	24.7	820	175	0.552	216	0.062	9	0.998	1095	1211	90.4
+5.0/-1.0	24.7	644	181	0.732	152	0.069	7	0.998	900	977	92.1
+5.0/-5.0	22.9	702	339	0	194	1	4	0.997	917	1235	74.3
+5.0/-10.0	21.0	-	-	-	-	-	-	-	519	-	-
Control											
+5.0/-0.5	18.1	1066	457	0.062	517	0.632	169	0.995	1780	2040	87.2
+5.0/-1.0	18.2	887	589	0.684	549	0.049	58	0.998	1683	2025	83.1
+5.0/-5.0	16.4	881	463	0.048	669	0.731	35	0.999	1706	2013	84.8
+5.0/-10.0	16.6	-	-	-	-	-	-	-	1081	-	-

Denaturation of *Spirulina* components and gel formation has been reported as a complex phenomenon, as a consequence of the presence of protein-pigment (phycocyanin) complexes (Chronakis, 2001). Gels submitted to slower cooling rates generally presented higher G'_{24h} and G'_{eq} values. In fact, for systems cooled slowly, there is more time to achieve dynamic equilibrium of the gel network, *i.e.* gel structure maturation, leading to a more structured network on cooling (Nunes *et al.*, 2006a). However, for the systems under study, the rheological properties (G'_{24h}, G'_{eq}) of the gels cooled at -1 and -5ºC/min are not substantially different. This presents an advantage considering that lesser time (17 min instead of 85 min) will be needed to achieve a similar final product. Gels subjected to the fastest cooling rates (-10ºC/min) showed much lower G'_{24h} values (control: 1081 Pa, *Spirulina*: 519 Pa). The maturation kinetic curve of these gels is also quite different, being not well adjusted to eq. 5.5. In these cases, G' increases rapidly up to a maximum and then slightly decreases, reaching an almost constant equilibrium value. This behaviour resembles a structural relaxation phenomenon, especially if considering that the rapid cooling would not let the solution to reach equilibrium at each temperature step, as referred before (Tosh and Marangoni, 2004).
On the contrary, in the case of *Haematococcus* gels, a general tendency for increasing G'_{eq} or G'_{24h} with decreasing cooling rates was not observed, inclusively the highest values were attained for the faster cooling rates. This suggests that the addition of *Haematococcus* promotes a structural reinforcement of the gel matrix that is rather independent of the cooling rate, under these gel setting conditions (90ºC/5min, +5ºC/min).

5.3.4. Binary systems - Microalgal biomass interaction in biopolymer gelled systems

In complex mixed systems it is difficult to determine the influence of microalgal addition, since there are many factors and process taking place simultaneously in the same system. Accordingly, it was decided to study microalgal biomass interaction with each biopolymer in simple binary gels (microalga/biopolymer). Ternary systems composed by microalga/pea protein/κ-carrageenan and microalga/pea protein/starch have also been studied. The biopolymer concentrations had to be increased to reach minimum gelling concentration, but the proportion microalga/biopolymer was kept the same as in the mixed gel formulation (section 5.2.2.1).

5.3.4.1. Microalgae / Pea protein systems

Pea protein dispersions at 12% appear to be near the limiting-concentration for gelling to occur (Batista *et al.*, 2005; Nunes, 2006). In some experiments (replicates), the gel formed was sometimes weaker and at other times stronger suggesting that at this concentration level the gels were not formed in a systematic manner. This has also been reported by Batista *et al.* (2005) when pea protein dispersions at 12.5% gelled in the rheometer device but not in the refrigerator.

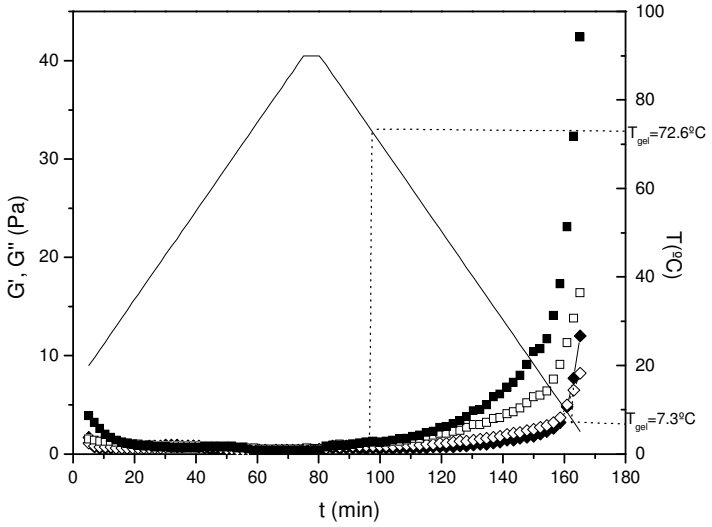


Figure 5.20. Evolution of the viscoelastic functions *G´* (closed symbol) and *G´´* (open symbol), of 12% pea protein dispersions along thermal treatment (T - line).

Figure 5.20 presents the evolution of the viscoelastic moduli along thermal treatment for different replicates of 12% pea protein dispersion samples. A decrease in *G´* and *G´´*, when heating the samples from 20ºC up to 90ºC, was followed by an increase upon cooling to 5ºC. This is the general behaviour of gels from globular protein systems, as reported by several authors (*e.g.* van Vliet *et al.*, 2002). However, it is clear that different sol-gel transition (*G´>G´´*) temperatures were found for replicates of the same material system. In one replicate, the

transition was identified at 73ºC, whereas in another replicate the transition was detected at only 7.3ºC, resulting in a much weaker gel. This can be confirmed from the microscope photographs in Figure 5.21, where one image (Figure 5.21a) shows a continuous dense network of protein aggregates (bright areas) and the other a discontinuous and disrupted structure (Figure 5.21b).

When *Spirulina* was added to the above system, a slight reinforcement of the pea protein gel structure was observed (Figures 5.21c-d), *i.e.* the brighter density of the red areas on the picture, especially when comparing Figures 5.21b and 5.21d. This reinforcement of the gel structure is confirmed by the rheological results shown in Figure 5.22b, where *G'* and *G"* values are slightly higher for the pea protein/*Spirulina* binary gel. In general, the microalgae appear to be embedded (red coloured) within the protein network and much denser in the case of Haematococcus (Figure 5.21e).

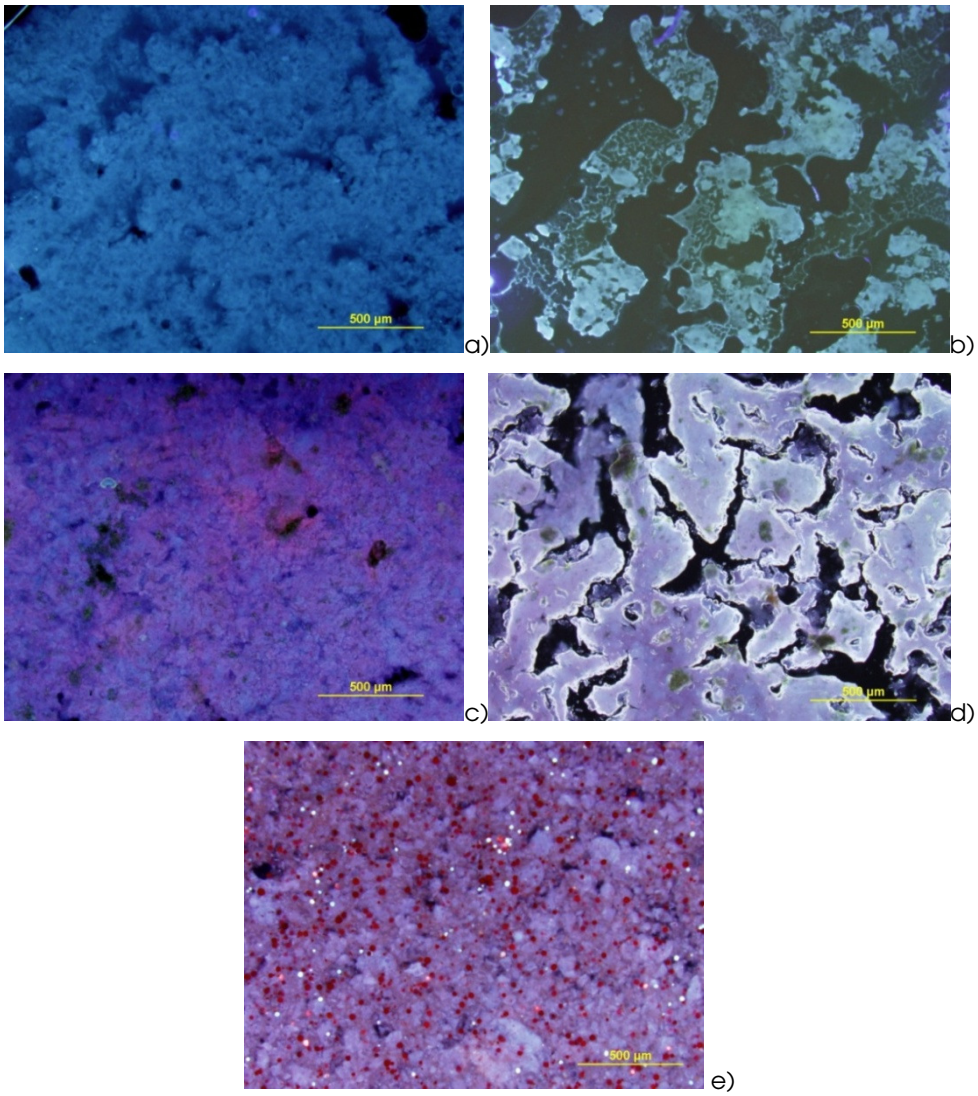


Figure 5.21. Fluorescence microscopy images of 12% pea protein gel systems (a-b) with 2.25% *Spirulina* (c-d) and *Haematococcus* (e) microalgal biomass addition (DAPI filter).

Adding *Haematococcus* causes a much more pronounced reinforcement of gel strength, as can be observed from the rheological data from Figure 5.22. In general, gel maturation kinetic curves are characterized by a marked increase in *G´* in the first few hours, followed by a slower evolution of this modulus along time. This behaviour is typical of biopolymer gelation processes (Clark *et al.*, 2001), where there is a continuous reorganization of the gel network due to the formation and rupture of entanglements between the polymeric chains of the macromolecules.

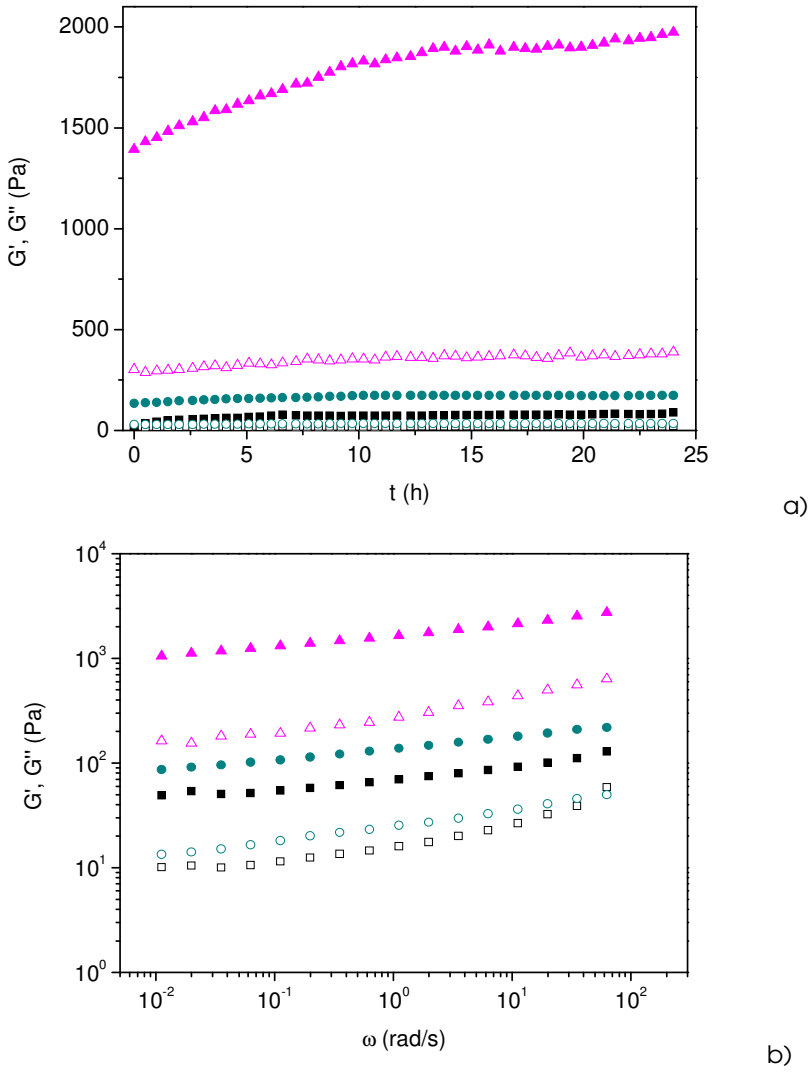


Figure 5.22. Maturation kinetics curves (a) and mechanical spectra (b) at 5ºC, of 12% pea protein gel systems (■) with 2.25% *Spirulina* (●) and *Haematococcus* (▲) microalgal biomass addition. *G´* (closed symbol), *G´´* (open symbol).

At the end of the 24 h maturation period, the pea protein/*Haematococcus* system presented a *G´* value around 2000 Pa, one order of magnitude higher than the pea protein simple gel and the pea protein/*Spirulina* gel system (Figure 5.22a). These results are well supported by the mechanical spectra (Figure 5.22b), where it is observed that, in all cases, *G´* is higher than *G"* although a much stronger gel is obtained by adding *Haematococcus*. However, the

SAOS functions frequency dependence is not modified by microalgae addition, indicating that pea protein is the dominant biopolymer in the gel network.

The impact of microalgal biomass addition can be related to modifications in the pH and salt content of the gels, by affecting pea protein electrostatic interactions during the aggregation mechanism. These should be more relevant in the case of *Spirulina* biomass which presents higher ionic content and a higher pH. However, pH values are always above pea protein isoelectric point (4.5), not affecting the global charge of this biopolymer which remains negative. In the case of *Haematococcus*, the structural reinforcement action could also be related to its high fat content (41%), as referred previously (section 5.3.2).

5.3.4.2. Microalgae / κ-Carrageenan systems

κ-Carrageenan forms a very weak gel at 0.75% which should be near its critical gelling concentration (Nunes, 2006; Nickerson and Paulson, 2004) in the absence of added ions. The sol-gel transition of κ-carrageenan dispersions is characterized by a sharp increase in *G'* upon cooling, and in parallel with a marked decrease in phase angle (δ). For the κ-carrageenan 0.75% system, this transition occurred in the 5-7ºC temperature range. However, when adding *Spirulina* and *Haematococcus* the gelation temperature increased to 30ºC and 25ºC, respectively (results not shown). This means that gel formation occurs earlier during the cooling process, making it possible to obtain κ-carrageenan gels at room temperature through the addition of microalgae biomass. In all cases, the resulting microalgae/κ-carrageenan maturated gels present impressively enhanced rheological properties, with *G'* values two orders of magnitude higher than the simple κ-carrageenan gel (Figure 5.23a). However, *Haematococcus* gel maturation curves are not typical, showing a decrease in G', probably because the gel was too hard and brittle, disturbing the rheological data measurement. The mechanical spectra of these systems is presented in Figure 5.23b, confirming the strong nature of the gel imparted by both microalgae.

κ-Carrageenan gels are formed through intermolecular association (junction zones) of double helices into stable structured aggregates (Morris *et al.*, 1980). The formation and aggregation of these double helices is induced by cooling and is highly dependent on the presence of electrolytes even at very low concentrations (Morris *et al.*, 1980; Rochas and Landry, 1987). Potassium cations (K^+) have a specific affinity to κ-carrageenan and are usually the most effective in inducing gellification, followed by divalent cations such as Ca2+ and then by other monovalent cations such as Na^+ (Chen *et al.*, 2002).

Microalgal biomass contains significant amounts of minerals (section 3.3.1), derived from its intrinsic chemical composition and from culture media residues. Systems with 0.75% κ-carrageenan and 3.75% microalgal biomass include 0.08% K^+ (0.02M), 0.03% Ca^{2+} (0.01M) and 0.26% Na^+ (0.1M) for *Spirulina* and 0.03% K^+ (0.01M), 0.01% Ca^{2+} (0.002M) and 0.21% Na^+ (0.1M)

for *Haematococcus*. This can explain the contribution of microalgal addition to the huge increase in κ-carrageenan gels rheological moduli. This is in agreement with previous research conducted by other authors, such as Chen and co-workers (2002) where the formation of very weak κ-carrageenan gels was observed for concentrations between 0.7% and 1.4% in the absence of salts. After the addition of low levels of potassium and calcium, the gels rheological moduli (*G´*, *G"*) increased considerably, indicating a higher degree of gel structure.

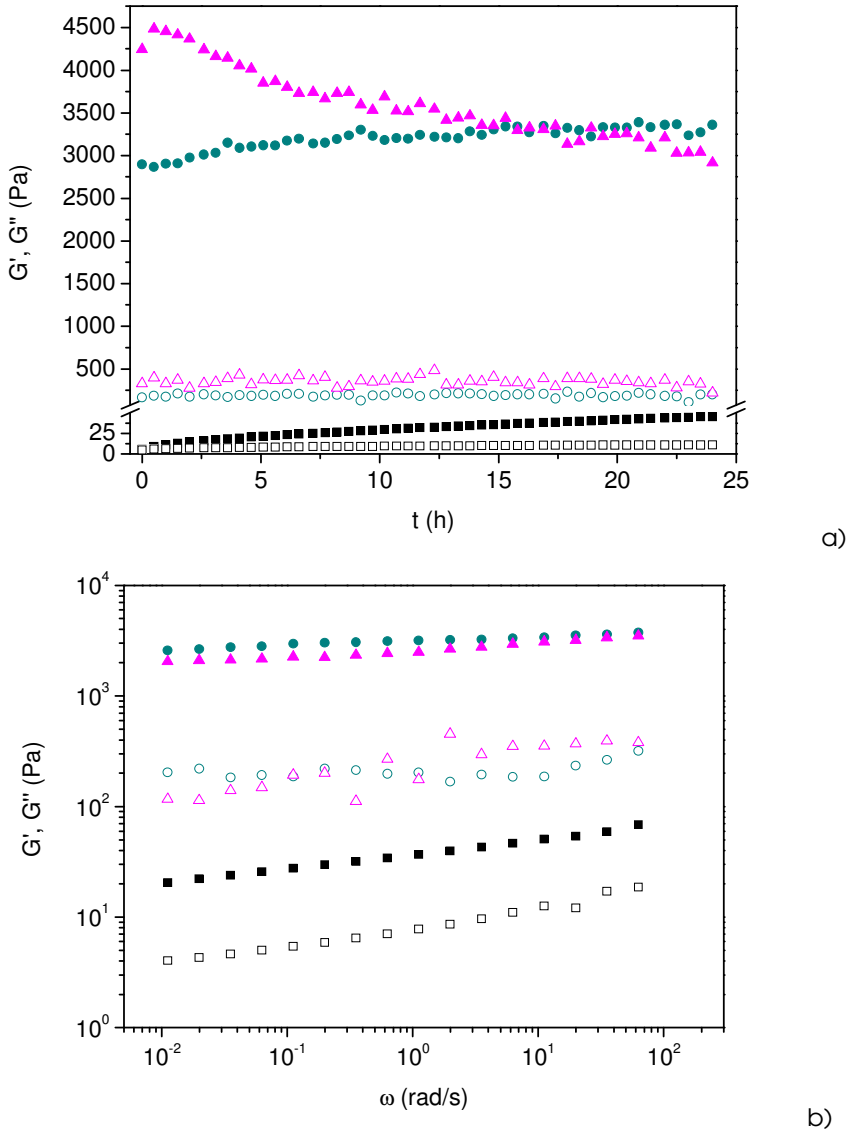


Figure 5.23. Maturation kinetics curves (a) and mechanical spectra (b) at 5ºC, of 0.75% κ-carrageenan gel systems (■) with 3.75% *Spirulina* (●) and *Haematococcus* (▲) microalgal biomass addition. *G´* (closed symbol), *G´´* (open symbol).

κ-carrageenan/microalgae gel structures can be observed in Figure 5.24. κ-carrageenan was not detected in the fluorescence microscope since there is no fluorescence marker available for non-covalent staining. It is however possible to observe the κ-carrageenan/ microalga systems, since *Haematococcus* and *Spirulina* present autofluorescence enabling

κ-carrageenan to be observed by contrast (dark areas). The images in Figures 5.24a and 5.24c were obtained with a DAPI-UV filter, and the ones in Figures 5.24b and 5.24d with a TRITC filter. The latter enables the collection of light emitted by the sample at higher wavelengths, enhancing the contrast when microalgae are present in the gel system.

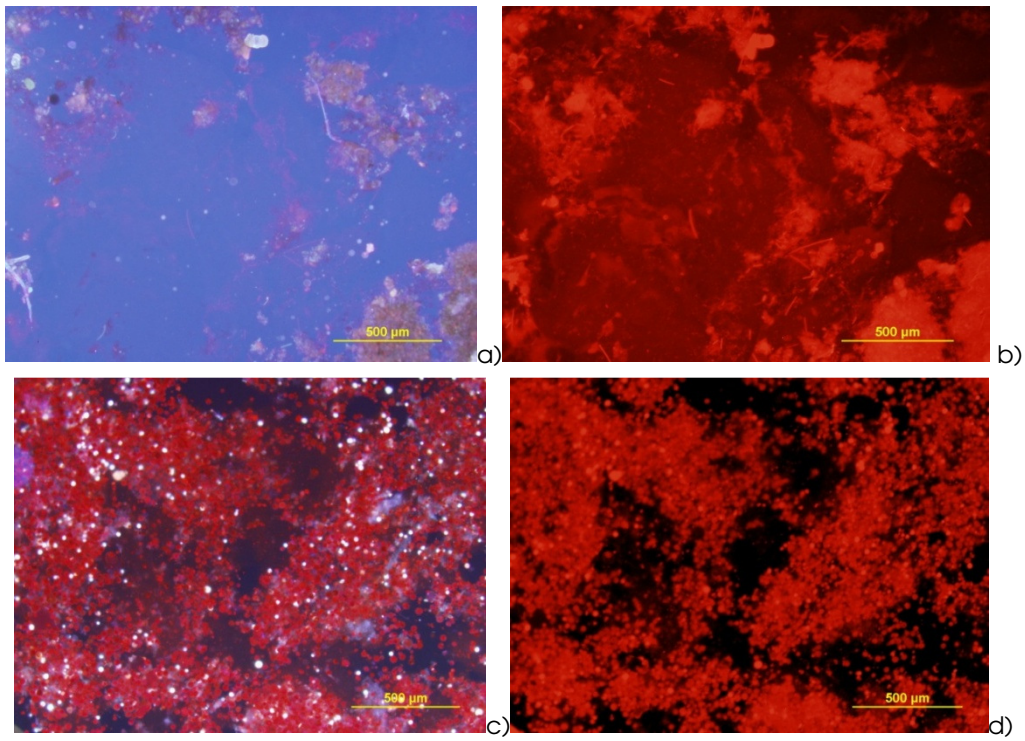


Figure 5.24. Fluorescence microscopy images of 0.75% κ-carrageenan gel systems with 3.75% *Spirulina* (a-b) and *Haematococcus* (c-d) microalgal biomass addition (DAPI and TRITC filters).

5.3.4.3. Microalgae / starch systems

A marked increase in *G'* was observed by heating 5% starch dispersions to 66.6ºC (Figure 5.25a), corresponding to starch gelatinization. This phenomenon is related with the swelling of the starch granules, and subsequent amylose solubilisation, which causes an increase in the system's viscosity (Morris, 1990). The addition of *Haematococcus* and *Spirulina* to this system causes a slight increase in the gelatinization temperature to 70.2ºC and 73.8ºC, respectively. This indicates that microalgal biomass interferes with the starch gelatinization process perhaps by competing for water binding sites during the granules' hydration process.

In the case of *Haematococcus*/starch systems this setback was overcome in the cooling ramp, resulting in a strongly maturated gel, with *G'* and *G"* values higher than the simple starch gel (Figure 5.25a). This can be related to a concentration effect of starch as a result of exclusion from the microalgae domain. It is also expected that *Haematococcus* high fat content (41%) can play a significant role in the gels' rheological properties, as discussed previously for microalgae - pea protein systems (section 5.3.4.1).

Alternatively, *Spirulina*/starch systems, presented much lower *G´* values than the control sample. This is in accordance with previous results which reported the formation of weaker gels when *Spirulina* was added to pea protein/κ-carrageenan/starch mixed gels, particularly for faster gel setting conditions (section 5.3.3.2).

As observed for pea protein and κ-carrageenan systems, the mechanical spectra of starch systems show the same frequency dependency with or without the addition of microalgal biomass (Figure 5.25b).

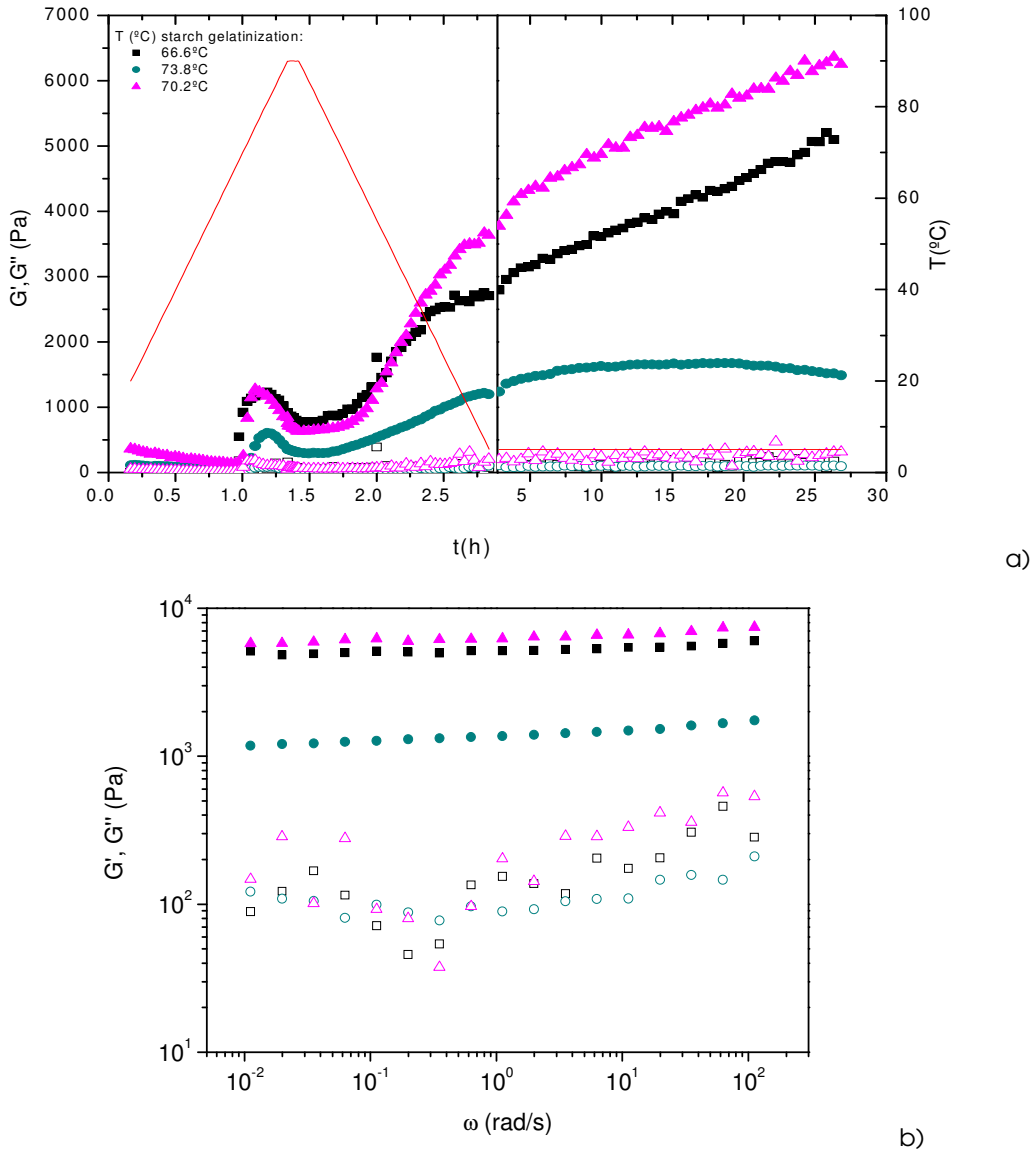


Figure 5.25. Evolution of *G´* and *G´´* along thermal treatment and 24 h maturation at 5ºC (a) and mechanical spectra (b) at 5ºC, of 5% starch gel systems (■) with 1.5% *Spirulina* (●) and *Haematococcus* (▲) microalgal biomass addition. *G´* (closed symbol), *G´´* (open symbol), *T* (line).

Microscope images (Figure 5.26) show large particles of microalgae that could imprint discontinuities in the starch network, especially in the case of *Spirulina*, leading to a more fragile gel structure. Unlike the other microalgae studied (section 5.3.2) *Spirulina* is a

cyanobacteria, and therefore its prokaryotic cells lack a rigid cell-wall which could lead to higher water absorption rates by its cellular components (mainly proteinaceous), destabilizing the starch gelation mechanism.

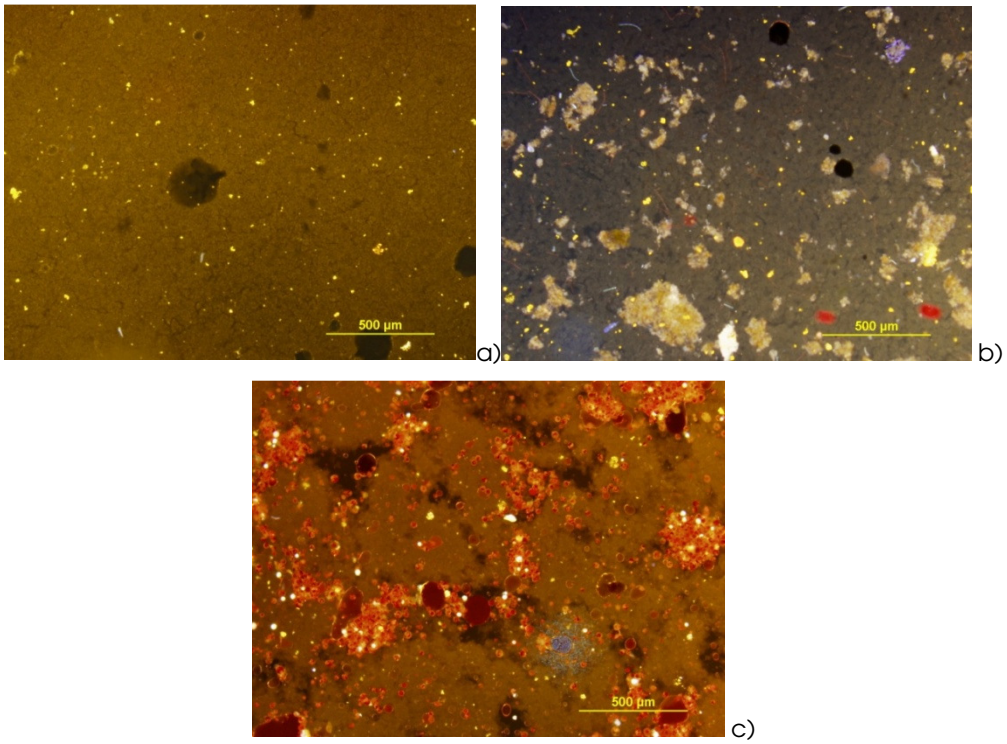


Figure 5.26. Fluorescence microscopy images of 5% starch gel systems (a) with 1.5% *Spirulina* (b) and *Haematococcus* (c) microalgal biomass addition (DAPI filter).

5.3.4.4. Microalgae / Pea Protein / κ-Carrageenan systems

Pea protein/κ-carrageenan binary systems form well structured gels, at low concentrations for both biopolymers, suggesting a synergistic effect. The rheological behaviour of the mixed gel is similar to the simple κ-carrageenan gel, namely the gelation temperature at 7.3ºC, the shape of the maturation curves and mechanical spectra (Figure 5.27). This suggests that κ-carrageenan constitutes the continuous phase of the mixed system, which is in accordance with the findings from Nunes (2006) regarding pea protein and κ-carrageenan as being phase separated, forming two independent networks dispersed in one another.

Rheological results (Figure 5.27) for pea protein/κ-carrageenan systems are surprising, since the addition of microalgae to this binary system results in weaker gels, as opposed to their behaviour when applied separately reinforcing each biopolymer gel individually.

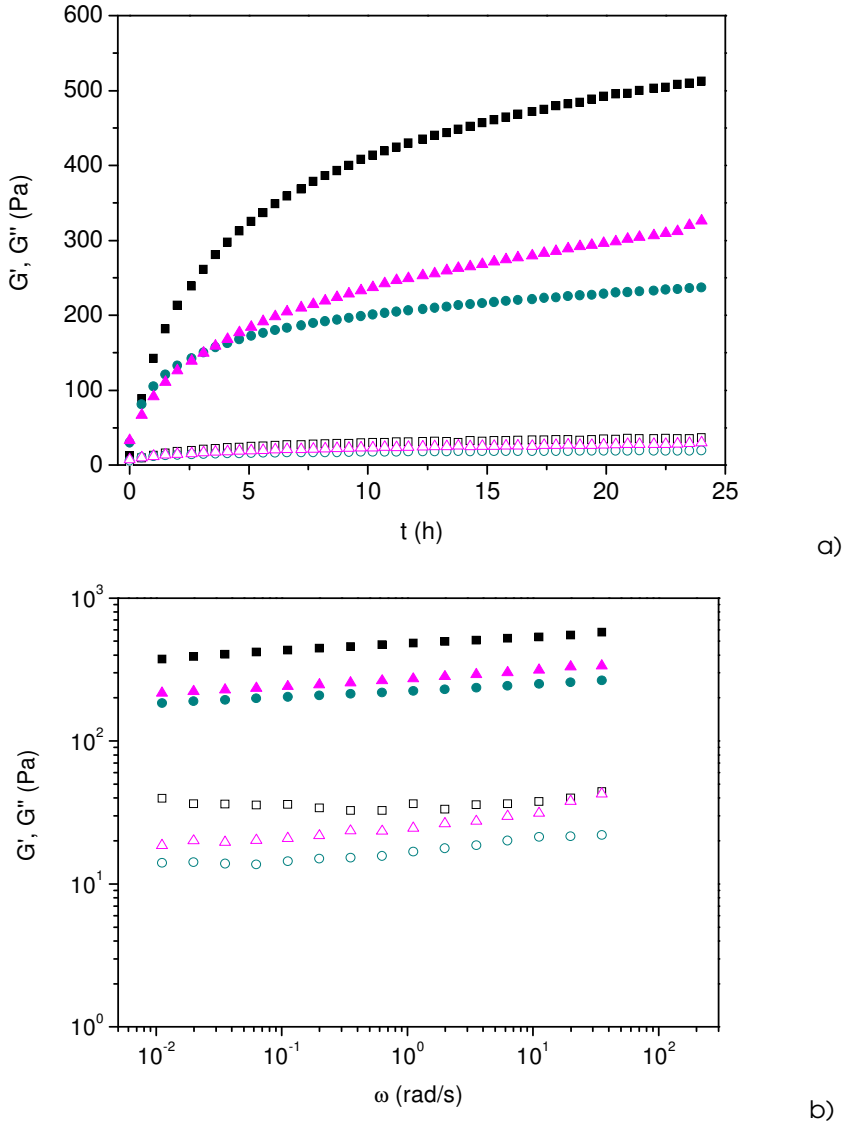
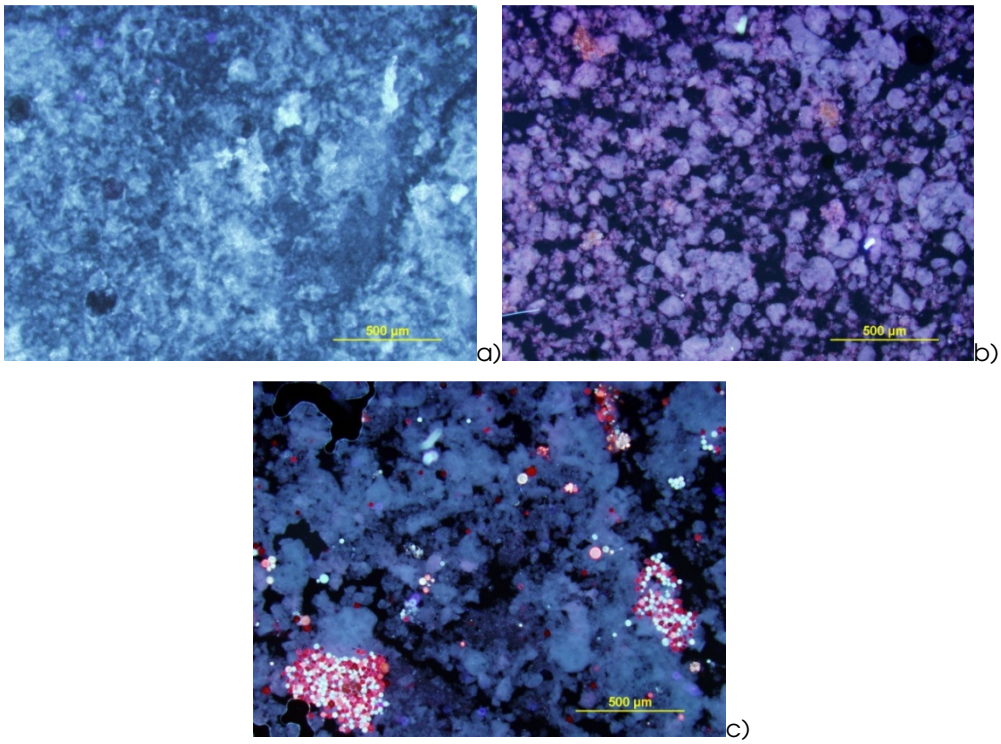


Figure 5.27. Maturation kinetics curves (a) and mechanical spectra (b) at 5ºC, of 4 % pea protein/ 0.15% κ-carrageenan gel systems (■) with 0.75% *Spirulina* (●) and *Haematococcus* (▲) microalgal biomass addition. *G´* (closed symbol), *G´´* (open symbol).

Microscope images (Figure 5.28) suggest that phase segregation becomes more evident upon microalgae addition, negatively affecting the biopolymers' interaction. Therefore, protein-polysaccharide thermodynamic incompatibility seems to be affected by the presence of microalgal biomass, which may be related to the volume exclusion effect as a consequence of macromolecular competition for space in the solution (Tolstoguzov, 2003). This could have a negative impact on the gel structure considering the low biopolymer levels (4% pea protein, 0.15% κ-carrageenan, 0.75% microalga) used in this study.



a) b) c)

Figure 5.28. Fluorescence microscopy images of 4% pea protein/0.15% κ-carrageenan gel systems (a) with 0.75% *Spirulina* (b) and *Haematococcus* (c) microalgal biomass addition (DAPI filter).

5.3.4.5. Microalgae / Pea Protein / Starch systems

Pea protein/starch binary systems also formed well structured gels, at low concentrations for both biopolymers, suggesting a synergistic effect. When heating these systems from 20ºC to 90ºC, a marked increase in *G'* is observed at 73.8-75.6ºC (Figure 5.29a). This temperature corresponds to the starch gelatinization temperature, which is higher compared to the simple 5% starch gel (66.6ºC), as reported in section 5.3.4.3. The effect of pea protein or *Spirulina* (section 5.3.4.3) addition to starch gels is characterized by similar gelatinization temperatures. This suggests that hydration of starch granules' is impaired by pea protein and *Spirulina* to the same extent, demonstrating that *Spirulina's* protein fraction should be competing with starch granules for hydration as in *Spirulina*/starch gelling systems.

Pea protein/starch systems presented stronger gels in the presence of both microalgae. *Haematococcus*/pea protein/starch systems presented starch gelatinization at 73.8ºC (no effect from *Haematococcus a*ddition), and resulted in a stronger gel with increasing viscoelastic functions (even after 24 h maturation) (Figure 5.29). The addition of *Spirulina* retarded starch gelatinization to 81-83ºC, and the gel seems to be fully maturated before 24 h period, presenting higher *G'* values when compared to the control gel.

From section 5.3.4.3, a destabilizing effect was noted after *Spirulina* addition to the starch gel. However, the pea protein/starch synergistic effect in microalgae/pea protein/starch systems seem to be stronger than the *Spirulina*/starch destabilizing mechanism, resulting in gels with higher viscoelastic properties (Figure 5.29).

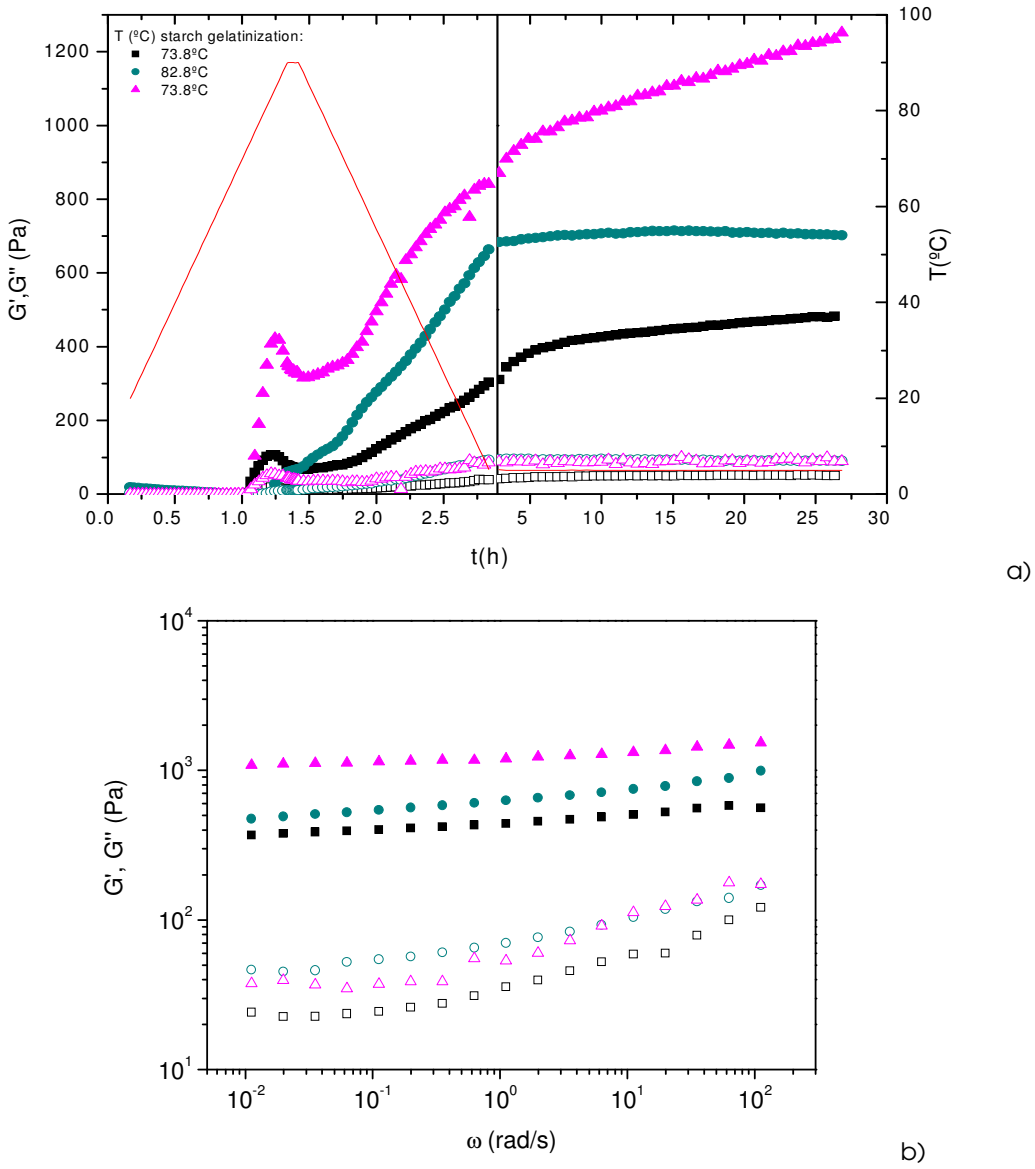


Figure 5.29. Evolution of *G´* and *G˝* along thermal treatment and 24 h maturation at 5ºC (a) and mechanical spectra (b) at 5ºC, of 6% pea protein/3.75% starch gel systems (■) with 1.125% *Spirulina* (●) and *Haematococcus* (▲) microalgal biomass addition. *G´* (closed symbol), *G˝* (open symbol), *T* (line).

Microscope images (Figure 5.30) present densely packed continuous structures, which are in accordance with the rheological results. From these results it seems clear that protein/polysaccharide interaction plays a dominant role on the development of the gel microstructure, and will determine the extent and direction of the microalgae addition effect.

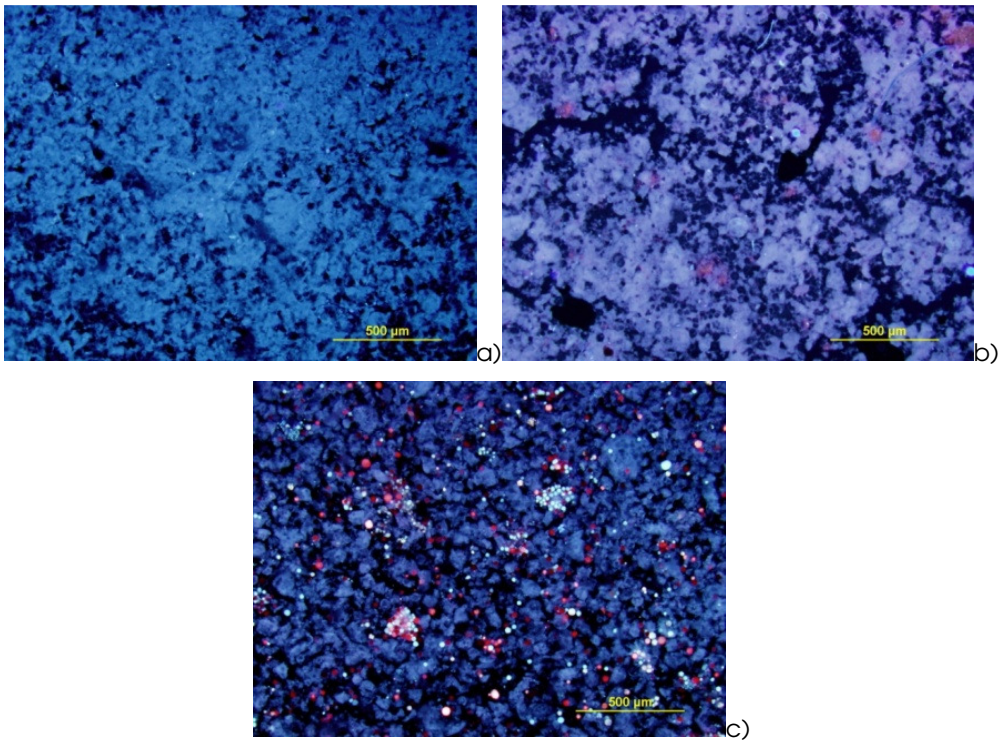


Figure 5.30. Fluorescence microscopy images of 6% pea protein/3.75% starch gel systems (a) with 1.125% *Spirulina*(b) and *Haematococcus* (c) microalgal biomass addition (DAPI filter).

5.4. CONCLUSIONS

Microalgal biomass seems to be a promising new ingredient with colouring effect, beneficial nutritional features and potential structuring abilities in colloidal gel food products.

Pea protein/κ-carrageenan/starch mixed gels were used as model systems for studying microalgal biomass impact. Gels with interesting colourations were attained, and it was evidenced that this matrix provided a good thermal resistance (up to 90ºC) of natural pigments and fatty acid bioactive molecules.

The incorporation of microalgae on these mixed gel systems seemed to be beneficial, especially for *Haematococcus pluvialis* (with an attractive "*strawberry-pink*" colour) which promoted a structural reinforcement expressed by improved rheological properties. This structural reinforcement action that may be related to its high fat content (41%), considering that fat droplets can act as active filler particles embedded in the gel matrix as observed for milk gelled systems.

However, the addition of *Spirulina* promoted a drastic reduction on the gels rheological parameters. This should be related with a thermodynamic incompatibility between the microalgal protein and the other components of the mixed gelled system.

The linear viscoelastic properties of pea/κ-carrageenan/starch mixed gel systems was highly dependent on the gel setting conditions, including temperature/time of thermal processing and heating/cooling rates. Increasing temperature (70-90ºC, 5 min) resulted in more structured gels, while the effect of time (5-30 min, 90ºC) was less pronounced. Higher values of the viscoelastic functions were achieved upon heating and/or cooling the mixed biopolymer suspensions at lower rates. However, the time length required for the process, and the subsequent costs involved, should be considered. The addition of microalgal biomass promoted some modifications on the gel structure, although the response to gel setting conditions followed in general the behaviour of the control gel. *Haematococcus* gels were less dependent on gel setting conditions; while for *Spirulina* using lower heating/cooling rates and more extensive thermal treatments enabled to overcome the structural drawback evidenced previously. In fact, *Spirulina* addition also resulted in stronger gel systems, except in the case of starch where it seems to inhibit the gelatinization process. This could be related with a competition for water binding sites by *Spirulina* protein molecules, hindering starch granules hydration.

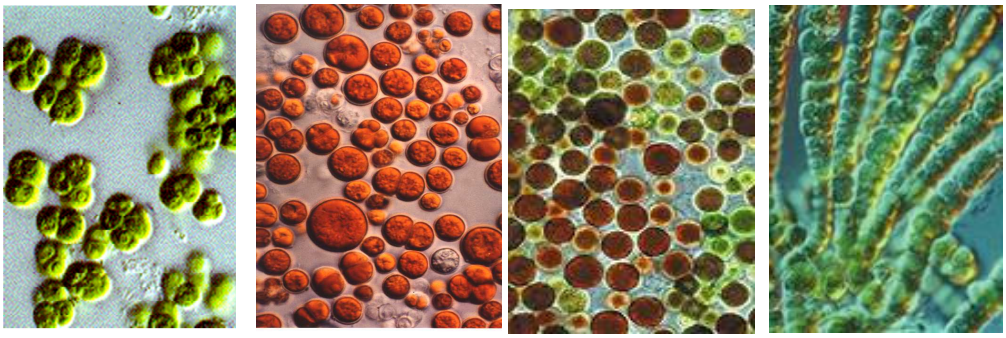
For all the microalga/biopolymer systems studied, it was observed that protein and polysaccharide biopolymers, alone or in binary combinations, are responsible for the formation of the gel structure and resulting rheological behaviour. In most cases, microalgae seem to be embedded in the gel's network, causing denser microstructures with improved rheological parameters. However, the fact that microalgal biomass contains significant amounts of ions means that this should also have a significant impact on the biopolymers'

interaction associated with the gelling mechanism, particularly in the case of κ-carrageenan gels. Fluorescence optical microscopy proved to be a simple and effective technique to observe these materials' microstructures, with microalgae being easily detected due to their natural pigments' autofluorescence. The microscopy results correlated well and supported the rheological findings.

The results presented in this chapter have been published in two articles in peer-review international journals (*Food Hydrocolloids*, *Journal of Food Engineering*), one article by invitation in *Nutrition and Food Science Journal*, one chapter of the book *Gums and Stabilisers in the Food Industry 14* (Appendix A), and presented in several national and international conferences, including the *International Symposium on Food Rheology and Structure* (ISFRS2009) and *Food Colloids* (*FC2010*) conference series among others (Appendix B).

CHAPTER 6

General conclusions



6. GENERAL CONCLUSIONS

Using microalgal biomass, and natural pigments, in model colloidal food systems - emulsions and gels - proved to be an efficient and innovative procedure, which simultaneously enables original and stable colourations, via health-beneficent natural pigments, and microstructural reinforcement, as observed through the emulsion and gel rheological behaviour. General conclusions and main considerations that can be drawn from the present thesis are presented below, regarding the main topics under study.

Microalgal biomass physico-chemical characterization:

- Microalgae can show different biomass profiles according to their origin, but mainly due to their environmental culture conditions. Of the six microalgae studied, the major differences in physicochemical composition were in fact related to the induction of carotenogenesis process in *Chlorella* (orange) and *Haematococcus*, as it was evidenced from Principal Component Analysis. Nevertheless, each microalga presented a typical biomass nutrient profile, allowing the selection of desired physicochemical characteristics for specific food technology applications.
- Thermogravimetric analysis indicated that carotenogenic microalgae, which have higher fat content, are more resistant to thermal treatments. This may be very useful considering some intensive technologies used in food processes, and the thermal sensitivity of carotenoids and other bioactive molecules that are naturally encapsulated within the microalgae cell walls.

Impact of microalgal biomass, and natural pigments, incorporation in pea protein-stabilized emulsions:

- Lutein and phycocyanin pigments imparted markedly different effects, on the emulsion structure, according to the pigment nature, particularly the affinity to the aqueous (continuous) or to the oil (disperse) phase, and their interactions with the emulsifier molecules at the interface. Lutein addition modified the nature of the oil phase originating emulsions with lower values of the rheological parameters analysed and weaker textural properties, but similar *DSD* results, compared to the control emulsion. Emulsions containing phycocyanin showed rheological and texture parameters that generally increase linearly with phycocyanin concentration.
- Phycocyanin provided an intense blue colouration, which although unexpected in a food product, is valuable considering that stable natural blue colourings are hard to find.

- Higher colour stabilities were attained for biomass concentrations below 1.0%, for all microalgae studied. Among them, *Haematococcus* proved to be the more effective colouring agent (3.0% total pigments), providing an attractive and intense *"strawberry pink"* tonality for low incorporation levels.
- The addition of microalgal biomass proved to be beneficial in terms of enabling lesser oil content in emulsion formulations, without disturbing their rheological and texture properties. These results support the potential use of microalgae material to act as a fat mimetic, besides the possible advantages as a colouring agent.
- *Spirulina* is the less effective microalgae, either as colouring or texturizing agent. Duller green colourations were attained, even at high biomass concentrations, and less significant effects on firmness and viscosity improvements were observed.

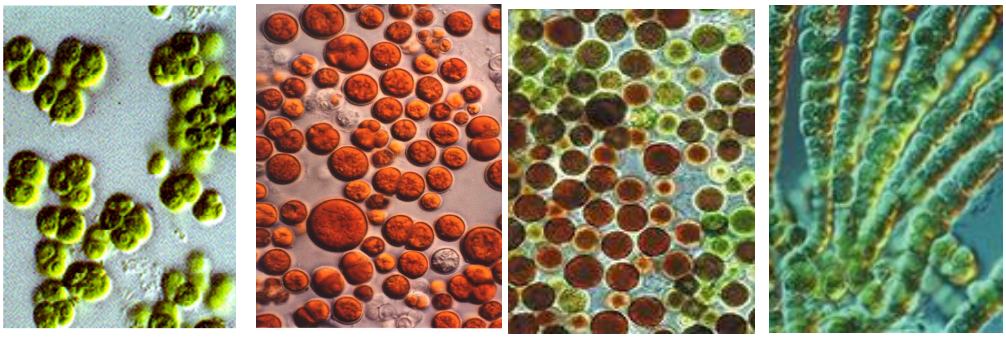
Impact of microalgal biomass and natural pigment, incorporation in pea protein/κ-carrageenan/starch gel systems:

- Gels with interesting colourations and good thermal resistance (up to 90ºC) were attained.
- The addition of phycocyanin and lutein natural pigments to gel formulations promoted inverse effects to those observed in oil-in-water emulsions. This demonstrates that using a particular natural pigment to colour food products can produce much different texturizing or de-structuring effects depending on the type of colloidal system.
- The incorporation of microlgae on mixed gel systems, especially *Haematococcus pluvialis,* promoted a structural reinforcement expressed by improved rheological properties. This structural reinforcement action may be related to its high fat content, considering that fat droplets can act as active filler particles embedded in the gel matrix.
- The addition of *Spirulina* promoted a drastic reduction on the gels rheological parameters. In fact, *Spirulina* seems to inhibit the starch gelatinization process, which is attributed to a competition for water binding sites by *Spirulina* protein molecules, hindering starch granule hydration.
- The linear viscoelastic properties of pea/κ-carrageenan/starch mixed gel systems were highly dependent on gel setting conditions, including temperature/time cycles applied during thermal processing and heating/cooling rates. The addition of microalgal biomass promoted some modifications on the resulting gel structure, although the response to gel setting conditions followed, in general, the behaviour of the control gel (without microalgae).

- For all the studied microalga/biopolymer systems, it was observed that protein and polysaccharide biopolymers, alone or in binary combinations, are responsible for the formation of the gel structure and resulting rheological behaviour. In general, microalgae seem to be embedded in the gel network, originating denser microstructures with improved rheological parameters, in most cases. Fluorescence microscopy results correlated well and supported the rheological findings.

CHAPTER 7

References



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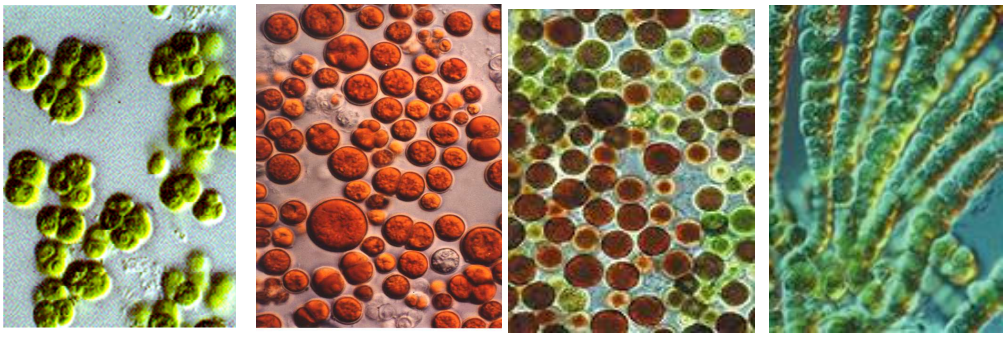
APPENDICES



A. PUBLICATIONS IN SCIENTIFIC JOURNALS AND BOOKS WITH PEER REVIEW

The work presented on this thesis has been published in the following papers and book chapters:

Chapter 2

GOUVEIA, L.; BATISTA, A.P.; SOUSA, I.; RAYMUNDO, A.; BANDARRA, N. (2008). Microalgae in novel food products. In *Food Chemistry Research Developments*. Ed. K.N. Papadopoulos. Nova Science Publishers, Inc. pp. 75-111. (ISBN: 978-1-60456-262-0).

Chapter 3

BATISTA, A.P.; GOUVEIA, L.; BANDARRA, N.M.; FRANCO, J.M.; RAYMUNDO, A. Screening of microalgal biomass profiles for use in novel food products. *Submitted to Algal research* (March 2012).

Chapter 4

BATISTA, A.P.; RAYMUNDO, A.; SOUSA, I.; EMPIS, J. (2006) Rheological characterization of coloured oil-in-water food emulsions with lutein and phycocyanin added to the oil and aqueous phases. *Food Hydrocolloids,* **20**(1), 44-52. (doi:10.1016/j.foodhyd.2005.02.009)

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B. PRESENTATIONS IN SCIENTIFIC MEETINGS AND RESULTING PUBLICATIONS IN PROCEEDINGS/ABSTRACTS BOOKS

The work presented on this thesis has also been presented in several national and international scientific reunions, which are listed below, as divided into main themes:

General results on the characterization and applications of microalgae:

BATISTA, A.P.; SOUSA, I.; RAYMUNDO, A.; BANDARRA, N.M.; GOUVEIA, L. (2007). Microalgae bioactive components for innovative food products development. *Book of Abstracts of the 37th WEFTA Annual Meeting "Seafood: source of health and well-being"*. Pág. 134 (S3.14). Lisboa, Portugal. (*Short Communication*).

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