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DEVELOPMENT OF BIOPROCESSES FOR THE UPGRADING OF FISH BY-PRODUCTS

DOCTORAL THESIS

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Resumen

1. INTRODUCCIÓN

1.1 Sub-productos de pesca: descartes

La industria pesquera genera un cantidad importante de sub-productos siendo los descartes la principal causa del mal uso de los recursos pesqueros (Davies et al., 2009). En este contexto, se define descarte como "la porción de la materia orgánica total de origen animal en la captura, la cual es desaprovechada, o vertida al mar por cualquier razón; sin incluir desechos del procesado del pescado a bordo" (Kelleher, 2005). Esta definición comprende a especies marinas de interés comercial que son descartadas debido a que no presentan la talla mínima para su desembarco o exceden el volumen fijado por la cuota de pesca para la especie. Asimismo, otra importante fracción de los descartes está formada por las denominadas capturas accesorias: animales marinos capturados accidentalmente tales como peces sin valor comercial, mamíferos marinos o incluso gaviotas. Por tanto, los descartes se producen fundamentalmente por: a) razones políticas debido a las regulaciones respecto a tallas mínimas de desembarco y cuotas máximas de captura por especie; y b) causas económicas ya que son los mercados los que determinan las especies de interés comercial y los que originan el "high-grading" (descarte de pescado comercial de talla legal y con poco valor). Por otra parte, existen factores locales que pueden intensificar la generación de descartes como la diversidad de especies en el área de pesca y la selectividad de las artes de pesca empleadas (Kelleher, 2005). Para el período 1992-2001, Kelleher (2005) estima un tasa global de descarte, referida a las capturas totales, del 8 %, lo que da lugar a un volumen anual de descartes de 7.3 millones de toneladas.

1.1.1 Descartes en la costa norte del mar de Alborán

España es un país eminentemente marítimo en el que el sector pesquero contribuye de manera importante a la economía nacional (MAPA, 2006). El volumen de capturas de la flota española ascendió a más de 865,000 toneladas en 2008, lo que supone un 14 % del total de capturas de los 27 Estados Miembros (UE, 2010). Las actividades pesqueras se concentran principalmente en el Atlántico Noroeste y el Mediterráneo, con el 44 y 16 % de las capturas respectivamente. Particularmente, en la región surmediterránea o costa norte del mar de Alborán se realiza el 42 % de las capturas andaluzas, teniendo una importancia significativa en el total de las capturas de la flota española (Consejería de Agricultura y

Pesca, 2009). Esta región se alarga desde el Estrecho de Gibraltar hasta Cabo de Gata con una extensión total de 250 km, una superficie de 11,000 km^2 y una profundidad entre 0 y 800 m. Además, comprende un total de 11 puertos de descarga: Algeciras y La Línea (provincia de Cádiz); Estepona, Marbella, Fuengirola, Málaga y Caleta de Vélez (provincia de Málaga); Motril (provincia de Granada); Adra, Roquetas de Mar y Almería (provincia de Almería) (García et al., 2012). En esta pesquería predominan las artes de arrastre de fondo, con 135 buques representando el 22 % de las capturas, y de cerco, con 89 buques que realizan el 64 % de las capturas (Consejería de Agricultura y Pesca, 2010). Aunque no existe una cuantificación precisa de los descartes en la zona, estudios preliminares (FROM, 2008) revelan unas tasas de descarte del 23 y 10 % para el arrastre y el cerco, respectivamente. Entre las especies más descartadas, se encuentran especies comerciales como la sardina (*Sardina pilchardus*), el jurel (*Trachurus mediterraneus*) y el aligote (*Pagellus acarne*) (Fig. 1). Estos descartes se deben a requerimientos de talla mínima, 11 cm para la sardina y 12 cm para el jurel y el aligote (R.D. 1615/2005), a restricciones de cuota y a prácticas comerciales como el "high grading". Otras especies como la pintarroja (*Scyliorhinus canicula*) y la boga (*Boops boops*) (Fig. 2), altamente presentes en la captura, son normalmente descartadas debido a su bajo valor comercial (Carbonell et al., 1997; CDPM, 2005).

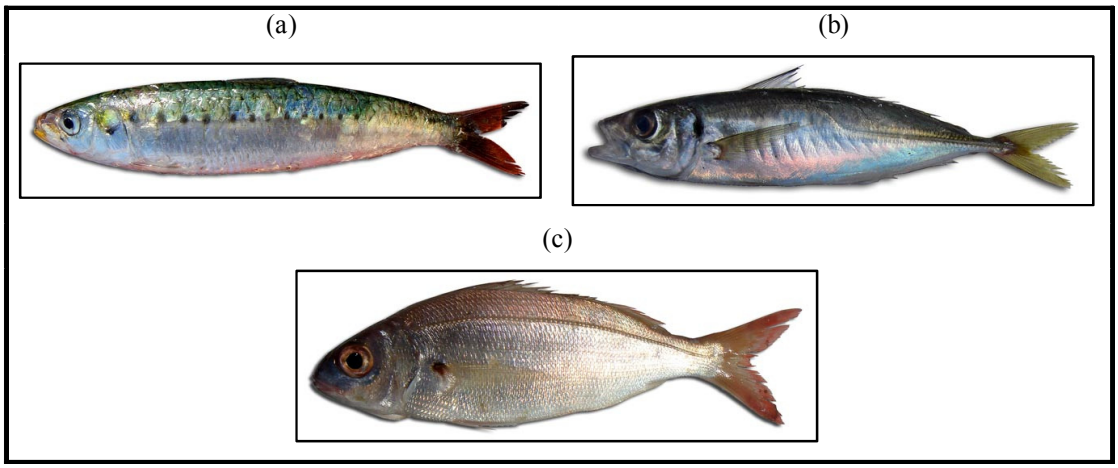


Figura 1. (a) *Sardina pilchardus*, (b) *Trachurus mediterraneus* y (c) *Pagellus acarne*. (Fotos: A.M. Arias)

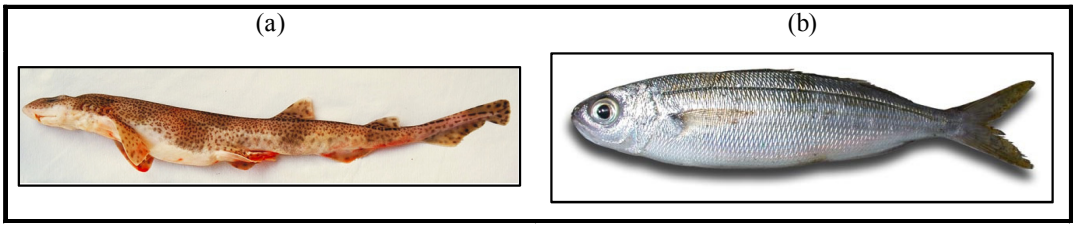


Figura 2. (a) Scyliorhinus canicula (Foto: FONDOPES) y (b) Boops boops (Foto: A.M. Arias).

Las capturas en esta región han disminuido prácticamente a la mitad desde 1990 (43,000 toneladas) hasta 2011 (22,000 toneladas) (Consejería de Agricultura y Pesca, 2011). Esto, debido principalmente a la sobreexplotación de las pesquerías, pone de manifiesto la necesidad de hacer un uso óptimo de los recursos pesqueros disponibles. En este sentido, la reducción de los descartes así como su posterior aprovechamiento se manifiestan como medidas estrictamente necesarias a llevar a cabo.

1.1.2 Consecuencias de los descartes y soluciones técnicas para su reducción

La generación de descartes, además de suponer un consumo irresponsable de los recursos pesqueros, con las negativas consecuencias económicas que ello acarrea, también conlleva un importante impacto ecológico sobre el hábitat marino. Esto es debido a que la gran mayoría de los individuos descartados están muertos o moribundos cuando se devuelven al mar. Ello favorece la abundancia de especies depredadoras que pueden, a largo plazo, alterar la estructura de las cadenas tróficas (Groenewold y Fonds, 2000). Por ejemplo, en el caso de la pesca de arrastre, la supervivencia de peces con vejigas natatorias es prácticamente nula debido a la expansión de la misma a medida que se iza la red, un 50% de los crustáceos y hasta un 98% de los cefalópodos no consiguen sobrevivir a la recogida y clasificación, siendo devueltos muertos al mar (Bozzano y Sardà, 2002). Por otra parte, el descarte de individuos juveniles pone en riesgo la durabilidad de una pesquería (Jensen *et al*., 1988).

Por todo ello, la Comisión Europea está en proceso de aprobar una reforma de la política pesquera común en la que se insta a una práctica de cero-descartes en todas las pesquerías europeas. Esta reforma propone una reducción de los descartes en tres etapas: i) especies pelágicas, incluido el Mediterráneo, para 2014; ii) especies demersales tales como bacalao, merluza y lenguado para 2015; y iii) resto de especies para 2016. Entre las medidas a implementar para su consecución se encuentran las siguientes: a) mejora de la selectividad

de todas las artes de pesca, b) obligación para que los pescadores muevan la zona de operaciones cuando se detecte un nivel de capturas incidentales por encima del umbral máximo fijado, c) eliminación de las restricciones en cuanto a talla mínima de desembarco, d) alto nivel de cobertura de observadores a bordo de los barcos, y e) incentivar económicamente la salida comercial de desembarcos de juveniles y capturas accesorias (e.g. producción de harina de pescado) (EU, 2011). No obstante, los descartes de juveniles (de venta prohibida) y de especies no comerciales son sustratos de bajo coste para la obtención de productos con mayor valor añadido tales como aceite de pescado e hidrolizados proteicos con propiedades bioactivas, de gran aplicación en la industria alimentaria y farmacéutica.

1.2 Aceite de pescado

El aceite de pescado se diferencia del resto de aceites vegetales y animales por su alto contenido en ácidos grasos poliinsaturados omega-3 (Hamilton & Rice, 1995). Estos ácidos grasos poliinsaturados (PUFA) son producidos por el fitoplancton marino y se incorporan a los peces a través de la cadena alimentaria (Shahidi, 1998). Los principales omega-3 PUFA contenidos en el aceite de pescado son el alfa linolénico (C18:3n-3, ALA), el eicosapentaenoico (C20:5n-3, EPA), el docosahexaenoico (C22:6n-3, DHA) y en menor medida el docosapentaenoico (C22:5n-3, DPA).

Los ácidos grasos omega-3 producen numerosos efectos beneficiosos sobre la salud humana, particularmente el EPA y DHA, los cuales han sido perfectamente documentados. El primero de ellos, el EPA, previene enfermedades relacionadas con la circulación sanguínea como la hipertensión, la trombosis cerebral y los ataques de corazón (Lees & Karel, 1990; Simopoulos, 1991). Además, se ha demostrado que este PUFA produce una mejora en la respuesta antiinflamatoria y alérgica del organismo (Uauy & Valenzuela, 2000). Por otra parte, el DHA desempeña un papel muy importante en el desarrollo del cerebro y la retina (Ward & Singh, 2005). Otros estudios indican el uso de omega-3 PUFA en la prevención de enfermedades mentales (Ross et al., 2007) y algunos tipos de cáncer (Sidhu, 2003).

Aunque EPA y DHA pueden ser sintetizados por el organismo humano, a partir del ácido graso esencial ALA mediante reacciones enzimáticas de desaturación y elongación, esta conversión tiene una baja eficiencia. De ahí que estos ácidos grasos omega-3 deban ser también ingeridos mediante la dieta (Colussi et al., 2007). Como consecuencia, el aceite de

pescado, considerado la fuente más importante de EPA y DHA, ha encontrado numerosas aplicaciones en la industria alimentaria y nutracéutica (Muggli, 2007).

1.2.1 Oxidación lipídica

El proceso de oxidación lipídica se ve influenciado por varios factores como son la disponibilidad de oxígeno, la temperatura, la luz, la presencia de iones metálicos y de enzimas lipooxigenasas en el aceite. Además, la composición del aceite también juega un papel fundamental en la oxidación de este, siendo la degradación por oxidación proporcional a su contenido en PUFA o presencia de hidrógenos bis-alílicos (Frankel, 2005). Por ello el aceite de pescado, debido a su alto contenido en ácidos grasos poliinsaturados, es altamente susceptible de ser oxidado.

Aunque existen tres mecanismos posibles de oxidación lipídica, autooxidación (inducida por el oxígeno triplete, O_2), fotooxidación (inducida por el oxígeno singlete, $^1O_2$) y oxidación enzimática (inducida por lipooxigenasas), sólo los dos primeros son significativos (Frankel, 2005). Por tanto, serán los discutidos en este apartado.

La Fig. 3 muestra de forma simplificada las reacciones implicadas en estos dos mecanismos de oxidación. Primeramente, la fotooxidación inducida tiene lugar en presencia de oxígeno, luz y fotosensitizadores (Sens). Entre los fotosensitizadores presentes en el aceite destacan pigmentos como la clorofila, hemoproteínas y riboflavina. Estos pigmentos (Sens) absorben luz y pasan a un estado electrónicamente excitado (^{3}Sens). Posteriormente, los fotosensitizadores excitados electrónicamente (^{3}Sens) reaccionan con el O_2 para obtener oxígeno singlete ($^1O_2$). Esta especie excitada del oxígeno molecular es muy electrofílica y reacciona rápida y directamente con los ácidos grasos insaturados para producir hidroperóxidos. Debido a esta alta reactividad, los hidroperóxidos producidos por la reacción del oxígeno singlete juegan un papel importantísimo en la iniciación de la oxidación lípidica por autooxidación (Min & Boff, 2008).

Por otro lado, la autooxidación transcurre mediante un mecanismo de reacción en cadena comprendiendo las etapas de iniciación, propagación y terminación. Inicialmente, los hidroperóxidos (LOOH) presentes como impurezas o producidos en la fotooxidación inducida se descomponen por acción del calor y/o iones metálicos dando lugar a especies radicalarias.

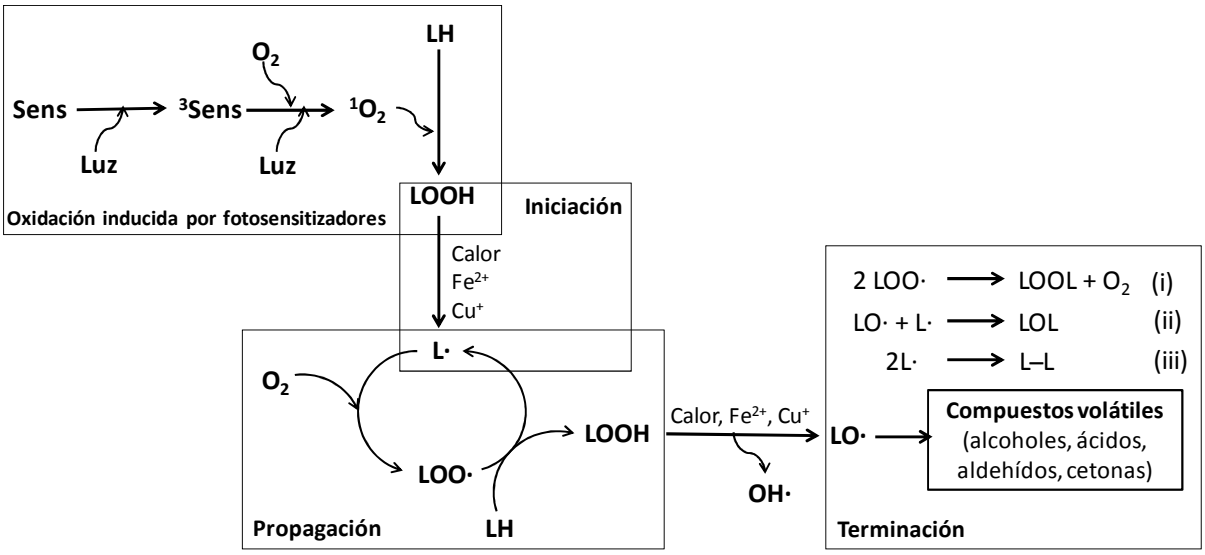


Figura 3. Descripción simplificada de las reacciones de oxidación lipídica. Adaptado de Muggli (2007)

Estos radicales libres junto con iniciadores (calor e iones metálicos) capturan un hidrógeno de un lípido insaturado (LH) para formar un radical alquilo lipídico (L·). A continuación, los radicales alquilo lipídicos (L·) reaccionan con oxígeno para formar radicales peroxilo (LOO·). Estos a su vez reaccionan con una molécula de un lípido insaturado (LH) para formar hidroperóxidos (LOOH) y obtener al mismo tiempo un nuevo radical alquilo ($L^{\cdot}$) que sufrirá este mismo proceso. Finalmente, los hidroperóxidos son descompuestos en presencia de iones metálicos o por la acción del calor a radicales alcoxilo (LO·), los cuales mediante β-fragmentación y reacciones sucesivas dan lugar a compuestos secundarios de oxidación tales como alcoholes, ácidos, aldehídos y cetonas. Además, los radicales libres reaccionan entre sí para dar otros compuestos no radicalarios. Estos se derivan de reacciones de condensación a baja temperatura, (i) en Fig. 3, y de reacciones de polimerización a elevada temperatura y baja concentración de oxígeno, (ii) y (iii) en Fig. 3 (Frankel, 2005).

Mientras que los peróxidos o productos primarios de oxidación no tienen sabor ni olor, los productos secundarios de oxidación o volátiles presentan un fuerte sabor y olor a rancio (Jacobsen & Nielsen, 2007). La Tabla 1 muestra los volátiles más frecuentes formados por autooxidación a partir de omega-3 PUFA.

Tabla 1. Productos secundarios de la oxidación de omega-3 PUFA. Adaptado de Let (2007)

Tipo de compuesto	Volátil	Tipo de compuesto	Volátil
Aldehído	Propanal	Alcohol	1-penten-3-ol
	2-propenal		(E)/(Z)-2-penten-1-ol
	(E)-2-butenal	Cetona	1-penten-3-ona
	(E)/(Z)-2-pentenal	Otros	2-etil-furano
	(E)-2/(Z)-3-hexenal		
	(Z)-4-heptenal		
	(E,E)-2,4-hexadienal		
	(E,E)/(E,Z)-2,4-heptadienal		
	(E,Z)-2,6-nonadienal		

1.2.2 Extracción y Refino

Actualmente, el aceite de pescado se extrae principalmente de especies pelágicas como la anchoa, arenque, sardina y capelán. Aunque también se puede obtener a partir de hígado de especies no grasas como el bacalao, de sub-productos del procesado de atún (e.g. cabezas) y de pequeños crustáceos como el krill (Bimbo, 2007a).

No obstante, el aceite de pescado crudo, generalmente extraído por el método de prensado en húmedo (FAO, 1986), contiene una serie de compuestos como fosfolípidos, ácidos grasos libres, productos de oxidación, dioxinas, trazas metálicas, pigmentos y otros que lo hacen inadecuado para ser utilizado en alimentación humana. Por ello, este aceite requiere un proceso de refino que lo habilite para usos alimentarios (Rubio-Rodríguez et al., 2010).

El refino convencional químico incluye las etapas de: a) desgomado donde se retiran fosfolípidos mediante adicción de agua y/o ácido fosfórico o cítrico, b) neutralización de los ácidos grasos libres con sosa, c) decolorización donde se separan pigmentos, posibles dioxinas y productos de oxidación mediante el empleo de tierras activadas, y e) desodorización para la eliminación de restos de productos volátiles de oxidación (Fig. 4).

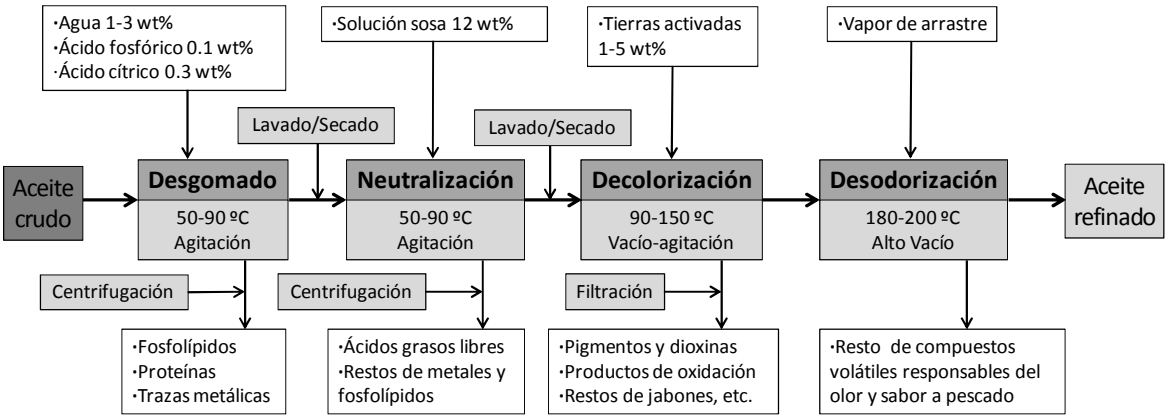


Figura 4. Refino convencional de aceite de pescado

La calidad de un aceite refinado viene determinada entre otros parámetros por su acidez, contenido en peróxidos (indicado mediante el índice de péroxidos, PV) y en productos secundarios de oxidación (indicado por el índice de anisidina, AV, o determinado mediante cromatografía de espacio de cabeza). Valores aceptables de estos parámetros son los siguientes: acidez < 3 mg KOH/g aceite, PV < 5 meq O_2/kg aceite, AV < 20 y totox (2×PV+AV) < 26 (Akman, 2005). No obstante, cuando el aceite de pescado se destina a la producción de alimentos enriquecidos en omega-3 se requiere una calidad más alta. En este sentido, Jacobsen et al. (2009) indican que la acidez y el PV deberían de estar por debajo de 0.1-0.2 % oleico y 1 meq O_2/kg aceite, respectivamente. En cuanto a los volátiles, estos deben ser reducidos al mínimo ya que su fuerte olor y sabor a pescado es detectable por la nariz humana incluso a concentraciones de ppb (Let et al., 2003). Por tanto, la etapas de decolorización (donde se remueven la mayoría de los productos de oxidación, así como pigmentos y restos de jabones y fosfolípidos), y de desodorización (donde se remueven los restos de volátiles que confieren el sabor y olor a rancio) son las más importantes en el proceso de refino (Sathivel, 2010).

1.2.3 Estabilización

La adicción de antioxidantes es uno de los métodos más comunes y eficientes para prevenir la oxidación de aceite de pescado rico en PUFA. Atendiendo a su mecanismo de actuación, los antioxidantes se clasifican en dos grandes grupos (Frankel, 2005):

a) Antioxidantes primarios: este tipo de antioxidantes inhibe o evita las fases de iniciación y/o propagación de la reacción en cadena. Esto se consigue mediante la estabilización de los radicales peroxilo y alcoxilo debido a la transferencia de hidrógeno o electrones por parte del antioxidante a estos radicales. El antioxidante produce un radical relativamente estable, el cual prácticamente no reacciona con lípidos. A esta clase de antioxidantes pertenecen compuestos sintéticos como el butil-hidroxitolueno (BHT), el butil-hidroxi-anisol (BHA) y compuestos naturales como los tocoferoles y otros polifenoles de plantas.
b) Antioxidantes secundarios: estos antioxidantes pueden actuar como quelantes de metales (e.g. ácido cítrico, fosfórico y el ácido etilendiaminotetracético, EDTA) y atrapadores de oxígeno (e.g. palmitato de ascorbilo). Además, estos antioxidantes suelen presentar efectos sinérgicos con los antioxidantes primarios por lo que son usados de forma conjunta (Aubourg et al., 2004; Olsen et al., 2005).

Entre otros análisis, el test Rancimat es comúnmente utilizado para determinar la estabilidad oxidativa de aceites estabilizados (Drusch et al., 2008). Aunque también se emplea en aceites refinados o crudos (Méndez, et al., 1996).

1.2.4 Incorporación a alimentos

El mercado de los alimentos enriquecidos en omega-3 PUFA ha crecido considerablemente en la última década (Sloan, 2006). Ello es debido a que, a pesar de los efectos beneficiosos de estos ácidos grasos en la salud humana, el consumo de pescado por parte de la población occidental es aún insuficiente (Horn et al., 2012a).

La microencapsulación y la pre-emulsión del aceite de pescado han sido estudiadas para prevenir la oxidación de los omega-3 PUFA cuando estos son incorporados a alimentos. La microencapsulación es un proceso más costoso y generalmente se utiliza en productos sólidos tales como pan o en productos en polvo como fórmulas infantiles (Jacobsen & Nielsen, 2007). Por otra parte, la incorporación de aceite de pescado pre-emulsionado podría ser más ventajosa en productos líquidos o semilíquidos, debido a mejoras en el mezclado, y ha sido desarrollada con éxito en leche (Let al., 2007). No obstante, en este mismo estudio se encontraron resultados negativos cuando se evaluó en yogurt y aliños de ensalada.

Entre los factores que afectan a la oxidación de lípidos en emulsiones (pH, tamaño y distribución de gotas, reología, carga electrostática de las gotas y condiciones de emulsificación entre otros) uno de los más importantes es el tipo de emulsionante empleado. Ello se debe a que este determina la estructura y espesor de la interfase que es el lugar de contacto entre los lípidos y los agentes prooxidantes (McClements & Decker, 2000). Los emulsionantes son sustancias anfifílicas capaces de interactuar en la interfase aceite-agua reduciendo la tensión interfacial, lo que permite estabilizar la emulsión. Emulsionantes comúnmente empleados son macromoléculas como las proteínas (e.g. caseína) y pequeñas moléculas con capacidad surfactante (e.g. fosfolípidos) (Jacobsen et al., 2009). Estos han sido utilizados con éxito en la estabilización de emulsiones de aceite de pescado tal y como describen los trabajos de Horn et al. (2012b) y Lu et al. (2012a), respectivamente. Por otra parte, también se han evaluado combinaciones de caseína y fosfolípidos como emulsionantes, las cuales parecen mejorar las propiedades de la interfase (Fang & Dalgleish, 1993).

Además, propiedades antioxidantes han sido descritas tanto para la caseína como para los fosfolípidos, lo que ayudaría a prevenir la oxidación lipídica. La caseína contiene varios residuos fosforilados de serina que ejercen actividad quelante (Díaz et al., 2003). Por otra lado, el mecanismo antioxidante de los fosfolípidos no ha sido aún determinado aunque se les atribuyen las siguientes propiedades: a) degradación de hidroperóxidos inhibiendo su acumulación (Lee et al., 1981; Saito & Ishihara, 1997), b) actividad quelante para el fosfatidilinositol (Pokorný, 1987), c) efectos sinérgicos con alfa-tocoferol, principalmente para la fosfatidiletanolamina (Oshima et al., 1993; Bandarra et al., 1999), y d) formación de pirroles con propiedad antioxidante a través de la reacción de Maillard (Hidalgo et al., 2005; Lu et al., 2012b).

1.2.5 Producción de biodiesel

El aceite de pescado extraído de sub-productos puede presentar muy alta acidez y/o estar fuertemente oxidado, principalmente debido al rápido deterioro de esta materia prima por enzimas y bacterias (Wu & Bechtel, 2008). Por tanto, su refino daría lugar a bajos rendimientos, fundamentalmente por pérdidas en la etapa de neutralización (Sathivel et al., 2003). Además, este aceite puede presentar un bajo contenido en omega-3 PUFA (<20 wt%) lo que también resultaría en una baja productividad en un proceso de obtención de concentrados omega-3, teniendo por tanto reducido interés para usos alimentarios y farmacéuticos (Bimbo, 2007b). Por todo ello, el aceite de pescado de baja calidad ha sido propuesto como materia prima de bajo coste para la producción de biodiesel (Zhang & El-Mashad, 2006).

El biodiesel (alquil ésteres) es un biocombustible que se obtiene, a partir de aceites y/o grasas, generalmente mediante el proceso de transesterificación (Fig. 5). Catalizadores básicos como la sosa y alcoholes baratos como el metanol son normalmente utilizados (Knothe et al. 2005). La norma europea EN 14214 determina las propiedades (pureza en alquil ésteres, viscosidad, características en frío, estabilidad a la oxidación, etc.) que debe tener el biodiesel para ser empleado como 100 % fuel o mezclado con otros fueles. En general, biodiesel con buenas propiedades ha sido obtenido a partir de aceite de pescado (El-Mashad et al., 2008; Fan et al., 2010; Costa et al., 2013; Fadhil & Ali, 2013). No obstante, este tipo de biodiesel presenta un baja estabilidad oxidativa debido al alto contenido en PUFA del aceite de pescado (Lin & Lee, 2010).

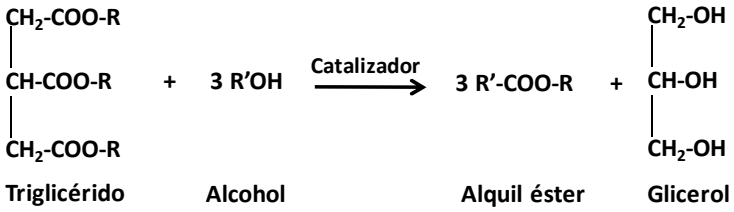


Figura 5. Transesterificación de triglicéridos

1.3 Hidrolizados de pescado

Los sub-productos de pesca presentan proteínas de alta calidad las cuales contienen la mayoría de los aminoácidos esenciales requeridos en una buena alimentación (Rustad, 2006). Las proteínas musculares del pescado se clasifican en tres grupos: a) proteínas sarcoplásmicas, solubles en agua, tales como mioglobina y enzimas (20-35 wt%); b) proteínas miofibrilares, solubles en soluciones salinas concentradas, como miosina, actina, tropomiosina y troponina (65-75 wt%); y c) proteínas del estroma, insolubles en soluciones salinas concentradas, las cuales representan un porcentaje bajo (2-3 wt%), excepto en elasmobranquios que pueden alcanzar hasta el 10 wt% (Torres et al., 2006).

La hidrólisis (química o enzimática) de estas proteínas es un proceso efectivo para la extracción del material proteico presente en los sub-productos de pesca (Kristinsson, 2006). En particular, la hidrólisis enzimática se presenta como la técnica más ventajosa debido a los siguientes aspectos: a) se lleva a cabo en condiciones moderadas de temperatura y pH, b) evita reacciones secundarias debido a la alta especificidad de las enzimas, y c) no deteriora las propiedades nutritivas de los productos resultantes (Guerard, 2006).

En la hidrólisis enzimática, las proteasas catalizan la ruptura del enlace existente entre los aminoácidos que componen la cadena peptídica, consumiendo una molécula de agua por cada enlace roto (Ec. 1):

$$P1\text{-}CO\text{-}NH\text{-}P2 + H_2O \Rightarrow P1\text{-}COOH + NH_2\text{-}P2 \qquad (1)$$

Por tanto, la ruptura continuada de enlaces peptídicos produce la degradación de la proteína origen dando lugar a especies de menor peso molecular tales como peptonas, péptidos y aminoácidos (Adler-Nissen, 1986).

Los hidrolizados enzimáticos se aplican en numerosas aplicaciones alimentarias gracias a sus mejoradas propiedades tecnológicas, entre las que destacan una mejora de la

solubilidad, capacidad de retención de agua, propiedades emulsionantes y espumantes y baja alergenicidad (Kristinsson & Rasco, 2000). No obstante, la producción de hidrolizados con propiedades bioactivas ha ganado gran relevancia en los últimos años debido al mayor valor añadido de estos productos (Thorkelsson et al., 2009).

En este sentido, numerosos estudios han demostrado la presencia de péptidos con actividad antioxidante en los hidrolizados de especies marinas como el arenque (Sathivel et al., 2003) o la caballa (Wu et al., 2003). Además, se han aislado fracciones peptídicas de origen marino con actividad antihipertensiva a partir de especies como el lenguado (Jung et al., 2006) o el salmón (Ono et al., 2006). Otras bioactividades también han sido descritas en hidrolizados de especies marinas tales como antimicrobiana (Fleury et al., 2008) y anticolesterolémica (Naqash et al., 2011). Las dos primeras son objeto de investigación en esta tesis doctoral y serán expuestas con más detalle a continuación.

1.3.1 Actividad antioxidante

Los antioxidantes, además de ser clave en la prevención de la oxidación lipídica en alimentos, también tienen un papel importante en la prevención de la oxidación a nivel fisiológico. La respiración celular da lugar a la formación de radicales libres (e.g. OH·) los cuales se pueden acumular en el organismo debido a una deficiencia de antioxidantes naturales (e.g. vitamina C). Estos radicales libres actúan como iniciadores de reacciones de oxidación en cadena que causan daño celular, deterioro de proteínas y mutaciones en ADN entre otros (Lee et al., 2004). Todo ello puede desencadenar en enfermedades crónicas tales como cáncer, diabetes y enfermedades inflamatorias y neurodegenerativas (Butterfield et al., 2002).

Los antioxidantes actúan inhibiendo o retardando las reacciones de oxidación mediante: a) la captación de radicales libres, y/o b) la quelación de iones metálicos o la modificación del potencial redox de estos, lo que previene la formación de nuevas especies radicalarias (Laroque et al., 2008). Tradicionalmente se han empleado para tales fines compuestos sintéticos como el butirato de hidroxianisol (BHA) y el butirato de hidroxitolueno (BHT). Sin embargo, y aunque estos productos presentan una alta capacidad antioxidante, su uso en alimentación es cada vez menos común debido a posibles efectos tóxicos (Ito et al., 1985). De ahí que haya un interés creciente en la obtención de antioxidantes naturales sin efectos secundarios.

Entre las fuentes naturales investigadas, las proteínas de pescado han sido ampliamente evaluadas para la obtención de hidrolizados enzimáticos con actividad antioxidante (Samaranayaka & Li-Chan, 2011). Buenos resultados han sido obtenidos a partir de especies como el arenque, *Clupea harengus*, (Sathivel et al., 2003); la caballa, *Scomber austriasicus*, (Wu et al., 2003); la merluza (hoki), *Johnius belengerii*, (Mendis et al., 2005); el carbonero, *Pollachius virens*, (Chabeaud et al., 2009); la alacha, *Sardinella aurita*, (Bougatef et al., 2010); y el sable negro, *Aphanopus Carbo*, (Batista et al., 2010). La actividad antioxidante de los hidrolizados depende en gran medida del tamaño molecular de los péptidos y de su composición y secuencia de aminoácidos. La mayoría de péptidos antioxidantes presentan entre 2 y 20 aminoácidos, con residuos hidrofóbicos tales como valina, glicina y prolina que favorecen la captación de radicales libres solubles en lípidos (Harnedy & FitzGerald, 2012). Otros aminoácidos como tirosina, triptófano, metionina, lisina, cisteína e histidina suelen estar presentes en la secuencia de los péptidos bioactivos debido a su capacidad para donar electrones o protones y estabilizar a los radicales libres (He et al., 2013). Todas estas características del hidrolizado vienen determinadas por: a) tipo de matriz a hidrolizar (e.g. especie, músculo, pieles y otros), b) especificidad de la proteasa empleada, c) condiciones de hidrólisis (temperaruta, pH y relación enzima/sustrato), y d) por la extensión de la reacción de hidrólisis (Laroque et al., 2008). La Tabla 2 recoge algunos de estos aspectos para los trabajos nombrados anteriormente.

Tabla 2. Ejemplos de hidrolizados con actividad antioxidante obtenidos a partir de proteínas de pescado

Especie	Material	Enzima	Secuencia	Mecanismo	Referencia
Arenque	Pez entero, cuerpo, cabeza, gónadas	Alcalasa	n.e.	1	Sathivel et al., 2003
Caballa	Filetes	Proteasa N	n.e.	1, 2, 3	Wu et al., 2003
Merluza (Hoki)	Piel	Tripsina	His-Gly-Pro-Leu-Gly-Pro-Leu	1, 2	Mendis et al., 2005
Carbonero	Músculo	Alcalasa	n.e.	1, 2	Chabeaud et al., 2009
Alacha	Cabeza y vísceras	Extracto de vísceras de *Sardina. Pilchardus*	Leu-His-Tyr	1, 2, 3	Bougatef et al., 2010
Sable negro	Cabeza, pieles, vísceras,recortes	Protamex	n.e.	2, 3	Batista et al., 2010

n.e.: no especificada; 1: Inhibición de oxidación lipídica; 2: Captación de radicales libres; 3: Poder reductor.

1.3.1 Actividad antihipertensiva

La hipertensión afecta a un tercio de la población adulta y es un factor de riesgo que puede dar lugar a enfermedades cardiovasculares como arritmias, embolias cerebrales e infartos (Kearney et al., 2005).

Uno de los principales métodos para combatir la hipertensión es la inhibición de la enzima convertidora de la angiotensina, ECA, (Puig, 2002). La ECA (EC 3.4.15.1) es una metalopeptidasa que contiene Zn^{2+} y que necesita cloruros para activarse. Esta enzima está presente en numerosas partes del organismo (cerebro, pulmones, hígado, riñones, testículos entre otras), principalmente ligada al endotelio vascular en forma de ectoenzima (Murray & FitzGerald, 2007). Su papel en la regulación de la prensión sanguínea se debe a que interviene en el sistema renina-angiotensina, en el que transforma el decapéptido inactivo Angiotensina I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) en el potente vasoconstrictor Angiotensina II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). La Angiotensina II además favorece la secreción de aldosterona que activa la reabsorción de agua y Na^+ por los túbulos renales lo que conlleva un aumento del volumen sanguíneo y, por consiguiente, de la presión arterial. Por otra parte, en el sistema calicreína-cinina, la ECA cataliza la degradación de la bradiquinina, un péptido que tiene propiedades vasodilatadoras (Li et al., 2004). La Fig. 6 muestra de forma simplificada los mecanismos de actuación de la ECA.

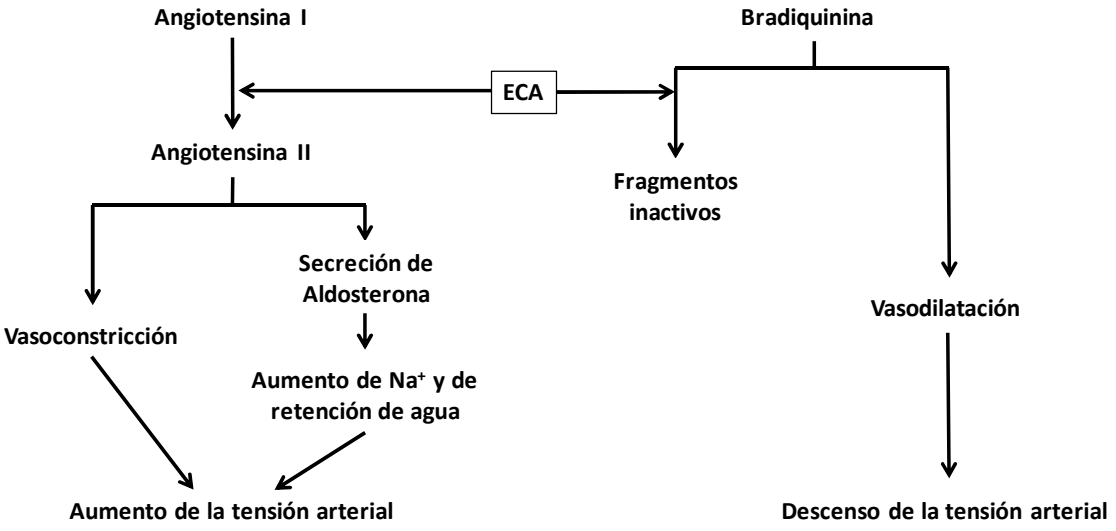


Figura 6. Mecanismos de actuación de la ECA. Adaptado de Li et al. (2004)

Actualmente, se han desarrollado compuestos sintéticos capaces de inhibir la actividad de la ECA tales como captopril, enalapril, alacepril y lisinopril. Sin embargo, el uso

continuado de estos fármacos resulta en efectos negativos como tos seca, deterioro del sentido del gusto o erupciones cutáneas (Wijesekara & Kim, 2010). Por ello, existe la necesidad de encontrar compuestos naturales capaces de inhibir la ECA sin que tengan efectos secundarios.

En este sentido, se han obtenido numerosos péptidos derivados de proteínas de pescado con buenas propiedades antihipertensivas (Martínez-Maqueda et al., 2012). Los péptidos inhibidores de ECA están formados por 2-12 aminoácidos y suelen presentar aminoácidos hidrofóbicos (e.g. Phe, Trp, Tyr, Pro) en una de las 3 posiciones extremas del C-terminal. Esto favorece la interacción con las tres zonas hidrofóbicas situadas en el centro activo de la enzima. En esta línea, la ECA muestra poca afinidad por inhibidores hidrofílicos como aquellos que presentan aminoácidos dicarboxílicos (e.g. Glu) en el C-terminal. Por otra parte, la carga positiva de aminoácidos como Lys y Arg también contribuye a la potencia inhibidora cuando estos se encuentran en el C-terminal. Otros estudios estructura-actividad manifiestan que la actividad inhibidora de péptidos con Pro en el C-terminal aumenta cuando el aminoácido adyacente es hidrofóbico (Li et al., 2004).

Por tanto, al igual que para la actividad antioxidante, la composición y secuencia de aminoácidos de los péptidos inhibidores de ECA dependerá de las características del material hidrolizado, del tipo de enzima empleada así como de las condiciones utilizadas en la reacción de hidrólisis. La Tabla 3 presenta algunos péptidos antihipertensivos derivados de proteína de pescado, así como su valor de IC_{50} (concentración de proteína que inhibe la actividad de ECA al 50 %).

Tabla 3. Ejemplos de hidrolizados inhibidores de ECA obtenidos a partir de proteínas de pescado

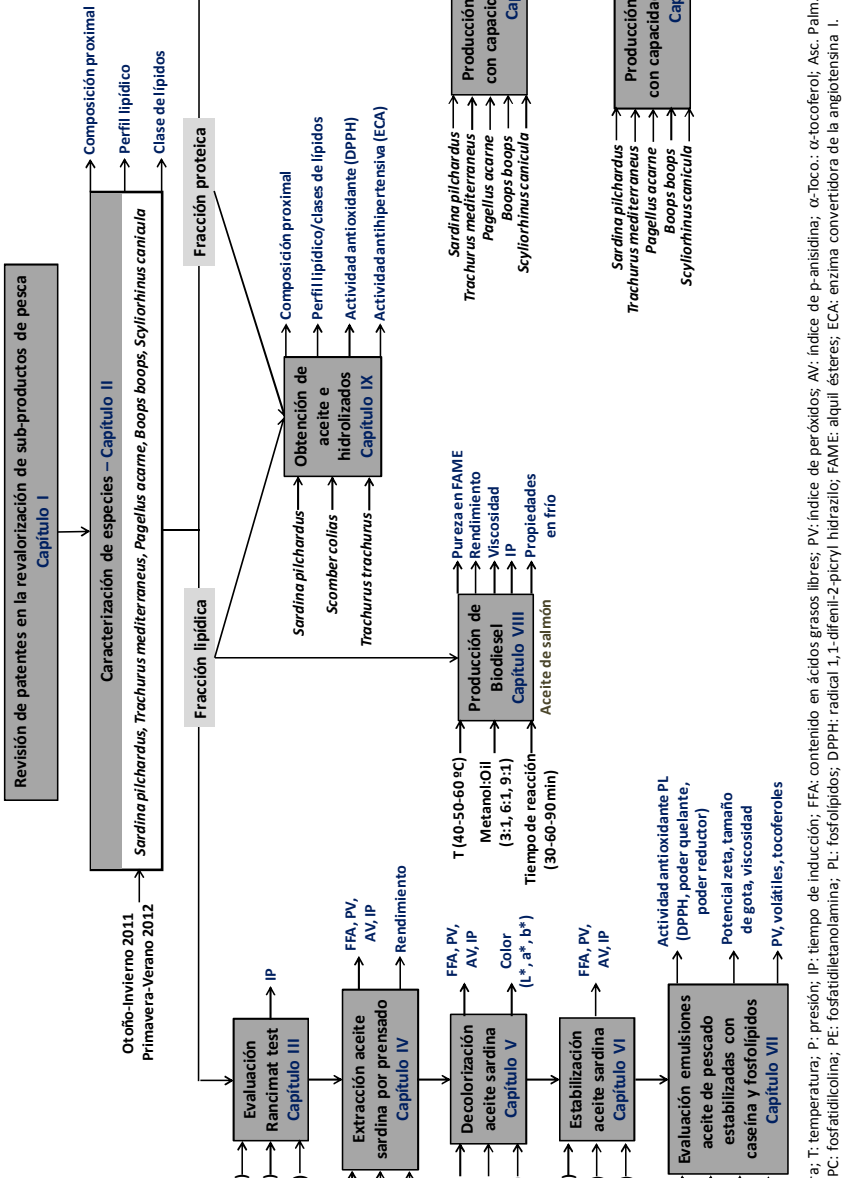
Especie	Material	Enzima	Secuencia	IC_{50} (µg/mL)	Referencia
Sardina	Músculo	Proteasa alcalina	n.e	260	Matsui et al., 1993
Aligote	Escamas	Proteasa alcalina	Gly–Tyr Val–Tyr Gly–Phe Val–Ile–Tyr.	570	Fahmi et al., 2004
Limanda	Recortes	α-quimotripsina	Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu	28.7*	Jung et al., 2006
Alacha	Cabeza y vísceras	Extracto de vísceras de *Sardina. Pilchardus*	n.e.	1200	Bougatef et al., 2008
Sepia	Músculo	Extracto de vísceras de *Sepia officinalis*	Val-Tyr-Ala-Pro Val-Ile-Ile-Phe Met-Ala-Trp	1000	Balti et al., 2010

n.e.: no especificada; *: IC_{50} del péptido

2. OBJETIVOS

El objetivo general de esta tesis doctoral es el desarrollo de bioprocesos para la revalorización tanto de la fracción lipídica como proteica de especies de descarte en el Mar de Alborán. Para el cumplimiento de este objetivo general se plantean los siguientes objetivos concretos (Fig. 7):

1. Revisión de patentes en la revalorización de sub-productos de pesca (capítulo I).
2. Caracterización de 5 especies de descarte (*Sardina pilchardus*, *Trachurus mediterraneus*, *Pagellus Acarne*, *Boops Boops* y *Scyliorhinus canicula*) y evaluación de su potencial como materia prima para la producción de aceite de pescado rico en omega-3 PUFA (capítulo II).
3. Estudio de la influencia de los parámetros del test Rancimat en la determinación de la estabilidad oxidativa de aceite de pescado (capítulo III).
4. Optimización del proceso de extracción de aceite de sardina por prensado hidráulico (capítulo IV).
5. Optimización del proceso de decolorización de aceite de sardina (capítulo V).
6. Optimización del proceso de estabilización de aceite de sardina mediante la adicción de antioxidantes (capítulo VI).
7. Evaluación de la capacidad antioxidante de fosfosfolípidos y estudio de la influencia de combinaciones caseína-fosfolípidos como emulsionantes en la estabilidad física y oxidativa de emulsiones de aceite de pescado (capítulo VII).
8. Optimización de la producción de biodiesel a partir de aceite de pescado (capítulo VIII).
9. Caracterización lipídica de 3 especies de descarte (*Sardina pilchardus, Scomber colias* y *Trachurus trachurus*) y evaluación de la actividad antihipertensiva y antioxidante de sus hidrolizados (capítulo IX).
10. Estudio de la actividad antioxidante de hidrolizados obtenidos a partir de las 5 especies de descarte enumeradas en el objetivo 2 (capítulo X).
11. Estudio de la actividad antihipertensiva de los hidrolizados obtenidos a partir de las 5 especies de descarte enumeradas en el objetivo 2 (capítulo XI).



Q: caudal de aire; M: masa de muestra; T: temperatura; P: presión; IP: tiempo de inducción; FFA: contenido en ácidos grasos libres; PV: índice de peróxidos; AV: índice de p-anisidina; α-Toco.: α-tocoferol; Asc. Palm.: palmitato de ascorbilo; CAS: caseinato de sodio; LC: lecitina; PC: fosfatidilcolina; PE: fosfatidiletanolamina; PL: fosfolípidos; DPPH: radical 1,1-difenil-2-picryl hidrazilo; FAME: alquil ésteres; ECA: enzima convertidora de la angiotensina I.

Figura 7. Diagrama de flujo de la investigación desarrollada en esta tesis doctoral

4. RESULTADOS Y DISCUSIÓN

4.1 Revisión de patentes en la revalorización de sub-productos de pesca

Numerosos procesos han sido patentados en las últimas décadas para la revalorización de sub-productos de pesca. En este sentido, destacan aquellos para la producción de hidrolizados enzimáticos y aceite de pescado.

Las aplicaciones de mayor importancia de los hidrolizados de proteína de pescado (FPH) son la alimentación humana y su utilización como ingredientes bioactivos. En la producción de FPH como aditivos o para consumo directo destacan las patentes que utilizan enzimas añadidas o exógenas como la patente US 2002/0182290A1 (Munk, 2002) que describe un proceso donde se emplea Protamex^{TM} para la producción de FPH, los cuales pueden ser incorporados a filetes de salmón mejorando su calidad. Además, existen otros procesos patentados que utilizan enzimas endógenas, contenidas en vísceras, como los procesos descritos en WO 03/066665A2 (Pyntikow et al., 2003) y US 2005/0244567A1 (Carlsson, 2005). Por otra parte, se ha patentado el uso de otras combinaciones enzimáticas que den lugar a FPH con reducido sabor amargo. En este grupo se tienen las patentes EP 0518999B1 (Dambmann et al., 1995) que propone el uso de una proteasa producida a partir de una cepa de *Bacillus Licheniformis*, que da lugar a péptidos hidrofílicos con residuos de ácido glutámico y aspártico en el C-terminal; y la patente US 2006/7070953B1 (Bjarnason & Benediktsson, 2006) que utiliza enzimas contenidas en vísceras de bacalao (tripsina, quimotripsina y otras) que permiten llevar a cabo la hidrólisis a 15-20 ºC dando lugar un hidrolizado sin amargor, el cual puede ser incorporado a sopas o salsas ya que conserva su sabor y olor.

En los últimos años, la producción de hidrolizados de pescado con propiedades bioactivas ha ganado un especial interés debido al mayor valor añadido de estos productos. Por ello, se han patentado diferentes procesos para la producción de péptidos con actividad antihipertensiva tales como los descritos en US 2007/007179793 (Ewart et al., 2007) que propone la hidrólisis de sub-productos de salmón con Protease S Amano; y en EP 1092724B1 (Hiroyuki, 2008) que utiliza termolisina para hidrolizar sub-productos de caballa, sardina, bonito y bacalao. Ambos procesos presentan una etapa final de ultrafiltración para obtener un producto con péptidos de un tamaño menor a 3,000 Da. Por

otra parte, también se ha patentado la producción de hidrolizados de pescado con propiedad antioxidante. En este aspecto, la patente WO 2004/071202A1 (Hagen & Sandness, 2004) describe la hidrólisis de sub-productos de salmón con Protamex^{TM} para la producción de un hidrolizado capaz de reducir la oxidación lipídica en filetes de pescado.

Por otro lado, las patentes en la producción de aceite de pescado se clasifican en aquellas que tratan la extracción, el refino, la estabilización y la incorporación de este aceite a alimentos. Diferentes metodologías han sido patentadas para la extracción de aceite: a) extracción por prensado en húmedo con desactivación de lipooxigenasas por adicción de ácido fosfórico, US 6190715B1 (Crowther et al., 2001); b) extracción con solventes a baja temperatura de fosfolípidos a partir de vísceras de atún y bonito, US 7189418B2 (Hiratsuka et al., 2007); c) extracción con fluidos supercríticos a partir de una materia prima con bajo contenido de aceite (e.g. harina de pescado), EP 0917876A1 (Blasco-Piquer & Mira-Ferri, 1999); y d) extracción por hidrólisis enzimática con proteasas, US 4976973 (Shirakawa et al., 1990). En el refino, destacan los procesos patentados para la eliminación de compuestos volátiles de oxidación, tales como la destilación molecular a alto vacío, US 4623488 (Takao, 1986); la adsorción cromatográfica en columna de gel de sílice, US 5023100 (Chang & Bao, 1991); y la extracción con CO_2 supercrítico, US 4692280 (Spinelly & Stout, 1987). Con respecto a la estabilización de aceite de pescado, se ha patentado la adicción de fructosa, US 5116629 (Schroeder & Muffett, 1992), y de vinagre y zumo de cítricos, US 6235331 (Kataoka & Kiyohara, 2001), para la estabilización de emulsiones como aliños de ensaladas o yogurt; y la adicción de antioxidantes naturales de naranja, manzana y mango para la estabilización de zumos de estas frutas enriquecidos con aceite de pescado, US 0202679 (Mathisen, 2009). Por último, se han desarrollado procesos de microencapsulación que previenen la oxidación y enmascaran el sabor y olor del aceite de pescado cuando este es incorporado a alimentos, US 48956725 (Martin et al., 1990) y US 5456985 (Zgoulli et al., 1995).

4.2 Caracterización de 5 especies de descarte (*Sardina pilchardus, Trachurus mediterraneus, Pagellus Acarne, Boops Boops y Scyliorhinus canicula*) y evaluación de su contenido en omega-3 PUFA

Los análisis de composición proximal indican que el contenido en proteína de las especies permaneció prácticamente constante durante el año, variando entre 15.5 wt% para la boga y 23.1 wt% para la pintarroja (Tabla 7). No obstante, el contenido en lípidos fue muy diferente entre especies y entre estaciones para una misma especie. Contenidos lipídicos máximos fueron observados en primavera (aligote 5.1 wt%, pintarroja 2.7 wt% y boga 2.5 wt%) y verano (sardina 13.6 wt% y jurel 6.2 wt%) cuando hay abundancia de alimento en el medio marino (Orban et al., 2011). Por otra parte, contenidos mínimos fueron observados tras el período de desove (primavera para la sardina e invierno para el resto de especies), debido a que la grasa es consumida por el pez como aporte energético (Krzynowek, 1992). El contenido en cenizas también permaneció relativamente constante a lo largo del año, mientras que el contenido en humedad varió de forma inversa al contenido en lípidos (Tabla 7).

En los aceites extraídos por prensado para cada una de las especies, los ácidos grasos más abundantes fueron los poliinsaturados, variando entre 33.0 y 45.1 wt%, principalmente constituidos por EPA y DHA. El contenido en EPA+DHA de los aceites fue superior a 20 wt% en todas las estaciones, destacando el aceite de pintarroja con un contenido en DHA de hasta el 20 wt%. Además, todos los aceites presentaron excelentes ratios n3/n6, siendo máximo para la sardina (14.9-16.7) seguido del jurel (11.9-13.5) (Tabla 8).

En cuanto al contenido en omega-3 de las especies, los resultados indicaron que la sardina y el jurel son extraordinarias fuentes de omega-3 en verano y otoño presentando hasta 3000 y 1300 mg/100 g pez, respectivamente. Estas fueron seguidas del aligote y pintarroja que en primavera presentaron valores de 960 y 650 mg/100 g pez, respectivamente (Tabla 9).

Con respecto a la clase de lípidos, los triglicéridos fueron los más abundantes con porcentajes mayores del 78 wt% para todos los aceites, siendo hasta del 96 wt% para el aceite de pintarroja. Por otra parte, el contenido en colesterol de los aceites varió entre 3.3 wt% para la pintarroja y 8.8 wt% para el aligote, mientras que fosfolípidos fueron detectados solamente en el aceite de sardina (0.8 wt%) (Tabla 10).

4.3 Evaluación de la influencia de los parámetros del test Rancimat en la determinación de la estabilidad oxidativa de aceite de pescado

Los datos experimentales indican que a valores constantes de caudal de aire (Q, L/h) y masa de muestra (M, g), el índice de estabilidad oxidativa (OSI, h), equivalente al tiempo de inducción (IP, h), disminuye considerablemente al aumentar la temperatura (T, ºC). No obstante, influencias tan claras en el OSI no fueron observadas en el caso del caudal de aire ni de la masa de muestra (Tabla 11).

Primeramente, los valores de OSI se ajustaron a un modelo cuadrático (Eq. 4), obteniendo un R^2 de 0.9971. Tras el análisis ANOVA (Tabla 12), tanto los efectos lineales de la temperatura y el caudal de aire como el efecto cuadrático de la temperatura resultaron estadísticamente significativos ($p<0.05$). A partir del modelo anterior y empleando la técnica de superficies de respuesta, se obtuvieron gráficas de contorno donde los valores de OSI fueron representados frente a Q y M a cada valor de T estudiado (Fig. 9). Se observa que las líneas de contorno siguen una curva, aunque esta se hace menos pronunciada conforme aumenta la temperatura. Este hecho puede deberse a un cambio en el mecanismo de oxidación a mayores valores de temperatura, favoreciéndose reacciones secundarias de los compuestos no volátiles tales como reacciones de polimerización (Frankel, 2005). La optimización del modelo cuadrático propuesto resulta en un mínimo para el OSI de 1.61 h obtenido para Q=25 L/h, M=6.91 g y T=88.26 ºC (se muestra como un círculo en la Fig.9). Estos resultados denotan que existe un óptimo para la relación caudal de aire-masa de muestra que favorecería las condiciones de saturación dentro del recipiente de reacción.

Por otra parte, debido a la falta de significancia de los efectos cuadráticos (excepto para la temperatura) y de los efectos cruzados, los valores de OSI fueron ajustados a un modelo lineal (Eq. 5). De acuerdo a trabajos previos (Reynhout, 1991; Farhoosh, 2007), el logaritmo decimal de OSI fue elegido para conseguir un mejor grado de ajuste, obteniéndose un R^2=0.9958. En este modelo, como en el cuadrático, tanto la temperatura como el caudal de aire resultaron significativos ($p<0.05$) (Tabla 13). Para el aceite de hígado de bacalao estudiado, se obtuvo un coeficiente de temperatura de -3.29×10^{-2} ºC^{-1}, muy similar al valor encontrado para otros aceites de pescado por Méndez et al. (1996).

4.4 Optimización del proceso de extracción de aceite de sardina por prensado hidráulico

Las muestras de aceite extraído presentaron prácticamente el mismo perfil lipídico, siendo los PUFA los ácidos grasos más abundantes (35 wt%). La composición en EPA del aceite fue del 13 wt% y en DHA del 11 wt%.

En cuanto a la calidad del aceite extraído (Tabla 14), destaca su bajo índice de acidez (0.25-1.23 % oleico) comparado con los valores reportados por otros autores (2.9 % oleico), quienes emplearon cabezas de atún (Chantachum et al., 2000) y sub-productos de arenque como materia prima (Aidos et al., 2001). Además, las muestras de aceite tenían una concentración baja de productos primarios de oxidación, variando el PV entre 0.33-5.75 meq/kg aceite. En cuanto a los productos secundarios de oxidación, prácticamente no tuvo lugar la descomposición de hidroperóxidos, variando el AV entre 0-1.98. Esto se debe fundamentalmente a la baja temperatura utilizada en el pretratamiento ya que valores mayores de PV (30 meq/kg) fueron obtenidos por Chantachum et al. (2000) y de AV (8.9) por Aidos et al. (2001) quienes precalentaron a 95 ºC. Por otra parte, los valores de IP (1.09-2-06 h) no se correspondieron con los valores de PV y AV, excepto para los experimentos llevados a cabo a 55 ºC los cuales dieron lugar a los menores tiempos de inducción. Con respecto al rendimiento en aceite, un amplio rango de valores (1.16-12.78 %) fue obtenido en función de las condiciones de extracción utilizadas (Tabla 14).

Los valores experimentales fueron ajustados a modelos cuadráticos, obteniendo valores de R^2 en torno a 0.9 para FFA, PV y rendimiento, 0.78 para AV y 0.76 para IP (Tabla 15). El análisis ANOVA puso de manifiesto la influencia significativa ($p<0.05$) del efecto lineal de la temperatura en todas las variables de salida, mientras que el efecto lineal de la presión no resultó significante en ninguna y el del número de etapas sí lo fue para AV y rendimiento. (Tabla 15).

Posteriormente, se llevó a cabo la optimización individual mediante superficies de respuesta de FFA y PV, como parámetros indicadores de la calidad del aceite, y del rendimiento, como variable indicadora de la eficiencia del proceso (Fig. 13). Un mínimo de FFA (0.25 % oleico) fue encontrado para 55 ºC, 60 bar y una etapa de prensado. De estos resultados se deduce que el precalentamiento a 55 ºC podría desnaturalizar las lipasas endógenas de la sardina, disminuyendo por tanto la hidrólisis de triglicéridos a ácidos grasos libres (Mukundan et al., 1985). Además, el mínimo número de etapas es preferible

ya que disminuye el tiempo de contacto del aceite con el agua de prensado. En cuanto al mínimo de PV (0.29 meq/kg), este fue encontrado razonablemente a la menor temperatura de pretratamiento (5 ºC) y a la menor presión (60 bar) y número de etapas. Al igual que para FFA, el número mínimo de etapas reduce el tiempo de prensado lo que disminuye la oxidación del aceite. Por último, el máximo rendimiento (12.47 %) se obtuvo a la máxima temperatura de pretratamiento (55 ºC), a la mínima presión (60 bar) y a un número intermedio de etapas (2). Calentar a 55 ºC conlleva una mayor ruptura de los tejidos celulares que recubren los depósitos de grasa y además favorece la coagulación de proteínas lo que también ayuda a la liberación del aceite (FAO, 1986). Por otra parte, parece existir una combinación óptima de presión y etapas de prensado (60 bar y 2 etapas) que no produce una compactación excesiva de la torta, permitiendo por tanto un flujo óptimo de aceite hacia el exterior.

Finalmente, la optimización simultánea de FFA, PV y rendimiento resultó en un conjunto de soluciones óptimas (Frente de Pareto) que satisfacen todos los objetivos (Tabla 16). Además, en la Tabla 16 se recogen los valores de las condiciones del proceso de extracción necesarias para obtener un determinado rendimiento de aceite con una calidad concreta. No obstante, se debe mencionar que valores de w_3<0.4 no son recomendables para no obtener un valor de rendimiento demasiado bajo (<11.06 %). Ello implica operar a 55 ºC, 60 bar y dos o una etapa de prensado.

4.5 Optimización del proceso de decolorización de aceite de sardina

En este trabajo se evaluó el proceso de decolorización de aceite de sardina previamente desgomado y neutralizado. El aceite de partida tenía un bajo valor de acidez (0.17 % oleico) y peróxidos (2.36 meq/kg aceite), aunque presentaba un alto contenido de productos secundarios de oxidación, AV (77). Además, presentaba un color marrón oscuro debido principalmente a su alto grado de deterioro.

La decolorización con tierras ácidas activadas (Tonsil 278), produjo un descenso en la acidez del aceite cuando se empleó la mayor concentración de tierras ensayada (5 wt%) (Tabla 17). En cuanto al contenido en peróxidos, el PV del aceite disminuyó hasta valores no detectables a alta concentración de tierras (5 wt%) y alta temperatura (130 ºC), debido a la degradación de hidropéroxidos. No obstante, a menores concentraciones de tierras (1-3

wt%) y menor temperatura (90-110 ºC), el PV del aceite no se redujo sino que incluso aumentó. Con respecto a los productos secundarios de oxidación, todas las muestras de aceite decolorado presentaron un AV menor que el del aceite de partida (Tabla 17). Este hecho pone de manifiesto la capacidad de las tierras utilizadas para la adsorción de este tipo de compuestos (Rossi et al., 2003, Sathivel, 2010). Los valores de IP no muestran una tendencia clara, aunque los aceites decolorados a 130 ºC, empleando 5 wt% de tierras y largos tiempos de contacto (40-60 min) parecen tener mejor estabilidad oxidativa (IP=0.95 h) (Tabla 17).

En relación a las medidas de color, el proceso de decolorización resultó en aceites con mayor luminosidad, valores de L* hasta de 83.61. El valor del parámetro a* fue disminuido indicando una adsorción de compuestos rojamente coloreados. El valor del parámetro croma, que representa la intensidad del color, fue reducido; mientras que el valor del tono aumentó con la concentración de tierras. Se obtuvieron valores de tono en el rango del amarillo-naranja (79.53-90.76) (Tabla 17).

Los datos experimentales se ajustaron satisfactoriamente a modelos de segundo orden, con valores de R^2 en torno a 0.9 excepto para el modelo de FFA (Tablas 18 y19). El análisis ANOVA (Tablas 18 y 19) indicó una influencia significativa del efecto lineal de la concentración de tierras en todas las variables medidas (FFA, PV, AV, IP, L*, a* y b*) y en las calculadas (totox, croma y tono) y de la temperatura en FFA, PV, AV, IP, totox y L*. El efecto cuadrático de la concentración de tierras también resultó significativo en L*, b*, y croma; y el del tiempo en FFA, PV, AV y L*.

Por tanto, para la optimización del proceso de decolorización se eligieron las parámetros de totox, indicando la calidad del aceite en términos de productos de oxidación, y de croma y tono como parámetros indicativos del color. La optimización individual de estas variables (Fig. 15) resultó en:

a) Un mínimo para totox (21.38) a 130 ºC, 5 wt% de tierras y 60 min. Este hecho se debe a que la mayor temperatura evaluada permite una mayor descomposición de hidroperóxidos a compuestos volátiles los cuales se adsorben mejor a mayor concentración de tierras. Por otra parte, largos tiempos de contacto favorecen la adsorción de los compuestos secundarios de oxidación (Sathivel, 2010).
b) Un máximo para el valor del tono (89.19) a la máxima concentración de tierras (5 wt%), a largos tiempos de contacto (56.6 min) y a un valor bajo de temperatura

(99.2 ºC). En cuanto al croma, un mínimo (81.76) fue localizado a 109.4 ºC, 5 wt% de tierras y 49.4 min. Estos resultados indican una posible mejor activación de las tierras empleadas, en cuanto a la adsorción de compuestos coloreados se refiere, a valores moderados de temperatura (99-110 ºC).

En la optimización conjunta de totox, chroma y hue-angle, las características requeridas para el aceite decolorizado determinarán la combinación de pesos (w_1, w_2 y w_3) para cada una de las variables individuales. Ello implica la elección de una solución óptima dentro del frente de Pareto obtenido (Tabla 20) y de un valor concreto para las condiciones del proceso (Fig. 16). Por ejemplo, si se desea obtener un aceite de calidad en términos de productos de oxidación, un valor de w_1<0.4 no es recomendado. Ello implicaría operar a 130 ºC, 5 wt% de tierras y en un rango de tiempo de 54 a 60 min, obteniendo un valor de totox <21.83.

4.6 Optimización del proceso de estabilización de aceite de sardina mediante la adicción de antioxidantes

La purificación del aceite de sardina crudo por adsorción en columna cromatográfica de alúmina-sílice redujo su acidez (FFA de 6.26 a 0.14 % oleico) y su contenido en productos secundarios de oxidación (AV de 17.18 a 2.17). No obstante, el valor de peróxidos permaneció prácticamente constante (PV de 2.7 meq/kg aceite). En cuanto al perfil lipídico, este no cambió, con un contenido en EPA de 19.7 wt% y en DHA de 9.4 wt%.

Durante el tiempo de almacenamiento, la acidez de las muestras de aceite estabilizado no aumentó sino que descendió (Tabla 21), debido probablemente a una oxidación de los ácidos grasos libres (Aidos et al., 2001). En la mayoría de los casos la adicción de antioxidantes condujo a PV menores que los obtenidos para las muestras control (Tabla 21). A tiempos cortos de almacenamiento (10 días), concentraciones bajas-medias de alfa-tocoferol (50-550 ppm) combinadas con una alta adicción de palmitato de ascorbilo (450 ppm) y ácido cítrico (50 ppm) resultó en los menores valores de PV, 20.83 y 18.20 meq/kg aceite. Para tiempos largos (30 días), una baja adicción de alfa-tocoferol (50 ppm) combinada con una alta de palmitato de ascorbilo (450 ppm) y ácido cítrico (50 ppm) produjo la máxima protección frente a la formación de hidroperóxidos, PV de 31.55 meq/kg aceite. (Tabla 21). La alta adicción de palmitato de ascorbilo se justifica por su efecto sinérgico con el alfa-tocoferol a bajas concentraciones de este (50-550 ppm). El

palmitato de ascorbilo es capaz de regenerar el radical derivado del alfa-tocoferol, mejorando por tanto su actividad antioxidante y previniendo que este radical participe en otras reacciones de oxidación (e.g. regeneración de radical peroxilo) (Frankel et al., 1994; Olsen et al., 2005). No obstante, este efecto no fue observado a alta concentración de alfa-tocoferol (1050 ppm), la cual tuvo un efecto prooxidante en las muestras de aceite estabilizado favoreciendo la formación de hidroperóxidos. Resultados similares fueron reportados por (Kulås & Ackman, 2001a; Zuta & Simpson, 2007).

En cuanto a los productos secundarios de oxidación, tanto para tiempos cortos y largos de almacenamiento, concentraciones medias de alfa-tocoferol combinadas con altas de palmitato de ascorbilo y ácido cítrico (50 ppm) dieron lugar a la máxima reducción de descomposición de hidroperóxidos, AV de 4.94 y 12.87 respectivamente (Tabla 21). Este hecho se debe principalmente a la capacidad del alfa-tocoferol de convertir hidroperóxidos en compuestos estables y a una mejor regeneración del alfa-tocoferol a altas concentraciones de palmitato de ascorbilo (Kulås & Ackman, 2001b; Frankel, 2005). Con respecto al tiempo de inducción, no se observó una relación directa de este con los valores de peróxidos y anisidina. Este hecho es debido a la alta temperatura empleada en el método Rancimat, que da lugar a mecanismos de oxidación diferentes a los producidos a temperatura ambiente (Drusch et al., 2008). El perfil lipídico de las muestras de aceite no cambió durante los 30 días de almacenamiento.

Los datos experimentales se ajustaron a modelos cuadráticos, obteniendo valores de R^2 en torno a 0.9 o superiores, excepto para el modelo de FFA (Tabla 22). El análisis ANOVA indicó la significancia del efecto lineal de la concentración de alfa-tocoferol en PV, totox e IP y del efecto lineal de la concentración de palmitato de ascorbilo en PV y totox. El efecto cuadrático de la concentración alfa-tocoferol y el efecto interactivo de la concentración de alfa-tocoferol y palmitato de ascorbilo también tuvieron una influencia significativa en PV, AV, totox e IP (Tabla 22).

Para la optimización del proceso, las variables PV y AV fueron elegidas como funciones objetivo indicando el deterioro por oxidación sufrido por las muestras de aceite estabilizado. La optimización mediante superficies de respuesta de sus modelos cuadráticos dio lugar a los valores mínimos indicados en la Tabla 4.

Tabla 4. Óptimos de PV y AV a 10 y 30 días de almacenamiento

10 días		**PV** (18.56 meq/kg aceite)	**AV** (2.64)
	α-tocoferol (ppm)	207	478
	Palmitato de ascorbilo (ppm)	450	390
30 días		**PV** (33.56 meq/kg aceite)	**AV** (15.34)
	α-tocoferol (ppm)	50	493
	Palmitato de ascorbilo (ppm)	450	450

De los resultados obtenidos se observa un efecto contradictorio de la concentración de alfa-tocoferol en la optimización individual de PV y AV. Por tanto, se llevó a cabo una optimización simultánea, a partir de la cual se obtuvieron un conjunto de soluciones óptimas (frente de Pareto) que satisfacen la optimización de cada una de las variables individuales, PV y AV a 30 días, en un cierto grado. Se observa que al darle un peso al PV menor de 0.6, el valor de AV desciende (de 21.98 a 15.34) a expensas de un acentuado incremento del valor de PV (de a 34.13 a 46.22). Ello implica pasar de adicionar una concentración baja de alfa-tocoferol (50 ppm) a una concentración alta (493 ppm) (Tabla 23).

4.7 Evaluación de la capacidad antioxidante de fosfolípidos y estudio de la influencia de combinaciones caseína-fosfolípidos como emulsionantes en la estabilidad física y oxidativa de emulsiones de aceite de pescado

Los tres fosfolípidos evaluados, lecitina (LC), fosfatidilcolina (PC) y fosfatidiletanolamina (PE), presentaron similar inhibición de DPPH, 95 % para soluciones 1 wt% de fosfolípidos en etanol (Fig. 19a). Este tipo de actividad antioxidante se atribuye principalmente a la capacidad de donar protones por parte de los grupos amino, especialmente en el caso de PC y PE (Bandarra et al., 1999). No obstante, la inhibición de DPPH por parte de los tres fofolípidos fue considerablemente inferior a la actividad de un antioxidante sintético como el BHT. En cuanto a la actividad quelante de iones metálicos, Zambiazi et al. (1998) indican capacidad quelante para los grupos amino en los fosfolípidos PC, PE y fosfatidilserina (PS) y para el azúcar inositol en fosfatidilinositol (PI). En este estudio PC exhibió la mayor capacidad para quelar iones metálicos Fe^{2+} (98 % al 1 wt% en etanol), seguido de PE (71 %) y LC (42 %) (Fig. 19b). Estos resultados podrían explicar aquellos reportados por: a) Bandarra et al. (1999), que indicaron mayor prevención en la oxidación

de aceite de sardina refinado por parte de PC que PE o alfa-tocoferol; y b) Cardenia et al. (2011), que encontraron que PC fue más efectiva que PE en la inhibición de la oxidación lipídica de emulsiones 1 wt% de aceite de soja en agua a pH 7. Aunque los tres fosfolípidos presentaron una actividad quelante baja en comparación con EDTA, este tipo de compuestos pueden ser un tipo de quelantes únicos debido a su capacidad para actuar en la fase hidrófoba (Cardenia et al., 2011). Por último, ninguno de los fosfolípidos ensayados resultó en un poder reductor significante, con absorbancias menores de 0.2 para concentraciones 1 wt% en etanol (Fig. 19c).

En cuanto a la estabilidad física, las emulsiones estabilizadas con CAS+LC y CAS+PC resultaron ser las más estables físicamente, con menores tamaños de gota a día 1 ($D_{3,2}$=210 nm y $D_{3,2}$=212 nm, respectivamente) (Tabla 25), y sin modificaciones en su distribución de partículas después de 14 días de almacenamiento. La menor estabilidad de la emulsión con sólo caseína se debe a que se necesita una mayor cantidad de emulsionante (al menos 1 wt%) para producir emulsiones estables durante 2 semanas de 10 wt% aceite de pescado a pH 7 (resultados no publicados). Por otra parte, la inestabilidad de la emulsión de CAS+PE puede deberse al bajo balance hidrofílico-hidrofóbico (HLB) de PE a pH neutro, lo que lo hace un emulsionante menos apropiado para estabilizar emulsiones de aceite en agua (Carlsson, 2008).

Con respecto a la estabilidad oxidativa, el análisis de componentes principales (ACP) indicó que las emulsiones más oxidadas en términos de productos primarios de oxidación fueron CAS+PC y CAS+PE (Fig. 20). Esto fue confirmado por los resultados experimentales del análisis de peróxidos, obteniéndose un mayor valor de PV para las emulsiones CAS+PC (PV-14 de 24.7 meq/kg aceite) y CAS+PE (PV-14 de 21.1 meq/kg aceite), seguidas de la emulsión CAS (PV-14 de 19.9 meq/kg aceite) y la emulsión CAS+LC en último lugar (PV-14 de 12.9 meq/kg aceite) (Fig. 21). Teniendo en cuenta los compuestos secundarios volátiles de oxidación, el ACP indicó la emulsión CAS como la más oxidada, con la mayoría de los volátiles en el cuadrante cuarto (Fig. 21). Esto fue confirmado por la medida experimental de volátiles, cuyos resultados también pusieron de manifiesto que la emulsión CAS+LC fue la más oxidativamente estable con incrementos menores en la concentración de volátiles derivados de la oxidación de omega-3 PUFA tales como 1-penten-3-ol y 1-penten-3-ona (Fig. 22).

No obstante, las propiedades físicas de la emulsión CAS+LC no explica su mejor estabilidad oxidativa debido a que: i) esta emulsión presenta el potencial zeta más negativo

(-32.9 mV), lo que incrementa la atracción de iones metálicos favoreciendo la oxidación lipídica (Hu et al., 2003); ii) también tiene el menor tamaño de gota ($D_{3,2}$=210 nm) lo que implica mayor superficie de contacto con los iones metálicos y da lugar a emulsiones con menos estabilidad oxidativa (Jacobsen et al., 2000); iii) LC no presenta mejores propiedades antioxidantes que el resto de fosfolípidos estudiados; y iv) el contenido en proteína de la fase acuosa es similar al resto de emulsiones con fosfolípidos, por lo que la actividad quelante de la caseína no debe ser mayor en esta emulsión. Por tanto, otros factores tales como las propiedades de la interfase pueden ser los responsables de esta mejorada estabilidad oxidativa. En este sentido, el aumento del contenido en proteína en la fase acuosa de las emulsiones con fosfolípidos, con respecto a la emulsión estabilizada sólo con caseína, indica la incorporación de fosfolípidos a la interfase. Ello puede dar lugar a que la caseína presente en la interfase adopte una conformación con más proyección hacia la fase acuosa, lo que conduce a un incremento en el grosor de la interfase (Fang & Dalgleish, 1993). En el caso de LC, su contenido en fosfolípidos con carga como PI y PA, los cuales son más solubles en agua pudiendo alcanzar antes la interfase (Rydhag & Wilton, 1981), podría dar lugar a diferente estructura y grosor de la capa interfacial de la emulsión. Sin embargo, este razonamiento necesita ser investigado más a fondo para ser confirmado.

4.8 Optimización de la producción de biodiesel a partir de aceite de pescado

El proceso de producción del biodiesel se llevó a cabo en dos etapas debido a la alta acidez del aceite de partida. En la primera etapa se realizó una esterificación ácida de los ácidos grasos libres, obteniendo una acidez menor de 2 mg KOH/g aceite antes del proceso de transesterificación alcalina. El biodiesel obtenido, empleando metanol y 1 wt% de NaOH como catalizador, presentó un alta pureza en FAME, variando entre 93.08 y 95.39 wt% (Tabla 27). Su composición en metil ésteres de ácidos grasos saturados fue de 17.7 wt% y en poliinsaturados de 33.2 wt%. No obstante, no se alcanzó el valor especificado en la normativa europea (EN 14214) para ser usado como 100 % fuel (96.5 wt% pureza en FAME).

El rendimiento del proceso varió entre 71.19 y 83.06 % (Tabla 27). Sin embargo, mejores resultados fueron obtenidos por otros autores, 99 % (El-Mashad et al., 2008) y 96 % (Fadhil & Ali, 2013), empleando menores concentraciones de catalizador (0.5 wt%). De

acuerdo a Fadhil & Ali (2013), concentraciones de catalizadores alcalinos superiores a 0.5 wt% favorece la saponificación de triglicéridos. En este sentido, los jabones formados se disuelven en el glicerol y además mejoran la solubilidad de los metil ésteres en esta fase, lo que conlleva importantes pérdidas (Vicente et al., 2004).

Por otra parte, el proceso de transesterificación fue efectivo en la reducción de la viscosidad cinemática del aceite, desde 45.34 mm^2/s para el aceite de partida a 6.05-6.66 mm^2/s para las muestras de biodiesel, ambas medidas a 30 ºC (Tabla 27). Los valores obtenidos fueron mejores que los reportados Lin & Li (2009) para biodiesel producido a partir de otro tipo de aceite de pescado (7.2 mm^2/s medida a 40 ºC). Este hecho se explica principalmente al mayor contenido en SFA de este tipo de aceite (37.06 wt%). Además, el biodiesel obtenido estuvo cerca de cumplir los valores exigidos en la normativa europea (3.5-5 mm^2/s a 40 ºC). Con respecto a la estabilidad oxidativa, bajos valores de IP fueron encontrados para las muestras de biodiesel (1.05-2.22 h). Ello es debido al alto contenido en PUFA de este tipo del aceite empleado. De ahí que, para cumplir la normativa europea (mínimo IP de 6 h), se debe llevar a cabo la adicción de antioxidantes (Lin & Lee, 2010) o la mezcla con otros aceites más estables, e.g. aceites vegetales (Costa et al., 2013).

En cuanto a las propiedades en frío, las muestras de biodiesel presentaron un punto de fusión, determinado mediante DSC, en el rango -73.60 ºC a 3.83 ºC. Los valores de COM no variaron significativamente, entre 3.31 y 3.83 ºC (Tabla 27), como consecuencia de que las muestras de biodiesel presentaron prácticamente el mismo perfil lipídico. Chiou et al. (2008) reportaron un valor similar de COM para biodiesel producido a partir de aceite de salmón (3.1 ºC) pero uno inferior para biodiesel producido a partir de aceite de maíz (1.7 ºC), debido a su menor contenido en ácidos grasos saturados. La acidez del biodiesel producido fue menor de 0.31 mg KOH/g aceite en todos los casos, lo que cumple las especificaciones de la norma europea (<0.5 mg KOH/g aceite).

Lo datos experimentales fueron ajustados a modelos de segundo orden, obteniéndose valores de R^2 por debajo de 0.71 para todas la variables estudiadas (Tabla 28). No obstante, los datos calculados de rendimiento, viscosidad, IP y COM estaban dentro de una desviación del ±10 % con respecto a los datos experimentales (Fig. 24). Por tanto, se llevó a cabo la optimización mediante superficie de respuesta de estas variables, obteniéndose lo siguiente (Fig. 25):

a) Un máximo de rendimiento (83.6 %) a 40 ºC que reduce la saponificación de triglicéridos, a máximo ratio metanol aceite (9:1) que favorece la conversión de triglicéridos a alquil ésteres y a máximo tiempo de reacción (90 min).

b) Un mínimo de viscosidad (6.30 mm^2/s) a 60 ºC, relación molar metanol a aceite de 5.15:1 y tiempo de reacción de 55.52 min. La máxima temperatura aumenta la pureza en FAME del biodiesel lo que reduce su viscosidad.

c) Un máximo de IP (1.99 h) a 60 ºC, relación molar metanol a aceite de 9:1 y 30 min. Aunque la temperatura máxima ensayada favorece la oxidación del biodiesel, también favorece la mezcla de este con el metanol en exceso lo que puede reducir en cierta medida el proceso oxidativo. Obviamente, el menor tiempo de proceso reduce la exposición del biodiesel a agentes causantes de la oxidación (e.g. calor, oxígeno y luz).

d) Un mínimo para COM (3.48 ºC) a 60 ºC, relación molar metanol a aceite de 3.74:1 y 90 min. El valor de COM para el biodiesel depende principalmente de su contenido en SFA (Knothe, 2005). En este estudio, las 27 muestras de biodiesel presentan prácticamente el mismo contenido en SFA por lo que este aspecto no puede explicar el óptimo encontrado.

4.9 Caracterización lipídica de *Sardina pilchardus, Scomber colias y Trachurus Trachurus,* y evaluación de la actividad antihipertensiva y antioxidante de sus hidrolizados

Aparte de las especies caracterizadas en el apartado 4.2, en el Mar de Alborán también se descarta otra especie de jurel (*Trachurus trachurus*), principalmente debido a restricciones de cuota y requerimientos de tamaños mínimos (FROM, 2008), y una especie no muy comercial como la caballa (*Scomber colias*), capturada accidentalmente en la pesca de arrastre y cerco (Carbonell et al., 1997).

La caracterización de estas especies puso de manifiesto que en esta época del año (mes de Mayo), el jurel presentó el mayor contenido lipídico (7.8 wt%) seguido de la sardina (4.4 wt%) y la caballa (2.5 wt%) (Tabla 29). No obstante, el aceite de sardina fue el que presentó un mayor contenido en EPA (12.96 wt%) y en DHA (17.63 wt%), seguido del de caballa (EPA+DHA = 27 wt%) y jurel (EPA+DHA = 22 wt%) (Tabla 30). Los aceites estuvieron compuestos principalmente por triglicéridos (TAG>97 wt%).

La hidrólisis de las tortas de prensado resultó en grados de hidrólisis (DH) satisfactorios (Fig. 26). El mayor DH fue obtenido para el jurel (21 %), seguido de la sardina (18 %) y de la caballa (14 %). Para todas las especies y tratamientos enzimáticos, la actividad antihipertensiva de los hidrolizados se incrementa rápidamente durante los primeros 30-60 min de reacción. Posteriormente, este valor (65-70 % de inhibición de ECA) permanece constante o disminuye ligeramente (Fig. 27). El tratamiento simultáneo con subtilisina y tripsina produjo los hidrolizados con mayor capacidad antihipertensiva para caballa (IC_{50}=345 μg proteína/mL) y jurel (IC_{50}=364 μg proteína/mL), mientras que el tratamiento subtilisina (2h) y tripsina (2h) dio lugar al hidrolizado con mayor actividad inhibidora de ECA para sardina (IC_{50}=400 μg proteína/mL). Estos hidrolizados presentaron un DH entre 13 y 16 %, lo que corresponde a una longitud media de la cadena peptídica (PCL) entre 6 y 8 aminoácidos (Tabla 31). Los resultados obtenidos están en el rango de los valores indicados por otros autores. En este sentido, Matsui et al. (1993) reportaron un valor de IC_{50} de 260 μg/mL para un hidrolizado de sardina.

Por último, los hidrolizados de las tres especies resultaron ser buenos inhibidores de DPPH con porcentajes de inhibición de 35 % para la caballa, 40 % para la sardina y 45 % para el jurel (Tabla 32). Tanto el tratamiento simultáneo como el secuencial de subtilisina y tripsina dieron lugar a los mejores resultados. Lo hidrolizados con mejor propiedad inhibidora de DPPH presentaron valores de PCL entre 5 y 7 aminoácidos (Tabla 32). Valores similares fueron reportados para hidrolizados de sardina, inhibición de DPPH entre 15-55% (Bougatef et al., 2010); aunque mejores resultados fueron obtenidos para caballa, 80 % inhibición, a expensas de mayores tiempos de hidrólisis (Wu et al., 2003).

4.10 Estudio de la actividad antioxidante de hidrolizados obtenidos a parir de especies de descarte (*S. Pilchardus, T. mediterraneus, P. acarne, B. boops, S. canicula*)

La hidrólisis enzimática de la proteína de estas especies dio lugar a diferentes valores de DH en función de la especie y del tratamiento enzimático. Los mayores valores de DH se obtuvieron para la torta de jurel (18.0-21.0 %) seguidos del músculo de pintarroja (17.3-19.2 %). Para la torta de aligote y boga se encontraron valores intermedios (15.3-17.6 %), mientras que los menores valores de DH fueron obtenidos para los hidrolizados de torta de sardina (13.2-14.9 %) (Tabla 33).

Con respecto a la influencia del DH en la actividad inhibidora de DPPH, se encontró que esta depende del material hidrolizado y de la combinación de enzimas utilizadas. Así, como ejemplos de tendencias diferentes, se tienen los hidrolizados de sardina y jurel. Mientras que los hidrolizados de sardina presentan máximos de actividad antioxidante en los períodos de actuación de subtilisina y tripsina en los tratamiento secuenciales (Fig. 30b), el hidrolizado de jurel aumenta su capacidad antioxidante con el DH hasta que esta se mantiene constante en un determinado valor (Fig. 30a). Además, se observa que la adicción de la segunda enzima produce un incremento en la actividad antioxidante de los hidrolizados, siendo mayor para la sardina y jurel. Estos dos comportamientos distintos de la actividad antioxidante de los hidrolizados de proteína de pescado en función de su valor de DH también han sido descritos previamente por otros autores (Jao & Ko, 2002; Wu et al., 2003).

Los hidrolizados finales presentaron diferente contenido en proteína, variando desde 60.7 wt% para sardina hasta 89.5 wt% para pintarroja. Igualmente, su contenido en lípidos varió de 25.3 wt% para sardina a 4.6 wt% para pintarroja (Tabla 33). Entre los lípidos presentes destaca el contenido en triglicéridos, especialmente en el caso de hidrolizados de sardina y jurel donde este valor alcanza el 74%. También se encuentran altos porcentajes de ácidos grasos libres, particularmente en el caso de especies menos grasas como aligote y boga (hasta del 36%). No obstante, los hidrolizados de pintarroja presentan un contenido insignificante de triglicéridos y ácidos grasos libres, mientras que la mayor parte de sus lípidos están constituidos por fosfolípidos (54.5 wt%) y colesterol (35.2 wt%) (Tabla 34). Estos resultados están en la línea de aquellos reportados por Daukšas et al. (2005). El análisis de cromatografía de filtración en gel indicó que los hidrolizados presentaban más un 80 wt% de sus péptidos/aminoácidos por debajo de 150 Da. Los hidrolizados de pintarroja al ser de músculo presentaron un perfil de elución distinto al resto, con más péptidos en el rango 150-450 Da (21 wt%) y menos por encima de 450 Da (2%) y en el rango 50-150 Da (16 %) (Fig. 31).

En cuanto a su actividad antioxidante, los hidrolizados que presentaron mayor capacidad secuestrante del radical DPPH fueron los de sardina (IC_{50}=0.91 mg protein/mL) y jurel (IC_{50}=1.47 mg proteína/mL) obtenidos con el tratamiento tripsina (2h) y subtilisina (2h) (Tabla 35). Ello puede deberse a que la adicción en segundo lugar de subtilisina puede incrementar la liberación de péptidos con aminoácidos hidrofóbicos en el C-terminal, lo que contribuye a la actividad inhibidora de DPPH (Je et al., 2009). Los hidrolizados con

mayor actividad quelante de Fe^{2+} fueron los de sardina (independientemente del tratamiento enzimático) y los de pintarroja con los dos tratamientos secuenciales, ambos presentaron un valor de IC_{50} de 0.32 mg proteína/mL (Tabla 35). La actividad quelante de estos hidrolizados puede estar relacionada con la existencia de péptidos conteniendo histidina. Se ha indicado que este aminoácido exhibe actividad quelante a través de su anillo de imidazol (Bougatef et al., 2009). Por otra parte, los hidrolizados con un mayor poder reductor fueron los de sardina y boga (independientemente del tratamiento enzimático empleado) con un absorbancia mayor de 0.7 a concentraciones de 20 mg proteína/mL, mientras que el hidrolizado de pintarroja presentó la actividad reductora más baja. La adicción simultánea de subtilisina y tripsina resulta en el hidrolizado de sardina con menor poder reductor, mientras que esto ocurrió en el caso de la boga con el tratamiento secuencial subtilisina (2h) y tripsina (2h) (Fig. 32). Los resultados de poder reductor obtenidos están en la línea de aquellos reportados para hidrolizados de sable negro (Batista et al., 2010) y de sub-productos de merluza (Pires et al., 2013).

4.11 Estudio de la actividad antihipertensiva de hidrolizados obtenidos a parir de especies de descarte (*S. Pilchardus, T. mediterraneus, P. acarne, B. boops, S. canicula*)

La influencia del DH en la actividad inhibidora de ECA es dependiente del material a hidrolizar y del tratamiento enzimático empleado. Dos tendencias fueron observadas: i) incremento de la actividad inhibidora con el DH hasta alcanzar un valor máximo donde se mantiene prácticamente constante (observado para el tratamiento simultáneo), y ii) obtención de máximos y posterior ligero descenso de la actividad inhibidora tanto en los períodos de hidrólisis con subtilisina y tripsina de los tratamientos secuenciales. Además, la adicción de una segunda enzima en los tratamientos secuenciales incrementa ligeramente la actividad inhibidora, siendo este incremento más notable para el jurel y la pintarroja (Fig. 33).

Los hidrolizados finales que presentaron una mayor actividad antihipertensiva fueron los de jurel y pintarroja, con valores de IC_{50} variando entre 279 y 398 μg proteína/mL. En segundo lugar se encuentran los hidrolizados de aligote y sardina, con valores de IC_{50} en el rango de 375-489 μg proteína/mL. Por último, los hidrolizados de boga presentaron mayores valores de IC_{50}, variando entre 637-768 μg proteína/mL (Tabla 36). Estos

resultados están en la línea de aquellos reportados para músculo de sardina (IC_{50} = 260 μg/mL) (Matsui et al., 1993), pero son considerablemente mayores que los obtenidos por Ono et al. (2006) para hidrolizados de salmón (IC_{50} = 38 μg/mL).

La mayor actividad inhibidora de ECA de los hidrolizados de pintarroja puede estar relacionada con su alto contenido en colágeno, el cual es rico en prolina. Se ha descrito que la presencia de este aminoácido no polar en el C-terminal de péptidos pequeños favorece la inhibición de ECA (Byun & Kim, 2001). En el caso del jurel, los altos valores de DH obtenidos, los cuales implican la presencia de un mayor número de péptidos de pequeño tamaño, podrían ser la causa de la mayor actividad inhibidora de estos hidrolizados. Con respecto al tratamiento enzimático, tanto para jurel como para pintarroja, los hidrolizados obtenidos con el tratamiento simultáneo de subtilisina y tripsina presentaron la máxima inhibición de ECA. Este hecho está en la línea de los resultados obtenidos para caballa y jurel (*T. trachurus*) en el capítulo IX.

Mediante cromatografía de filtración en gel se identificaron 7 fracciones para el hidrolizado de jurel obtenido con la adicción simultánea de subtilisina y tripsina (Fig. 34a). De ellas, la fracción B (130-2350 Da) fue la que presentó la mayor actividad inhibidora de ECA con un IC_{50} de 85 μg/mL, lo que supuso un incremento de 3.3 veces la actividad inhibidora del hidrolizado (Tabla 37). Por otra parte, para el hidrolizado de pintarroja se identificaron 5 fracciones (Fig. 34b). Entre ellas, las fracciones C (470-1210 Da) y D (58-470 Da) exhibieron la máxima actividad inhibidora de ECA, con valores de IC_{50} de 72 y 27 μg/mL respectivamente (Tabla 37). El valor de IC_{50} de la fracción D del hidrolizado de pintarroja, con una actividad inhibidora 11.2 veces superior al hidrolizado inicial, es incluso inferior al de un péptido activo purificado a partir de limanda (IC_{50} = 29 μg/mL) (Jung et al., 2006).

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Summary

1. INTRODUCTION

1.1 Fish by-products: discards

Fish industry generates a large amount of by-products, being discards the most significant waste of fish resources (Davies et al., 2009). In this context, discards are defined as "that proportion of the total organic material of animal origin in the catch, which is thrown away, or dumped at sea for whatever reason. It does not include post-harvest waste such as offal" (Kelleher, 2005). According to this definition, discards are comprised by commercial specimens which do not meet the minimum landing size or which exceed the quota restrictions. Besides, organisms caught incidentally such as non-target species, marine mammals or seabirds also represent a considerable fraction of the discards produced. Therefore, discards are mainly produced by: a) fishing policies including regulations on minimum landing size or fishing quota and b) economic reasons since market policies determine the consumers' acceptance towards some species, and are the origin of high grading practices (discards of commercial specimens meeting landing size requirements but with low value in the market). Moreover, there are other factors enhancing the occurrence of discarding practices, such as the diversity of fish species or the selectivity of fishing gears employed in a given fishery (Kelleher, 2005). For the period 1992-2001, Kelleher (2005) estimated a global discard rate of 8%, reported as the quotient between discards and total catches. Assuming this estimation, the yearly amount of total discards rises up to 7.3 million tons.

1.1.1 Discards in the northern coast of Alboran Sea

Spain is a country with a large seaboard, where fishing activities contribute in a large extent to national economy (MAPA, 2006). The volume of catches from Spanish fleet was more than 86,5000 tons in 2008, which represents more than 14 % of the overall catches of the 27 EU member states (EU, 2010). Fishing activities are mainly located in Northwest Atlantic and Mediterranean coasts, with 44 % and 16 % of national fish catches, respectively. Particularly, southern Mediterranean coast (or northern coast of Alboran Sea) is responsible for 42 % of catches in the region of Andalusia, a region which attaches great importance within the Spanish fishing fleet (Consejería de Agricultura y Pesca, 2009). Alboran Sea lies between strait of Gibraltar and cape of Gata, with an extension of 250 km,

a surface of 11,000 km^2 and a depth between 0 and 800 m. This coast comprises 11 commercial ports: Algeciras and La Línea (province of Cádiz), Estepona, Marbella, Fuengirola, Málaga and Caleta de Vélez (province of Málaga); Motril (province of Granada); Adra, Roquetas de Mar and Almería (province of Almería) (García et al., 2012). Main fishing gears employed in this area are trawling, with 135 fishing vessels and 22 % of fishing catches, and purse seine, with 89 fishing vessels and 64 % of catches (Consejería de Agricultura y Pesca, 2010). Although there is a lack of complete data on discards in this area, some preliminary studies (FROM, 2008) allowed to estimate a global discard rate ranging from 10 to 23% for purse seine and trawling, respectively. Among the discarded species, some are target species such as sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*) and axillary seabream (*Pagellus acarne*) (Fig. 1). Discards from these species are due to minimum landing size requirements, e.g. 11 cm for sardine and 12 cm for horse mackerel and seabream (R.D. 1615/2005), fishing quota or high grading practices. Other discards comprise non targeted species of low commercial value, such as bogue (*Boops boops*) or small-spotted catshark (*Scyliorhinus canicula*) (Fig. 2) (Carbonell et al., 1997; CDPM, 2005).

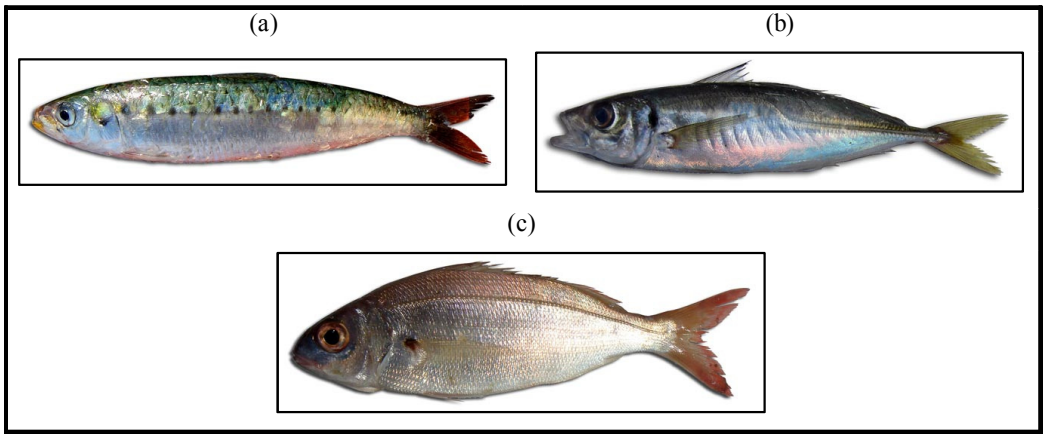


Figure 1. (a) *Sardina pilchardus*, (b) *Trachurus mediterraneus* and (c) *Pagellus acarne*. (Photo: A.M. Arias)

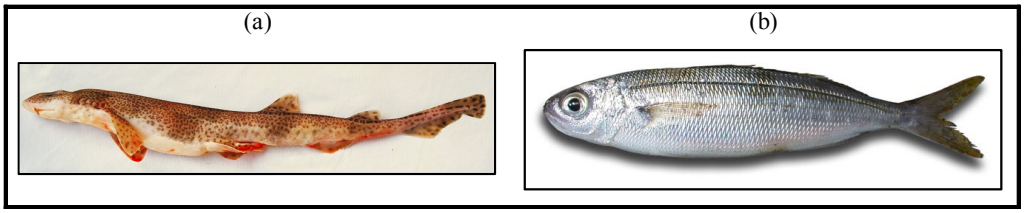


Figure 2. (a) *Scyliorhinus canicula* (Foto: FONDOPES) and (b) *Boops boops* (Photo: A.M. Arias)

Fish catches in this region have been reduced in a half from 1990 (43,000 tons) to 2011 (22,000 tons) (Consejería de Agricultura y Pesca, 2011). This fact, due to the overexploitation of fisheries, highlights the need for a better management of current fish stocks. To this regard, the progressive reduction of the amount of discards and the search for a better utilization of these materials need to be undertaken in the short term.

1.1.2 Consequences of discards and technical solutions for their reduction

Discards represent not only an irresponsible underutilization of marine stocks, which affects negatively future fishing production, but also they have a negative impact on marine ecosystem. Indeed, most of the individuals which are returned to the sea are died or dying, which alters the structure of trophic chains since it favors the presence of depredator species (Groenewold & Fonds, 2000). For instance, in the case of trawling, survival rates for finfish species drop to zero due to the air expansion in natatory bladders when hailing the nets. Similarly, 50 % crustaceans and 98 % cephalopods do not survive after the classification and discarding process on board, and are returned died to the sea (Bozzano & Sardà, 2002). Furthermore, discarding juvenile individuals reduces future yields and hence, economic incomes of fisheries (Jensen et al., 1988).

In view of the above, the European Commission is currently undertaking a depth reform in common fisheries policy, adopting a set of measures towards the complete elimination of discards in EU fisheries, the so-called zero-discard policy. This reform proposes a three-step strategy for the reduction of discards: i) pelagic species, including Mediterranean Sea, by 2014; ii) demersal species such as cod, hake and sole by 2015; and iii) rest of species, by 2016. Discard bans are accompanied by a set of technical measures, such a) improvement of the selectivity of fishing gears, b) obligation of fishing vessels to quit a given fishing ground when the occurrence of incidental catches exceeds a threshold value, c) avoidance of the current regulations on minimum landing size, d) higher coverage of observers on board, and e) economic incentives to bring onto the market juveniles and not target species (e.g. production of fish meal) (EU, 2011). Nevertheless, discards of juveniles specimens (whose sell out is currently forbidden) and non-target species are raw materials of low cost suitable for obtaining high valued products such as fish oil and fish protein hydrolysates exhibiting bioactive properties. Both products are of interest in food and pharmaceutical industries.

1.2 Fish oil

Fish oil presents a unique composition, different from that of vegetal and other animal oils, due to its high content of omega-3 polyunsaturated fatty acids (Hamilton & Rice, 1995). These polyunsaturated fatty acids (PUFA) are produced by the marine phytoplankton and are then incorporated into the fish through the food chain (Shahidi, 1998). The main omega-3 PUFA contained in fish oil are alpha linolenic acid (C18:3n-3, ALA), eicosapentaenoic (C20:5n-3, EPA), docosahexaenoic (C22:6n-3, DHA) and in a lesser extent docosapentaenoic (C22:5n-3, DPA).

Omega-3 PUFA, especially EPA and DHA, have been scientifically recognized as being responsible for numerous beneficial effects on human health. EPA prevents cardiovascular diseases such as hypertension, cerebral thrombosis and heart attacks (Lees & Karel, 1990; Simopoulos, 1991). Furthermore, this PUFA improves the anti-inflammatory and allergic response of the organisms (Uauy & Valenzuela, 2000). On the other hand, DHA plays an important role in the development of brain and eye retina (Ward & Singh, 2005). Other studies have reported the potential of these PUFA in the prevention of mental diseases (Ross et al., 2007) and some types of cancer (Sidhu, 2003).

Although humans can synthesize EPA and DHA from the essential fatty acid ALA, through desaturation and elongation reactions, the rate of conversion is low. Thus, these omega-3 PUFA must be also ingested through the diet (Colussi et al., 2007). As a consequence, fish oil, which is the most important source of EPA and DHA, has received much attention by the food and pharmaceutical industries (Muggli, 2007).

1.2.1 Lipid oxidation

Lipid oxidation is highly influenced by several factors such as oxygen, light and heat exposure; and the presence of metal ions and lipoxygenases enzymes in the oil. Besides, oil composition plays a key role in its oxidation, increasing the oxidative spoilage of the oil with the availability of bis-allylic hydrogens (Frankel, 2005). Therefore, fish oil, due to its high content of PUFA, is extremely susceptible to oxidative degradation.

Although there are three possible mechanisms of lipid oxidation, autoxidation (induced by molecular oxygen, O_2), photoxidation (induced by singlet oxygen, $^1O_2$) and enzymatic oxidation (induced by lipoxygenases), only the two firsts are significant (Frankel, 2005). Hence, they will be the ones discussed in this section.

Fig. 3 shows the reactions involved in the autoxidation and photoxidation processes. Photoxidation takes place in the presence of oxygen, light and photosensitizers (Sens). Photosensitizing properties have been described for chlorophyll, hemeproteins and riboflavin. These pigments absorb light adopting an excited state (^{3}Sens). Then, the electronically excited photosensitizers react with O_2 to yield singlet oxygen ($^1O_2$). This excited species of oxygen is highly electrophilic and rapidly reacts with unsaturated lipids to produce lipid hydroperoxides. As consequence of the high reactivity of singlet oxygen, the hydroperoxides produced by photoxidation plays an important role in the initiation of lipid autoxidation (Min & Boff, 2008).

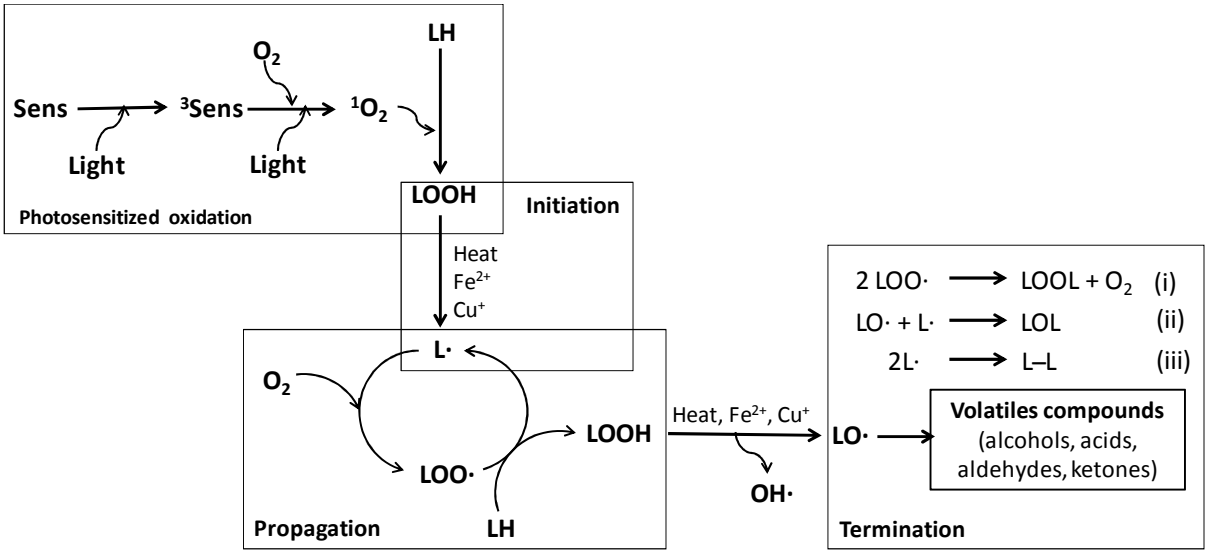


Figure 3. Mechanisms of lipid oxidation. Adapted from Muggli (2007)

Furthermore, autoxidation proceeds by a free radical chain mechanisms, comprising the stages of initiation, propagation and termination. Firstly, the hydroperoxides (LOOH), present as impurities or produced by photoxidation, are decomposed by the action of heat and/or metal ions to yield radical species. These radicals together with initiators (heat, light and metal ions) have the potential to extract a hydrogen radical from an unsaturated lipid (LH) to obtain a lipid free radical (L·). Then, free lipidic radicals react with oxygen so as to produce peroxyl radicals (LOO·). Subsequently, peroxyl radicals will react with a new lipid molecule to form hydroperoxides (LOOH) and another alkyl radical (L·), whereby the propagation can be continuously repeated. Finally, in the presence of metals ions or high temperatures hydroperoxides are decomposed to alkoxy radicals (LO·), which undergo homolytic β-scission and successive reactions to yield secondary oxidation products such as alcohols, acids, aldehydes and ketones. In addition, free radicals interact with each other

to form non-radical products. These stable products can be produced by condensation reactions at low temperature, (i) in Fig. 3, and by polymerization reactions at high temperature and low oxygen concentration, (ii) and (iii) in Fig. 3 (Frankel, 2005).

Hydroperoxides or primary oxidation products are tasteless and odorless; however, the secondary volatile oxidation products are responsible for the off-flavors (Jacobsen & Nielsen, 2007). Table 1 shows the principal volatile products derived from the autoxidation of omega-3 PUFA.

Table 1. Volatile oxidation products derived from oxidation of omega-3 PUFA. Adapted from Let (2007)

Type of compound	Volatile	Type of compound	Volatile
Aldehyde	Propanal	Alcohol	1-penten-3-ol
	2-propenal		(E)/(Z)-2-penten-1-ol
	(E)-2-butenal	Ketone	1-penten-3-one
	(E)/(Z)-2-pentenal	Other	2-ethyl furane
	(E)-2/(Z)-3-hexenal		
	(Z)-4-heptenal		
	(E,E)-2,4-hexadienal		
	(E,E)/(E,Z)-2,4-heptadienal		
	(E,Z)-2,6-nonadienal		

1.2.2 Extraction and refining

Currently, fish oil is extracted mainly from pelagic species such as anchovy, herring, sardine and capelin. Nevertheless, fish oil can be also obtained from the liver of white lean fish such as cod, from by-products of tuna processing (e.g. heads), and from small crustaceans such as krill (Bimbo, 2007a).

Crude fish oil, which is generally obtained by the wet pressing method (FAO, 1986), contains non-triglycerides, such as phospholipids, free fatty acids, oxidation products, dioxines, metal ions, pigments and insoluble impurities that reduce the oil quality. Thus, in order to meet the standards of safety and an acceptable shelf life, this oil requires a refining treatment (Rubio-Rodríguez et al., 2010).

The conventional fish oil-refining includes the stages of: a) degumming for removal of phospholipids by the addition of water and/or phosphoric or citric acid, b) neutralization of free fatty acids with sodium hydroxide, c) bleaching with activated clays which adsorb

oxidation products, coloured compounds and dioxins, and e) deodorization by vacuum distillation of traces of volatiles compounds such as aldehydes and ketones (Fig. 4).

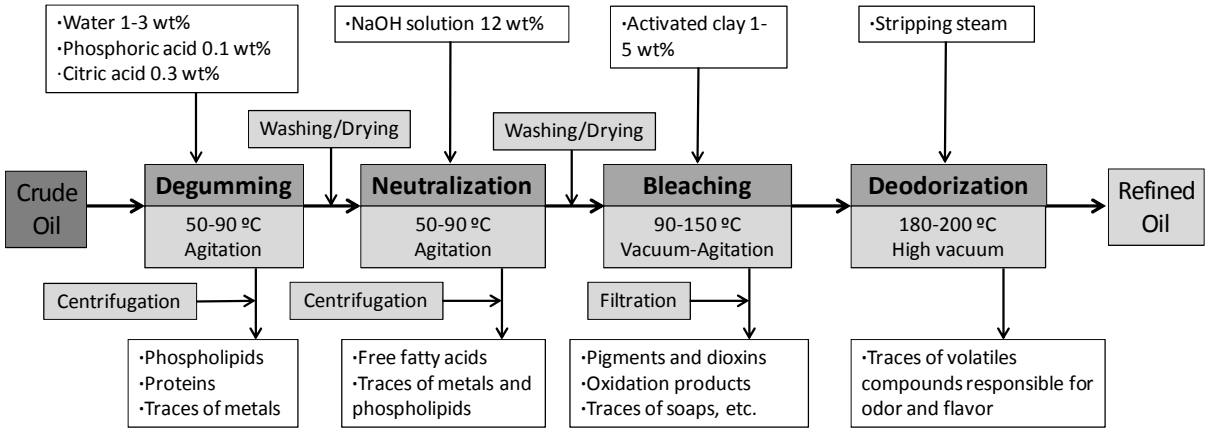


Figure 4. Chemical refining of fish oil

The quality of a refined oil is indicated, among other characteristics, by its acidity, hydroperoxides content (measured by the peroxide index, PV) and its content of secondary oxidation products (measured by the p-anisidine index, AV, or determined by headspace chromatography). The following are acceptable values for these paremeters: acidity < 3 mg KOH/g oil, PV < 5 meq O_2/kg oil, AV < 20 and totox (2×PV+AV) < 26 (Akman, 2005). However, fish oil which is produced in order to be incorporated into food products must be of high quality. To this regard, Jacobsen et al. (2009) reported maximum values for acidity and PV of 0.1-0.2 % oleic and 1 meq O_2/kg oil, respectively. With regard to volatiles, their content should be as low as possible since they have a low treshold value, being even detected at ppb levels (Let et al., 2003). Therefore, the bleaching (which removes most of the oxidation products, as well as pigments, and traces of soaps and phospholipids) and the deodorization (which removes traces of volatiles which are responsible for the flavor deterioration of the oil) are the most important stages for the refining of fish oil (Sathivel, 2010).

1.2.3 Stabilization

The addition of antioxidants is the most common strategy employed for the prevention of the oxidation of fish oil. Antioxidants can act by different mechanisms, being classified in two main groups (Frankel, 2005):

a) Primary antioxidants: these antioxidants inhibit the initiation and propagation stages of the free radical chain reaction. It is achieved by the stabilization of

peroxyl and alkoxy radicals by hydrogen or electrons donation. Besides, they produce a stable and relatively unreactive antioxidant radical. To this group belong synthetic compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), and natural compounds such as tocopherols and other polyphenols extracted from plants.

b) Secondary antioxidants: this type of antioxidants can act by a set of mechanisms such as binding metal ions (e.g. citric acid, phosphoric acid, and ethylenediamine tetraacetic acid) and scavenging oxygen (e.g. ascorbyl palmitate). Moreover, secondary antioxidants have shown synergic effects with primary antioxidants; thus, mixture of them are normally used (Aubourg et al., 2004; Olsen et al., 2005).

Among the existing analyses, the Rancimat test is commonly employed to determine the resistance to oxidation of stabilized oils (Drusch et al, 2008). In addition, this method has also been carried out for the determination of the oxidative stability of refined and crude fish oil (Méndez et al., 1996).

1.2.4 Incorporation into food

Functional foods containing omega-3 PUFA have resulted in one of the fastest-growing food product categories (Sloan, 2006). It is due to the fact that in spite of the numerous health effects of these fatty acids, the intake of fish by the occidental population is still low (Horn et al., 2012a).

Common strategies developed to protect these oxidatively unstable lipids when incorporating them into food is the application of omega-3 PUFA delivery systems such as microencapsulated fish oil powders or fish oil-in-water emulsions. The microencapsulation process is expensive and is normally used in solids products such as bread or in powdered products such as infant formula (Jacobsen & Nielsen, 2007). On the other hand, the employment of emulsified omega-3 oils is more suitable for liquid or semi-liquid foods due to handling/mixing issues and it has been successfully developed in milk (Let et al., 2007). However, negative results were obtained by the same authors when evaluating the incorporation of fish oil emulsions in yoghurt and salad dressings.

Among the factors affecting lipid oxidation in emulsions (pH, droplet size, viscosity, charge of the interphase, emulsification conditions, etc.), the emulsifier employed has a great importance. It is due to the fact that the emulsifier determines the structure and thickness of the interfacial layer, which is the place of contact between lipids and

prooxidative components (McClements & Decker, 2000). Emulsifiers are amphiphilic molecules which can act at the interface oil-water reducing the surface tension and, thus stabilizing the emulsion. Common emulsifiers are macromolecules such as proteins (e.g. caseins) and smaller surfactant molecules such as phospholipids (Jacobsen et al., 2009). They have been successfully employed to stabilize fish oil emulsions as described by the studies of Horn et al. (2012b) and Lu et al. (2012a), respectively. Besides, combinations of caseins and phospholipids have also been evaluated, resulting in interfacial layers with improved properties (Fang & Dalgleish, 1993).

Additionally, antioxidant properties have been described for caseins and for phospholipids. Caseins contain several phosphorylated serine residues that have been suggested to exhibit metal chelating activity (Diaz et al., 2003). With respect to phospholipids, although their antioxidant mechanism is not completely elucidated, the following functions have been attributed to them: a) degradation of hydroperoxides inhibiting their accumulation (Lee et al., 1981; Saito & Ishihara, 1997), b) chelating activity for phosphatidylinositol (Pokorný, 1987), c) synergism with alpha-tocopherol, primarily by phosphatidylethanolamine (Oshima et al., 1993; Bandarra et al., 1999; Lee & Choe, 2011), and d) formation of Maillard-type products, such as pyrroles, between amino phospholipids and oxidation products which have protective effect against oxidation (Hidalgo et al., 2005; Lu et al., 2012a).

1.2.5 Biodiesel production

Fish oil extracted from fish wastes may present a high content of free fatty acids and oxidation products. It is mainly due to the presence of enzymes and bacteria which rapidly deteriorate fish by-products (Wu & Bechtel, 2008). Hence, the refining of this low quality oil would result in low yields, principally because of high losses in the neutralization stage (Sathivel et al., 2003). Besides, oil obtained from fish wastes might have a low amount of EPA and DHA (<20 wt%). This fact also leads to low yield of omega-3 concentrates, considerably reducing the application of this oil in the pharmaceutical and functional food fields (Bimbo, 2007b). Therefore, poor quality fish oil has been suggested as a low cost feedstock for the production of biodiesel (Zhang & El-Mashad, 2006).

Biodiesel (alkyl esters) is a renewable fuel produced from the transesterification of fats and oils (Fig. 5). In this process, alkaline catalysts such as NaOH and cheap alcohols such as methanol are commonly employed (Knothe et al., 2005). The European standard EN 14124

indicates the properties (alkyl esters content, viscosity, cold flow properties, oxidative stability, etc.) required for biodiesel in order to be employed as 100 % diesel fuel or blended with other fuels. In general, biodiesel with a good quality has been obtained from fish oil (El-Mashad et al., 2008; Fan et al., 2010; Costa et al., 2013; Fadhil & Ali, 2013). Nevertheless, this type of biodiesel normally exhibits a low oxidative stability due to the high PUFA content of fish oil (Lin & Lee, 2010).

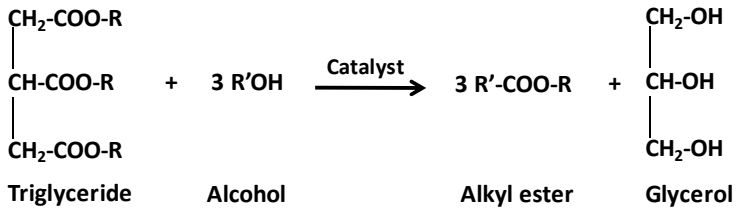


Figure 5. Transesterification of triglycerides

1.3 Fish protein hydrolysates

Fish by-products contain high quality proteins with an excellent amino acids composition (Rustad, 2006). Fish proteins are mainly divided into three groups: a) sarcoplasmic proteins such as myoglobin and enzymes that are soluble in water (20-35 wt%), b) myofibrillar proteins such as myosin, actin, tropomyosin and troponin which are soluble in concentrated saline solutions (65-75 wt%), and c) stroma proteins which are insoluble in concentrated saline solutions and represent a low percentage of fish proteins (2-3 wt%), except for elasmobrachii, up to 10 wt% (Torres et al., 2006).

Chemical or enzymatic hydrolysis of proteins is an effective process for the recovery of protein material from fish by-products (Kristinsson, 2006). Particularly, enzymatic hydrolysis has been reported as the most appropriated technique due to the following advantages: a) the reaction is carried out at mild conditions of pH and temperature, b) side reactions are avoided because of the high specificity of the enzymes, and c) the products obtained have a high nutritional value (Guerard, 2006).

Enzymatic hydrolysis of proteins is catalyzed by proteases, which cleave peptide bonds between two amino acids consuming a molecule of water per each bond cleaved (Eq. 1).

$$P1\text{-}CO\text{-}NH\text{-}P2 + H_2O \Rightarrow P1\text{-}COOH + NH_2\text{-}P2 \qquad (1)$$

Therefore, the continuous cleavage of peptide bonds breaks down proteins into products of lower molecular weight such as peptones, peptides and amino acids (Adler-Nissen, 1986).

Protein hydrolysates have numerous applications in the food industry due to their technological properties such as improved solubility, water holding capacity, emulsifying and foaming properties and low allergenicity (Kristinsson & Rasco, 2000). Nevertheless, the production of fish protein hydrolysates exhibiting bioactive properties has gained an increasing interest in the last decade due to the added value of these products (Thorkelsson et al., 2009).

In this regard, several studies have reported protein hydrolysates with antioxidant activity obtained from fish species such as herring (Sathivel et al., 2003) and mackerel (Wu et al., 2003). Besides, peptide fractions with antihypertensive activity have been purified from fish species such as yellowfin sole (Jung et al., 2006) and salmon (Ono et al., 2006). Other bioactivities have been also described for fish protein hydrolysates such as antimicrobial (Fleury et al., 2008) and anticholesterolemic (Naqash et al., 2011). The first two bioactivities, antioxidant and antihypertensive, are the main focus of this Ph.D. thesis and they will be further discussed.

1.3.1 Antioxidant activity

Antioxidants compounds, apart to inhibit or retard lipid oxidation reactions in food, also have a key role on the prevention of oxidation at physiological level. Cell respiration generates free radicals (e.g. OH·) which can accumulate in the organisms due to a deficiency of natural antioxidants (e.g. vitamin C). These radicals act as initiators or propagators of chain reactions, which result in the damage of lipid membranes, structural proteins, enzymes and DNA structure (Lee et al., 2004). Several studies have related the accumulative oxidative damage to the occurrence of several chronic diseases such as cancer, diabetes, inflammatory and neurodegenerative diseases (Butterfield et al., 2002).

In order to prevent oxidation reactions, antioxidants can act by different mechanisms: a) scavenging of free radicals, and b) binding metal ions or modifying their redox potential, which prevent the formation of new radical species (Laroque et al., 2008). Traditionally, synthetic compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been employed as additives to prevent oxidation. However, although they exhibit a high antioxidant activity, their use in food has begun to be restricted due to their potential hazardous effects (Ito et al., 1985). Thus, there is an increasing interest in finding natural antioxidants with reduced side effects.

Among the existing natural sources, fish proteins have been widely evaluated as raw material for the production of fish protein hydrolysates with antioxidant activity (Samaranayaka & Li-Chan, 2011). In the last decade, several authors have reported a strong antioxidant activity for fish protein hydrolysates obtained from different species such as herring, *Clupea harengus*, (Shativel et al., 2003); hoki, *Ohnius belengerii*, (Mendis et al., 2005); saithe, *Pollachius virens*, (Chabeaud et al., 2009); sardinelle, *Sardinella aurita*, (Bougatef et al., 2009); and black scabbardfish, *Aphanopus Carbo*, (Batista et al., 2010). The antioxidant activity of a hydrolysate depends on the molecular size of the peptides as well as on their amino acids composition and sequence. In this regard, antioxidant peptides normally contain between 2 and 20 amino acids, with hydrophobic residues such as valine, glycine and proline which enhance the scavenging of lipid soluble radicals (Harnedy & FitzGerald, 2012). Other amino acids such as tyrosine, tryptophan, methionine, lysine, cysteine and hystidine are commonly present in the sequence of antioxidant peptides due to their capacity to donate protons and electrons which stabilize free radicals (He et al., 2013). The release of antioxidant peptides is highly influenced by: a) properties of the matrix hydrolysed (e.g. species, muscle, skin, etc.), b) specificity of the protease employed, c) hydrolysis conditions (temperature, pH and enzyme/substrate ratio), and d) extent of the hydrolysis reaction (Laroque et al., 2008). Table 2 shows some of these aspects for the previously commented studies.

Table 2. Examples of fish protein hydrolysates exhibiting antioxidant activity

Species	Raw material	Enzyme	Sequence	Mechanisms	Reference
Herring	Whole fish, body, head, gonads	Alcalase	n.e.	1	Sathivel et al., 2003
Mackerel	Fillets	Protease N	n.e.	1, 2, 3	Wu et al., 2003
Hake (Hoki)	Skin	Trypsin	His-Gly-Pro-Leu-Gly-Pro-Leu	1, 2	Mendis et al., 2005
Saithe	Muscle	Alcalase	n.e.	1, 2	Chabeaud et al., 2009
Sardinelle	Head and viscera	Extract from viscera of *Sardina. Pilchardus*	Leu-His-Tyr	1, 2, 3	Bougatef et al., 2010
Black scabbardfish	Head, skin, viscera, trimmings	Protamex	n.e.	2, 3	Batista et al., 2010

n.e.: not specified; 1: Inhibition of lipid oxidation; 2: Scavenging of free radicals; 3: Reducing power.

1.3.2 Antihypertensive activity

Hypertension, affecting up to one-third of the adult population, is a risk factor which can cause cardiovascular diseases such as arrhythmias, strokes and heart attacks (Kearney et al., 2005).

One of the most common methods to reduce blood pressure is the inhibition of the Angiotensin converting enzyme, ACE, (Puig, 2002). ACE (EC 3.4.15.1) is a chloride-dependent zinc metallopeptidase which is present in numerous parts of mammals organisms such as brain, lungs, liver, kidneys, testicles and others, predominantly as a membrane-bound ectoenzyme in vascular endothelial cells (Murray & FitzGerald, 2007). It plays a crucial role on the regulation of blood pressure since, in the renin-angiotensin system, ACE transforms the inactive decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) into the potent vasoconstrictor octapeptide, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Furthermore, Angiotensin II favors the secretion of aldosterone which activates the reabsorption of Na^+ and the retention of water by the renal tubes, increasing the blood pressure. In addition, in the kallikrein-kinin system, ACE catalyzes the degradation of bradykinin, a vasodilator nonapeptide (Li et al., 2004). Fig. 6 shows in a simplified way the mechanisms of action of ACE.

Currently, several synthetic compounds such as captopril, enalapril, alacepril and lisinopril have been developed for the inhibition of ACE. However, the prolonged use of these synthetic drugs causes some side effects such as cough, taste disturbances and skin rashes (Wijesekara & Kim, 2010). Therefore, there is an increasing interest in finding safer natural ACE inhibitors.

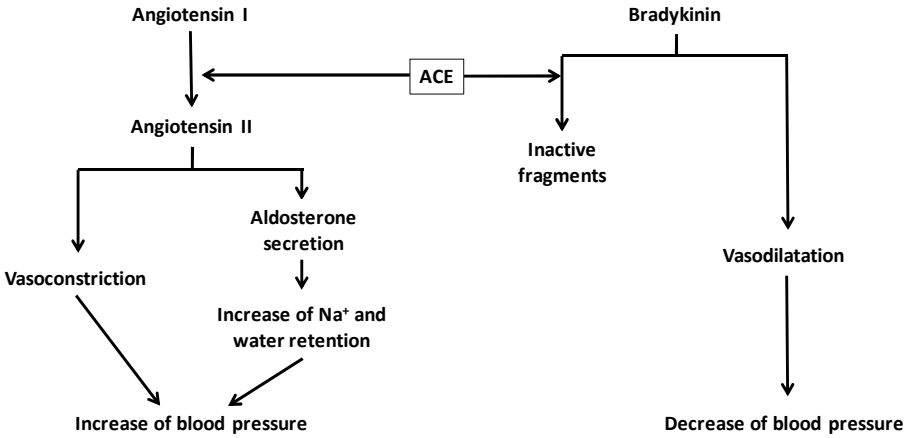


Figure 6. Mechanisms of action of ACE. Adapted from Li et al. (2004)

In this regard, fish protein hydrolysates have shown strong ACE-Inhibitory activity (Martínez-Maqueda et al., 2012). The ACE-inhibitory peptides are normally constituted by 2-12 amino acids and they normally contain hydrophobic residues (e.g. Phe, Trp, Tyr, Pro) in the C-terminal tripeptide sequence. This enhances the interaction of the peptide with the three hydrophobic zones placed at the active site of the enzyme. In this line, ACE shows weak or no affinity for hydrophilic inhibitors such as those which present dicarboxylic amino acids (e.g. Glu) at the C-terminal. Moreover, the presence of charge amino acids such as Lys and Arg at the C-terminal also contribute to the ACE-inhibitory potency. Other structure-activity studies suggest that the inhibitory activity of the peptides with C-terminal proline residue is improved with hydrophobic adjacent amino acids (Li et al., 2004).

Therefore, as in the case of antioxidant activity, the amino acids composition and sequence of the ACE-inhibitory peptides depends on the properties of the raw material hydrolyzed, the type of enzyme employed and the reaction conditions. Table 3 shows several peptides derived from fish proteins exhibiting ACE-inhibitory activity. Their IC_{50} values (protein concentration which inhibits the activity of ACE by half) are also shown.

Table 3. Examples of fish protein hydrolysates exhibiting antihypertensive activity

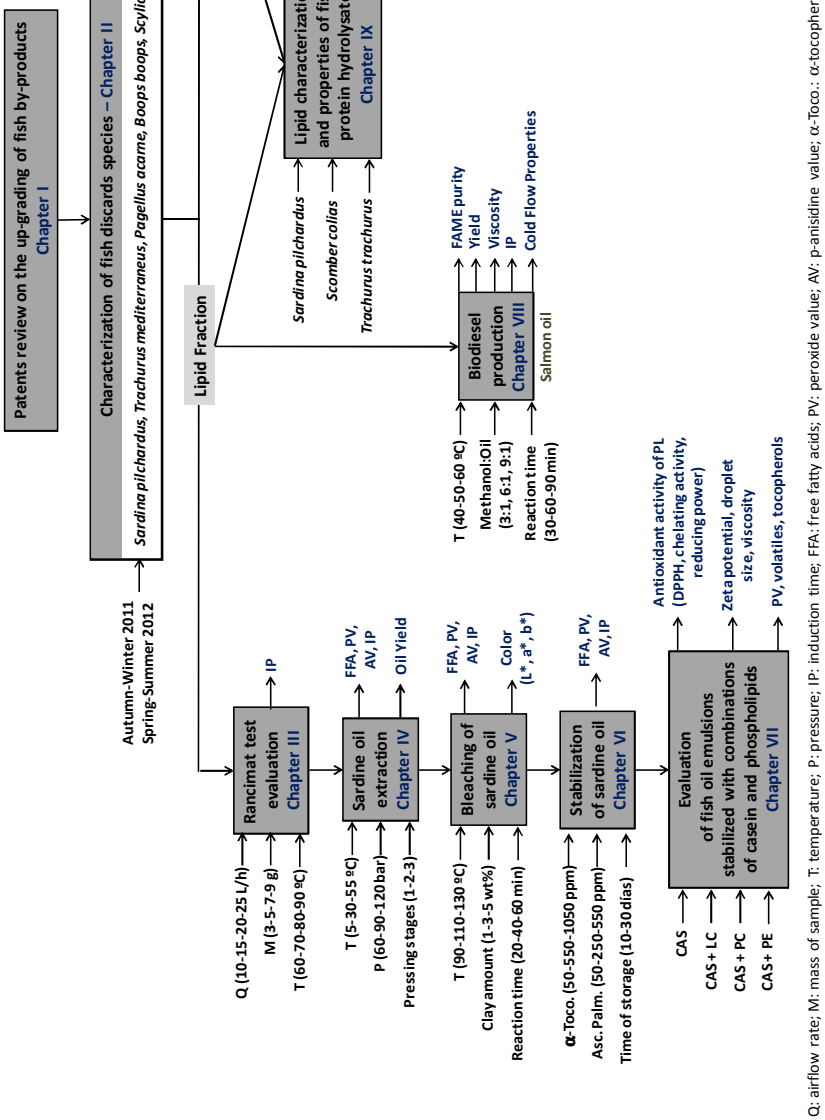
Species	Raw Material	Enzyme	Sequence	IC_{50} (µg/mL)	Reference
Sardine	Muscle	Alkaline protease	n.e	260	Matsui et al., 1993
Axillary seabream	Scales	Alkaline protease	Gly–Tyr Val–Tyr Gly–Phe Val–Ile–Tyr.	570	Fahmi et al., 2004
Yellowfin sole	Trimmings	α-chymotrypsin	Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu	28.7*	Jung et al., 2006
Sardinelle	Head, viscera	Extract from viscera of *Sardina. Pilchardus*	n.e.	1200	Bougatef et al., 2008
Cuttlefish	Muscle	Extract from viscera of *Sepia officinalis*	Val-Tyr-Ala-Pro Val-Ile-Ile-Phe Met-Ala-Trp	1000	Balti et al., 2010

n.e.: not specified; *: IC_{50} of the peptide

2. OBJECTIVES

The main objective of this Ph.D. thesis was to develop bio-processes for the up-grading of the lipid and protein fractions of fish discards species in the Alboran Sea. In order to achieve this main objective, the research carried out was divided into the following specific goals (Fig. 7):

1. Patents review on the up-grading of fish by-products (chapter I).
2. Characterization of 5 fish discards species (*Sardina pilchardus*, *Trachurus mediterraneus*, *Pagellus Acarne*, *Boops Boops* and *Scyliorhinus canicula*) and evaluation of their potential as raw material for the production of fish oil rich in omega-3 PUFA (chapter II).
3. Evaluation of the influence of the Rancimat test parameters on the determination of the oxidative stability of fish oil (chapter III).
4. Optimization of sardine oil extraction by hydraulic pressing (chapter IV).
5. Optimization of bleaching conditions for sardine oil (chapter V).
6. Optimization of antioxidants addition for the stabilization of sardine oil (chapter VI).
7. Evaluation of the antioxidant activity of phospholipids and of the influence of casein-phospholipids combinations as emulsifiers on the physical and oxidative stability of fish oil emulsions (chapter VII).
8. Optimization of biodiesel production from fish oil (chapter VIII).
9. Lipid characterization and properties of protein hydrolysates obtained from 3 fish discards species in the Alboran Sea (*Sardina pilchardus, Scomber colias* and *Trachurus Trachurus*) (chapter IX).
10. Evaluation of the antioxidant activity of protein hydrolysates obtained from the 5 fish discards species commented in objective 2 (chapter X).
11. Evaluation of the antihypertensive activity of protein hydrolysates obtained from the 5 fish discards species commented in objective 2 (chapter XI).



Q: airflow rate; M: mass of sample; T: temperature; P: pressure; IP: induction time; FFA: free fatty acids; PV: peroxide value; AV: p-anisidine value; α-Toco.: α-tocopherol; Asc. Palm.: ascorbyl palmitate; CAS: sodium caseinate; LC: lecithin; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PL: phospholipids; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical; FAME: alquil esters; ACE: Angiotensin I-converting enzyme.

Figure 7. Flow diagram of the research carried out on this Ph.D. thesis

4. RESULTS AND DISCUSSIONS

4.1 Recent patents review on the up-grading of fish by-products

Numerous processes have been patented in the last decades for the up-grading of fish by-products. In this regard, it should be highlighted those focused on the production of fish protein hydrolysates and fish oil.

The most important applications of fish protein hydrolysates (FPH) are as ingredients for food and as bioactive compounds. In the production of FPH as food additives, a common practice is the addition of exogenous proteases to carry out the hydrolysis reaction as described in the patent US 2002/0182290A1 (Munk, 2002). This patent employs Protamex^{TM} for the production of FPH which can be incorporated into salmon fillets in order to improve their quality. Other processes employ endogenous enzymes contained in fish viscera such as those provided in patents WO 03/066665A2 (Pyntikow et al., 2003) and US 2005/0244567A1 (Carlsson, 2005). Moreover, different enzymatic treatments have been employed in order to obtain FPH with reduced bitter taste. In this sense, the patent EP 0518999B1 (Dambmann et al., 1995) discloses a process using a protease produced from a strain of *Bacillus Licheniformis*, in order to obtain hydrophilic peptides with glutamic and aspartic acids at the C-terminal. With the same purpose, the patent US 2006/7070953B1 (Bjarnason & Benediktsson, 2006) indicates the employment of enzymes from cod intestines (trypsin, chymotrypsin and others) which hydrolyse at mild conditions, 15-20 ºC, yielding hydrolysates deprived of bitterness. These hydrolysates maintain their flavor and odor and can be incorporated to fish soups and sauces.

In the last years, a great interest has been developed in the production of FPH exhibiting bioactive properties due to the higher added value of these products. In that issue, the patent US 1007/007179793 (Ewart et al., 2007) describes the hydrolysis of salmon leftovers with Protease S Amano for the production of FPH with antihypertensive activity. Another process to obtain ACE-inhibitory peptides from mackerel sardine, bonito and cod by-products using thermolysin has been patented as EP 1092724B1 (Hiroyuki, 2008). Both processes incorporate a final ultrafiltration stage in order to obtain a product containing peptides below 3,000 Da. Additionally, the patent WO 2004/071202A1 (Hagen & Sandness, 2004) provides a process to produce FPH from salmon by-products using Protamex^{TM}, which retard lipid oxidation when incorporated into fish fillets.

The patents on the production of fish oil are classified according to their objectives into the following categories: a) extraction, b) refining, c) stabilization, and d) incorporation into food.

Different processes have been patented for the extraction of fish oil: a) wet reduction method together with deactivation of lipoxygenases by the addition of phosphoric acid, US 6190715B1 (Crowther et al., 2001); b) low-temperature solvent extraction of phospholipids from viscera of tuna and bonito, US 7189418B2 (Hiratsuka et al., 2007); c) extraction of oil with supercritical fluids from raw materials with a low oil content (e.g. fish meal), EP 0917876A1 (Blasco-Piquer & Mira-Ferri, 1999); and d) extraction by enzyme proteolysis, US 4976973 (Shrirakawa et al., 1990). For the refining of fish oil, it should be highlighted the processes patented for the removal of volatiles oxidation products, such as molecular distillation at high vacuum, US 4623488 (Takao, 1986); size-exclusion by means of a silica gel column, US 5023100 (Chang & Bao, 1991); and the extraction with supercritical CO_2, US 4692280 (Spinelly & Stout, 1987). With regard to the stabilization of fish oil, it has been patented the addition of fructose, US 5116629 (Schroeder & Muffett, 1992), and vinegar and juice from citrus fruits, US 6235331 (Kataoka & Kiyohara, 2001), for the stabilization of fish oil emulsions to be incorporated in salad dressings or yoghurt. Likewise, another patent procedure describes the addition of natural antioxidants from orange, apple and mango for the stabilization of juices fortified with fish oil, US 0202679 (Mathisen, 2009). Finally, several processes have been reported for the microencapsulation of fish oil, which eliminates its unpleasant taste and smell when it is incorporated into food, as those described in US 48956725 (Martin et al., 1990) and US 5456985 (Zgoulli et al., 1995).

4.2 Characterization of 5 fish discards species (*Sardina pilchardus, Trachurus mediterraneus, Pagellus Acarne, Boops Boops and Scyliorhinus canicula*) and evaluation of their content in omega-3 PUFA

The proximate composition results indicated that the protein content of the species remained practically constant along the year, ranging from 15.5 wt% for bogue to 23.1 wt% for small-spotted catshark (Table 7). On the other hand, the lipid content changed considerably among species and also significant variations were noticed for the same

species in different seasons. Maximum lipid content was observed in spring (axillary seabream 5.1 wt%, small-spotted catshark 2.7 wt% and bogue 2.5 wt%) and summer (sardine 13.6 wt% and horse mackerel 6.2 wt%), since in these seasons there is an abundant availability of food resources in the aquatic environment (Orban et al., 2011). On the contrary, minimum lipid content was found after the spawning period (spring for sardine and winter for the rest of species). This finding is due to the fact that during the spawning the fat is employed by the fish as energy source (Krzynowek, 1992). The ash content was practically constant along the year, whereas the moisture content was found to correlate inversely with the lipid content (Table 7).

A wide variety of fatty acids were detected in the five fish oils extracted. The major fatty acids identified were PUFA ranging from 33.0 to 45.1 wt%, mainly constituted by EPA and DHA. The content of EPA+DHA of the oils was higher than 20 wt% in all the seasons, highlighting the oil from small-spotted catshark with DHA content up to 20 wt%. Besides, the five oils showed excellent ratio of n3/n6, being maximum for sardine (14.9-16.7), followed by horse mackerel (11.9-13.5) (Table 8).

With regard to the omega-3 content of the species, it was confirmed that sardine and horse mackerel were excellent sources of omega-3 PUFA in summer and autumn, having values of EPA+DHA up to 3,000 and 1,300 mg/100 g fish, respectively. They were followed by axillary seabream and small-spotted catshark, which exhibited a content of EPA+DHA in spring of 960 and 650 mg/100 g fish, respectively (Table 9).

In terms of lipid class composition, all the oils exhibited a composition in triglycerides higher than 78 wt%, being considerable higher for small-spotted catshark, 96 wt%. The cholesterol content of the oils ranged from 3.3 wt% for small-spotted catshark to 8.8 wt% for axillary seabream. On the other hand, phospholipids were detected only in sardine oil (0.9 wt%) (Table 10).

4.3 Evaluation of the influence of the Rancimat test parameters on the determination of the oxidative stability of fish oil

The results obtained indicated that at constant values of airflow rate (Q, L/h) and sample weight (M, g), the oxidative stability index (OSI, h), equivalent to the induction period (IP, h), considerably decreased when increasing temperature, (T, ºC). However, the influence of airflow rate and sample weight on OSI was not as evident (Table 11).

Firstly, the experimental data of OSI were fitted to a quadratic model (Eq. 4), obtaining a R^2 value of 0.9971. The ANOVA analysis indicated that the OSI was highly dependent on the linear effects of temperature and airflow rate, with p-values lower than 0.05 (Table 12). By means of this model and employing response surface methodology, contour maps were generated where the OSI levels were plotted against Q and M at each value of T (Fig. 9). It was observed that the OSI followed a curved surface, although the contour lines became less pronounced as temperature increased. This finding may suggest a change in the mechanism of fish oil oxidation with the increase of temperature, favoring secondary reactions of the non-volatile products such as polymer formation (Frankel, 2005). By optimization of the quadratic model, an optimum value for OSI (1.61 h) was obtained for Q = 25 L/h, M = 6.91 g and T = 88.26 ºC (shown as a circle in Fig. 9). This finding denotes that may exist an optimum relation for sample-weight and airflow rate of M = 6.91 g and Q = 25 L/h, which guaranteed an adequate saturation level in the reaction vessel.

The lack of significance for both quadratic (except for temperature) and interaction effects in the complete quadratic model suggested fitting the experimental data to a linear model (Eq. 5). According to previous studies (Reynhout, 1991; Farhoosh, 2007), the decimal logarithm of OSI values were employed in order to obtain a better fit, R^2 of 0.9958. As for the quadratic model, the linear effect of temperature and airflow rate were statistically significant ($p<0.05$) (Table 13). For the cod liver oil studied, it was found a temperature coefficient of -3.29×10^{-2} ºC^{-1}, which is similar to the values reported by Méndez et al. (1996) for other fish oils.

4.4 Optimization of sardine oil extraction by hydraulic pressing

The oils extracted from the different batches presented similar fatty acid profiles. Polyunsaturated fatty acids were the major fatty acids identified (~35 wt%), mainly eicosapentaenoic (~13 wt%) and docosahexaenoic (~11 wt%) acids.

With regard to the quality of the oils extracted (Table 14), they had a low content of free fatty acid, ranging from 0.25 to 1.23 % oleic. These values were considerably lower than those reported for oil extracted from tuna heads (Chantachum et al., 2000) and from herring by-products (Aidos et al., 2001). Moreover, the oils showed a low content of primary oxidation products, with PV between 0.33 and 5.75 meq/kg oil. Additionally, the decomposition of hydroperoxides did not take place to a large extent, AV varying from 0 to 1.98. These findings are due to the low pretreatment temperature employed since higher

PV (30 meq/kg oil) was obtained by Chantachum et al. (2000) and higher AV (8.9) by Aidos et al. (2001) when preheating at 95 ºC. On the other hand, a direct correspondence between IP and PV and AV was only observed for the batches carried out at 55 ºC, which showed the lowest IP. Considering the oil yield, different values (1.16-12.78 %) were obtained depending on the extraction conditions employed (Table 14).

The experimental data for each measured variable were fitted to a quadratic model, obtaining coefficients of determination, R^2, around or higher than 0.90 except for AV (0.78) and IP (0.76) (Table 15). ANOVA analysis reveals that all the output variables were dependent on the linear effect of temperature ($p<0.05$), whereas the linear effect of pressure was not significant for any of them. Regarding the number of pressing stages, its linear effect was statistically significant on AV and yield.

Secondly, the individual optimizations of FFA and PV, as indicators of the oil quality in terms of acidity and oxidation products, and of yield, as variable showing the efficiency of the process, were carried out (Fig. 13). A minimum of FFA (0.25 % oleic) was found at 55 ºC, 60 bar and one pressing stage. These results denoted that preheating at 55 ºC may denature endogenous lipases of sardine, thus reducing the hydrolysis of triglycerides to free fatty acids (Mukundan et al., 1985). Besides, employing a reduced number of pressing stages decreased the contact time between oil and stickwater, which minimized the FFA content of the final oil. Considering PV, a minimum (0.29 meq/kg oil) was found at the lowest pretreatment temperature (5 ºC), 60 bar and one pressing stage. As for FFA, the lowest number of pressing stages reduced the processing time minimizing the oxidation of the oil. Moreover, a maximum for yield (12.47 %) was obtained at the highest pretreatment temperature (55 ºC), minimum pressure (60 bar) and two pressing stages. The highest preheating temperature improved the breaks down of the walls of the fat cells and the coagulation of proteins, enhancing the oil extraction. Besides, it seems to exist an optimum combination of pressure exerted and number of pressing stages (60 bar and two stages), which do not compact excessively the press cake allowing the release of the press liquor from the pores.

Finally, the multi-objective optimization of FFA, PV and yield allowed to obtain a set of non inferior solutions (Pareto Front) which satisfied the three objectives to an adequate degree (Table 16). Besides, Table 16 shows the processing conditions which should be employed in order to obtain the yield of oil desired with the required quality. Nevertheless, it should be noted that values of $w_3<0.4$ are not recommended in order to not obtain a

considerably low yield (<11.06 %). It implies to work at 55 ºC, 60 bar and two or one pressing stages.

4.5 Optimization of bleaching conditions for sardine oil

This study evaluates the bleaching of previously degummed and neutralized sardine oil (DNSO). Although this oil presented a low acidity (0.17 % oleic) and low PV (2.35 meq/kg oil), its content of secondary oxidation products was considerably high (AV of 77). Besides, the oil showed a dark brown color, attributable to its advanced state of rancidity. In terms of hydroperoxides content, for bleached oils processed at the highest clay amount (5 wt%) and temperature (130 ºC), no significant PV was obserbed (Table 17). Nevertheless, carrying out the bleaching process using lower concentrations of clay (1-3 wt%) at low process temperatures (90 – 110 ºC) did not reduce the PV of the oil; on the contrary, these conditions even enhanced the formation of peroxides. Considering the secondary oxidation products, an AV reduction was found for all the bleached oils (Table 17). This fact denoted the capacity of the acid-activated earths to adsorb these types of compounds (Rossi et al., 2003; Sathivel, 2010). Although the values of the Rancimat induction period, IP, did not follow a clear trend, it was noticed that the oils bleached at 130 ºC, employing a clay amount of 5 wt% and a long time of contact (40-60 min) showed a higher oxidative stability (IP=0.95 h) (Table 17).

With regards to the color measurements, the bleaching process increased the lightness of the oil, reaching L* values up to 83.61. Besides, the activated earth adsorption process effectively reduced a* value indicating that a decrease in red color occurred. Chroma values of the bleached oils, which represent the intensity of color, were reduced, while hue-angle value increased with the concentration of the activated earth. The hue-angle values were in the range of 79.53-90.76 which implies yellow-orange color for the bleached oils (Table 17).

The experimental data were satisfactory fitted to second order models, with values of R^2 around 0.9 except for the model of FFA (Tables 18 and 19). The ANOVA analysis indicated that the linear effect of clay amount was statistically significant ($p<0.05$) for all the measured variables (FFA, PV, AV, IP, L*, a* and b*) as well as for the calculated ones (totox, chroma, and hue-angle). Regarding temperature, the output variables FFA, PV, AV, IP, totox and L* were highly dependent on its linear effect. On the other hand, the

quadratic effects were found to be significant only for clay percentage in the cases of L*, b* and chroma; and time in the cases of FFA, PV, AV and L* (Tables 18 and 19).

For the optimization of the bleaching process, totox value was chosen as measured variable to indicate the total oxidation of the oils, while hue-angle and chroma were selected as output variables indicating the final color of oil samples. The following optimum values were obtained (Fig. 15):

a) A minimum value for totox (21.38) at 130 ºC, 5 wt% and 60 min. This finding is explained because: i) working at the maximum temperature assayed led to the highest rate of peroxides decomposition to secondary oxidation products, and ii) these secondary oxidation products were better adsorbed when employing a high concentration of activated clay. Besides, long contact time maximized the adsorption of secondary oxidation products (Sathivel, 2010).

b) A maximum for hue-angle (89.19) at low temperature (99.2 ºC), adsorbent concentration of 5 wt% and long contact time (56.6 min). Considering chroma a minimum value (81.76) was found at 109.4 ºC, 5 wt% and 49.4 min. These results may indicate that at moderate temperature (99-110 ºC) the activated clay (Tonsil 278) is better activated in terms of colored compounds removal.

In the simultaneous optimization of totox, chroma and hue-angle, the desired characteristics for the bleached oil will determine the weight given to each objective variable (w_1, w_2, w_3). This implies to choose an optimal solution from the Pareto Front generated (Table 20) and to work at the specific processing conditions shown in Fig. 16. For instance, a value of w_1<0.4 is not recommended in order to obtain a bleached oil with a good quality in terms of oxidation products (totox<21.83). It implies to operate at the following conditions: 130 ºC, clay amount of 5 wt% and process time ranging from 54 to 60 min.

4.6 Optimization of antioxidants addition for the stabilization of sardine oil

The purification of sardine oil by chromatographic adsorption on alumina-silica column led to a significant reduction of the FFA content (from 6.26 to 0.14 % oleic) and of the content of secondary oxidation products (AV from 17.18 to 2.17). However, the content of

peroxides remained practically constant (PV of 2.7 meq/kg oil). Regarding the fatty acid profile, no significant differences were observed between crude and purified oil.

During the storage period, the FFA content of the stabilized oils did not increase but even suffered a slight decrease (Table 21). It may be due to the fact that FFA were possibly oxidized since they are more susceptible to oxidation than esterified fatty acids (Aidos et al., 2001).

In most of the cases, it was revealed that the addition of antioxidants led to stabilized oils with lower PV than the control samples (Table 21). For short time of storage (10 days), low-medium concentrations of alpha-tocopherol (50-550 ppm) combined with maximum addition of ascorbyl palmitate (450 ppm) and citric acid (50 ppm) resulted in the lowest PV, 20.83 and 18.20 meq/kg oil, respectively. For long time of storage (30 days), the addition of a low concentration of alpha-tocopherol (50 ppm), 450 ppm of ascorbyl palmitate and 500 ppm of citric acid, resulted in the maximum protection against hydroperoxides formation, PV of 31.55 meq/kg oil (Table 21). The high addition of ascorbyl palmitate was beneficial due to the synergic effect between ascorbyl palmitate and alpha-tocopherol. It was reported that ascorbyl palmitate can regenerate alpha-tocopherol from its radicals derivatives enhancing its activity and thus, reducing the amount of alpha-tocopherol radical available for participation in side reactions and further oxidation (Frankel et al., 1994; Olsen et al., 2005). Nevertheless, this effect was not observed at high concentration of alpha-tocopherol (1050 ppm), which had a prooxidant effect in terms of hydroperoxide formation. Similar results were reported by Kulås and Ackman (2001a) and by Zuta and Simpson (2007).

With regard to secondary oxidation products, for both short and long time of storage, medium concentration of alpha-tocopherol combined with a high addition of ascorbyl palmitate (450 ppm) and citric acid (50 ppm) allowed the maximum reduction in the decomposition of hydroperoxides, AV of 4.94 and 12.87 respectively (Table 21). It might be explained due to the fact that alpha-tocopherol can induce decomposition of hydroperoxides to stable products and that a better regeneration of alpha-tocopherol radical is produced at high concentration of ascorbyl palmitate (Kulås & Ackman, 2001b; Frankel, 2005).

Rancimat induction periods did not have a direct correspondence with PV and AV. This fact is attributed to the high temperature employed in the Rancimat test which did not

allow a comparison of the data obtained from this accelerated oxidation method and experiments at room temperature (Drusch et al., 2008). Regarding the fatty acid profile of the stabilized oils, no significant changes were observed after 30 days of storage.

The experimental data were fitted to quadratic models, obtaining R^2 values around 0.9 except for FFA (Table 22). The ANOVA analysis revealed that PV, totox and IP were highly dependent on the linear effect of alpha-tocopherol concentration ($p<0.05$). Regarding ascorbyl palmitate concentration, its linear effect was statistically significant just for PV and totox. Moreover, the quadratic effect of alpha-tocopherol and the interactive effect of alpha-tocopherol and ascorbyl palmitate were statistically significant in the cases of PV, AV, totox and IP (Table 22).

In order to optimize the stabilization process, the measured variables PV and AV were chosen as appropriate objective functions indicating the oxidative deterioration of the oil samples. The individual optimization of these variables by response surface methodology allowed to obtain the minimum values indicated in Table 4.

Table 4. Optimum values for PV and AV after 10 and 30 days of storage

10 days		**PV** (18.56 meq/kg oil)	**AV** (2.64)
	α-Tocopherol (ppm)	207	478
	Ascorbyl palmitate (ppm)	450	390
30 days		**PV** (33.56 meq/kg oil)	**AV** (15.34)
	α-Tocopherol (ppm)	50	493
	Ascorbyl palmitate (ppm)	450	450

From these results, it was denoted a contradictory effect of alpha-tocopherol concentration on the optimization of PV and AV. Thus, a bi-objective optimization was carried out in order to generate a set of non inferior solutions (Pareto Front) which satisfied both goals to an adequate degree. It was observed that when using a weight factor for PV (w) lower than 0.6, AV decreased from 21.98 to 15.34, implying a sharp increase of PV (from 34.13 to 46.22). This fact also indicated a change for the optimum alpha-tocopherol concentration (from 50 to 493 ppm) (Table 23).

4.7 Evaluation of the antioxidant activity of phospholipids and of the influence of casein-phospholipids combinations as emulsifiers on the physical and oxidative stability of fish oil emulsions

For the three phospholipids evaluated, lecithin (LC), phosphatidylcholine (PC) and phosphatidylethanolamine (PE), similar DPPH scavenging activities were obtained, 95.0 % inhibition for solutions 1 wt% phospholipid (Fig. 19a). This radical scavenging activity may be related to the proton donating capacity of the amino group of phospholipids, particularly for PE and PC (Bandarra et al., 1999). Nevertheless, it should be mentioned that the three phospholipids tested were found to be less effective DPPH scavengers than BHT. With regards to the metal-binding capacity of phospholipids, Zambiazi et al. (1998) attributed their metal-chelating properties to the amino group of PC, PE and PS and to the sugar moiety of PI. In the present study it was found that PC presented the highest capacity to bind Fe^{2+} ions (98 % for 1 wt% solutions) followed by PE (71 %), whereas LC exhibited the lowest metal-chelating activity (42 %) (Fig. 19b). These results are in the line of those reported by: a) Bandarra et al. (1999), who described that the addition of PC to refined sardine oil resulted in a higher protection against lipid oxidation when compared to the addition of PE and alpha-tocopherol; and b) Cardenia et al. (2011), who indicated that PC was more effective than PE at inhibiting lipid oxidation in 1 wt% stripped soybean oil-in-water emulsions at pH 7. Although all the phospholipids were considerably less effective than EDTA in binding metal ions, they may represent a unique type of chelator in case they can act in the lipid phase (Cardenia et al., 2011). Finally, the three phospholipids evaluated exhibited an insignificant reducing power when compared to ascorbic acid. For the three cases, absorbances at 700 nm lower than 0.2 were obtained for solutions 0.1 wt% (Fig. 19c).

With regard to the physical stability, the emulsions stabilized with CAS+LC and CAS+PC were the most physically stable. They had the lowest mean droplet size at day 1 ($D_{3,2}$=210 nm and $D_{3,2}$=212 nm, respectively) and they did not suffer any change in their particle size distribution after 14 days of storage (Table 25). The physical instability of the emulsion stabilized with only casein is due to the fact that a higher amount of emulsifier (up to 1 wt%) is required in order to avoid coalescence or flocculation in 10 wt% fish oil-in water emulsions prepared at pH 7 and stored during 2 weeks (not published results). In the case

of the emulsion 0.3CAS+0.5PE, the physical instability could be due to the lower hydrophilic-lipophilic balance (HLB) of PE at neutral pH, which makes it less appropriate to stabilize water-continuous emulsions (Carlsson, 2008).

Considering the oxidative stability of the emulsions, the principal component analysis (PCA) indicated that the emulsions CAS+PC and CAS+PE were the most oxidized in terms of primary oxidation products (Fig. 20). This interpretation of the model was confirmed by the PV values obtained at day 14: PV of 24.7 meq/kg oil for CAS+PC, PV of 21.1 meq/kg oil for CAS+PE, PV of 19.9 meq/kg oil for CAS and PV of 12.9 meq/kg oil for CAS+LC (Fig. 21). In regard with volatile oxidation products, the PCA loadings plot showed that most of the volatiles were located in the 4^{th} quadrant, indicating a higher concentration of volatiles in the CAS emulsion (Fig. 20). This was confirmed by the raw data which also indicated that the emulsion CAS+LC was the most oxidative stable since it showed the lowest increase in the concentration of volatiles derived from the oxidation of omega-3 PUFA such as 1-penten-3-ol and 1-penten-3-one (Fig. 22).

Nevertheless, the higher oxidative stability of the emulsion stabilized with CAS and LC cannot be explained by its physical data since: i) this emulsion had the most negative zeta potential (-32.9 mV), which would increase the attraction of transition metals ions to the interface enhancing lipid oxidation (Hu et al., 2003); ii) it also presented the lowest mean droplet size ($D_{3,2}$=210 nm), which implies larger surface for the contact between metal ions and hydroperoxides leading to less oxidative stable emulsions (Jacobsen et al., 2000); iii) LC did not show better antioxidant properties than the other phospholipids; and iv) the protein content in the aqueous phase of this emulsion was practically identical to the other emulsions with phospholipids, hence, the casein could not exert higher chelating activity in this emulsion. Therefore, these results suggested that the improved oxidative stability of the CAS+LC emulsion may be related to other factors such as the characteristics of the interface. In this regard, the increase observed for the protein concentration in the aqueous phase for the emulsions with phospholipids when compared with the CAS emulsion (Table 25), suggested the incorporation of phospholipids at the interface. This fact could lead casein to adopt a conformation that projects further into the aqueous phase, which results in an augmentation in the thickness of the interface (Fang & Dalgleish, 1993). In the case of LC, its content in charged phospholipids such as PI and PA, which are more soluble in water and are then more easily adsorbed at the oil-water interface (Rydhag & Wilton,

1981), may lead to different structure and thickness of the interfacial layer for the emulsion stabilized with LC. However, this requires further research in order to be confirmed.

4.8 Optimization of biodiesel production from fish oil

Due to the high acidity presented by the raw fish oil employed, a two-step production process was performed. The first step consisted of an acid-catalyzed pre-treatment to esterify the FFA which allowed to reduce the acid value below 2 mg KOH/g oil. In the second stage, the biodiesel was produced via alkaline transesterification (1 wt% NaOH) with methanol. The biodiesel samples obtained showed a high content of FAME, ranging from 93.08 to 95.39 wt% (Table 27). The content of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) amounted to 17.7 wt% and 33.2 wt%, respectively. However, it should be mentioned that the FAME values obtained are slightly lower than the minimum required by the European standard (EN 14214) for use as 100 % diesel fuel (96.5 wt%).

The biodiesel yields obtained varied from 71.19 to 83.06 % (Table 27). Nevertheless, higher yields were obtained by previous studies, 99 % (El-Mashad et al., 2008) and 96 % (Fadhil & Ali, 2013), which employed a lower concentration of alkaline catalyst, 0.5 wt%. According to Fadhil and Ali (2013), an alkaline catalyst (NaOH or KOH) concentration beyond 0.5 wt% enhanced the formation of soaps by triglyceride saponification. Owing to their polarity, soaps are dissolved into the glycerol phase increasing also the methyl ester solubility in the glycerol, which is a cause of yield loss (Vicente et al., 2004).

The transesterification process was effective in terms of kinematic viscosity reduction. It decreased from a value of 45.34 mm^2/s for the crude fish oil to 6.05-6.66 mm^2/s for the biodiesel samples (both measured at 30 ºC, Table 27). The biodiesel produced showed a lower viscosity than biodiesel obtained from different marine fish oil which exhibited a kinematic viscosity of 7.2 mm^2/s at 40 ºC (Lin & Li, 2009). It is explained by the higher SFA content of the oil employed by these authors (37.06 wt%). Thus, the biodiesel samples obtained were close to meet the European standard (3.5-5 mm^2/s at 40 ºC) since reduced viscosities would have been obtained at 40 ºC.

With regard to the oxidative stability, low IP values were found for all the biodiesel samples (1.05-2.22 h). It is due to the high PUFA content of the oil employed as raw material. Thus, in order to meet the value indicated by the European standard (minimum IP

of 6 h), the use of antioxidants (Lin & Lee, 2010) or the mixture of fish oil with more stable oils before transesterification (Costa et al., 2013) are required.

Considering the cold flow properties of the biodiesel samples, they presented a melting range of -73.60 to 3.83 ºC, which was determined by DSC. The COM values varied in a narrow range, from 3.31 ºC to 3.83 ºC (Table 27), since all the samples showed the same lipid profile. Chiou et al. (2008) reported a similar COM value for salmon oil methyl esters (3.1 ºC) but a lower one was reported by the same authors for corn oil methyl esters (1.7 ºC), which is explained by its lower saturated fatty acids content. Moreover, all the biodiesel samples showed an acid value below 0.31 mg KOH/g oil after the transesterification step, which is in agreement with the European standard (<0.5 mg KOH/g oil).

Then, the experimental data were fitted to second-order models, having R^2 values lower than 0.71 for all the output variables studied (Table 28). However, in the cases of yield, viscosity, IP and COM, the deviation between predicted and measured values was lower than ±10 % (Fig. 24). Thus, these variables were optimized by response surface methodology (Fig. 25), obtaining the following optimum values:

a) A maximum for yield (83.06 %) at 40 ºC, 9:1 methanol to oil ratio and 90 min. This low temperature reduced the saponification of triglycerides, while the high methanol to oil ratio and the long time of reaction favored the conversion of triglycerides to alkyl esters.

b) A minimum for viscosity (6.30 mm^2/s) at 60 ºC, 5.15:1 methanol to oil ratio and 55.52 min. The highest temperature led to biodiesel with a higher purity of FAME which implied a lower viscosity.

c) A maximum for IP (1.99 h) at 60 ºC, 9:1 and 30 min. Although increasing temperature favored the oxidation of FAME, it may also enhance the mixture of the produced FAME and the excess of methanol which could reduce the oxidative deterioration of the FAME during the production process. Considering reaction time, the lowest value of time assayed was found as optimum value since it implies a shorter contact time of FAME with oxygen at the processing temperature.

d) A minimum for COM (3.48 ºC) at 60 ºC, 3.74:1 and 90 min. The COM value of biodiesel mainly depends on the SFA composition of the fuel (Knothe, 2005). In this regard, no significant differences were found among the SFA content of the 27

samples obtained. Therefore, it cannot explain the location of the optimum at these processing conditions values.

4.9 Lipid characterization and properties of protein hydrolysates obtained from 3 fish discards species in the Alboran Sea (*Sardina pilchardus, Scomber colias and Trachurus Trachurus*)

Apart from the species mentioned in section 4.2, discards in the Alboran Sea also comprise another species of horse mackerel (*Trachurus trachurus*), mainly due to quota restrictions and minimum landing sizes requirements (FROM, 2008), and a non-target species such as mackerel, which appears as by-catch species in trawling and purse seine fisheries (Carbonell et al., 1997).

The proximate composition results indicated that, in this period of the year (month of May), horse mackerel presented the highest lipid content (7.8 wt%), followed by sardine (4.4 wt%) and mackerel (2.5 wt%) (Table 29). On the other hand, sardine oil exhibited the highest content of EPA (12.96 wt%) and DHA (17.63 wt%), followed by mackerel (EPA+DHA = 27 wt%) and horse mackerel (EPA+DHA = 22 wt%) (Table 30). All the oils extracted were mainly constituted by triglycerides (TAG>97 wt%).

The enzymatic hydrolysis of the press cakes led to satisfactory degree of hydrolysis (DH) (Fig. 26). The highest DH was obtained by horse mackerel hydrolysates (up to 21 %), followed by sardine hydrolysates (up to 18 %) and mackerel (up to 14 %). For all the combinations between species and enzymatic treatments the ACE-inhibitory activity increased sharply within the first 30 - 60 min of reaction. Above this point, ACE inhibition values (65-70 %) remained constant or presented a slight decreasing trend (Fig. 27). The simultaneous addition of subtilisin and trypsin resulted in the mackerel and horse mackerel hydrolysates exhibiting the highest ACE-inhibitory activity, IC_{50} values of 345 and 364 μg protein/mL respectively. In the case of sardine, the sequential addition of subtilisin and trypsin led to the hydrolysate with the lowest IC_{50} value (400 μg protein/mL). These three hydrolysates presented a DH between 13 and 16 %, corresponding to an average peptide chain length (PCL) of 8 and 6 amino acids, respectively (Table 31). These results are in agreement with those reported by previous studies. In this sense, Matsui et al. (1993) obtained a sardine hydrolysate exhibiting an IC_{50} value 260 μg protein/mL.

Furthermore, the hydrolysates produced from the three press cakes evaluated also exhibited satisfactory DPPH scavenging activities. The highest DPPH radical inhibition was showed by the hydrolysates of horse mackerel (45 %), followed by the hydrolysates of sardine (40 %) and mackerel (35 %) (Table 32). The simultaneous treatment as well as the sequential addition of subtilisin and trypsin resulted in the hydrolysates (PCL of 5-7 amino acids) with the highest antioxidant activity (Table 32). Similar results were described for sardine hydrolysates, exhibiting a DPPH inhibition of 15-55 % (Bougatef et al., 2010). On the other hand, Wu et al. (2003) reported a DPPH scavenging activity of 80 % for mackerel hydrolysates after 10 h of hydrolysis.

4.10 Evaluation of the antioxidant activity of protein hydrolysates obtained from discarded fish species (*S. Pilchardus, T. mediterraneus, P. acarne, B. boops, S. canicula)*

Different DH values were obtained depending on the raw material hydrolysed and on the enzymatic treatment employed. The highest DH were obtained for the horse mackerel hydrolysates (18.0-21.0 %), followed by the hydrolysates of small-spotted catshark (17.3-19.2 %). The hydrolysates of axillary seabream and bogue showed medium values of DH (15.3-17-6 %), whereas the lowest DH values were found for sardine hydrolysates (13.2-14.9 %) (Table 33).

With regard to the influence of DH on the DPPH scavenging activity, different trends were found depending on the species and on the enzymatic treatment used. For instance, the hydrolysates of sardine exhibited maximum values of DPPH inhibition in the periods of hydrolysis with subtilisin and trypsin in the sequential treatments (Fig. 30b). On the other hand, the hydrolysates of horse mackerel showed an increasing DPPH scavenging activity with DH for these treatments (Fig. 30a). Moreover, it was observed that the addition of a second enzyme led to an increase in the DPPH inhibitory activity of the hydrolysates, which was more notorious in the cases of sardine and horse mackerel. These two different trends for the antioxidant activity of fish protein hydrolysates with DH were also previously reported (Jao & Kao, 2002; Wu et al., 2003).

The final hydrolysates presented a protein content ranging from 60.7 wt% for sardine to 89.5 wt% for small-spotted catshark. Conversely, the highest lipid content was obtained for sardine hydrolysates (25.3 wt%) and the lowest for small-spotted catshark hydrolysates

(4.6 wt%) (Table 33). Triglycerides were the most abundant lipids in the hydrolysates produced from the press cakes, especially in the cases of sardine and horse mackerel (74 wt%). Furthermore, a high content of free fatty acids was obtained, particularly for axillary seabream and bogue (up to 36 wt%). On the other hand, the hydrolysates of small-spotted catshark had a negligible content of triglycerides and free fatty acids, while most of their lipids are constituted of phospholipids (54.5 wt%) and cholesterol (35.2 wt%) (Table 34). These results are in line with those reported by Daukšas et al. (2005). The molecular weight profiles indicated that the hydrolysates were mainly constituted of small peptides, more than 80 % of their peptides/amino acids were below 150 Da. The small-spotted catshark hydrolysates showed a rather different peptide profile from the hydrolysates prepared from the press cake of the bony fish. It presented lower percentages of peptides higher than 450 Da (2 %) and in the range of 50-150 Da (16 %) but higher percentages of peptides in the range of 150-450 Da (21 %) (Fig. 31).

Considering their antioxidant properties, the highest DPPH scavenging activity was exhibited by the final hydrolysates of sardine (IC_{50}=0.91 mg protein/mL) and horse mackerel (IC_{50}=1.47 mg protein/mL). Both were obtained by the sequential treatment trypsin (2h) and subtilisin (2h) (Table 35). It may be due to the fact that adding subtilisin as second enzyme may favor the cleavage at the C-terminal of hydrophobic residues which can contribute to the DPPH inhibitory activity (Je et al., 2009). The highest chelating activity was found for the hydrolysates of sardine (independently of the enzymatic treatment) and for the hydrolysates of small spotted cathskar obtained by the sequential treatments. They exhibited and IC_{50} value 0.32 mg protein/mL (Table 35). This binding metal capacity may be related to the presence of peptides containing histidine, which has been reported to exert metal chelating activity through its imidazole ring (Bougatef et al., 2009). Fig. 32 shows that sardine and bogue hydrolysates exhibited the highest reducing power with absorbances higher than 0.7 at a concentration of 20 mg protein/mL, whereas small-spotted catshark hydrolysates presented the lowest. It should be also mentioned that the simultaneous addition of subtilisin plus trypsin was the least appropriate enzymatic treatment for the production of sardine protein hydrolysates with a high reducing power, whereas the sequential treatment subtilisin (2h) and trypsin (2h) led to the bogue hydrolysate with the lowest reducing power (Fig. 32). The reducing power results obtained in this work were similar to that reported for black scabbardfish (Batista et al., 2010), and that those found for hake by products (Pires et al., 2013).

4.11 Evaluation of the ACE-inhibitory activity of protein hydrolysates obtained from discarded fish species (*S. Pilchardus, T. mediterraneus, P. acarne, B. boops, S. canicula)*

The influence of DH on the ACE-inhibitory activity of the hydrolysates depends on the raw material hydrolyzed and on the enzymatic treatment used. The following trends were observed: i) increase of ACE-inhibitory activity with DH until attaining a maximum value, then, it remained practically constant when increasing DH (observed for the simultaneous treatment); ii) the antihypertensive activity of the hydrolysates reached maximum values and then decreased slightly in the sequential periods of hydrolysis with subtilisin and trypsin (observed for the sequential treatments). Furthermore, the addition of a second enzyme, which was carried out in the sequential treatments, led to an increase in the ACE-inhibitory activity of the hydrolysates which was more noticeable in the cases of horse mackerel and small-spotted catshark (Fig. 33).

The final hydrolysates of small-spotted catshark and horse mackerel hydrolysates exhibited the highest ACE-inhibitory activity with IC_{50} values ranging from 279 to 398 μg protein/mL. They were followed by axillary seabream and sardine hydrolysates with IC_{50} values varying from 375 to 489 μg protein/mL, whereas bogue hydrolysates presented the lowest ACE-inhibitory activity with IC_{50} values in the range of 637-768 μg protein/mL (Table 36). These results are in line with those reported for sardine muscle (IC_{50}=260 μg/mL) (Matsui et al., 1993); but they are considerable higher than those obtained by Ono et al. (2006) for hydrolysates of salmon (IC_{50}=38 μg/mL).

The superior ACE-inhibitory activity exhibited by the hydrolysates of small-spotted catshark may be due to its high collagen content, which is rich in proline. Small peptides containing proline at the C-terminal have been reported to play a significant role in the inhibition of ACE (Byun & Kim, 2001). In the case of horse mackerel hydrolysates, the high DH values obtained, which imply the presence of a higher number of small peptides, may be the reason for their strong antihypertensive activity. With regard to the enzymatic treatment, the simultaneous addition of subtilisin and trypsin resulted in the hydrolysates of horse mackerel and small-spotted catshark with the highest ACE-inhibitory activity. This finding was in agreement with the results obtained for mackerel and horse mackerel (*T. trachurus*) in chapter IX.

Thus, the horse mackerel and small-spotted catshark hydrolysates obtained with the simultaneous treatment were further fractionated by size-exclusion chromatography. For horse mackerel hydrolysate, fraction B (130-2350 Da) exhibited the highest ACE-inhibitory activity, with an IC_{50} value of 85 μg/mL (a 3.3-fold increase of activity compared with that of the hydrolysate). In the case of small-spotted catshark, fractions C (470-1210 Da) and D (58-470 Da) showed the lowest IC_{50} values, 72 and 27 μg/mL respectively (Table 37). The IC_{50} value obtained for fraction D (11.2 pufication-fold), is even lower than the one reported for a purified peptide from yellowfin sole (IC_{50} = 29 μg/mL) (Jung et al., 2006).

5. REFERENCES

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I. Recent Patents on the Up-Grading of Fish by-Products*

The fish and aquaculture activities produce an important volume of by-products due to discards and processing on board and in land. These residues (whole fish, fillet cuts, bones, heads, viscera and tails) require to be processed in order to not create a significant environmental problem and to maintain the economic viability of the fisheries sector. The present paper reviews a list of recent patents on fish by-products applications, specifically on the products with the highest added value, such as fish protein hydrolysates and fish oil. Initially, it is presented an assessment of the processes and techniques employed to obtain fish protein hydrolysates, which were classified by their utilization: ingredients for food, products for animal feeding, bioactive compounds, fertilizers, cosmetics and nutrient media for bacterial growth in specific industrial processes. Secondly, the different methods to extract the oil from the fish, to refine the fish oil avoiding undesirable taste and odor and to improve the stability of the mentioned oil, were surveyed. Finally, procedures for the microencapsulation of the oil obtained and its incorporation into food compositions were described.

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1. INTRODUCTION

Fishing is a prehistoric activity and has an essential role in the human society due to, among other aspects, its significant nutrients contribution to the diet.

According to the latest FAO data (FAO, 2008), the world fish production is 143.6 million tons from which 92 million tons corresponded to marine capture and 51.6 million tons corresponded to aquaculture. This total amount implies an increase of more than 8% with respect to the year 2000, acquiring the aquaculture an essential position (around 30 %) with an increasing rate per year of 6.9 %. Table 5 shows the previously commented evolution of the world fish catch and aquaculture production since 2002.

Regarding to the European Union, Spain is the country with the largest fish capture and aquaculture production, having the highest number of ships and fishermen. The Spanish contribution is close to 1 million tons, distributed in 757,799 tons as catch and 221,921 tons as aquaculture (SMAFF, 2006).

Table 5. Evolution of the world fish production since 2002 (FAO, 2008)

Year	Total Production (Million tons)	Aquaculture (Million tons)	Catch (Million tons)
2002	133.6	40.4	93.2
2003	133.2	42.7	90.5
2004	140.5	45.9	94.6
2005	142.7	48.5	94.2
2006	143.6	51.7	92

However, an important fraction of the whole catches is not used satisfactorily and three separated reasons can be stated: discards, leftovers on board and in land. Discards are defined by the FAO as “that proportion of the total organic material of animal origin in the catch, which is thrown away, or dumped at sea for whatever reason. It does not include plant material and post harvest waste such as offal. The discards may be dead or alive” (Kelleher, 2005).

According to this definition, discards may be organisms caught incidentally, such as finfish, crustaceans, molluscs, sea mammals and seabirds; and commercial species which

are not taken onshore due to its low commercial value, quota restriction or minimum landing sizes (EU, 2007). This practice reaches values of 7.3 million tonnes per year, around 8 % of the total catches (Kelleher, 2005), having negative consequences such as the waste of marine resources and causing an important ecological impact on the marine organism's food chain due to most of discards are dumped at sea dead or dying.

In this framework, international organisms such as the European Commission have proposed several measures in order to reduce discards such as the use of more selective fishing tackle and the introduction of discard regulations and bans. These measured have been incorporated into the national fishery regulations, e.g. recent regulations from the Spanish Ministry of Agriculture, Fishery and Foods. Nevertheless, taking into account that discards can be reduced but not eliminated, the following activities proposed by a FROM project (FROM, 2008) should be implemented:

a) The fitting-out of the ships, the fish ports and the fish markets in order to gain a good conservation of the by-catch.

b) The development of suitable industrial processes to up-grade the discards by its transformation into commercial landed products.

Furthermore, currently, a significant part of the fish captured is processed on board producing leftovers such as heads, guts, fish bones and skin. In the case of this fraction is returned into the sea, ecological (spill of organic matter), environmental (discharge of toxic compounds) and toxicological (parasites from viscera, i.e. Anisakis) problems may be generated (Blanco, et al., 2007).

In addition, an increasing amount of leftovers as consequence of the processing in land is being obtained. Due to the changes produced in the consumer's life style that prefers more elaborated frozen preparation and pre-cooked dishes, an increasing proportion of the total fish landings undergoes any processing before being consumed, generating a larger quantity of wastes (SMAFF, 2006).

The fish residues mentioned above require to be processed in order to not create a significant environmental problem and to maintain the economic viability of the fisheries sector (Gildberg, 2002). Most of these wastes are employed to produce fish meal, fish silage or fertilizer, which are products with a low economical value (Kim & Mendis, 2006). Nevertheless, according to recent studies, fish leftovers can be processed into up-graded products such as fish protein hydrolysates (FPH) and high quality fish oil which

contain a variety of biomolecules with potential health benefits. As a result, a higher profitability can be obtained with these bioactive compounds, encouraging also the full utilization of the fish raw material (Kristinsson, 2006).

In this regard, this paper reviews the different processes and techniques for fish protein hydrolysates and fish oil production through a list of patents issued during the past decades.

2. FISH PROTEIN HYDROLYSATES

High quality proteins, which may supply the essential amino acids required in a balanced nutrition, are presented in seafood wastes. Traditionally, acid or alkaline conditions have been used to hydrolyse these proteins (Bjarnason & Benediktsson, 2006). However, enzymatic hydrolysis has been found to present many advantages when compared to the conventional chemical hydrolysis, as shown in Table 6.

Table 6. Comparison between chemical and enzymatic hydrolysis. Adapted from Guerard (2006)

Process	Specificity	Advantages	Disadvantages
Acid/alkaline hydrolysis	Random process	• Fast reaction • Complete hydrolysis • Low cost • High solubility	• High temperatures • Molecular weight out of control • Large amount of salt • Undesirable side reactions
Enzymatic hydrolysis	Unique specificity of action of the enzyme(s)	• Control of the molecular weight • Mild reaction conditions • Attractive functional product characteristics (solubility, dispersibility, foaming, capacity and foam stability) • Control of the properties of the resulting products • Few side reactions • No destruction of amino acids • High nutritional value	• Cost of enzyme(s) • Subsequent deactivation of the enzyme(s) • Complex process

Nowadays, enzymatic hydrolysis is a well-established method employed to up-grade fish proteins and to produce specialty products from them such as fish protein hydrolysates, which may be used in many applications, especially where water-holding capacity, water solubility, emulsifying and foaming abilities are important (Shahidi, 2006).

By using this method, the fish proteins are hydrolysed by the addition of enzymes and then, they are separated from the rest of components with the aid of other processes such as filtration and centrifugation. Although many different processes have been reported to produce fish protein hydrolysates by enzymatic hydrolysis, according to Kristinsson (2006) all of them present the following general steps: mincing the raw material, homogenization in water, temperature equilibration, pH adjustment, enzyme addition, enzyme inactivation, cooling, recovery of protein and concentration/drying. In order to have a good control over the process and to obtain a final product of consistent and good quality, mainly, five factors need to be carefully considered: substrate concentration, enzyme-substrate ratio, pH, temperature and time (Adler-Nissen, 1986). These parameters are widely commented and explained in the first part of this paper for each patent studied.

The hydrolyzed proteins obtained have not only good functional properties but also retain the nutritional value of the original proteins and therefore, they are mainly used as ingredients for food and for animal feeding (Dambmann, 1995). However, the recent interest in fish protein hydrolysates has shifted to their bioactive properties and so, to their use as bioactive compounds (Kristinsson, 2006). Moreover, the fish proteins hydrolysates have other interesting applications such as cosmetics, pharmaceutical products and as nutrient media in specific industrial processes where bacterial growth is required.

Following, it is presented an assessment of the processes and techniques employed to obtain fish proteins hydrolysates classified by their different utilizations stated above. Nevertheless, this first part of the review is mainly focused on the three most important FPH applications: ingredients for food, products for animal feeding and bioactive compounds.

2.1 FPH as ingredients for food

Protein intake is quite important in humans for strength, immunity and general health (Donaldson & Lang, 2007). In that context, fish and fish by-products play an important

role due to the fact that they are significant sources of high quality proteins containing an excellent amino acid composition (Bjarnasson & Benediktsson, 2006).

By enzymatic hydrolysis, functional properties of fish proteins are improved, which is an imperative requirement when they are used as ingredients in food products. Functionality of food proteins is defined as: "those functional and chemical properties which affect the behavior of proteins in food systems during processing, storage, preparation and consumption" (Kinsella, 1976). Solubility is the most interesting functional property of fish protein hydrolysates since many other of their functional properties such as emulsification and foaming depend on it. As a consequence, solubility has a tremendous importance in FPH applications, determining their use (Kristinsson & Rasco, 2000).

Myofibrillar proteins comprise 65 to 75 wt% of the total protein in fish meat, being the primary food proteins of fish (Kristinsson & Rasco, 2000). While intact myofibrillar proteins are only highly soluble at high and low pH, fish protein hydrolysates differ from them in having a high solubility at a wide range of pH and ionic strength (Kristinsson, 2006). This is due to the fact that upon enzymatic hydrolysis the proteins are broken down into smaller peptides. It produces a reduction in the molecular weight and an increase in the number of polar groups which enables more water-protein interactions (Sathivel & Bechtel, 2006).

The stated high solubility of FPH at very different conditions is an advantage of great consequence because it allows adding effectively these FPH to food systems (Kristinsson & Rasco, 2006). In that issue, the embodiment 22 of the US patent 2002/0182290A1 (Munk, 2002) provides a process to treat a homogenized mixture at 55 ºC of water and thoroughly minced fish bodies such as salmon, sardine, mackerel and tuna with a protease giving a slurry. The mixture is prepared in a ratio 0.8/1 on weight basis for water/fish raw material respectively. This step is required in order to have a good mixing and to allow good access for the enzyme. In the invention, one gram of Protamex 1.5 MG^{TM}, a bacillus protease complex from Novozymes AS, per kilogram of fish raw material is added to the homogenized mixture at 55 ºC in order to produce the hydrolysis of the fish protein during 45 minutes. After that, the enzymes are deactivated by heating to 95 ºC during 15 minutes. The fish bones present in the slurry are removed by sieving and then, this slurry is divided into an oil phase, a water phase and a sediment phase by means of a continuous decanter. The water phase containing the hydrolysed fish proteins, after be concentrated and dried, can be injected into raw fish meat such as salmon fillets in order to improve their quality.

Having a good control over the five parameters which determine the degree of hydrolysis (substrate concentration, enzyme-substrate ratio, pH, temperature and time) and employing the suitable enzyme preparation are critical aspects to produce fish proteins hydrolysates with the appropriate properties required.

The degree of hydrolysis (DH) is defined as the percentage of the total peptide bonds which are cleaved in the course of the enzymatic hydrolysis (Adler-Nissen, 1986). Assessing the DH is an efficient method employed to measure the extent of the hydrolysis process resulting in an unequivocal determination of the FPH properties for a protein-enzyme system given (Kristinsson & Rasco, 2000).

Different methods have been employed in order to quantify the DH such as pH-stat technique, osmometer technique, tricholoroacetic (TCA) method and trinitrobenzenesulfonic acid (TNBS) method. Among them, the pH-stat technique is the most useful for industrial applications, although it is only suitable under alkaline conditions. Despite the osmometer technique is a reliable method, it is not frequently utilized. On the other hand, the TCA and the TNBS methods are quite convenient when the working pH is in the range of 3-7, where the pH-stat technique is useless (Kristinsson & Rasco, 2000).

Regarding to the enzyme preparation, as stated above, its choice is a quite crucial step when producing fish protein hydrolysates. Usually, commercial enzymes are employed due to the fact that they allow a good control over the hydrolysis process such as in the mentioned patent US2002/0182290A1 (Munk, 2002). These marketable preparations may contain enzymes with endopeptidase activity, in order to obtain many peptides but relatively few free amino acids, enzymes with exopeptidase activity when the opposite result is required, many free amino acids an few large peptides, or a combination of both types of enzymes (Kristinsson, 2006).

On the other hand, as a consequence of the resulting high prices of the added or also called exogenous enzymes, proteases present in fish viscera may be utilized to carry out the hydrolysis. In this regard, the patent US 2005/0244567A1 (Carlsson, 2005) discloses a continuous method to produce fish protein hydrolysates with low fat content utilizing endogenous enzymes from cold-blooded fish species. The raw material, fish offal and by-products from the fishing industry, requires being grinded in order to increase the working surface and to release the endogenous enzymes contained in it. Before the mixture obtained

is sent to the hydrolysis reactor, the oil is removed by centrifugation reducing considerably the fat content. In the reactor, under vigorous stirring, the optimal conditions for the enzymation are kept constant: the pH is adjusted to 7.6-8.2 by adding water containing calcium and the temperature is increased up to 45 – 48 ºC. After the enzymes have been deactivated by heating at 75 ºC, the solids and the remained oil are separated from the hydrolysate using a screen device followed by a three-phase decanter. In addition, the inventor proposes to apply an UV method after the deactivation of the enzymes in order to prevent the growth of microorganisms. Finally, previous to the concentration by vacuum distillation at 65-70 ºC and to the spray-drying process required to make the final product more storage-stable, the hydrolysate is pumped through a membrane filter which allows the isolation of peptides with a size smaller than 10,000 Daltons. The filter used in the invention has a longer life, a lower running cost and a higher efficiency compared with traditional filtering processes employed in this field; it is due to the fact that the enzymes are retained in the filter causing the breakdown of non-filtered proteins and peptides. The FPH produced can be used for human consumption either as an additive or as an independent product.

In the same context, the patent WO 03/066665A2 (Pyntikow et al., 2003) describes the use of endogenous enzymes from the guts and entrails of cold water fish species to manufacture FPH in large scale. As in the patent [20], the raw material (salmon, mackerel or trout leftovers) is grinded and mixed with the enzyme in a ratio 1:1 by weight. Deionized water is added into the bioreactor, the temperature is set to 45 ºC, and an alkaline pH is adjusted by the addition of NaOH. After 3 hours, the hydrolysis has been carried out, and the enzyme is deactivated by heating at 95 ºC during 10 minutes, then, the mixture is cooled up to 60 ºC. The aqueous phase formed is separated by centrifugation and it is further refined. This inventor, contrary to the membrane filter utilized in the patent US 2005/0244567A1 (Carlsson, 2005), proposes to carry out, in two different tanks, an acid and an alkaline denaturation to maximize the removal of unwanted by-products of the hydrolysis reaction. Firstly, with the acid denaturation, where 50 % phosphoric acid is employed to reduce the pH up to 3.5, the high molecular weight peptides (glycoproteides, lipoproteides and nucleoproteides) are removed from the hydrolysate after heating at 95 ºC during 10 minutes and later centrifugation. Secondly, employing a 30 % $Ca(OH)_2$ solution, the pH is increased up to 8.5 and after heating and centrifugation a large fraction comprising peptides of molecular weights between 30,000 Da and 5,000 Da is separated.

The resulting product, after be concentrated and dried, can be used directly for human consumption or can be fractionated in its different nutrients (vitamins, minerals, amino acids and peptides) applying different techniques such as ultrafiltration or countercurrent extraction utilizing an organic phosphorous acid. In addition, this invention discloses a methodical system to control the overall process, including interlocks for safety and system shutdown.

Another example, patented as US 2004/0203134A1 (Pyntikow et al., 2004), discloses the production of FPH by enzymatic hydrolysis employing endogenous enzymes from cold water fish. The FPH obtained, containing 70-90 wt% free amino acids, 10-20 wt% high molecular weight peptides and 3-5 wt% vitamins, minerals and oils, may be incorporated to some specific food products, especially for feeding children and adolescents, in order to compensate their deficiency of essential amino acids, vitamins and trace elements. Moreover, these FPH might be used as food for elderly people which cannot develop a complete digestion. It is due to the fact that FPH food products are absorbed from the gastrointestinal tract directly into the blood without the requirement of a previous digestion.

As mentioned before, endogenous enzymes are mainly obtained from cold water fish species. These species are preferred due to the higher robustness of their fermentative system, which is forced to trigger proteolysis at a lower temperature. As a consequence, when using this type of enzymes, the hydrolysis process in the bioreactor may be carried out under mild conditions of pH and temperature enabling the obtaining of final products with enhanced nutritional properties. Therefore, this high activity and the low cost of these endogenous enzymes are two quite interesting reasons to consider when choosing the enzyme preparation. However, enzymatic procedures using fish endogenous proteases cannot be efficiently controlled. This is due to the fact that the visceral material can vary substantially in activity and enzyme levels depending on the fish species, the season of the year and other factors (Kristinsson, 2006). This problem has a tremendous significance when producing FPH for food use where the consistency of the final product is of great importance.

One general problem which arises in the FPH production and which considerably constraint their incorporation into food products is the development of a bitter taste during the enzymatic hydrolysis process (Adler-Nissen, 1986). This phenomenon is explained due to the cleavage of proteins at amino acids with hydrophobic side chains, resulting in the

formation of peptides with exposed hydrophobic side chains, which are typically concealed in the interior of proteins and longer peptides due to their tertiary structure (Adler-Nissen, 1986). A variable affecting the extent of bitterness is the degree of hydrolysis, leading to less bitterness at limited and extended hydrolysis. Another factor to be taken into account is the enzyme used. Enzyme preparations having specific activity will be required in order to limit the development of bitterness, enabling at the same time the production of FPH with good functional properties. In that issue, the patent EP 0518999B1 (Dambmann, 1995), reveals a process to obtain a fish protein hydrolysate with non bitter-taste by the use of a protease produced by a strain of *Bacillus Licheniformis*, which is specific for glutamic and aspartic acid residues. This enzyme is capable of providing a satisfactory limited hydrolysis of protein due to the formation of hydrophilic peptides with C-terminal glutamic or aspartic acid residues. This fact results in a final hydrolysate with improved palatability which can be incorporated, until a maximum of 30 % by weight, into a food product. Another embodiment of the present invention describes the possibility to carry out a second hydrolysis with a selective enzyme to hydrophilic amino acids such as trypsin which specifically cleaves peptide bonds at lysine and arginine residues. The product obtained may be used as protein enrichment for beverages.

In the same regard, the patent US 2006/7070953B1 (Bjarnason & Benediktsson, 2006) provides a no continuous process to produce a fish protein hydrolysate from fish and seafood by-products where a proteolytic composition derived from the intestines of cold water fishes, such as cod, is employed. This enzyme preparation, comprising trypsin, chymotripsin, elastase, aminopeptidase and carboxypeptidase, cleaves specific amino acids bonds releasing hydrophilic peptides which results in final hydrolysates deprived of bitterness. Moreover, due to the high activity of these proteases at low temperature, the hydrolysis can be carried out at mild conditions, 15-20 ºC, enabling the maintenance of flavor and odor from the original material in the end product. As consequence, the FPH produced is quite useful when preparing fish soups and sauces.

Another example aiming to improve the palatability of the resulting FPH is patented as WO 2007/086762 A1 (Donaldson & Lang, 2007). In this invention a pasteurized protein rich liquid extract is obtained by forming an aqueous slurry containing the fish protein source and hydrolyzing the fish protein with an enzyme complex extracted from kiwifruit. This enzyme preparation is composed of thiol cysteine proteases being commercially produced under the brand name of Proactinase by Vital Food Processors, Limited of

Auckland, New Zealand. The hydrolysis is carried out at a temperature between 30-35 ºC for at least six hours and then the mixture is heated to 60 ºC to deactivate the enzyme. Then the pasteurized hydrolysate, which has a high degree of digestibility, is quite suitable to be utilized as ingredient in foods.

A different method to debitter FPH consists in the application of a second hydrolysis with exopeptidases enabling the conversion of bitter peptides to flavour-neutral substances. In view of the foregoing, the patent EP 0406598 A1 (Kwon & Vadehra, 1991) provides a batch process to debitter an enzymatically hydrolysed protein comprising the incubation of a slurry of the FPH with a culture of a food grade microorganism, preferably *Lactobacillus helveticus*, which is capable of producing dipeptidases, tripeptidases, aminopeptidases and carboxypeptidases which hydrolyse the bitter tasting peptides to substances with a pleasant flavour. To be efficient, the slurry should have a solid concentration in the range of 10-30 % by weight and the food microorganism ought to contain 10^4-10^8 cell/g. The hydrolysis is carried out under agitation during at least 12 hours at a temperature between 35-50 ºC and at a pH of 6. After deactivation of the enzyme at 70 ºC, the resultant product is cooled and dried being ready for its incorporation into foods.

Finally, the last factor that should be taken into consideration when producing FPH intended for human consumption is the presence of collagen which comes from the fish skin and bones. The hydrolysate containing collagen may result too viscous hampering that the evaporation stage could be carried out efficiently. This fact does not allow the reduction in water content of the end product limiting therefore, its storage and usefulness. In order to solve this problem, the patent US 2009/0123965A1 (Wahl, 2009) discloses a batch process where, after the aqueous phase containing the hydrolysed protein obtained by enzymatic hydrolysis has been separated from the oil and the solid phases, the water soluble proteins undergo a quick cooling up to 20-22 ºC. At this temperature and without agitation, the collagen precipitates at the bottom of the specific clearing sump where the operation is carried out. The remaining water soluble proteins are drained out from the sump and sent to the concentration and evaporation stages after which, the dry matter content of the FPH may be increased up to a 60 wt% making more suitable the product for storage and shipping. The collagen is removed from the sump after be converted into liquid by heating at 65 ºC and may be used for other purposes. Usually the collagen is employed as raw material to produce gelatine. Due to the fact that the gelatine has a very low nutritive value due to it lacks of the essential amino acid Trp, it is mainly used as

functional ingredient rather than nutritional component in food applications (Regenstein & Zhou, 2006). In this regard, the patent US 2005/0124034 A1 (Hiroyuki & Takayuki, 2005) provides a method to produce gelatine peptides free of odour and with a light colour from fish skin, specially skin from Alaskan pollack and Pacific cod. The process comprises the steps of washing the fish skin with 0.5-2 wt% salt water and obtaining an extract from the fish skin using fresh water in a neutral pH at 50-100 ºC. The extract is filtered for the removal of insoluble material and further digests with an endoprotease enzyme. The resulting product is concentrated under a reduced pressure, treated with carbon activated and dried by the spray-drying technique. Such gelatine hydrolysates obtained may be used in beverages where a reduced gelling tendency is required, even at low temperature.

2.2 FPH for animal feeding

Protein is an essential component in any feed preparation, which also should content other ingredients such as lipids, carbohydrates, salts, minerals and vitamins (Pedersen & Standal, 2006). Fish protein has been utilized historically, often in the form of fish meal, for feeding many different kind of animals such as fish, pigs, poultry, cattle and fur animals due to its high essential amino acids content, among other aspects (Bechtel, 2006). However, fish meal, which is obtained from fish materials by a heating, pressing, separation and drying processes, is not the best suited protein form for the uptake of the animal. To solve this disadvantage, FPH are being used as replacements of fish meal and any other sources of protein due to the fact that they enable a better protein uptake by the animal enhancing therefore, its growth. As known, fish protein hydrolysates have a high level of digestibility due to their considerable content of peptides and free amino acids which is of great importance in feed composition, especially for very young animals whose digestive system is not fully developed (Bechtel, 2006).

With regards to this issue, the patent US 2008/0020097 A1 (Pedersen & Standal, 2006) describes a batch process to produce FPH from whole fish, heads, backbones and intestines from salmon, trout, pollack and herring in order to replace fish meal in feed compositions. The raw material utilised is mixed with water in the ratio 1:1 by weight and the mixture is heated in a reactor with agitation to 55 ºC. The hydrolysis reaction is carried out for 45 minutes employing Protamex^{TM}, a mixture of a neutral protease and an alkaline protease available from Novozymes A/S, which is added in a ratio 1:1000 by weight. A degree of hydrolysis between 15-20 % is reached. After deactivation of the enzyme by heating at 95

ºC for 15 minutes, the bone material is removed by sieving. Following, the three phases obtained in the hydrolysis process, aqueous, solid and oil phases, are separated using a three phase decanter such as Wesffalia SC35. Then the protein fraction is evaporated using a falling film evaporator, Niro Atomizer FF 150, and spray dried using a Niro Atomizer P-6 spray drier. The FPH obtained may be incorporated into a feed composition replacing the fish meal and might constitute the 2-16 wt% of its dry matter. This fact causes an increase in the growth rate of animals as it has been tested by the inventors in a study with salmon fish.

In order to produce FPH for the same application, feeding animals, the patent US 1990/4,976,973 (Yoichi et al., 1990) discloses an improved method where the partially hydrolysed proteins of the FPH obtained scarcely undergo thermal denaturalization. Commercial species such as sardine, mackerel, herring and alaska pollack are used as starting material, mixed with protease enzymes such as trypsin, papain and aminopeptidases which enable carrying out the hydrolysis reaction under at low temperature, 45-50 ºC. Although the FPH produced has a similar composition to the ones manufactured by other conventional methods, it is obtained from a milder procedure which minimizes the nutritive losses.

In addition, an improvement in the quality of the end product is achieved when incorporating into the FPH manufacturing process an ultrafiltration (UF) and nanofiltration (NF) stages as described in the patent US 2008/0020097A1 (Torp & Torrissen, 2008). In this invention, by combining UF and NF, the removal of minerals, especially sodium and chloride, non protein nitrogen compounds and other not desired products from enzymatic degradation is carried out. Moreover, more than 70 % of the water contained in the aqueous phase obtained after the hydrolysis reaction is removed in the UF and NF stages. This results in significant energy savings up to 50 % of the costs of standard evaporation. The FPH produced is intended as a feed product especially for porcine and bovine (dairy cow) species but it may be fed all types of animals, such as domesticated feline and canine species.

2.3 FPH bioactive compounds

Recent studies on fish protein hydrolysates have demonstrated that they may provide significant physiological benefits to living systems, including humans (Kristinsson, 2006). Some of the FPH positive effects on humans such as the reduction of plasma cholesterol,

plasma homocysteine and hepatic triacylglycerols concentration are stated in the patent US 2007/0142274A1 (Rolf, 2007). In this invention, it is provided a method to produce FPH from salmon by-products having a preventive and therapeutic effect on stenosis, atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke and fatty liver. The hydrolysis of the flesh remnants on salmon bone frames is carried out at 55 ºC and a pH of 6.5 during 60 minutes employing Protamex^{TM} as enzyme. After the deactivation of the enzyme complex, the aqueous fraction, free of bones, is separated from the oil and the solid phase in a three-phase decanter and then, it is concentrated by using a falling film evaporator. Finally, this concentrate phase containing the soluble proteins is filtered through an ultramembrane with a nominal molecular weight limit of 10,000 Da and the permeate obtained is spray-dried in a Niro Atomizer. The FPH produced containing proteins in the range of 70-90 wt% may be commercialized as pharmaceutical or as a functional food.

In the last decade, a great interest has been developed in the potential use of FPH to lower the blood pressure by inhibition of Angiotensin-I converting enzyme (Kristinsson, 2006). Angiotensin-I converting enzyme (ACE) catalyzes the cleavage of inactive angiotensin-I into an active vasoconstrictor, angiotensin-II. Besides, ACE also catalyzes the degradation of the vasodilator, bradykinin. These two facts cause an increase in the blood pressure which can be avoided by the use of an ACE inhibitor such as some peptides contained in FPH. Therefore, FPH are clinically useful and may be employed for the prevention and treatment of hypertension. In that issue, the patent US 2007/007179793 B2 (Ewart et al., 2007) describes a batch process to produce this type of bioactive FPH from salmon leftovers which comprise at least one peptide from the group consisting of: Leu-Ala-Phe, Leu-Thr-Phe, Ile-Ile-Phe, Leu-Ala-Tyr, Ile-Ala-Tyr, Val-Phe-Tyr, Tyr-ala-Tyr, Val-Leu-Trp, Ile-Ala-Trp, Tyr-Ala-Leu and Tyr-Asn-Arg. The salmon frames are de-boned and grinded and then, water is added in a ratio 1:1 by weight. A previous denaturing of the endogenous enzymes is carried by heating at 70 ºC for 10 minutes. In order to carry out the hydrolysis, a type of bacillolysin, Protease S Amano obtainable from Amano Enzyme USA Company Limited, is employed in a ratio of 2.6 % w/w. This type of enzyme preferentially catalyzes the hydrolysis of peptide bonds on the C-terminal side. The Protease S Amano optimally works at 50 ºC and a pH of 8 so, maintaining these conditions during 7 hours the degree of hydrolysis achieved is about 17 %. After deactivation of the enzyme by heating and separation of the aqueous fraction from the oil and solid phases formed during the

hydrolysis by centrifugation, the aqueous phase is further processed by ethanol precipitation, ultrafiltration or reverse-phase chromatography. This is a crucial step because high molecular weight peptides and protein fragments are removed which improves the effectiveness of the end product. Finally, the protein soluble fraction is concentrated with a rotary evaporator and spray-dried to yield a powdered protein hydrolysate. The obtained FPH has been demonstrated to be a potent inhibitor of ACE by determination of the IC_{50} value, and to reduce mean blood pressure in spontaneous hypertensive rats. This end product may be used in a variety of products such as pharmaceutical or nutraceutical products, dietary supplements, nutritional supplements, food ingredients and beverages.

In the same context, the patent EP1092724B1 (Hiroyuki, 2008) is also devoted to the production of peptides with ACE inhibitory activity, starting from different raw material (mackerel, sardine, cod and bonito by-products) and employing thermolysin as enzyme. The hydrolysis is carried out at 60 ºC and a pH 7 with an E/S ratio 1 wt%. As in the previous patent, the inventor proposes to filter the aqueous fraction through an ultramembrane with a fractionation molecular weight of about 3,000 Da. This is due to the fact that it is not desirable to have a concentration higher than 5 wt% of peptides with a molecular weight at least of 5,000 Da in the final product because they give a bitter taste, a brown/yellow hue and they do not enhance the ACE inhibition. Therefore, the peptides obtained by this procedure, having a good color, no bitterness and a nice after taste, can be incorporated into medical supplies, foods and special foods for health care.

Another process to produce ACE inhibitor peptides from bonito leftovers utilizing thermolysin has been patented as US 2003/0170373 A1 (Le Blanc, 2003)). A further advantage of the obtained hydrolysate, katsuobushi oligopeptide, is that it enhances the salty taste of sodium chloride and may be used to replace salt in foods and beverages which also contributes to reduce the blood pressure.

Furthermore, antioxidant peptides have been obtained from fish by-product such as capelin, mackerel (Kristinsson, 2006), yelowfin sole (Jung et al., 2004) and others. The term antioxidant is defined as “any substance that, when present at low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate” (Park et al., 2001). Antioxidant FPH control peroxidative damage in food and physiological systems by the scavenging of radicals formed during peroxidation and chelate transition metals, which are potent prooxidants. In this sense and considering the

several hazard caused by synthetic antioxidants such as 3-ter-butyl-4-hydroxyanisole (BHA), 3,5-di-tert-butyl-4-hydroxytoluene (BHT), tertiary-butylhydroxyquinone and propyl galate, the production and purification of safer antioxidant hydrolysates from marine leftovers is becoming a promising research area (Guerard et al., 2005). In that context, the patent WO 2004/071202 A1 (Hagen & Sandness, 2004) provides a process to produce FPH from salmon bones off-cut which control the peroxidative damage in foods. The raw material is homogenized with water in a ratio 1:1 by weight. The temperature of the mixture is raised up to 60 ºC prior to the addition of the enzyme preparation (Protamex^{TM} in a ratio 1:1000 based on weight) and the hydrolysis is conducted during 45 minutes. Following, the protease is deactivated by heating at 95 ºC for 15 minutes and the aqueous fraction is separated from the rest of phases formed during the hydrolysis. Finally, the obtained aqueous fraction is concentrated by evaporation up to a protein content of 40 wt%. Several test (TBARS method) carried out by the inventor demonstrate that when injecting the FPH produced to fish meat its rancidity is surprisingly reduced. As result, the use of conventional antioxidants in fish meat as an additive may be considerably diminished. Besides, the redness, lightness, yellowness and freshness of the fish meat are improved.

2.4 Other applications

Other applications for the FPH, different from their use as ingredients in feedstuffs or functional food products are covered in this section. Firstly, FPH may be also used as fertilizers for crops due to the fact that they are an excellent source of nitrogen because of its amino acid and peptide content. This nitrogen is easily adsorbed and utilized for the plants (Kristinsson, 2006). Regarding to that, the example three of the patent US 0203134 A1 (Pyntikow et al., 2004) considers the improving of potassium polyphosphate fertilizers effectiveness by the incorporation of FPH in the manufacturing process.

Another potential utilization for FPH is based on their incorporation to cosmetic creams and lotions. The components present in the FPH such as peptides, vitamins, amino acids cause a local nourishment for the skin cells which may prevent negative effects on human skin from natural and artificial environmental factors. Also patent US 0203134 A1 (Pyntikow et al., 2004) mentions the possibility of incorporating the FPH produced by its process into some cosmetics creams such as a shampoo.

Finally, FPH may be employed as a specialty growth media for microorganisms because of its free amino acids, vitamins and minerals content. According to the patent US 0203134 A1 (Pyntikow et al., 2004), biological industrial processes such as the production of methane from coal, the production, extraction and fermentation of natural gas and the manufacturing of pharmaceuticals can be enhanced by the addition of FPH into the reproduction phase, growth phase, and/or decline phase of the responsible microorganisms.

3. FISH OIL

The fat content of fish varies from 2-30 wt% depending on the type of species, diet, season, environment, and geographic variations. Composition of fish oil is different from that of other oils, owed to its unique content of unsaturated fatty acids with the first double bond between the third and fourth carbon atom, the so-called and mainly omega-3, also sometimes marked as n-3 fatty acids. In fish oil, the major omega-3 fatty acids are eicosapentaenoic acid (C20:5n-3, commonly called EPA) and docosahexaenoic acid (C22:6n-3, commonly called DHA). Both have been reported to promote several benefits on human health (Rubin & Rubin, 1991; Schroeder & Muffett, 1990a; Mathisen, 2009a). Among the properties of omega-3 fatty acids the best known are prevention of atherosclerosis, reduction of blood pressure and protection against arrhythmias, which are extensively reported in literature. However, only plants can synthesize fatty acids in the omega-6 and omega-3 series. Animal depend on getting these essential fatty acids through their diet, and a minimal intake of these compounds is essential for normal growth, health and reproduction.

According to the data reported by the IFFO for 2006 (Dumay, 2006), 87 % of fish oil production is intended for its use as feed ingredient in aquaculture, since fish oil is essential for the production of farmed carnivorous fish, particularly species such as salmonids and shrimp. On the basis of a global survey conducted between December 2006 and October 2007 (Tacon & Metian, 2008) concerning the use of fish meal and fish oil within compound aquafeeds over 50 countries, it is estimated that in 2006 the aquaculture sector consumed 835 thousand tons of fish oil. The top consumers of fish oil in 2006 were marine shrimp, followed by marine fish, salmon, Chinese carps, trout, and other freshwater crustaceans and fishes. Results from several studies (Tacon & Hasan, 2006) suggest that the use of fish meal and fish oil in compound aquafeeds will decrease in the long term,

estimating a decrease of 44.5 % for fish meal and 15.5 % for fish oil in the period from 2005 to 2020, owed to a combination of a decreasing market availability of fish meal and fish oil from capture fisheries, increasing market cost for these finite commodities and increased global use of cheaper plant and animal alternative protein and lipid sources.

Other usages for fish oil are their incorporation to feedstuffs for human or pet consumption and their industrial processing (mainly for pharmaceutical purposes). Although these applications provide final products with higher added value, they only account for 13 % of global fish oil production. The use of fish oil for human consumption calls for improved stabilization and refining techniques since the fish oil is highly susceptible to oxidation and the final products containing fish oil may develop unpleasant fishy odor, which limits its acceptability by consumers.

Following these general considerations, this section will cover the following tasks:

a) Processing aspects and innovations regarding fish oil extraction.

b) Refining of the oily fractions in order to remove undesirable odorous and oxidation-promoting substances from the crude oil.

c) Stabilization of the refined oil prior to its incorporation to food products to slow down oxidation processes and generation of unpleasant odors and flavors.

d) Use of fish oil as bioactive compound (e.g. antihypertensive, anti-inflammatory) making up the formulation of feed ingredients or processed to be consumed as pharmacological principle (e.g. in the form of fish oil capsules).

3.1 Fish oil extraction process

Fish oil can be produced by several methods, including high-speed centrifuge fractionation, low temperature solvent fractionation, supercritical fluid extraction and the so-called wet reduction method.

3.1.1 Wet reduction method

The latter was the first method developed for obtaining both fish meal and fish oil, and it continues to be the common procedure for manufacturing the bulk of the world's fish meal and oil. This method involves the cooking of the raw material in order to coagulate the proteins, which are easily separated from the liquid fraction by a pressing operation by means of a screw press. This operation yields a partially dewatered cake, containing 60-80

wt% of the dry matter and a liquid phase, which is decanted and separated into their constitutive phase, recovering the fish oil which is refined to remove any remaining impurities.

Earlier patents based on this method were focused on improving the recovery of soluble protein from the press liquor (Vincent, 1976) and the dewatered cake, rather than improving the yield and quality of the fish oil. For economic reason, such continuous plants should be designed for production capacities in the range of 200-3,000 tons/day of raw fish to maintain the viability of the process. Likewise, the fish oil recovery step should only be considered for fatty species, such as menhaden or sardine.

In that context, a simplified process was proposed able to treat smaller production volumes, where the previously milled fish is charged into an indirect heat exchanger equipped for heating, stirring and steam removal (Bladh, 1982a). The oily fraction is removed by attaching the heat exchanger to a three-phase decanter which starts once the fish material has reached the protein coagulation temperature (between 60-100 ºC). The main advantages of these procedures with respect to the traditional wet steam method are their availability for lower feed inputs and the lower energetic costs thanks to a better energy utilization.

A slight modification of this procedure is proposed in the US patent 4335146 (Bladh, 1982b), where the raw material undergoes an initial cooking step followed by a phase separation where the oily phase is recovered and the resulting sludge and stickwater are mixed and transferred to an evaporation unit where the suspension will attain a final dry content in the range of 50-80 wt%. The initial heating step can be carried out in a boiler, which requires longer residence times so the temperature should not exceed 55-60 ºC or a plug-flow indirect exchanger where the temperature can be risen up to 70-90 ºC during a shorter residence time (0.5 – 3 minutes), analogously to a pasteurization treatment. Both time-temperature combinations entail minimum detrimental in the nutritional quality of fish proteins and fish oil. After the heat treatment, the suspension is de-oiled by means of a three phase decanter or a modified centrifuge where an upstream oily phase is obtained, which is discharged in a storage tank for a further refining operation, and the stickwater and sludge are discharged through a common outlet or remixed after the centrifuge separation to form a suspension which is conveyed to the evaporating plant. It consists of a vertical heat exchanger tube provided with steam supply and rotating scrapers for the separation of the dried product. In order to fulfill the standards for the dried fish meal, in

terms of moisture content and protein quality, the residence time in the evaporation zone should be in the range of 2 – 6 minutes. This patent proposes a modification where the evaporation is carried out under vacuum, which results in minimal loses of nutritional quality since the fish mass is brought to a temperature not exceeding 60º C.

Although the two above mentioned methods permit the production of fish oil, they are designed with the aim of providing a high protein quality to the final fish meal, focusing on a better energy utilization and a minimal protein de-naturation. US patent 6214396 B1 (Barrier & Rousseau, 2001) describes a milder procedure with special care to the final quality of the recovered fish oil. The starting material for this procedure is a de-headed and eviscerated fish, which is fed into a flesh press. This unit consists in an endless screw and sieve able to retain the bones and the skin, while the fish flesh is discharged into a mixer where it is homogenized with cold water (below 15 ºC) in a proportion between 10 to 40 wt% depending on the viscosity of the final mixture. This mass is subsequently transferred to a decanter where the oily phase is separated from a de-oiled flesh (with a content of oil below 3 wt%.) which is conveyed to the packaging line. With regard to the fish oil, it is stored in a silo under inert atmosphere provided with liquid nitrogen in order to minimize lipid oxidation before being refined by passing through a rotary brush sieve and a final 2-phase centrifuge for removing sludges from the fish oil.

Driven by the positive health benefits provided by the polyunsaturated fatty acids contained in the fish oil, the US patent 6190715 B1 (Crowther et al., 2011) proposes a modified wet steam method able to obtain both fish meal and fish oil with minimal nutritive and organoleptical loses. Untreated fish oils and more specifically fish oils rich in omega-3 fatty acids develop an unsavory odor and flavor which can be initiated by lipid peroxidation catalyzed by enzymatic activity, such as lipoxygenase, peroxidase and ciclooxigenase. This lipid enzymatic degradation is also responsible for the diminution in the content of omega-3 fatty acids, and this process is especially inherent of fish oil from menhaden, salmon, sardine, anchovy and cod. The proposed procedure starts from a pressing operation to release the press liquor and includes an intermediary enzyme deactivation operation with the aim of slowing down the apparition of unsavory taste and smell in the fish oil. Enzyme deactivation is attained by addition of 85 % food grade phosphoric acid to the press liquor, followed by centrifugation where the fish oil is removed and the acid is recovered for a subsequent use.

3.1.2 Solvent extraction.

In solvent extraction, the raw material is treated with a suitable solvent, usually the lower carbon alkanes, at elevated temperatures and pressures. The resulting oil/solvent emulsion is then fractionated to recover the valuable phase and recycle the solvent for a further utilization. The bulk of the oil extraction processes in commercial use today employ hexane or heptane as solvents, commonly applied to the extraction of oil from plant materials (oil-seeds, cereal brans, fruits, beans and nuts). Despite its commercial success, conventional solvent extraction with organic liquid solvents has shown to be inefficient to treat raw materials with a high content in oil. In these cases, the solvents are not saturated in oil when leaving the extractor as a result of the slow kinetics of extraction involved, demanding a large consumption of solvent per unit of extracted oil (Blasco-Piquer & Mira-Ferri, 1999). Furthermore, after leaving the extraction unit the solvent must be thoroughly removed from the extracted oil in order to meet with the sanitary regulations (less than 3 ppm of solvent per unit mass of refined oil). This removal can only be achieved by distillation, increasing the energy consumption for the whole process. In the field of seafood products, conventional extraction has not been successfully implemented (Franke, 2001).

Solvent extraction has been successfully employed for the recovery of phospholipids from fish viscera (Hiratsuka et al., 2007). This process can provide a better utilization for the viscera from fatty fish species, such as the sardine, tuna or bonito, which are an available source for these lipids, comprising large percentages of polyunsaturated fatty acids such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. The raw material must be fresh or previously frozen, in order to minimize the phospholipid decomposition by the phospholipase enzymes naturally present in the fish viscera. The preserved raw material is heated by water bath or steam during 10 to 90 minutes and temperatures between 60 to 100 ºC in order to deactivate the catabolic enzymes of phospholipids present in the fish viscera and eliminate the *Bacilli colli* microorganisms, in response to the sanitary regulations. Higher temperatures or treatment times are not desirable to avoid phospholipid thermal denaturation. After the heat treatment, the lipid content is extracted with an organic solvent, such as ethanol, methanol, propanol, ethyleter, hexane or chloroform, in order to obtain an extract comprising a lipid mixture containing phospholipids and valuable amounts of DHA and EPA fatty acids. After removal of the remaining solvent of the extract stream, the separation of the phospholipidic fractions can be performed by

chromatography. Two DHA-bonded phospholipids are of interest, the phosphatidylserine, which has proved to have beneficial effects against brain disorders and the phosphatidylethanolamine, which exhibits antioxidant effects.

3.1.3 Supercritical extraction

A large improvement to the conventional solvent extraction was provided by the supercritical extraction technology. As mentioned above, lipid extraction with organic solvents requires high solvent consumption and long times of contact with the oil-bearing material owing to the small solubility of the extraction agents. The kinetics of extraction is improved by increasing the temperature, which results in lipid denaturation and loss of functional properties. Supercritical fluid extraction relies on the utilization of solvents, which are under gas form at room conditions, but are brought above their critical values of pressure and temperature. They are drawing an increasing attention since they present the following advantages with respect to other solvent or physical separation processes:

a) Higher solubility. Above critical conditions, the solvents increase considerably their solvant power. This property reduces the time of contact between the solvent and solute and thus the amount of solvent per unit of extract.

b) Selectivity. Small changes in the pressure and temperature of the supercritical fluid cause large variations in their density and other physical properties, which render these fluids suitable for solvent fractionation, due to their high selectivity.

SCF extraction has been particularly useful for obtaining aromatic and lipid components from plant tissues. A first attempt to extend this technique to animal tissues was reported (Kamarei, 1988), where a three-step supercritical extraction was performed on several animal tissues in order to recover different fractions (e.g. lipids, proteins, vitamins and flavors). Although this method can be applied to several animal materials, the patent does not contain any reference to fish tissues.

The supercritical extraction of fish oil may be worthwhile in tissues where its low content makes unviable its recuperation by other traditional methods. The extraction of fish oil from fishmeal was issue of a European Patent Application, EP 0917876 A1 (Blasco-Piquer & Mira-Ferrer, 1999) where 200 g of fishmeal where treated with the solvent mixture comprising 170 g. of hexane and 60 g of CO_2 at 250 bar and 50 ºC. After extraction and subsequent concentration of the extract by vacuum evaporation, 13 g of fish oil were obtained, containing 8 wt% of EPA and 8 wt% of DHA.

Based on the high selectivity of supercritical fluids, this technique can be employed to concentrate the polyunsaturated fatty acid moiety in fish oil (Stout & Spinelli, 1987). To this purpose, the fish oil was previously transesterified with a lower alkanol to form a mixture of lower alkyl fatty acid esters which are subsequently separated by supercritical CO_2 fractionation. Since lower molecular weight components are more soluble in supercritical CO_2 than higher molecular weight fractions, fatty acids between 20 and 22 carbon atoms (containing 19-23 wt% EPA) tend to remain in the raffinate, while extracts will be rich in lower molecular species below 18 carbon atoms.

3.1.4 Enzymatic extraction

Among the different methods able to recover oil from fish proteinaceous materials, enzymatic extraction is a promising technology which can be performed at mild conditions implying lower operational costs and minimal heat denaturation. Early patents reporting this technology are found in 1962 (Ehlert, 1962) where high quality oil is extracted from fish or fish offal via enzyme proteolysis. The raw material is previously mechanically disintegrated to form a homogeneous pulp which was transferred to the reactor vessel, where it is mixed with the substrate of a fermented dead culture of lactic acid bacterial. Such a substrate contains proteolytic enzymes which are not present in fresh cultures containing living bacteria, but they are released after the final fermentation and death of the cells. These enzymes start a quick hydrolysis of the proteins, thus facilitating the release of oil from the adipose tissue. After 3 hours at a temperature between 20 to 50 ºC, the liquefied mixture is separated by centrifugation in order to recover the oily fraction and a sludge which can be subsequently dried-atomized to obtain a protein-rich powder of high nutritional value.

Traditional methods to obtain the so-called fish meat extracts employ a preliminary disintegration step to finely divide the raw material, prior to being transferred to the reaction vessel. This step requires great labor power and promotes the release and activation of the autolytic enzymes present in fish viscera, which are responsible for the decomposition of fats to low-molecular weight fatty acids having putrid smell. This undesirable effect can be overcome by feeding the whole fish in the reaction vessel, without being subjected to a grinding pre-treatment and adjusting the pH of the reacting mixture in order to inactivate the autolytic enzymes. With this regard, US patent 4036993 (Ikeda & Takasaki, 1977) proposes a 2-step hydrolysis process for production of fish meat extracts and edible fish oil from commercial fish species such as saurel, mackerel, sardine,

mackerel pike, walleye pollack, atka mackerel and krill. The whole fish bodies are charged in the reactor vessel, mixed with an equal quantity of water and heated above 60 ºC to deactivate autolytic enzymes naturally present in fish tissues, prior to undergo a two-step enzymatic hydrolysis involving alkaline and acid proteases.

More recent patents use whole fish bodies without a grinding pre-treatment prior to the hydrolysis reaction to avoid the adverse effects above mentioned, but they simplify the enzymatic extraction to a single step. For instance, US patent 4861602 (Uchida & Shirakawa, 1989) discloses an enzymatic treatment with endogenous enzymes at an enzyme-substrate ratio between 0.005 to 1 wt% and a temperature between 30 – 60 ºC. The gain in soluble nitrogen (increase in the amount of nitrogen in the final product related to that present in the starting material) was reported to be a key parameter to control the oil releasing from the raw material. A value below 3 wt% favors the release of oil and its further separation from the slurry, while most of the proteins remain attached to the fish bones. A similar procedure for fish oil extraction was proposed (Shirakawa et al., 1990), comprising a single step hydrolysis reaction where the resulting slurry is passed through a 5 – 15 mm-mesh sieve to remove fish bones prior to the phase separation. The filtered slurry is then fed into a decanter to remove the sludge from the liquid, which is subsequently pumped into a centrifuge to separate the fish oil from the aqueous phase containing the soluble proteins.

3.2 Fish oil refining methods

The health-promoting effects of omega-3 fatty acids have boosted the production of fish oil intended for human consumption. Unfortunately, untreated fish oils and more specifically those containing omega-3 fatty acids have an unsavory fish odor and flavor. Furthermore, crude oils are highly susceptible to lipid oxidation and degradation processes which decrease their content of available omega-3. Therefore, crude oils must be submitted to a refining process before obtaining edible oil intended for animal or human consumption. Refining comprises a set of operations, such as degumming, neutralizing, bleaching and deodorizing, aiming to remove undesirable substances from the crude oil which may impart unpleasant odor, flavor and color or promote lipid oxidation processes. Bleaching removes soaps, trace metals, sulphurous compounds, and part of more stable pigments and pigment-breakdown products. At present, fish odor is removed by adsorption to active carbon, active clay, diatomite and like, molecular distillation or steam distillation.

Although the beneficial effects provided by the regular intake of fish oil, its definitive implantation as an ingredient in the composition of animal or human feedstuffs is restrained by the presence of unpleasant fishy odors and the low stability of the crude oil. In this regard, deodorization is a key process to avoid undesirable odors in the fish oil, thanks to the removal of odor precursor or oxidizing species. The bulk of the different deodorization procedures found in literature are based on the removal of phospholipids or low molecular amines from the fish oil, both responsible for the appearance of fishy odors and rancid tastes.

Phospholipids cannot be removed from the fish oil by physical procedures (chemical extraction or centrifugation) since they remain solubilized in the lipid fraction. Nevertheless, they can be transposed into water-soluble compounds by reaction with monosodium glutamate (MSG). According to the proposed procedures, (Park & Lee, 1997; Lee & Lee, 1001) the fish oil is stirred with water and MSG during 30-60 minutes at 40-60 ºC, in the presence of urea as catalyst. Urea avoids lipid interesterification or other side reactions, enhancing the recovery of water-soluble phospholipids. After the treatment, the transposed phospholipids can be easily separated from the mixture by centrifugation, leading to a deodorized fish oil deprived of phospholipids.

Molecular distillation has been proposed to remove low molecular amines, naturally present in the fish oil, which react in the course of time with aldehydes and ketones formed during storage and handling of the fish oil, leading to the formation of odorous substances Moreover, emission of fishy odors is enhanced by an increasing content of polyunsaturated fatty acids, such as EPA. This drawback was overcome in US 4623488 (Takao, 1986), which describes a process to obtain a refined fish oil with a minimal content in EPA higher than 20 wt% and almost free of fishy odor substances. The starting fish oil is treated with a food-grade polyhydric alcohol, such as glycerol or dipropylene glycol. The amines present affinity for the hydroxyl groups of the alcohol, and they have similar boiling point, which accelerates and facilitates the amine removal by molecular distillation. Monoglycerine is added to the emulsion in order to facilitate the dispersion of the polyalcohol into the fish oil. In the following step, the mixed oil is deodorized by molecular distillation under vacuum conditions (10 – 100 mmHg) and temperatures between 90 to 150 ºC. After three distillation stages, amines and other volatile substances are separated from the fish oil together with polyhydric alcohol, obtaining a deodorized fish oil with a removal of odorous substances in the range of 90 – 98 %.

Nevertheless, vacuum distillation cannot remove some minor undesirable compounds such as cholesterols and pesticides, and separations performed at higher temperatures may favor undesirable side reactions, such as the formation of polymers, conjugated dienes, trans-isomers and other positional isomers imparting unpleasant odors and flavors. In this regard, a patented procedure (Chang & Bao, 1991) combines vacuum distillation at low temperatures with size-exclusion by means of a silica gel column. This procedure assures a significant removal of the high boiling and more polar volatile flavor components from the oil with minimum thermal damage and formation of sub-products.

Another attempt to overcome the adverse effects of traditional deodorizing treatments was made in patent US 4692280 (Spinelly & Stout, 1987), which describes the use of supercritical carbon dioxide at 1,070-10,000 psi and 35 – 95 ºC, which exhibited good selective properties in the purification of the fish oil, since it extracted the volatile compounds responsible for the fishy odor as well as the products from lipid autoxidation. The mild temperatures employed during the extraction limited lipid autoxidation and degradation processes on the polyunsaturated fatty acids, while the inert atmosphere created by the CO_2 avoids oxidation reactions. An additional advantage provided by supercritical extraction is that CO_2 is easily recycled after extraction by heating the mixture at room temperature and venting the volatiles.

Other refining processes, such as hydrogenation, are not focused on the removal of undesirable substances, but on the conversion of these odor precursors into more stable compounds against odor formation and oxidative processes. However, a hydrogenation treatment will result in a decrease of the content of the polyunsaturated fatty acids in the fish oil, which results in lower nutritional quality. Aiming to reduce these undesirable effects, US patent 5693835 (Konishi et al., 1997) proposes a partial hydrogenation for fish oils rich in polyunsaturated fatty acids, such as those from sardine, mackerel, skipjack or tuna. The fish oil is mixed in a reaction vessel with a reduced catalyst, preferably a nickel-based one. The hydrogen is supplied at a pressure of 3 kg/cm^2 and the reacting mixture is heated between 90 – 150 ºC during 5 to 30 minutes. The reaction is stopped by cooling down to 20 ºC and the catalyst is separated from the partially hydrogenated oil by absorption on active clay and further filtration. By this procedure, fish odor components or precursors are converted into other chemical components giving no fishy odor, and the stability of the fish oil against oxidation is enhanced.

3.3 Stabilization methods for fish oil formulations

A refining method is not enough to prevent re-appearance of the fish or rancid odors after the incorporation in a foodstuff or pharmaceutical product. Unpleasant odors may re-appear as a consequence of the oxidative processes of highly unsaturated fatty acids such as DHA or EPA during preservation. The stabilization of the fish oil against the oxidation and the formation of rancid flavors is a key process prior to its incorporation as an ingredient in food products. Traditionally, hydrogenation has been proposed for this purpose, since it decreases the degree of polyunsaturation of the fish oils and improves their physical properties with a view to their transformation into margarines. Nevertheless, this technique decreases the amount of available polyunsaturated fatty acids, such as DHA or EPA, which are known to promote health benefits. Given the progressive incorporation of fish oil in a variety of food products (canned fish, capsules, salad dressings, etc.), there is a continuing need for developing stabilization methods which do not alter the content of polyunsaturated fatty acids. Besides the improvements made in the refining process, to assure a final fish oil deprived of undesirable odors and flavors, the main approaches found in literature attempt to find adjuvants which can be added to the deodorized fish oil to provide additional stability and enlarge the self-life of the final food product.

Among other reducing sugars, the incorporation of minor amounts of fructose to deodorized nonhydrogenated fish oil has shown to protect the fish oil against rancidic oxidative processes as well as stabilize the oil/water emulsions, so this approach is suitable for the incorporation of fish oil in salad dressings, mayonnaises, etc. Other reducing agents such as glucose or xylose have not been found to provide the same benefits at relatively low levels and without excessive sweetness. To this regard, some patents deal with the processing of several food emulsions (salad dressings, yogurt or cheese food emulsions) containing variable amounts of nonhydrogenated fish oil which was stabilized by the addition of modest amounts of fructose with a weight ratio of fructose to fish oil 0.01:1 (Schroeder & Muffett, 1990a; Schroeder & Muffett, 1990b). The same attempt was extended to the processing of meat food products (Schroeder & Muffett, 1992) containing fish oil stabilized with fructose in combination with other stabilizers such as Tertiary Butylhydroquinone (TBHQ), Butylated Hydroxyamisole (BHA) or Butylated Hydroxytoluene (BHT). The field of application was the preparation of luncheon meats, such as ham loaf or salami, as well as sausages (bologna, fresh pork sausage, etc.), where

the starting meat material should be deprived of any fish flesh or other fish proteinaceous material able to alter the stability of the fish oil.

The addition of vinegar and juice from citrus fruits has also been reported to promote the stability and preservation of fish oil emulsions (Kataoka & Kiyohara, 2001). According to the patented procedure, 100 parts of fish oil were mixed with 2 – 70 weight parts of vinegar of citrus juice. The emulsion was stirred at 10 – 25 ºC during 1 – 30 minutes. After settling, the mixture was separated in an upper oily layer and an aqueous phase which was removed by centrifugation. The oily layer was recovered and preserved by the addition of vegetable oil before being used as ingredient of fish oil emulsions such as salad dressings. Likewise, another patented procedure used the citrus flavonoid naringin, a natural pigment extracted from grapefruit pulp and peel, as a stabilizing agent against both odor and flavor degradation (Bakal et al., 2002). The stabilizing agent was added in a weight proportion between 0.003 to 0.02 % related to the fish oil, in combination with vegetable oil as preservative. The stabilized fish oil was used as ingredient for a pourable Italian salad dressing.

In an attempt to combine the beneficial effects of a regular intake of fish oils and the presence of antioxidant compounds in fresh fruits, patent US 0202679A1 (Mathisen, 2009b) proposes a manufacturing method to obtain a preparation comprising 2-15 wt% fish oil with up to 50 wt% of juice containing naturally present antioxidants, preferably selected from the group of rosehips, mango, orange or apple. Fruits from these groups contain hundreds of pigments and non-pigment compounds which not only increase the stability of the fish oil, but also may play an important role in preventing diseases related with oxidative cellular damage, such as cancer or brain degenerating disorders such as Parkinson's or Alzheimer's diseases. This composition is completed by adding potassium ascorbate and tocopherol as preservatives, together with a variety of sweetener, flavoring and emulsifier agents to acquire a final consistency varying from drinkable to creamy like yoghurt.

3.4 Incorporation of fish oil into feedstuffs

Supported by several studies, a regular intake of fish oils, especially those rich in fatty acids from the omega-3 family, has proved to promote significant benefits on the immune system, as well as against cardiovascular and brain degenerating disorders. Even if their beneficial effects are largely proved, the typical diet today lacks of omega-3 fatty acids.

The Health authorities recommend eating fish 4 times per week to supply the correct dose of fish oil, which is not affordable or desirable for many families. In order to redress this imbalance between fish requirements in diet and fish consumption, many attempts have been done to convert commonly available food products (such as margarines, mayonnaise, dressings) into functional foods by adding fish oil. Another field of application is the elaboration of nutrient supplements, mostly under the form of fish oil capsules.

For instance, fish oil has been added to margarines to increase their content in polyunsaturated fatty acids. Margarines are traditionally produced from solid fats and oils, which are solids at normal temperature, as well as hardened oils, which are liquid at normal temperature but have been subjected to hydrogenation to reduce their content of unsaturated fatty acids and thus acquire a harder consistency. Margarines obtained from solid fats or hardened oils have a low nutritive value, which can be improved by adding fish oils in rich polyunsaturated fatty acids. A typical production method for margarine containing fish oil, as disclosed in patent US 4764392 (Yasufuku et al., 1988), uses sardine oil as starting material, which after refining contains 10-40 wt% of EPA and 5-20 wt% of DHA. This oil is preferably added in amounts of 5-15 wt% to the total product. A larger amount of fish oil will impart a fishy taste to the margarine whereas a content of fish oil below 2 wt% of the total product does not provide a balanced composition of polyunsaturated fatty acids. The margarine is obtained by melting a solid fat or hardener oil and stirring with the desired amount of the refined fish oil and oil-soluble flavor and colorant. To obtain a margarine with the desired hardness, the solid fat is used in an amount of 0.5 – 2 parts by weight to 1.0-1.8 parts of fish oil. This oily material is kept between 50-70 ºC before being mixed with an aqueous solution containing an emulsifying agent in an amount of 0.1-2.0 wt%. Both liquids are mixed in a proportion of 0.1-0.4 part by weight of aqueous material to 1 part of oil. Finally, the emulsion is cooled between 4 – 5 ºC for solidification to obtain the margarine containing fish oil.

The main constraint to a further incorporation of fish oil in functional feedstuffs is the rapid and easy oxidability of fish oil, compared to other vegetal oils. In this regard, a manufacturing procedure under inert atmosphere comprising short holding times preserves the sensory attributes of the fish oil. The contact with oxygen should be minimized during the blending process, to retard and minimize the apparition of fishy or oxidized flavors in the final product. This blending method under inert atmosphere was employed successfully in some patents, covering the manufacturing of an enteral nutritional product (Deville et

al., 1996) or a drink containing probiotics (Mathisen & Mathisen, 2008), among others. The former invention discloses a method for blending fish oil with a food slurry containing carbohydrates and proteins. The fish oil was stored under nitrogen atmosphere before being directly discharged throughout the slurry, and the mixture was homogenized at a temperature not greater than 4 ºC, followed by a heat treatment to obtain the final product. With respect to the incorporation of fish oil in a functional drink, the fish oil is blended under inert atmosphere with an aqueous solution to form an oil-in-water emulsion comprising 5-20 wt% of fish oil. Through this invention it has been found that addition of probiotic bacteria from the genus *Lactobacillus* to the starting fish oil, the aqueous solution or the final emulsion, preferably *Lactobacillus acidophilus*, stabilizes the oxidation of fatty acids in the final product. Such final product containing the oil-in-water emulsion and the probiotics should be filled onto suitable airtight containers at a temperature of 0-10 ºC.

In response to the recommendations made by the Health authorities and the increasing concern of consumers towards the benefits of a regular intake of fish oils, food supplements are a user-friendly preparation able to supply the recommended daily dosage of omega-3 fatty acids. They overcome the common drawbacks and associated to the consumption of fish or liquid fish oil (unpleasant odors or flavors, low acceptance, high prices) while preserving the properties of the fish oil against lipid oxidation or other detrimental processes.

To this regard, patent US 48956725 (Martin et al., 1990) discloses a method to produce microcapsules containing fish oil which eliminate the unfortunate problems of the unpleasant taste and smell, as well as the aftertaste. The microcapsules are formulated from an emulsion of fish oil and 25 wt% suspension of ethyl cellulose (an enteric coating) in ammonium hydroxide. The emulsion is atomized into an acidic solution using an inert gas such as nitrogen or argon obtaining particles with a size between about 0.5 to 100 microns. In addition, by microencapsulating the fish oil following this method, the normal oxidation of polyunsaturated fatty acids is inhibited and the encapsulated fish oil is released in the intestine where is easily absorbed.

Another attempt to microencapsulate fish oil is reported in the patent US 5456985 (Zgoulli et al., 1995). These inventors proposed the use of a gastro-resistant polymer (gums, synthetic or natural resins which are physiologically acceptable) as coating material to encapsulate fish oil. The method comprises the direct atomization in a drying tower of an oil/water emulsion formed by the fish oil and the aqueous solution of gastro-resistant

polymer in order to obtain microcapsules with a size between 25-100 microns which may be incorporated into food supplements as tablets, granules, and others.

4. CURRENT & FUTURE DEVELOPMENTS

Although wastes from the fish and the aquaculture industry have been traditionally transformed into by-products having low added value such as fish meal, fish silage and fertilizers, currently, researchers and developers have achieved a better utilization of fish leftovers by processing them into up-graded by-products, FPH and high quality fish oil.

As this review states, applications where FPH are advantageously utilized due to their technological properties such as ingredients for food and animal feeding have been widely developed in the last decade and several processes employing enzymatic hydrolysis for the FPH obtaining have been patented. On the other hand, future uses of FPH lie in specialty products and markets. In this regard, methods to produce ACE inhibitor and antioxidant peptides from fish by-products have gained an increasing interest. Nevertheless, a further research and development in this field is required in order to scale up economically these processes for the production of bioactive compounds in a repeatable manner.

Likewise, fish oil is another fraction of interest which can be extracted from fish materials of high availability such as fish by-products or wastes from fish processing activities. An increasing number of scientific works support that a regular intake of fish oils (especially those containing omega-3 fatty acids) has a protective effect against cardiovascular and brain degenerative disorders. Driven by the health-promoting effects of fish oil, in combination with the continuous improvements in the extraction techniques and the low costs of the raw material, the production of fish oil, intended for human and animal consumption, has spread over the past decades. The increasing incorporation of fish oil in food and nutrition supplements (e.g. oil capsules) calls for further research on the current extraction, refining, stabilization and enriching procedures, in order to obtain fish oil formulations able to supply the dietary requirements for omega-3 fatty acids, deprived of unpleasant fishy odor and taste and stable against oxidative degradation.

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II. Discarded Species in the West Mediterranean Sea as Sources of Omega-3 PUFA*

Five discarded fish species in the Alboran Sea, namely axillary seabream (*Pagellus acarne*), small-spotted catshark (*Scyliorhinus canicula*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*) and bogue (*Boops boops)* were evaluated as novel sources for the production of omega-3 PUFA. The lipid content of the five species varied significantly within the different seasons, being maximum in spring for axillary seabream, small-spotted catshark and bogue (5.1, 2.7, 2.5 wt%, respectively) and in summer for sardine and horse mackerel (13.6 and 6.2 wt%, respectively). Sardine and horse mackerel presented also the maximum amount of EPA+DHA, 3,000 and 1,300 mg/100 g fish, respectively. Their oils exhibited a composition of EPA+DHA higher than 23 wt% and they were mainly composed by triacylglycerols. Axillary seabream, small-spotted catshark and bogue presented a lower amount of EPA+DHA, 960, 650 and 157 mg/100 g fish respectively, but their oils also exhibited a composition of EPA+DHA higher than 20 wt%. Particularly important was the composition of DHA, 20 wt%, of the oil extracted from small-spotted catshark. Therefore, the five discarded species studied were found to be valuable raw material for the production of fish oil presenting a high content in EPA and DHA.

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1. INTRODUCTION

The Alboran Sea is the portion of the west Mediterranean Sea lying between the Spanish southern coast and the north of Morocco. Spanish fleet in this area traditionally targets species such as sardine, horse mackerel, hake, blue whiting, goatfish, octopus and some shrimp species, employing trawling and purse seine as major fishing gears (MAPA, 2005). According to last estimations of the Regional Agricultural and Fisheries Council, fish captures in the Alboran Sea have decreased in almost a half during the period between 1990 (43,000 tons) and 2008 (24,000 tons), which reveals the overexploitation of fisheries in this area (CAP, 2009).

The depletion of fish stocks, which is a general trend worldwide, has promoted the setting up of new fisheries policies aiming to ensure the sustainability of extractive fishing (FAO, 1995). Fishing regulations are reinforced by several technical measures, such as the improvement of the selectivity of fishing gears (e.g. larger minimum mesh sizes, squared mesh nets, sort grids) to minimize the occurrence of by-catch or juvenile species in the catch (Sardà et al., 2004). Other measures pursue to increase control on the compliance of fisheries regulations which can be achieved not only by external supervisors, but also by taking advantage of satellite techniques (Antelo et al., 2011). Finally, an essential pillar of current fisheries policies is the need to reduce and progressively eliminate discards. Discards are that portion of total catch which is not retained for sale, but dumped at sea for any reason. It comprises juvenile species, individuals below minimum landing size and non-target species of low commercial value (by-catches) (Kelleher, 2005). Given the high mortality rates of discarded fish (e.g. nearly all finfish species, 98% of cephalopods, half of cruscateans) (Bozzano & Sardà, 2002), discard practices reduce the stock of juveniles and spawning biomass, which has a negative impact on future productivity (Jensen et al., 1988). Beside this, the adverse effect of discards on marine trophic chains has been reported in literature (Bozzano & Sardà, 2002).

Recent studies on Spanish Mediterranean fisheries estimate an average discard rate of 23% for trawling and 10% for purse seine (FROM, 2008). Most of discards in the Alboran Sea are composed by target species, discarded by minimum landing-size requirements, quota restrictions or high-grading practices (i.e. market considerations). This is the case of commercial species such as blue whiting, axillary seabream, horse mackerel and sardine,

whose harvest generates more than 375 tons per year (unpublished results). For instance, despite the negligible discard rates of sardine in the west Mediterranean Sea (0.1 – 0.2% of total catches), the mass of sardines discarded yearly is significant (Carbonell et al., 1997). Other species, such as small-spotted catshark or bogue, highly present in fish catch, are fully discarded due to their lack of commercial value (CDMP, 2005).

Recent EU regulations aim at avoiding progressively discards practices in European fisheries until their complete prohibition by 2016 (EU, 2002; EU, 2007; EU, 2011), in the framework of the new Common Fisheries Policy. So far, several discard-reduction pilot projects are in force in some specific EU fisheries (Catchpole & Gray, 2010).

Discard bans must be accompanied by the commercial promotion of non-target species, as well as technical solutions to obtain added-value products from fish discards and by-products, such as fish protein hydrolysates (Folador et al., 2006) or polyunsaturated fatty acids (Barnathan, 2008), highly demanded for nutraceutical and pharmaceutical applications. To this regard, research studies exploring the nutritive value or up-grading capabilities of non-commercial fish species have been receiving increasing attention. For instance, Orban et al. (2011) evaluate the nutritional properties of the lipid fraction from bogue and horse mackerel, which are commonly discarded in Italian trawl fisheries. Antelo et al. (2012) revised the best available decontamination techniques for fish discards and by-products intended to fish oil or fish meal production.

Given the decline in fish harvest, fish by-catch and by-products have become a source of great interest for the extraction of fish oil (Bimbo, 2007; Nichols et al., 2010). Unlike lipids from animal or vegetal origin, fish lipids are rich in polyunsaturated fatty acids (PUFA) belonging to the omega-3 family, such as eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA). Over last decade, a great deal of research has been expended on the role of dietary omega-3 fatty acids in the prevention of cardiovascular diseases such as arrhythmias, hypertension or coronary heart disease (Ruxton et al., 2005; Harris et al., 2008), inhibition of inflammatory processes (Calder, 2006) and the fetal development of brain and eye retina (Hornstra, 2001).

Current technologies, such as pressing, high-speed centrifuge fractionation or solvent extraction, enable to obtain fish oil from fish materials of high availability such as non-commercial species, fish by-products or wastes from fish processing (García-Moreno, et al., 2010). When implementing a fish oil extraction process, a major concern which should

be addressed is the availability of marine lipid sources throughout the year. Indeed, both lipid content and fatty acid composition of many species undergo seasonal variations, influenced by the stage of maturity, spawning cycle or the environmental and feeding conditions, among others (Børresen , 1992).

In this work, five discarded species in the Alboran Sea, axillary seabream (*Pagellus acarne*), small-spotted catshark (*Scyliorhinus canicula*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*) and bogue (*Boops boops),* were evaluated as sources for the production of fish oil rich in omega-3 PUFA. Their proximate and fatty acid compositions were studied in all seasons. This study is a first step towards the valorization of this underutilized biomass.

2. MATERIALS AND METHODS

2.1 Raw material

Axillary seabream (*Pagellus acarne*), small-spotted catshark (*Scyliorhinus canicula*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*) and bogue (*Boops boops*) were chosen for this study as species discarded by the Spanish fleet in the Alboran Sea. The raw material was provided once per season during a year (autumn-winter 2011 and spring-summer 2012) by the fishing harbor of Motril (Spain). It was kept in ice during the transportation and pressed in the same day. Averages of length and weight of the fish were as follows: axillary seabream 14.4±0.3 cm and 118.2±15.5 g, small-spotted catshark 38.2±4.1 cm and 218.4±56.6 g, sardine 15.2±2.8 cm and 56.5±24.7 g, horse mackerel 16.5±0.8 cm and 62.1±13.2 g and bogue 15.0±0.1 cm and 78.7±2.6 g. For the analysis of lipid classes, fish caught in March 2013 was employed as raw material.

2.2 Proximate chemical composition

The proximate composition of the fish species was determined according to the official methods of the AOAC (AOAC, 2006). Moisture and ash content were estimated gravimetrically by heating the samples until attaining constant weight at 105ºC and 550ºC, respectively. Total nitrogen was determined by the Kjeldahl method, and the content of crude protein was then obtained by employing a nitrogen-to-protein conversion factor of

6.25. Total lipid content was evaluated according to the Soxhlet semi-continuous extraction method. Results were expressed as wt%.

2.3 Oil extraction

For each species, approximately 2 kg of whole fish (included viscera and gonads) were preheated at 40 ºC for 30 minutes by means of a water bath model Digiterm 100 (Selecta, Spain). Then, they were fed into an electric press model ESP-K (Sanahuja, Spain) where they were submitted to three consecutive pressing steps until attaining a final pressure of 120 bar. The press liquor released during the operation was collected and then centrifuged at 20,000×g in order to recover the oily phase.

2.4 Fatty acid profile

The oils obtained were methylated by direct transesterification following the method of Lepage and Roy (1984) modified by Rodríguez-Ruiz et al. (1998). These methyl esters were then analyzed according to the method described by Camacho-Paez et al. (2002) with an Agilent 7890A gas chromatograph (Agilent Technologies, S.A.) connected to a capillary column of fused silica Omegawax (0.25 mm × 30 m, 0.25 μm standard film; Supelco, Bellefonte, PA), and a flame-ionization detector. Matreya (Pleasant Gap, PA) n-3 PUFA standard (catalogue number 1177) was used for the qualitative fatty acid determination. Nonadecanoic acid (19:0) (Sigma-Aldrich) was used as internal standard for quantitative determination of fatty acids. Each sample was analyzed in triplicate. Results were expressed on a weight percentage basis.

2.5 Lipid class composition

The lipid classes of the oils extracted were determined by thin-layer chromatography (TLC) according to Batista et al. (2010). Plates coated with 0.25 mm silica gel G were developed with hexane/diethyl ether/acetic acid (65:35:1, v/v/v). Then, they were sprayed with 10% phosphomolibdic acid in ethanol and heated at 120 ºC for 5 min. Lipid class identification was made by comparison with standards (Sigma-Aldrich). For quantification purposes, the TLC plates were scanned (GS-800 densitometer, Bio-Rad) and analyzed with Quantity One analysis software (Bio-Rad). Each sample was analyzed in triplicate. Results were expressed on a weight percentage basis.

2.6 Statistical analysis

The Statgraphics software (version 5.1) was used to carry out one-way analysis of variance (ANOVA) on the data. For each species, mean seasonal values were compared by means of the Tukey's test. Differences between means were considered significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Proximate composition

The proximate compositions of axillary seabream (*Pagellus acarne*), small-spotted catshark (*Scyliorhinus canicula*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*) and bogue (*Boops boops*) caught in distinct seasons in the Alboran Sea are reported in Table 7. Regarding the ash content, it was practically constant for the five species along the year studied. It varied from 1.6 to 4.5 wt%, except for axillary seabream where values of ash content higher than 5 wt% were obtained. Considering the protein content for axillary seabream, sardine, horse mackerel and bogue, it varied from 15.5 to 19.1 wt%. These results are in line with previous works which reported the following protein contents: 17.3 - 18.2 wt% for sardine (*Sardina pilchardus*) (Zotos & Vouzanidou, 2012), 18.9 - 20.7 wt% for horse mackerel (*Trachurus trachurus*) and 18.4 – 20.3 wt% for bogue (*Boops boops*) (Orban et al., 2011). In the case of small-spotted catshark, protein values higher than 20 wt% were observed. However, it should be noted that elasmobranchs have a high content of nonprotein nitrogen in the form of trimethylamine oxide (TMAO) and urea. Therefore, an overestimate of the actual protein content may have occurred when employing a conversion factor of 6.25 to convert total nitrogen to crude protein (Økland et al., 2005).

According to the fat content and considering the four categories reported by Ackman (1989), the results from Table 7 denoted that small-spotted catshark and bogue were lean fish (< 4 wt%), axillary seabream and horse mackerel were semi fatty fish (< 8 wt%) and sardine was fatty fish (> 8 wt%). It was observed that the lipid content changed considerably among species and also significant variations were noticed for the same species at different seasons. It is widely known that the lipid content of fish species depends on several factors such as the stage of maturity, the sex, the spawning cycle, the environment and the nutritional condition of the animal (Børresen, 1992). Especially, the

lipid content is strongly related to the spawning period in which the lipids are employed as energy source. Thus, after this period, the lipid content of the fish is reduced until a minimum level (Krzynowek , 1992). For axillary seabream, small-spotted catshark, horse mackerel and bogue, the spawning periods were identified in autumn (Nannini et al., 1997; Tsikliras et al., 2010). As a consequence, these species exhibited the lowest lipid content in winter, 1.3, 1.1, 1.7 and 0.5 wt%, respectively. On the other hand, sardine has the spawning period in winter (Abad & Giraldez, 1989), hence the lowest lipid content for this species was obtained in spring, 0.6 wt%. In Table 7, it is also shown that axillary seabream and bogue also reduced their protein content after the spawning period. This fact revealed that these species, apart from the lipids, require additional energy sources during the reproductive process and the proteins are employed for this purpose. This phenomenon is characteristic for lean and semi fatty fish species and it was also noticed by Zotos et al. (2012) when working with hake (*Merluccius merluccius*).

In spring and summer seasons there is an abundant availability of food resources in the aquatic environment (Orban et al., 2011). As a result, axillary seabream, small-spotted catshark and bogue presented the highest lipid content in spring, 5.1, 2.7, 2.5 wt%, respectively, whilst sardine and horse mackerel exhibited the highest lipid content in summer, 13.6 and 6.2 wt%, respectively. Moisture was found to correlate inversely with the lipid content for the five species, obtaining the lowest values for moisture when the species had the highest fat content.

Table 7. Seasonal changes of the proximate composition of the five discarded fish species studied

Composition (wt%)	Axillary seabream				Small-spotted catshark				Sardine				Horse mackerel				Bogue			
	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.
Moisture	73.1	76.6	71.4	72.8	77.1	76.3	74.2	75.8	64.6	73.3	78.2	63.9	73.5	75.4	74.4	70.9	78.7	77.9	75.2	77.0
Ash	5.5	5.7	5.4	5.1	1.6	2.0	3.3	3.0	3.0	3.1	3.8	2.7	3.6	3.5	3.1	3.1	3.9	4.5	4.2	3.0
Protein	17.6	15.9	19.1	18.6	20.3	20.3	22.0	23.1	16.0	16.9	18.3	18.2	16.9	16.8	18.5	19.0	17.0	15.5	17.5	16.4
Lipid	3.7	1.3	5.1	4.0	2.5	1.1	2.7	1.9	11.3	2.1	0.6	13.6	5.0	1.7	4.9	6.2	0.8	0.5	2.5	1.8

3.2 Fatty acid composition

As lipid content, fatty acid composition also varies with species, sex, age, season, reproductive status, temperature of the water and food availability (Gruger, 1967). Table 8 shows the fatty acid profile of the oils extracted from axillary seabream, small-spotted catshark, sardine, horse mackerel and bogue in different seasons. It should be noted that for axillary seabream no oil was extracted in autumn and, that from bogue, oil was extracted only in summer. It is explained due to technical problems during the extraction together with the low lipid content of the raw material in these cases.

A wide variety of fatty acids were detected in the five fish oils extracted. The major fatty acids identified were polyunsaturated fatty acids (PUFA) ranging from 33.0 to 45.1 wt%, mainly eicosapentaenoic (C20:5n-3) and docosahexaenoic (C22:6n-3), followed by docosapentaenoic (C22:5n-3). Then, saturated fatty acids (SFA), varying from 22.4 to 31.4 wt%, were found as the second most abundant constituents of the oils for axillary seabream, sardine, horse mackerel and bogue. Within this group, the predominant fatty acids were palmitic (C16:0), stearic (C18:0) and myristic (C14:0). Monounsaturated fatty acids (MUFA) such as oleic (C18:1n-9), palmitoleic (C16:1n-7), erucic (C22:1n-9), vaccenic (C18:1n-7) and gadoleic (C20:1n-9) were also identified. Their content in the oils varied from 19.5 to 34.9 wt%. These results are in line with previous studies on the fatty acids profile of marine species (Bandarra et al., 1997; Özogul & Özogul, 2007, Orban et al., 2011; Zotos & Vouzanidou, 2012). In the case of small-spotted catshark, MUFA were more important than SFA due to the high content of oleic acid exhibited by this oil (15.3 - 22.1 wt%).

The five oils studied presented a significant concentration of omega-3 PUFA, particularly EPA and DHA. In Table 8, it is shown that the content of EPA and DHA varied significantly ($p<0.05$) in the oil extracted from each species with seasons. It was generally observed that after the spawning, when minimum lipid content for the species was obtained, the oils exhibited the highest amount of EPA+DHA whilst the lowest content in MUFA was found at this stage. For instance, sardine oil presented a MUFA content of 23.6 % in summer and it was reduced to 19.5 % in spring, whereas the percentage of EPA+DHA increased from 24.2 to 32.8 % at the same interval, Table 8. It is explained due to the fact that, during the spawning, MUFA were employed as energy source. On the contrary, PUFA were maintained because of their important role as constituents of the cell membrane phospholipids and their significant contribution to the reduction of the mean

melting point of total lipids (Zotos & Vouzanidou, 2012). Moreover, from the results in Table 8, it is also revealed, for most of the species (axillary seabream, sardine and horse mackerel), that after the spawning period, an accentuated decrease of DHA percentage was produced with a proportional increase of EPA. This increase in EPA may result from fish diet, since after the spawning, fish start to feed eagerly and EPA is a major fatty acid of plankton. Besides, DHA is a significant component of membrane structural lipids and therefore its relative percentage can decrease (Bandarra et al., 1997).

In the period of maximum lipid content, the following omega-3 compositions were obtained for the oils of the five discarded species evaluated (Table 8): 10.2 wt% EPA and 10.8 wt% DHA for axillary seabream, 6.9 wt% EPA and 20.3 wt% DHA for small-spotted catshark, 14.4 wt% EPA and 9.8 wt% DHA for sardine and 12.7 wt% EPA and 11.1 wt% DHA for horse mackerel. For the oil of bogue, a content of 9.8 wt% EPA and 14.7 wt% DHA was observed in summer. These results denoted that, in that period, the concentration of EPA+DHA for the five oils were higher than 20 wt%, highlighting the content of EPA+DHA for the oil of small-spotted catshark (27.2 wt%), as consequence of its high concentration in DHA 18.2-20.3 wt%.

Extraordinary ratios of n-3/n-6 were obtained for the oils of the five species, Table 8. Sardine presented the highest ratio of n-3/n-6 (14.9 – 16.7), followed by horse mackerel (11.9-13.5), bogue (12.6), small-spotted catshark (10.8 – 11.6) and axillary seabream (6.0 – 7.7). Besides, for sardine and horse mackerel, the highest ratios of n-3/n-6 were obtained in summer, when these species presented the maximum lipid content. These results strongly support that the oils extracted from the five discarded fish species studied have an important content of omega-3 PUFA. This fact was well-known for the oils of sardine and horse mackerel which have been traditionally produced (Pikel & Jackson, 2010). Nevertheless, oils from axillary seabream and small-spotted catshark also resulted to exhibit a significant composition in EPA and DHA. These oils reached in spring, when the highest lipid content for these species was observed (Table 7), a total amount of EPA+DHA up to 21 and 27.2 wt%, respectively (Table 8). Of special relevance is the case of small-spotted catshark because, in spite of its low fat content (Table 7), the oil extracted for this species presented a high proportion of DHA (up to 20.3 wt%). As a result of this exceptional omega-3 profile, similar to the one presented by tuna oil, this oil may be an excellent functional ingredient for brain and children's health applications.

Table 8. Seasonal changes in the fatty acid profile (wt%) of the oils extracted from the five discarded fish species studied

Fatty Acid	Axillary seabream[1]			Small-spotted catshark				Sardine				Horse mackerel				Bogue[2]
	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Sum.
C14:0	4.8	4.0	4.5	1.8	2.9	1.7	1.8	6.5	6.2	5.9	7.2	6.5	6.0	6.0	6.8	6.3
C16:0	17.1	15.1	16.1	17.1	16.6	17.5	16.9	20.8	18.9	19.4	18.9	18.8	18.4	17.4	18.1	17.9
C16:1n-7	7.0	6.4	6.8	7.0	6.7	6.2	6.0	6.6	5.6	7.0	7.3	6.4	6.2	6.4	8.0	7.4
C16:2n-4	1.1	1.0	1.1	0.6	0.8	0.6	0.6	1.2	1.3	1.3	1.4	1.2	1.2	1.2	1.5	1.3
C16:3n-4	0.9	0.8	0.9	0.8	0.7	0.7	0.7	0.7	0.6	0.7	0.9	0.6	0.7	0.7	0.7	0.6
C16:4n-1	0.1	0.3	0.5	0.0	0.1	0.0	0.0	0.6	0.4	0.5	1.4	0.3	0.3	0.4	0.9	0.6
C18:0	7.1	6.3	6.2	3.5	3.7	3.7	4.1	4.1	4.5	3.5	3.7	5.1	4.9	4.6	4.4	5.0
C18:1n-9	14.4	14.0	13.2	22.1	15.3	20.7	18.4	7.9	8.8	7.5	8.1	13.6	12.7	15.0	12.0	13.1
C18:1n-7	4.6	4.6	4.4	4.2	4.0	3.8	4.4	3.3	2.9	3.3	3.2	2.6	2.8	3.1	2.6	2.6
C18:2n-6	1.4	2.5	1.4	1.1	1.4	1.1	1.0	1.5	1.5	1.5	0.9	1.7	1.9	1.3	1.2	1.5
C18:3n-3	0.3	0.7	0.6	0.5	0.7	0.6	0.5	1.2	1.0	1.2	0.6	0.9	1.3	0.8	0.9	1.3
C18:4n-3	0.9	1.1	0.9	0.4	0.9	0.4	0.5	2.3	1.6	2.1	1.9	1.4	1.8	1.0	1.8	2.6
C20:1n-9	3.5	1.7	1.4	3.1	2.4	1.2	3.0	2.6	2.3	0.9	3.7	3.0	2.6	3.1	3.6	2.2
C20:4n-6	2.5	2.2	2.2	1.5	1.8	1.7	1.9	1.0	1.1	1.1	0.9	0.9	1.1	1.0	1.0	1.1
C20:4n-3	0.2	0.6	0.5	0.6	0.9	0.7	0.7	1.1	1.0	1.3	0.9	0.7	0.9	0.7	0.7	1.0
C20:5n-3	**9.4^{a}**	**10.2^{b}**	**10.7^{b}**	**5.7^{a}**	**8.1^{b}**	**6.9^{c}**	**7.7^{d}**	**12.9^{a}**	**11.2^{b}**	**12.3^{c}**	**14.4^{d}**	**10.6^{a}**	**11.0^{b}**	**9.5^{c}**	**12.7^{d}**	**9.8**
C22:1n-9	0.4	1.0	1.4	1.7	1.2	1.2	1.6	2.4	3.1	1.7	5.0	5.2	3.7	5.4	6.9	2.0
C22:5n-3	4.9	4.3	4.2	3.4	4.2	3.0	3.2	2.8	2.9	2.7	2.8	2.4	2.6	2.7	2.3	2.8
C22:6n-3	**13.0^{a}**	**10.8^{b}**	**10.7^{b}**	**18.2^{a}**	**20.2^{b}**	**20.3^{b}**	**18.9^{c}**	**16.5^{a}**	**17.5^{b}**	**20.5^{c}**	**9.8^{d}**	**15.8^{a}**	**18.1^{b}**	**13.8^{c}**	**11.1^{d}**	**14.7**
Others	6.5	12.6	12.3	6.8	7.4	7.9	8.2	4.2	7.5	5.7	7.7	2.2	2.1	6.3	3.1	6.3
Total	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
SFA	29.0	25.4	26.8	22.4	23.2	22.9	22.8	31.4	29.6	28.7	29.7	30.4	29.2	27.9	29.3	29.1
MUFA	26.4	26.0	25.8	34.9	27.2	32.0	30.3	20.2	20.4	19.5	23.6	27.7	25.3	29.7	29.4	25.2
PUFA	34.6	34.4	33.6	32.9	39.8	36.0	35.6	41.6	40.2	45.1	35.9	36.6	40.9	33.0	34.6	37.2
n-6	3.9	4.6	3.6	2.6	3.2	2.8	2.9	2.5	2.6	2.6	1.8	2.6	3.0	2.3	2.2	2.6
n-3	28.7	27.7	27.5	28.9	35.1	31.9	31.4	36.7	35.3	40.1	30.4	31.9	35.7	28.4	29.3	32.1
n-3/n-6	7.3	6.0	7.7	11.2	11.0	11.6	10.8	14.9	13.6	15.5	16.7	12.2	11.9	12.5	13.5	12.6
EPA+DHA	**22.4^{a}**	**21.0^{a}**	**21.4^{a}**	**24.0^{a}**	**28.3^{b}**	**27.2^{c}**	**26.6^{d}**	**29.4^{a}**	**28.7^{b}**	**32.8^{c}**	**24.2^{d}**	**26.5^{a}**	**29.2^{b}**	**23.2^{c}**	**23.7^{c}**	**24.6**

Data are means of triplicate determinations, with standard deviation SD < 3%. For each species, values within a row with different superscript letters indicate significant differences ($p<0.05$). [1] Data on fatty acid composition are not available for autumn. [2] Data on fatty acid composition are only available for summer.

In table 9, it is shown the composition of fatty acids expressed in mg/100 g fish. These values were calculated by using the conversion factors indicated by Weihrauch et al. (1977) and by taking into account the lipid content (Table 7) and the fatty acid profiles (Table 8) of each species. Owing to seasonal changes in fat content and fatty acid profile, significant changes ($p < 0.05$) were also observed for the fatty acid composition expressed as mg/100 g fish. It was confirmed that sardine and horse mackerel were excellent sources of omega-3 PUFA in summer and autumn, having values of EPA+DHA up to 3,000 and 1,300 mg/100 g fish, respectively. Similar results, 2,600 mg/100 g edible flesh, were obtained by Zotos and Vouzanidou (2012) for sardine (*Sardina pilchardus*) and by Bandarra et al. (2001) for horse mackerel (*Tachurus trachurus*), 1,600 mg/100 of edible flesh. These species were followed by axillary seabream and small-spotted catshark, which exhibited a content of EPA+DHA in spring of 960 and 650 mg/100 g fish, respectively.

The lipid class composition of the oils extracted is presented in Table 10. The low polar compounds presented the highest percentage being triacylglycerols (TG) the most important. All the oils exhibited a composition in TG higher than 78 wt%, being considerable higher for small-spotted catshark, 96 wt%. This lipid class varies greatly with the fat content of the fish, reaching the highest percentage in the fatty season (Bandarra et al., 1997). The free fatty acids content (FFA) of the oils ranged from 10.2 wt% for horse mackerel to 15.6 wt% for bogue. The cholesterol composition of the oils varied from 3.8 wt% for small-spotted catshark to 8.8 wt% for axillary seabream. These results are in agreement with previous studies on the lipid class composition of sardine (*Sardina pilchardus*) (Bandarra et al., 1997) and horse mackerel (*Trachurus trachurus*) (Bandarra et al., 2001). In terms of polar lipids, phospholipids were detected only in sardine oil, 0.8 wt%. It seemed that the method employed to extract the oil did not allowed the extraction of these structural lipids. Therefore, these results revealed that most of the omega-3 PUFA contained on the oils would be presented as TG which are more resistant to oxidation (Wijesundera et al., 2008) and which are also more bioavailable for the human digestive system (Lawson & Hughes, 1988).

Table 9. Seasonal changes in the fatty acid profile (mg/100 g fish) of total lipids of the five discarded fish species studied

Fatty acid	Axillary seabream[1]			Small-spotted catshark				Sardine				Horse mackerel				Bogue[2]
	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Sum.
C20:5n-3	104.2^{a}	465.6^{b}	387.0^{c}	124.4^{a}	69.5^{b}	164.7^{c}	128.7^{d}	1347.1^{a}	201.0^{b}	53.5^{c}	1798.6^{d}	475.7^{a}	156.2^{b}	415.4^{c}	713.7^{d}	63.0
C22:6n-3	143.4^{a}	493.5^{b}	385.9^{c}	396.0^{a}	173.0^{b}	488.7^{c}	314.8^{d}	1714.9^{a}	314.9^{b}	89.4^{c}	1228.7^{d}	708.4^{a}	256.3^{b}	605.1^{c}	624.5^{d}	94.3
EPA+DHA	247.6^{a}	959.1^{b}	773.0^{c}	520.4^{a}	242.5^{b}	653.4^{c}	443.5^{d}	3062.0^{a}	515.9^{b}	142.9^{c}	3027.3^{d}	1184.2^{a}	412.5^{b}	1020.6^{c}	1338.2^{d}	157.3

Data are means of triplicate determinations, with standard deviation SD < 3%. For each species, values within a row with different superscript letters indicate significant differences ($p<0.05$). [1] Data on fatty acid composition are not available for autumn. [2] Data on fatty acid composition are only available for summer

Table 10. Lipid composition of the oils extracted from the five discarded fish species studied

Composition (wt%)	Axillary seabream	Small-spotted catshark	Sardine	Horse mackerel	Bogue
TG	78.3±1.8^{a}	96.2±2.1^{b}	78.7±1.4^{a}	84.3±1.5^{c}	78.2±1.2^{a}
FFA	12.9±0.9^{a}	nd	13.0±0.8^{a}	10.2±1.7^{b}	15.6±1.3^{c}
CH	8.8±1.8^{a}	3.8±2.1^{b}	7.5±1.4^{a,c}	5.5±2.1^{b,c}	6.2±0.7^{a,b,c}
PL	nd	nd	0.8±0.1	nd	nd

TG-Triacylglycerols; FFA-Free fatty acids; CH-Cholesterol; PL-Phospholipids; nd-not detected

Data are means of triplicate determinations with standard deviation. Values within a row with different superscript letters indicate significant differences ($p<0.05$).

4. CONCLUSIONS

Providing an added-value to discards is essential for the future management of Spanish Mediterranean fisheries. In this sense, the five discarded species evaluated were found to be a good source for the production of fish oil containing a high proportion of omega-3 PUFA.

The lipid content of the five species showed significant seasonal changes during the year. The minimum fat content coincided with the period after the spawning (spring for sardine and winter for the rest of the species) and the maximum with periods of high availability of food resources in the aquatic environment (spring for axillary seabream, small-spotted catshark and bogue and summer for sardine and horse mackerel). Sardine (*Sardina pilchardus*) and horse mackerel (*Trachurus mediterraneus*) exhibited the highest lipid content, 13.6 wt% and 6.2 wt% respectively, presenting also the highest amount of EPA+DHA, 3,000 and 1,300 mg/100 g fish, respectively. Their oils had a composition of EPA+DHA higher than 23 wt%.

Axillary seabream (*Pagellus acarne*) and small-spotted catshark (*Scyliorhinus canicula)*, in spite of their lower amount of EPA+DHA, 960 and 650 mg/100 g fish respectively, also represent good raw materials for the production of fish oil with a composition of EPA and DHA higher than 20 wt%. Interestingly, the oil extracted from small-spotted catshark presented a high content of DHA (up to 20 %). Thus, it may be an excellent functional ingredient for brain and children's health applications.

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III. Influence of the Parameters of the Rancimat Test on the Determination of the Oxidative Stability Index of Cod Liver Oil*

The operational parameters of the Rancimat method (airflow rate, sample weight and temperature) were studied to determine their effects on the oxidative stability index (OSI) of cod liver oil. To this end, experimental data were firstly fitted to a complete quadratic model and an ANOVA analysis was performed, which concluded that airflow rate and temperature were significant ($p < 0.05$). By means of this model and using response surface methodology in order to minimize the OSI, the optimal conditions for the three parameters of the Rancimat method were found to be Q = 25 L/h, M = 6.91 g and T = 88.26 ºC. The different trend obtained for the OSI at increasing temperatures supports that the oxidation mechanism of fish oil at the conditions studied may differ from the lipid oxidation mechanism at room temperature. Secondly, a simplified linear model was assayed, obtaining also that the influences of airflow rate and temperature were significant ($p < 0.05$). Moreover, the temperature contribution resulted to have the most important effect on the OSI, obtaining a temperature coefficient of -3.29×10^{-2} $ºC^{-1}$.

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1. INTRODUCTION

At present, the production of high quality fish oil has gained a great importance since it is considered the main natural source of omega-3 polyunsaturated fatty acids (Rubio-Rodríguez et al., 2012). Among these PUFAs, eicosapentaenoic acid (C20:5n-3, commonly called EPA) and docosahexaneoic acid (C22:6n-3, commonly called DHA) are particularly important since they have been reported to promote several benefits on human health (Lees & Karel, 1990; Uauy &Valenzuela, 2000).

As a consequence of its high degree of unsaturation, fish oil is very susceptible to oxidation. Due to the fact that the oxidation process causes the development of undesirable fishy off-flavors, which deteriorate the overall quality of the oil reducing considerably its use in both pharmaceutical and food applications (Jacobsen & Nielsen, 2007; Jacobsen et al., 2008), fish oil should be optimally produced, stored and packed. Moreover, addition of different types of antioxidants is carried out in order to protect fish oil against oxidation (Rubio-Rodríguez et al., 2010). Therefore, the measurement of the oxidative stability of fish oil is of great importance for researchers and manufacturers in order to control and optimize the production process and to predict the shelf-life of the final oil.

Determining the resistance of oils and fats to oxidation is a tedious and time-consuming analysis when carried out at room temperature. Thus, several accelerated methods employing high temperatures and air flow supply have been developed to assess oxidative stability in a relatively short period (Reynhout, 1991). Among these techniques, the Active Oxygen Method (AOM) (AOCS, 1980) has traditionally been used for such determinations. However, this method is labor-intensive, non reproducible and involves the use of toxic chemicals (Mateos et al., 2006). A superior accelerated analysis, the Rancimat test, was developed by Hadorn and Zurcher (1974), becoming the AOCS standard method Cd 12b-92 (AOCS, 1992) after the study carried out in 1991 by Jebe et al. (1993). As a result of this fact and due to its ease of use and reproducibility, the Rancimat test has been widely employed over the past two decades. Furthermore, the Rancimat method has been specially validated for oxidative stability determination on both bulk (Méndez et al., 1996) and microencapsulated fish oils (Velasco et al., 2009; Bustos et al., 2003).

The Rancimat method is based on the conductivity changes experienced by deionized water after collecting the volatile organic acids produced in the final steps of the

accelerated oil oxidation process (de Man et al., 1987; Jebe et al., 1993; Méndez et al., 1996). The time required to produce a sudden increase of the conductivity due to volatile acids formation, mainly formic acid, determines the oxidative stability index (OSI), which can be defined as a measure of the resistance to oxidation of a fat or oil. In addition, previous studies have demonstrated the correlation between the stability data obtained by the Rancimat test and those determined by other sensory and/or analytical methods (Gordon & Mursi, 1994; Coppin & Pike, 2001; Anwar et al., 2003).

Airflow rate, oil sample weight and temperature are the operational parameters that can be adjusted easily in the Rancimat method and may affect the determination of the OSI number (Farhoosh, 2007a; Farhoosh, 2007b). Although a number of studies (Reynhout, 1991; Hasenhuettl & Wan, 1992; Hill & Perkins, 1995; Farhoosh, 2007a; Kochhar & Henry, 2009) have investigated the individual or simultaneous effect of the parameters of the Rancimat test on the oxidative stability of vegetable oils, no comprehensive studies have been reported so far about the influence of these three parameters on the OSI evaluation of fish oil.

In the light of the above, the purposes of this work were mainly two: a) to assess, by means of experimental design and analysis of variance (ANOVA) techniques, the influence of each operational parameter upon the oxidative stability index of cod liver oil; and b) to develop, by means of response surface methodology (RSM), empirical models in order to correlate the oxidative stability index with the operational parameters involved in the Rancimat test. These models were then optimized to determine the combination of experimental factors which minimize the OSI.

2. MATERIALS AND METHODS

2.1. Materials

Partially refined cod liver oil employed was purchased from Acofarma (Barcelona, Spain), with the following declared properties: density 923 kg/m^3, refractive index 1.477, peroxide value 3.12 meq/kg oil, acidity value 0.16 % oleic, EPA 8.18 wt%, DHA 11.02 wt% and non-saponifiable content 0.39 %. The oil was kept in a bottle covered with aluminum foil at 4 ºC under nitrogen atmosphere. Antioxidants were not added during storage.

2.2. Apparatus

A Metrohm Rancimat model 743 (Methrom Instruments, Herisau, Switzerland) was employed for OSI determination. Before each run, the sample tubes were rigorously cleaned and immersed overnight in a hot solution of an alkaline detergent (3 wt%), rinsed off with distilled water and acetone and then dried in oven at 80 ºC. This procedure was carried out in order to avoid any contamination which could catalyze the autooxidation process. Also, electrodes, connecting tubes and measuring vessels were cleaned with alcohol and distilled water and were blown out with nitrogen.

2.3. OSI determination

A stream of filtered, cleaned and dried air was bubbled into the oil sample contained in a reaction vessel. This vessel was placed in an electric heating block, which was set at the desired temperature for each experimental run. Effluent air containing volatile organic acids from the oil sample were collected in a measuring vessel with 60 mL of distilled water. The conductivity of the water was continuously recorded and the OSI was automatically determined by the apparatus. Eight oil samples were analyzed in the equipment at the same time.

2.4. Experimental design

Three experimental factors were considered for this study: air flow rate (Q), sample weight (M) and temperature (T). They were varied according to a 4 x 4 x 4 full factorial design. To this end, each input factor was set at four levels, as follows: air flow rate (10, 15, 20 and 25 L/h), sample weight (3, 5, 7 and 9 g) and temperature (60, 70, 80 and 90 ºC). The stability oxidative index (OSI) was evaluated for a total of 64 determinations, as shown in Table 11.

2.5. Statistical analysis

The Statgraphics software (version 5.1) was used to generate the statistical analysis and the regression models. Firstly, the OSI was related to the input variables (air flow rate, sample weight and temperature) by a second degree polynomial according to Eq. 2:

$$OSI = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j}^{3} b_{ij} X_i X_j \qquad (2)$$

where the coefficients b_i and b_{ii} are related to the linear and quadratic effects, respectively, of each input factor on the OSI and the cross-product coefficients b_{ij} represent the interactions between two input variables.

Secondly, the modified variable log(OSI) was chosen to obtain an alternative correlation when fitting the data to a linear regression model, Eq. 3:

$$\log(OSI) = b_0 + \sum_{i=1}^{3} b_i X_i \quad (3)$$

Finally, the analysis of variance (ANOVA) tables were generated. The significance of all terms in the models was judged statistically by computing the p-value at a confidence level of 95%. The regression coefficients were then used to generate contour maps and find the optimal Rancimat parameters values which minimize the OSI. For this final purpose, response surface methodology approach was employed as described by Ba et al. (2007).

3. RESULTS AND DISCUSSION

The OSI results for the chosen experimental design are presented in Table 11. It can be seen that, at constant values of airflow rate and sample weight, the OSI values significantly decreased with temperature. As an example, at an airflow rate of 10 L/h and a sample weight of 3 g, OSI decreased from 18.03 h at 60 ºC to 1.97 h at 90 ºC. This behavior could be attributed to the acceleration produced in the decomposition of lipid hydroperoxides (Frankel, 2005).

However, at constant temperature, the influence of both airflow rate and oil sample weight on the OSI did not follow a clear trend. For instance, it is shown in Table 11 that at an airflow rate of 10 L/h and a temperature of 60 ºC, OSI fluctuated between 17.76 h at and 18.03 h when sample weight varied from 3 to 9 g. Similarly, at a sample weight of 3 g and a temperature of 60 ºC, OSI oscillated between 17.67 h and 18.12 h when airflow rate varies from 10 to 25 L/h.

Although at a first moment, it could be reasoned that OSI decreases with airflow rate and increases with sample weight, these phenomena were not observed in the results expressed in Table 11. It may be attributed to variations in the air-saturated conditions as

consequence of a turbulent status in the oil sample which results in more air escaping from the oil than used in lipid oxidation (Farhoosh, 2007a).

Table 11. Experimental design and measured values for the oxidative stability index

Run	Airflow rate, L/h	Sample size, g	Temperature, ºC			
			60	70	80	90
1 - 4	10	3	18.03	8.12	4.22	1.97
5 - 8	10	5	17.91	8.03	3.95	2.00
9 – 12	10	7	17.76	8.09	3.97	2.04
13 - 16	10	9	17.98	8.15	4.06	2.08
17 - 20	15	3	17.71	8.10	3.85	1.93
21 - 24	15	5	17.51	8.01	3.76	1.93
25 - 28	15	7	17.55	7.90	3.81	1.93
29 - 32	15	9	17.72	8.08	3.89	1.96
33 - 36	20	3	17.67	8.01	3.47	1.80
37 - 40	20	5	17.56	7.97	3.38	1.67
41 - 44	20	7	17.34	7.85	3.33	1.72
45 - 48	20	9	17.63	7.87	3.42	1.84
49 - 52	25	3	18.12	8.21	3.31	1.78
53 - 56	25	5	17.35	8.04	3.42	1.74
57 - 60	25	7	17.38	7.78	3.11	1.69
61 - 64	25	9	17.46	8.03	3.26	1.52

The experimental data were firstly fitted to a complete quadratic model. The polynomial coefficients for the surface response model (Eq. 4) were calculated by multiple regression:

$$\begin{aligned} OSI = {} & 155.61 - 1.99\times10^{-2}\cdot Q - 2.07\times10^{-1}\cdot M - 3.46\cdot T + \\ & + 1.49\times10^{-3}\cdot Q^2 + 1.47\times10^{-2}\cdot M^2 + 1.97\times10^{-2}\cdot T^2 \\ & - 3.12\times10^{-3}\cdot Q\cdot M - 5.41\times10^{-4}\cdot Q\cdot T + 9.21\times10^{-4}\cdot M\cdot T \end{aligned} \quad (4)$$

Statistical testing of the model was performed by the Fisher's statistical test for analysis of variance (ANOVA). By this approach, an associated probability (p-value) was evaluated for each term of the regression model. For a given confidence level (95% in our case), an effect with p-value higher than 0.05 was not considered statistically significant on the output variable. Table 12 shows that the OSI is highly dependent on the linear effects of temperature and airflow rate, with associated probabilities $p < 0.001$ and $p = 0.001$,

respectively. However, the influence of quadratic effect was found to be significant only for temperature ($p < 0.001$). The p-values for the remaining effects indicated that the interaction between experimental factors was not significant statistically ($p > 0.05$).

The goodness of the fit is revealed by Fig. 8, where it is plotted the observed OSI values against the calculated ones. These data are correlated by means of a regression line whose equation is inserted in the figure: the better the correlation, the higher the coincidence between this regression line and the diagonal. As shown in this figure, most of the experimental points are placed inside the region delimited by the dotted lines at both sides of the diagonal, which represent a deviation of ±10% between predicted and observed values. Indeed, the residuals presented a mean value (mean absolute value) of 0.2723 and a standard deviation (standard error of estimate) of 0.3567, as shown in Table 12.

Table 12. Analysis of variance for the quadratic model

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value
M, sample size	7.91E-02	1	7.91E-02	6.22E-01	0.4338
Q, airflow rate	1.46	1	1.46	1.15E+01	0.0013
T, temperature	2149.03	1	2.15E+03	1.69E+04	0.0000
M^2	2.22E-01	1	2.22E-01	1.75	0.1920
M·Q	9.72E-02	1	9.72E-02	7.64E-01	0.3859
M·T	3.39E-02	1	3.39E-02	2.66E-01	0.6078
Q^2	8.92E-02	1	8.92E-02	7.01E-01	0.4061
Q·T	7.33E-02	1	7.33E-02	5.76E-01	0.4511
T^2	247.394	1	2.47E+02	1.94E+03	0.0000
Total error	6.87	54	1.27E-01		
Total (corr.)	2405.34	63			

$r^2 = 0.9971$; Adjusted $r^2 = 0.9967$;
Standard error of estimate = 0.3567; Mean absolute error = 0.2723.

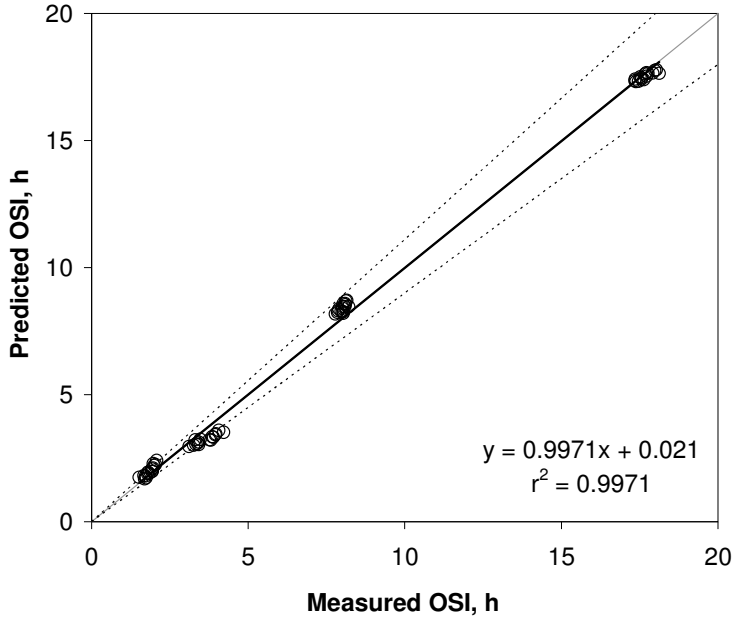

Figure 8. Correlation between predicted and measured values of the oxidative stability index

It can be concluded that the quadratic model assayed predicts adequately the influence of the experimental factors studied upon the response variable, with a determination coefficient $r^2 = 0.9971$. Likewise, the 0.9967 value of the adjusted r^2 was sufficiently good.

By means of this model and employing response surface methodology, contour maps were generated (Fig. 9), where the OSI levels were plotted against airflow rate (Q, L/h) and sample weight (M, g), setting temperature at 60, 70, 80 and 90 ºC. It was observed that the OSI followed a curved surface, owing to the significance of the temperature quadratic effect. However, the contour lines become less pronounced as temperature increases. This fact could support a change in the mechanism of fish oil oxidation with the increase of temperature, favoring secondary reactions of the non-volatile products such as polymer formation (Frankel, 2005), and thus differing from the standard lipid oxidation mechanism at room temperature (Martín et al., 2012).

By optimization of the quadratic model, an optimum value for the OSI = 1.61 h was found. It is shown as a circle point in the contour plots in Fig. 9. This optimum was located inside the experimental ranges of the three input variables: M = 6.91 g, Q = 25 L/h and T = 88.26 ºC. Although the sample weight resulted to be not significant on the OSI determination, an optimum for the relation sample weight - airflow rate exists, the values of M = 6.91 g and Q = 25 L/h being the appropriated conditions which guaranteed an adequate saturation level in the reaction vessel. Moreover, the optimum for the temperature, T = 88.26 ºC, was

found in the nearby of the maximum level assayed for this parameter. Therefore, when determining the OSI of fish oil and in order to minimize the time of analysis, the advisable values for the Rancimat parameters would be the following: M = 6.5-7 g, Q = 25 L/h, T = 85 - 90 ºC.

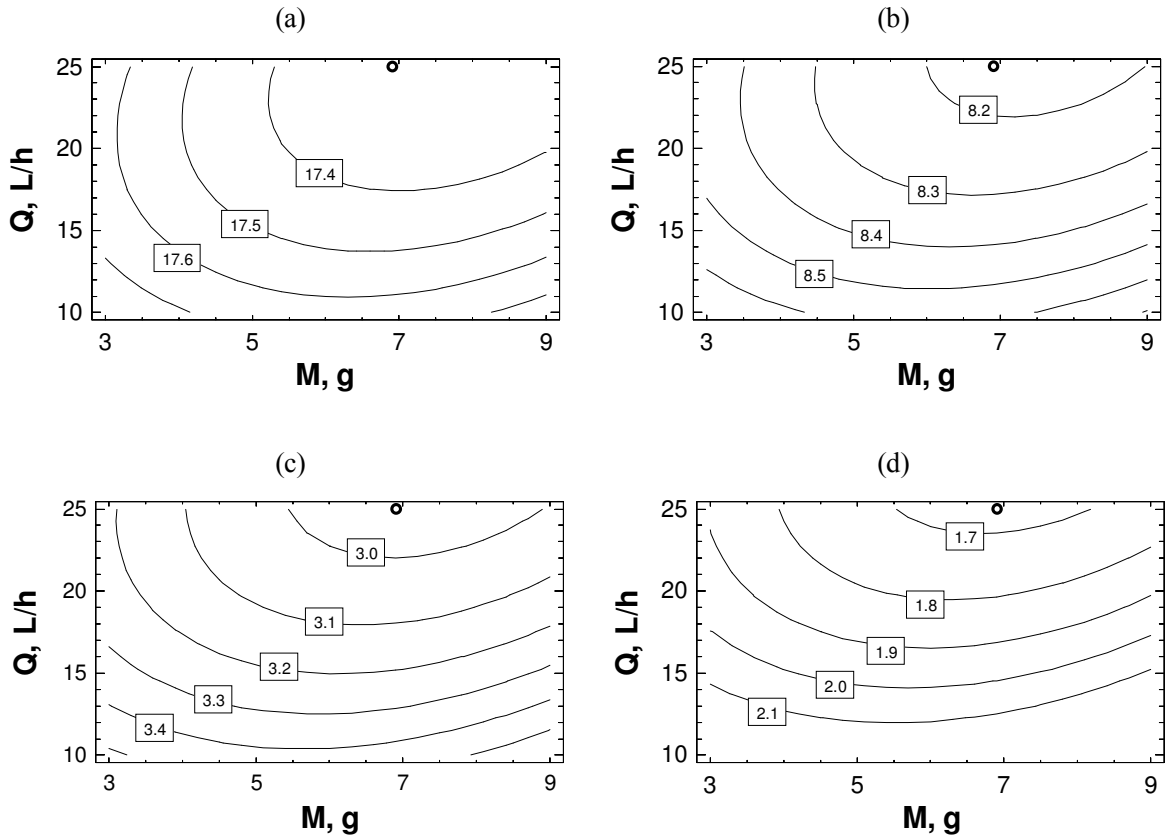

Figure 9. Contour plots for the oxidative stability index at (a) 60 ºC, (b) 70 ºC, (c) 80 ºC and (d) 90 ºC

The lack of significance for both quadratic (except for temperature) and interaction effects in the complete quadratic model suggested fitting the experimental data to a lower level model. To this purpose, the decimal logarithm of OSI values were fitted to a linear model, shown in Eq. 5:

$$\log(\mathrm{OSI}) = 3.27 - 3.29\times10^{-3}\cdot\mathrm{Q} - 1.02\times10^{-3}\cdot\mathrm{M} - 3.29\times10^{-2}\cdot\mathrm{T} \qquad (5)$$

The decimal logarithm was preferred to the raw variable in order to obtain a better degree of fit, according to previous scientific literature (Reynhout, 1991; Hasenhuettl et al., 1992; Farhoosh, 2007a; Farhoosh, 2007b). As in the case of the quadratic model, a complete ANOVA analysis was performed for the lineal model (Table 13). From the F-test, it was concluded that both temperature and airflow rate had a significant effect on the log(OSI),

with associated probabilities $p < 0.001$. Similarly to the quadratic model, the proposed linear model explains the variability of the data to a large extent. This is confirmed by the plot of measured OSI values versus predicted ones (Fig. 10), where most of the experimental data were arranged along the diagonal line, obtaining a coefficient of determination $r^2 = 0.9958$. This value coincided to that adjusted to the degrees of freedom, $r^2 = 0.9958$. The adjusted r^2 statistics is generally the best indicator of fit when comparing two models which involve a different number of coefficients. This is the case at hand, where the response variable was firstly fit to a complete quadratic and then to a linear model. The quadratic model presented a larger value for the adjusted r^2, although the difference between these estimates for the two models assayed was not considerable. It was concluded that both the quadratic and linear model could predict to an adequate degree the OSI values.

Table 13. Analysis of variance for the linear model

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value
M, sample size	3.37E-04	1	3.37E-04	5.59E-01	0.4578
Q, airflow rate	2.17E-02	1	2.17E-02	3.60E+01	0.0000
T, temperature	8.65	1	8.65	1.43E+04	0.0000
Total error	3.62E-02	60	6.03E-04		
Total (corr.)	8.71	63			

$r^2 = 0.9958$; Adjusted $r^2 = 0.9956$;
Standard error of estimate = 0.0245; Mean absolute error = 0.0193.

Regarding the residuals between observed and predicted values, they were distributed around a mean value of 0.0193 and presented a standard deviation of 0.0245. These estimates were almost 20-fold lower than those obtained for the quadratic model, which was attributed to the difference of scale between log (OSI) and the raw variable.

According to the regression model proposed, the generated contour maps, shown in Fig. 11, were comprised of straight lines. It was observed that log(OSI) decreased as airflow rate, sample weight and temperature increased. As a consequence, the minimum for the log(OSI), log(OSI) = 0.22, was obviously found at the maximum values of the three input variables assayed: M = 9 g, Q = 25 L/h and T = 90 ºC.

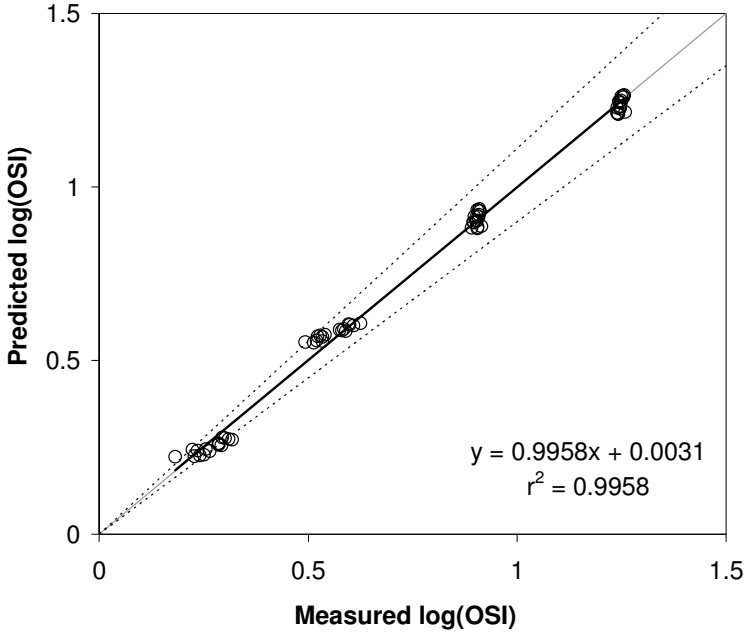

Figure 10. Correlation between predicted and measured values of the logarithm of the oxidative stability index

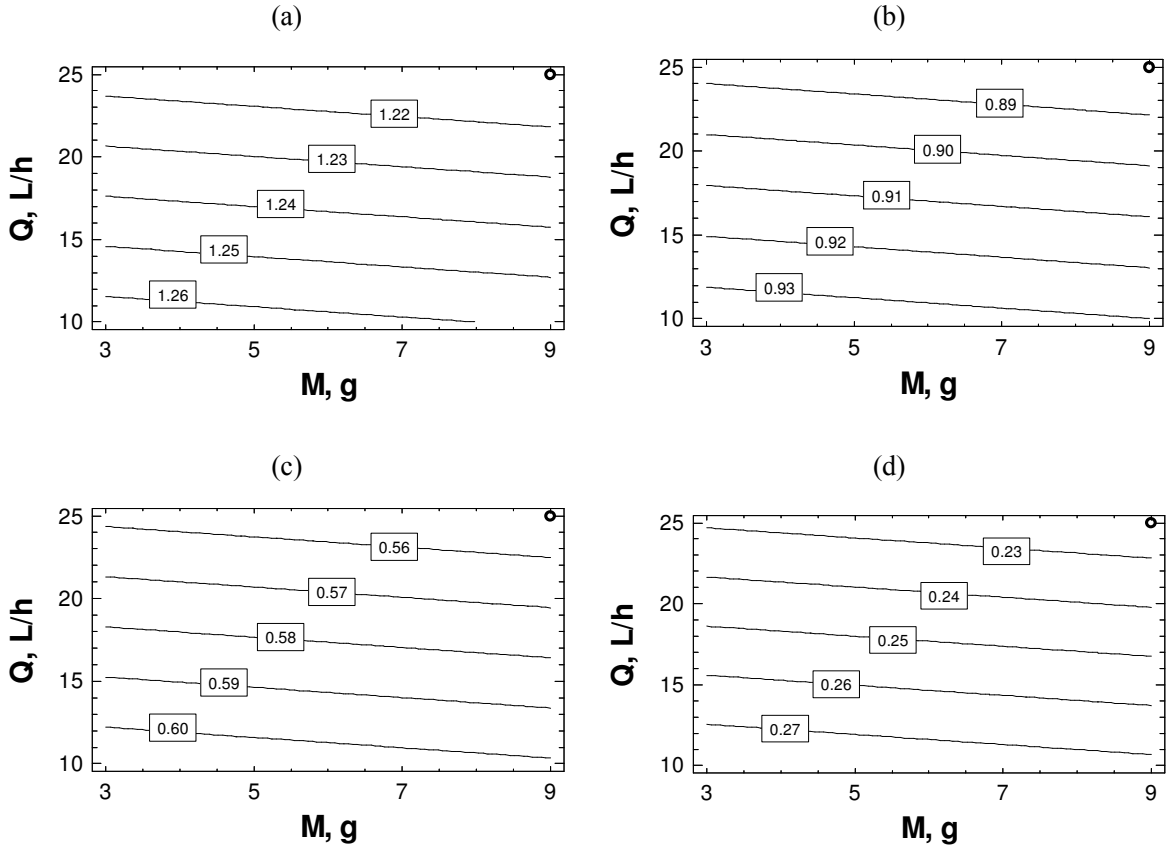

Figure 11. Contour plots for the logarithm of the oxidative stability index at (a) 60 ºC, (b) 70 ºC, (c) 80 ºC and (d) 90 ºC

Owing to its high F-value (Table 3), the temperature was by far the most influencing factor on the oxidative stability index of the cod fish oil. According to these results, the temperature coefficient obtained for cod liver oil was -3.29×10^{-2} ºC^{-1}. Hasenhuettl et al. (1992) reported temperature coefficient values for log(OSI) of vegetable oils between -2.78×10^{-2} and -3.15×10^{-2} ºC^{-1} (mean value: -3.01×10^{-2} ºC^{-1}). Reynhout (1991) and Farhoosh (2007a) reported similar values -3×10^{-2} ºC^{-1} and -3.12×10^{-2} ºC^{-1} for soybean oils. Méndez et al., (1996), employing the natural logarithm of OSI, ln(OSI), obtained a mean temperature coefficient for fish oil of -7.5×10^{-2} ºC^{-1} which is similar to our value (-7.57×10^{-2} ºC^{-1}), which was obtained by employing the decimal logarithm of OSI.

4. CONCLUSION

After evaluating the effect of the Rancimat parameters on the determination of the oxidative stability index of cod liver oil, it was found that both temperature and airflow rate were statistically significant upon the oxidative stability of the fish oil. By using response surface methodology, a minimum for the OSI (OSI = 1.61 h, at M = 6.91 g, Q = 25 L/h and T = 88.26 ºC) was obtained for the quadratic model. From this result, it was concluded that, when determining the OSI of fish oil and in order to minimize the time of analysis, the most appropriated values for the Rancimat parameters would be the following: M = 6.5-7 g, Q = 25 L/h, T = 85-90 ºC.

A simplified linear model was also assayed, which was able to explain the variability of the data to a large extent, with a determination coefficient $r^2 = 0.9958$. From this model, it was revealed that the temperature is by far the most influential factor affecting the OSI, decreasing considerably the OSI as temperature increases. A temperature coefficient of -3.29×10^{-2} ºC^{-1} was obtained, which was similar to other values reported in literature.

5. REFERENCES

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IV. Optimization of Sardine Oil Extraction by Hydraulic Pressing*

The aim of this study was to evaluate the influence of the processing conditions (pretreatment temperature: 5-55 ºC, pressure: 60-120 bar and number of pressing stages: 1-3) on the yield and quality (acidity, peroxide value, p-anisidine, Rancimat induction period) of the oil extracted from whole sardine by hydraulic pressing. Experimental factors were investigated by a designed experiment and optimized by response surface methodology. A maximum yield of oil, 12.47 %, was obtained at 55 ºC, 60 bar and 2 pressing stages. Regarding oil quality, it was found minimum values for acidity (0.25 % oleic at 55 ºC, 60 bar and 1 pressing stage) and for peroxide value (0.29 meq/kg oil at 5 ºC, 60 bar and 1 pressing stage). Hence, the opposite effect of pretreatment temperature and number of pressing stages on the yield of oil and on its oxidation parameters suggested applying the weighted-sum method as multi-objective optimization technique.

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1. INTRODUCTION

Fish oil is the most important source of omega-3 polyunsaturated fatty acids (Rubio-Rodríguez et al., 2010). These fatty acids, especially eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids, have been reported to play a beneficial role in human health (Lees & Karel, 1990; Uauay & Valenzuela, 2000; Ward & Singh, 2005). As a consequence, fish oil use has gained increasing attention over last decades in the pharmaceutical and food fields.

In order to meet the future needs of omega-3 market, fish by-catch and by-products have become sources of great interest for the extraction of fish oil (Bimbo, 2007a). In this sense, discards of small pelagic species such as sardine, mackerel and horse mackerel, which are estimated to be 50,000 tons per year (FAO, 2004), have a great potential as raw material for fish oil production. Among them, particularly interesting is sardine (*Sardina pilchardus*), a discarded species in the west Mediterranean Sea which has been reported to present a high lipid content, up to 13.6 wt%, and an omega-3 composition higher than 3,000 mg/100 g fish (García-Moreno et al., 2013).

Fish oil can be extracted from marine species by several methods such as low temperature solvent extraction (Moffat et al., 1993), enzymatic treatment with proteases (Dumay et al., 2004; Batista et al., 2009) and supercritical fluid extraction (Rubio-Rodríguez et al., 2008). Nevertheless, the wet reduction method, which includes the steps of cooking, pressing (by hydraulic batchwise or by continuous screw presses) and centrifugation (FAO, 1986), is still the most common process to obtain fish oil from fatty fish species (Chantachum et al., 2000; Aidos et al., 2003a).

The wet reduction method generates a dewatered press cake and press liquor containing oil, suspended matter and soluble compounds (FAO, 1986). Although this method allows to obtain high yields, the high temperature normally used in the cooking step (85-95 ºC for 15-20 min) facilitates the oxidative spoilage of the crude oil produced (Rubio-Rodríguez et al., 2012). It results in undesired oxidation products and free fatty acids which considerably reduce both the nutritional and the organoleptic properties of the oil (Frankel, 2005). In addition, an intense cooking treatment may also reduce the quality of the proteins remained in the press cake, decreasing their future applications (Šližyte et al., 2005). It was reported that the walls of the fat cells are broken down below 50 ºC and that the coagulation of the

fish protein is completed at about 75 ºC. Both facts considerably improve the oil extraction. Besides, the temperature of the cooking treatment also has influence on the performance of the pressing stage. Indeed, the intensity of the cooking treatment together with the pressure applied are related to the degree of tissue disruption in the subsequent pressing step, and therefore the amount of suspended solids in the press liquor. However, the optimum conditions for the extraction process are dependent on the raw material used (FAO, 1986).

Therefore, the aim of this paper was to optimize the processing conditions, including temperature, pressing stages and final pressure, for the extraction of fish oil from sardine by hydraulic pressing. Yield and quality of the oil extracted in terms of acidity and oxidation parameters (peroxide value, p-anisidine and Rancimat induction period) were investigated. Finally, a multi-objective optimization was performed in order to find a combination of experimental factors satisfying simultaneously both oil quality and yield.

2. MATERIALS AND METHODS

2.1 Materials

Raw sardines (*Sardina pilchardus*) were purchased from the fishing harbour of Motril (Spain) in July of 2012. They were kept in ice during the transportation and frozen at -20 ºC until use. All other chemicals and solvents used were of analytical grade.

2.2 Proximate chemical composition

The proximate chemical composition of the species studied was determined as described in the Materials and Methods section of chapter II. Measurements were carried out in duplicate.

2.3 Oil extraction procedure

The frozen sardines were thawed at 4 ºC overnight. For each experiment, approximately 2 kg of whole fish (including viscera and gonads) were pretreated at the desired temperature during 30 minutes by means of a water bath model Digiterm 100 (Selecta, Spain). Then, they were fed into an electric press model ESP-K (Sanahuja, Spain) where they were submitted to the selected pressing stages until attaining a chosen pressure. A previous

study (Pérez-Gálvez et al., 2012) reported that whole sardines should be employed instead of grinded material due to the fact that it resulted in higher volumes of collected press liquor. According to these authors, the grinded material acquired a doughy consistence, which enhanced the water holding capacity of the material.

The three experimental factors considered for this study were pretreatment temperature, number of pressing stages and pressing pressure. A face-centered central composite design including 3 central points was executed, in which each input variable was set at three levels: 5, 30 and 55 ºC for pretreatment temperature; 1, 2 and 3 as number of pressing stages; and 60, 90 and 120 bar for pressing pressure. After the pressing process, the press liquor released during the operation was collected and then centrifuged at 20,000×g at 25 ºC for 15 min in order to recover the oil. The yield of oil was reported as the quotient between the mass of oil recovered and the mass of sardines fed into the press. The oil samples were subsequently analyzed.

2.4 Fatty acid profile and lipid class composition

The fatty acid profile of the oils extracted was determined as described in the Materials and Methods section of chapter II. Each sample was analysed in triplicate.

The composition of the lipid fraction was determined by thin layer chromatography (TLC) according to Hita et al. (2009). Plates of silica-gel (Precoated TLC plates, SIL G-25; Macherey-Nagel, Sigma–Aldrich) were activated by heating at 105 ºC for 30 min. The samples were spotted directly on the plate by adding 100 µL of a solution of oil in hexane (0.02 mg/µL). The plates were developed in chloroform/acetone/methanol (95:4.5:0.5, v/v/v). Spots of each lipid were visualized by spraying the plate with iodine vapour in a nitrogen stream. Fractions corresponding to each lipid type were scraped from the plates and methylated and analysed as described above.

2.5 Determination of oil quality parameters

2.5.1 Free fatty acids

The free fatty acids (FFA) content of the oil samples was determined according to the standard ISO 660:2009. This method is based on the titration of the oil, suitably diluted with an ethanol-ethyl ether mixture, with a potassium hydroxide solution employing phenolphthalein as indicator. Results are expressed as percentage of oleic acid.

2.5.2 Peroxide and p-anisidine values

The peroxide value (PV) of the oil samples was determined according to the standard ISO 3960:2007. The PV method is based on the titration with a sodium thiosulfate solution of the oil diluted with an acetic acid-isooctane mixture and then treated with potassium iodide. Results are expressed as miliequivalents per kg of oil. Measurements were carried out in duplicate.

The anisidine value (AV) of the oil samples was determined according to the standard ISO 6885:2006. The AV method is based on the reaction of p-anisidine diluted in acetic acid with the α– and β–unsaturated aldehydes (primary 2-alkenals) present in the oil. Results are expressed as 100 times the increment of absorbance produced by this reaction, measured at 350 nm. Measurements were carried out in duplicate.

2.5.3 Oxidative stability

The Rancimat test was employed to determine the oxidative stability of the oil samples. A Metrohm Rancimat model 743 (Methrom Instruments, Herisau, Switzerland) was utilized. A stream of filtered, cleaned and dried air at a rate of 20 L/h was bubbled into 3 g of oil samples contained in reaction vessels. These vessels were placed in an electric heating block which was set at a temperature of 80 ºC. Effluent air containing volatile organic acids from the oil samples were collected in a measuring vessel with 60 mL of distilled water. The conductivity of the water was continuously recorded and the induction period (IP) was automatically determined by the apparatus. Rancimat induction period was expressed as resistance time (in hours) of the oil to oxidation. Measurements were carried out in duplicate.

2.6 Statistical analysis

The Statgraphics software (version 5.1) was employed to perform the statistical analysis. Firstly, second degree polynomials, Eq. 6, were generated to relate the output variables (Y: FFA, PV, AV, IP and Yield) to the input ones (X: pretreatment temperature, number of pressing stages and pressing pressure):

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j}^{3} b_{ij} X_i X_j \qquad (6)$$

Secondly, the analysis of variance (ANOVA) was performed. The significance of all terms in the models was judged statistically by computing the p-value at a confidence level of 95%.

2.7 Multi-objective optimization

Although higher temperature in the pretreatment step increased the yield of oil recovered, its effect on the oil oxidative stability was detrimental. In this work, a multi-objective optimization was carried out since it was desired to maximize the yield of oil extracted while minimizing its FFA content and PV.

A Pareto Front, defined as a set of non inferior solutions which satisfied the three objectives, was generated by the weighted-sum method (Halsall-Whitney & Thibault, 2006; Kim, 2004). This method consists of expressing a comprehensive objective function (OBJ) as a linear combination of the individual objectives (FFA, PV and Yield), by means of weight factors (w_i), which quantifies the relative importance given to the accomplishment of each individual objective, Eq. 7:

$$OBJ = w_1 \cdot (FFA) + w_2 \cdot (PV) + w_3 \cdot (-Yield) \quad (7)$$

where $w_1+w_2+w_3=1$ and $0 \leq w_i \leq 1$

It should be mentioned that: i) the contribution of yield is negative in order to pose the problem as a minimization of the objective function, and ii) the number of pressing stages is an integer variable. The Solver Tool, included in the MS Excel software, was chosen to carry out all the calculations required for the multi-objective optimization.

3. RESULTS AND DISCUSSION

3.1. Proximate chemical composition of sardines

The sardines employed in this work presented the following proximate composition: moisture 57.5±0.3 wt%, ash 2.7±0.0 wt%, protein 16.7±0.7 %w and lipid 17.7±1.4 wt%. These results are in agreement with our previous work which reported similar protein and ash content for sardine in all the seasons, 17 wt% and 3 wt% respectively (García-Moreno et al., 2013). However, the lipid content was reported to considerably vary within seasons,

reaching minimum values in March (0.6 wt%) and maximum in June (13.6 wt%). Thus, from these results, it was revealed that July, when sardine presented a lipid amount up to 17.7 wt%, is even a better month to obtain a higher yield of oil from this species.

3.2. Characterization of extracted fish oils

The oils extracted from the different batches presented similar fatty acid profiles. Polyunsaturated fatty acids were the major fatty acids identified (~35 wt%), mainly eicosapentaenoic (~13 wt%) and docosahexaenoic (~11 wt%) acids. Saturated fatty acids were found as the second most abundant constituents of the oils (~31 wt%), being the predominant fatty acids palmitic (~20 wt%), myristic (~6 wt%) and stearic (~4.7 wt%). Finally, monounsaturated fatty acids were also detected (~29 wt%). Within this group the major fatty acids were oleic (~12 wt%), palmitoleic (~7 wt%) and gadoleic (~4 wt%). These results are in accordance with previous studies on the fatty acids profile of sardine (*Sardina pilchardus*) (Bandarra et al., 1997; García-Moreno et al., 2013).

Likewise, all the extracted oils presented similar lipid class compositions. Triacylglycerols were the principal components of the oils (>99 wt%), whereas a low percentage of free fatty acids was obtained, below 1 wt%. Neither diacylglycerols nor monoacylglycerols were detected in the oils. However, García-Moreno et al. (2013) reported that sardine oil obtained by the same extraction process also presented cholesterol (7.5 wt%) and phospholipids (0.8 wt%). Although these compounds could be also contained in the extracted oils obtained in this work, they were not identified probably due to the different polarity of the mobile phase employed in the TLC analysis to develop the silica plates.

Table 14 depicts the experimental values of free fatty acid content (FFA), peroxide (PV), p-anisidine (AV) and induction period (IP) measured for the extracted oils. The yield of oil obtained for each batch is also shown.

Table 14. Experimental design and measured values for the response variables

Exp#	Temperature (ºC)	Pressure (bar)	Pressing stages	FFA (% oleic)	PV (meq/kg oil)	AV	IP (h)	Yield (%)
1	5	60	1	1.15	0.33	0.42	1.45	1.16
2	5	60	3	1.06	1.31	1.55	1.67	4.76
3	5	90	2	0.93	2.38	0.44	1.91	3.50
4	5	120	1	1.20	2.42	0.00	1.79	3.44
5	5	120	3	0.99	2.62	0.00	1.41	7.15
6	30	60	2	0.98	2.87	0.00	1.88	4.83
7	30	90	1	0.93	1.67	0.00	1.95	4.10
8	30	90	2	1.06	3.69	1.29	1.94	7.34
9	30	90	2	1.16	3.79	1.48	1.92	6.97
10	30	90	2	1.21	2.97	1.35	1.56	7.11
11	30	90	3	1.23	3.69	0.86	1.58	6.99
12	30	120	2	0.68	2.08	0.00	2.06	6.97
13	55	60	1	0.25	4.43	0.00	1.49	11.10
14	55	60	3	0.59	5.75	1.98	1.09	12.26
15	55	90	2	0.55	5.50	1.27	1.35	12.78
16	55	120	1	0.34	5.74	1.63	1.23	8.81
17	55	120	3	0.70	5.58	1.94	1.30	9.97

The FFA content of crude fish oil is an important quality parameter due to the fact that the value of the oil decreases when increasing its acidity (Bimbo, 2007b). Besides, free fatty acids are more susceptible to oxidation than esterified fatty acids, affecting negatively to oil quality (Aidos et al., 2001). FFA are generated from hydrolysis of triglycerides and phospholipids by lipases in the presence of moisture (Aidos et al., 2003b). Bimbo (1998) stated that the FFA content of crude fish oil usually ranges between 2 and 5 % oleic. Table 14 shows that the FFA content of all the extracted oils obtained in this work varied from 0.25 to 1.23 % oleic. From the results in Table 14, it was noticed that the oils with the lowest FFA content were obtained at the highest processing temperature assayed (55 ºC). It may be explained due to the fact that at temperature above 45 ºC lipases from sardine may become unstable, as described by Mukundan et al. (1985). It seems that the FFA content also increased with the number of pressing stages since it implied larger contact time of oil with stickwater which favored hydrolysis (Table 14). On the other hand, it was not observed a clear influence of pressing pressure on FFA. Higher FFA content (up to 2.9 % oleic) were reported by Chantachum et al. (2000) and Aidos et al. (2001) for oils produced from tuna heads and herring by-products, respectively. Although these authors employed higher cooking temperatures (95 ºC), the difference in the FFA values may be better due to the low quality of the raw material employed in those extraction processes (Aidos et al., 2003c).

In order to evaluate the quality of the extracted oils in terms of oxidation parameters, the PV of the oils was determined to quantify the primary oxidations products, whereas the AV was measured to assay the concentration of secondary oxidation products generated by decomposition of hydroperoxides. Furthermore, the Rancimat induction periods of the oils were determined aiming at evaluating their oxidative stabilities. Table 14 shows that the PV of the oils varied from 0.33 to 5.75 meq/kg oil. It was clearly observed that exposing the raw material to higher pretreatment temperature enhanced the formation of peroxides in the oil. This fact is also supported by the results reported by Chantachum et al. (2000), which extracted oil from tuna heads by heating at 95 ºC resulting in PV up to 30 mg/kg. Moreover, the increase in the number of pressing stages denotes a higher residence time of the oil in the press which also increase the oxidative spoilage of the oil (Aidos et al., 2003a). In addition, higher pressing pressure also favors the production of hydroperoxides. It may be related with the larger amounts of suspended solids in the press liquor, including iron-containing proteins which bring iron into closer proximity with oil (Decker & Xu, 1998). Considering secondary oxidation products, all the oils extracted presented insignificant AV which ranged from 0 to 1.98. As for PV, the highest AV was found at the highest temperature assayed. It is due to the fact that temperature contributes to the formation of hydroperoxides as well as their decomposition to secondary oxidation products (Frankel, 2005). The negative effect of temperature in AV was confirmed by the study of Aidos et al. (2001) which reported higher AV, up to 8.9, for oil extracted from herring by-products after preheating at 95 ºC for 8 min. With regards to Rancimat induction periods, a direct correspondence between IP and PV and AV was only observed for the batches carried out at 55 ºC, which showed the lowest IP. For instance, oil obtained in experiment 14 which had the highest PV and AV also showed the minimum IP (Table 14).

In the case of yield, values ranging from 1.16 to 12.78 % were obtained (Table 14). Lower yields were described for the extraction of oil by the same method from tuna heads, 4.8 %, (Chantachum et al., 2000) from herring by-products, 9.9 %, (Aidos et al., 2001) and from sardine discards, 2 %, (Pérez-Gálvez et al., 2012). In our study, working at the highest temperature assayed allowed to obtain the highest values of oil yield. It is due to the fact that at this temperature the lipid cells were completely ruptured. Besides, high temperatures reduce viscosity of the oil and tend to facilitate the flow from the solid phase (FAO, 1986). On the other hand, the influence of pressing stages and pressure on the yield

of oil was not that clear. Although increasing the number of stages and pressure should improve the release of press liquor, it led to a high compaction of the press cake which makes more difficult the expulsion of fluid (Pérez-Gálvez et al., 2012) and it also resulted in larger amount of suspended solids which can affect negatively to the separation of oil (FAO, 1986).

3.3. Statistical modeling

The experimental data for each measured variable were fitted to a quadratic model. Table 15 shows the polynomial coefficients for each surface response model calculated by multiple regression as well as their associated p-value. It was observed that all the output variables (FFA, PV, AV, IP and Yield) were dependent on the linear effect of temperature ($p < 0.05$). Similarly, T^2 was also significant on FFA and Yield. On the contrary, the pressure exerted had no significant effect upon the output variables. Indeed, only the interaction pressure-temperature was significant on Yield. Regarding number of pressing stages, its linear effect was statistically significant on AV and Yield. Table 15 also shows that the proposed quadratic models for FFA, PV and Yield explain the variability of the data to a large extent, with coefficients of determination, R^2, around or higher than 0.90. However, for AV and IP lower R^2 were obtained, 0.78 and 0.76 respectively.

In order to optimize the extraction process, FFA and PV were chosen as indicators of the oil quality in terms of acidity and oxidation products, whereas Yield was selected as output variable showing the efficiency of the process. In this sense, the goodness of the fit for these three variables was also proved by plotting the measured values against the predicted ones for FFA (Fig. 12a), PV (Fig. 12b) and Yield (Fig. 12c). The data were correlated by means of a regression line whose equation was inserted in each figure. Deviation of ±10 % between experimental and predicted values was shown as dotted lines.

Table 15. Polynomial coefficients and p-values for the response variables

	FFA (% oleic)		PV (meq/kg oil)		AV		IP (h)		Yield (%)	
	Coefficient	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*
Constant	3.63E-01	-	-7.39	-	-3.79	-	1.20	-	-1.08E+01	-
A: Temperature, ºC	-2.14E-03	0.0005	9.24E-03	0.0001	-8.85E-02	0.0353	2.58E-02	0.0372	1.56E-01	<0.0001
B: Pressure, bar	2.87E-02	0.8107	1.34E-01	0.1404	8.95E-02	0.8290	-4.76E-03	0.7695	1.41E-01	0.4927
C: Pressing stages	-4.54E-01	0.1900	2.30	0.0945	1.29	0.0395	6.64E-01	0.2524	5.14	0.0048
AA	-3.79E-04	0.0384	1.61E-03	0.0542	6.86E-04	0.2316	-4.86E-04	0.0564	2.88E-03	0.0193
AB	3.67E-05	0.6257	-3.77E-04	0.2996	5.93E-04	0.0512	-2.17E-05	0.8390	-1.54E-03	0.0121
AC	5.00E-03	0.0535	-1.00E-04	0.9924	5.80E-03	0.4691	-8.50E-04	0.7908	-2.50E-02	0.1131
BB	-1.63E-04	0.1586	-5.09E-04	0.3277	-4.74E-04	0.2342	4.00E-05	0.7948	-4.90E-04	0.4830
BC	-4.17E-04	0.8233	-9.42E-03	0.2996	-1.17E-02	0.1072	-5.42E-04	0.8390	4.58E-04	0.9693
CC	1.03E-01	0.3057	-2.54E-01	0.5790	3.80E-03	0.9911	-1.69E-01	0.2451	-7.96E-01	0.2230
R^2	0.8936		0.9173		0.7830		0.7591		0.9603	

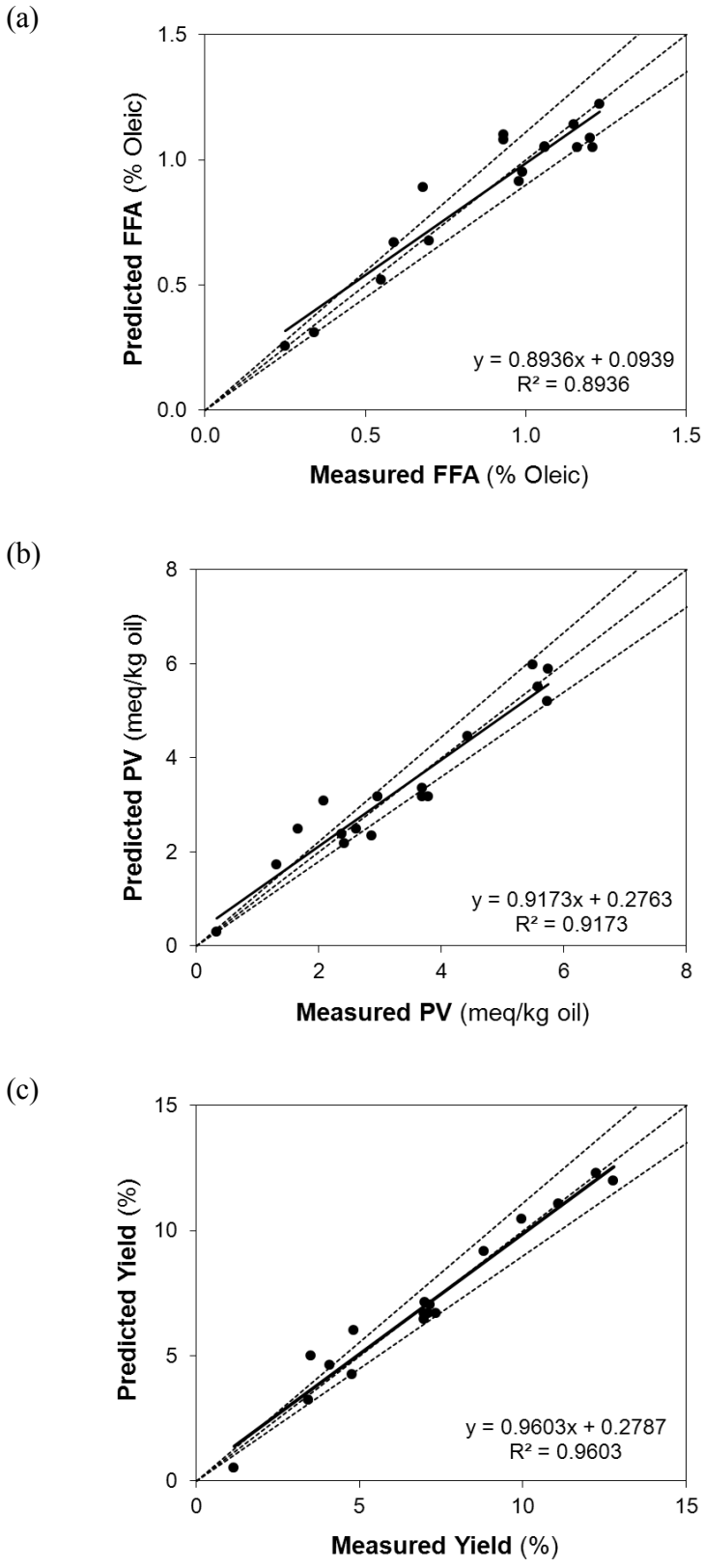

Figure 12. Correlation between predicted and measured values of (a) FFA, (b) PV and (c) Yield

Then, contour plots for FFA, PV and Yield were generated by means of the quadratic models obtained and using response surface methodology (Fig. 13). By optimizing the second-order models, optimum values, marked as circles in the contour plots (Fig. 13), were found for FFA, PV and Yield. Due to the fact that the number of pressing stages is an integer variable, it was set at the optimum value obtained for each variable: 1 for FFA (Fig. 13a) and PV (Fig. 13b) and 2 for Yield (Fig. 13c).

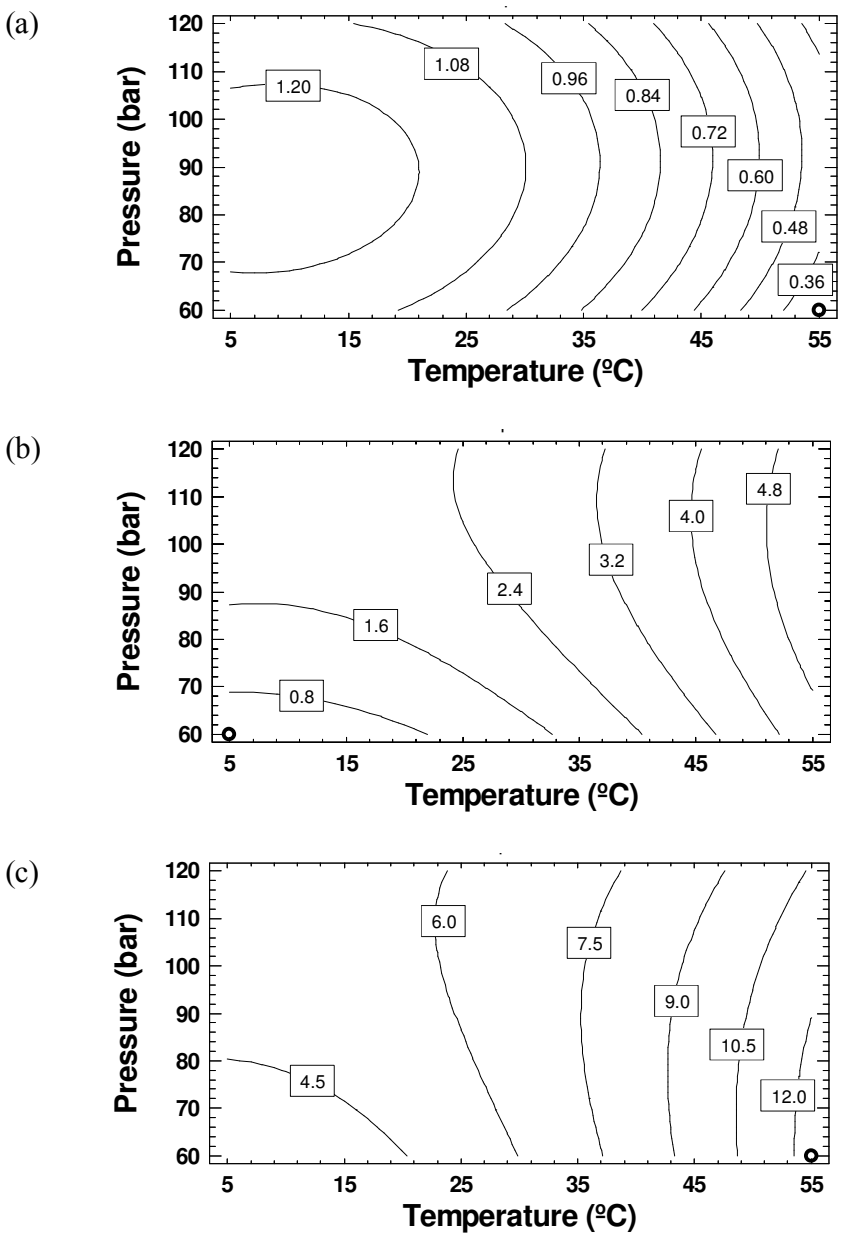

Figure 13. Contour plots for (a) FFA, (b) PV and (c) Yield

In Fig. 13a, the minimum value for FFA, 0.25 % oleic, was found at the lowest values assayed for number of pressing stages (one) and pressure (60 bar) and at the highest temperature (55 ºC). Reducing the number of pressing stages decreases the contact time between oil and stickwater minimizing the FFA content of the final oil. In addition, preheating at temperature above 45 ºC was preferred because this fact led to the denaturation of the lipases which contribute to hydrolysis. In the case of PV (Fig. 13b), a minimum value, 0.29 meq/kg oil, was located at the minimum levels assayed for the three input parameters, one pressing stage, 60 bar and 5 ºC. As for FFA, one pressing stage reduced the residence time of the oil in the press which minimized its oxidation. In contrast with FFA, for minimizing PV the lowest temperature assayed (5 ºC) was required in order to decrease the formation of hydroperoxides. Additionally, the minimum pressure employed (60 bar) resulted as optimum pressure for minimizing FFA and PV. It may be due to the fact that increasing the pressing pressure could enhance the stress suffered by the oil favoring its oxidation (Aidos et al., 2003a). Fig. 13c shows the maximum predicted value for Yield, 12.47 %, which was found at the medium value evaluated for number of pressing stages (two), the minimum value of pressure (60 bar) and the highest temperature (55 ºC). From this fact, it may be drawn that preheating at 55 ºC results in a maximum cell rupture and it also may coagulate some proteins enhancing the separation of oil from the solid phase (FAO, 1986). Moreover, the optimum of pressing stages may be attributed to the fact that a high degree of compaction was attained by the press cake after two pressing stages at 60 bar reducing the release of press liquor from the pores (Pérez-Gálvez et al., 2012).

From these results, it is indicated that different processing conditions (number of pressing stages and pretreating temperature) were required for the optimization of FFA, PV and Yield. It was observed that high temperature enhanced the yield oil but reduced its quality in terms of hydroperoxides concentration. Additionally, employing one pressing stage preserved the quality of the extracted oil while two pressing stages were preferred to maximize the yield of oil.

3.4. Multi-objective optimization

A multi-objective optimization technique was employed since simultaneous optimum of the three output variables selected (FFA, PV and Yield) were pursued.

Firstly, a Pareto Front, shown in Table 16, was generated using the weighted-sum method. It allowed to obtain a set of non inferior solutions which satisfied the three objectives to an adequate degree. Secondly, the Pareto Front was translated to the decision space, also shown in Table 16, by determining the optimum combination of input variables (temperature, pressure and number of pressing stages) for each selection of weight factors (w_1, w_2, w_3).

In rows 1-3 of Table 16, two of the three weight factors are given a value of zero; thus, the optimization of the objective function is equivalent to the optimization of a single objective. In rows, 4-30, a bi-objective optimization problem is faced since only one of the weight factors takes a value of zero. The following three situations are possible:

a) Bi-optimization of PV and Yield where w_1=0 (rows 4-12). It is shown that a decrease in PV (desired) implied a reduction of Yield (undesired). This trend was more evident at values of PV lower than 4.45 (w_3<0.4). The pressure was maintained constant at 60 bar and the temperature and the number of pressing stages decreased from 55 to 6.86 ºC and from 2 to 1, respectively. However, different conditions (5 ºC, 120 bar, three pressing stages) were obtained when w_2=0.7.

b) Bi-optimization of FFA and Yield where w_2=0 (rows 13-21). It is observed that when w_3 takes values ≥0.1 the individual optimum of Yield is always obtained. Then, the optimum extracting conditions were maintained constant at 55 ºC, 60 bar and two pressing stages. It is due to the fact that Yield had a greater importance on the objective function than FFA.

c) Bi-optimization of FFA and PV where w_3=0 (rows 22-30). It is noticed that a reduction of PV (desired) denotes an increase of FFA (undesired). This phenomenon is noticeable when changing w_1 from 0.9 to 0.8. Then, it was always found the optimum value of PV. The optimum pressure and number of pressing stages were kept at the same values, 60 bar and one respectively, whereas the temperature varied from the optimum for FFA (55 ºC) to the optimum for PV (5 ºC). This fact is also explained because PV also had a higher significance than FFA on the objective function.

Additionally, four solutions in which any weight takes the value of zero were obtained (rows 31-34).

Table 16. Set of optimal solutions and decision space for the multi-objective optimization problem

Row	w_1	w_2	w_3	FFA (% oleic)	PV (meq/kg oil)	Yield (%)	Temperature (ºC)	Pressure (bar)	Pressing stages
1	1.00	0.00	0.00	0.25	4.45	11.06	55.00	60	1.00
2	0.00	1.00	0.00	1.14	0.29	0.51	5.00	60	1.00
3	0.00	0.00	1.00	0.36	5.42	12.47	55.00	60	2.00
4	0.00	0.10	0.90	0.36	5.42	12.47	55.00	60	2.00
5	0.00	0.20	0.80	0.36	5.42	12.47	55.00	60	2.00
6	0.00	0.30	0.70	0.36	5.42	12.47	55.00	60	2.00
7	0.00	0.40	0.60	0.36	5.42	12.47	55.00	60	2.00
8	0.00	0.50	0.50	0.36	5.42	12.47	55.00	60	2.00
9	0.00	0.60	0.40	0.25	4.45	11.06	55.00	60	1.00
10	0.00	0.70	0.30	0.95	2.48	7.02	5.00	120	3.00
11	0.00	0.80	0.20	1.13	0.41	1.22	12.91	60	1.00
12	0.00	0.90	0.10	1.14	0.30	0.65	6.86	60	1.00
13	0.90	0.00	0.10	0.36	5.42	12.47	55.00	60	2.00
14	0.80	0.00	0.20	0.36	5.42	12.47	55.00	60	2.00
15	0.70	0.00	0.30	0.36	5.42	12.47	55.00	60	2.00
16	0.60	0.00	0.40	0.36	5.42	12.47	55.00	60	2.00
17	0.50	0.00	0.50	0.36	5.42	12.47	55.00	60	2.00
18	0.40	0.00	0.60	0.36	5.42	12.47	55.00	60	2.00
19	0.30	0.00	0.70	0.36	5.42	12.47	55.00	60	2.00
20	0.20	0.00	0.80	0.36	5.42	12.47	55.00	60	2.00
21	0.10	0.00	0.90	0.36	5.42	12.47	55.00	60	2.00
22	0.90	0.10	0.00	0.25	4.45	11.06	55.00	60	1.00
23	0.80	0.20	0.00	1.14	0.29	0.51	5.00	60	1.00
24	0.70	0.30	0.00	1.14	0.29	0.51	5.00	60	1.00
25	0.60	0.40	0.00	1.14	0.29	0.51	5.00	60	1.00
26	0.50	0.50	0.00	1.14	0.29	0.51	5.00	60	1.00
27	0.40	0.60	0.00	1.14	0.29	0.51	5.00	60	1.00
28	0.30	0.70	0.00	1.14	0.29	0.51	5.00	60	1.00
29	0.20	0.80	0.00	1.14	0.29	0.51	5.00	60	1.00
30	0.10	0.90	0.00	1.14	0.29	0.51	5.00	60	1.00
31	0.50	0.25	0.25	0.36	5.42	12.47	55.00	60	2.00
32	0.25	0.50	0.25	0.25	4.45	11.06	55.00	60	1.00
33	0.25	0.25	0.50	0.36	5.42	12.47	55.00	60	2.00
34	0.33	0.33	0.34	0.36	5.42	12.47	55.00	60	2.00

Hence, the required characteristics of the extracted oil in terms of acidity and hydroperoxides concentration as well as the yield of oil desired will determine the selection of a single optimum solution from the Pareto Front (optimum combination of pretreating temperature, pressure and number of pressing stages). Nevertheless, it should be noted that values of w_3<0.4 are not recommended in order to not obtain considerably low yield (<11.06 %). It implies to work at 55 ºC, 60 bar and two or one pressing stages.

4. CONCLUSIONS

The results shown in this work indicate the optimum conditions for the extraction of oil from whole sardine by hydraulic pressing. In order to obtain oil with minimum FFA content, 0.25 % oleic, the highest pretreatment temperature assayed (55 ºC) and the lowest pressure (60 bar) and number of pressing stages (one) were preferred. On the contrary, for minimization of hydroperoxides formation, PV of 0.29 meq/kg oil, the lowest pretreating temperature was required (5 ºC). It was revealed that choosing the minimum pressure and number of pressing stage reduced the oxidative stress in the oil and the residence time of the oil in the press which enhanced its quality in terms of acidity and oxidation parameters. Regarding efficiency of the extraction process, employing the highest pretratment temperature (55 ºC) and pressing at 60 bar using two pressing stages maximized the yield of oil, 12.49 %. Therefore, the conflicting behavior of pretreatment temperature and number of pressing stages towards the optimization of FFA, PV and Yield, motivated the generation of a set of non inferior solutions (Pareto Front) which satisfied the three objectives to an adequate degree.

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V. Optimization of Bleaching Conditions for Sardine Oil*

This work studies the influence of the operational conditions (temperature: 90-130 ºC, activated clay amount: 1-5 wt% and contact time: 20-60 min) on the bleaching process of degummed and neutralized sardine oil. The bleached oils were evaluated for free fatty acids, peroxide value, p-anisidine value, totox, oxidation stability (Rancimat) and color (CIELAB coordinates). Such measured variables were statistically modeled by full factorial experimental design and response surface methodology. An individual optimum value for totox of 21.38 (which minimizes oxidation products content) was obtained at 130 ºC, 5 wt% of clay amount and 60 min. Regarding the color quality, it was found a maximum value for hue-angle (89.19 at 99.2 ºC, 5 wt% of clay and 56. 6 min) and a minimum value for chroma (81.76 at 109.4 ºC, 5 wt% of clay and 49.4 min). The conflicting effect of temperature and time on the location of the individual optimum values motivated the generation of a Pareto front (set of non inferior solutions) employing the weighted-sum multi-objective optimization technique.

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1. INTRODUCTION

Small pelagic fish species such as sardine, mackerel and horse mackerel are a major source of fish oil presenting a high content of omega-3 polyunsaturated fatty acids (PUFA), including EPA (C20:5n-3) and DHA (C22:6n-3) (Shahidi, 2006). In the last decades, these omega-3 PUFA have received much attention in the scientific and industrial communities because of their benefits on human health, such as prevention of cardiovascular disease (Lees & Karel, 1990), improvement of the anti-inflammatory response (Uauy & Valenzuela, 2000) and development of brain and eye retina in infants (Ward & Singh, 2005).

Crude fish oil, which is generally obtained by the wet pressing method (FAO, 1986), contains non-triglycerides, such as phospholipids, free fatty acids, oxidation products, pigments and insoluble impurities that reduce the oil quality (Huang & Sathivel, 2010). Thus, in order to meet the standards safety and an acceptable shelf life, this oil requires a refining treatment.

The conventional fish oil-refining steps include: *degumming* (removal of phospholipids by the addition of phosphoric or citric acid), *neutralization* of free fatty acids with sodium hydroxide, *bleaching* with activated clays which adsorb oxidation products and pigments, and *deodorization* by vacuum distillation of volatiles compounds such as aldehydes and ketones (Rubio-Rodríguez et al., 2010).

Adsorption processes are particularly important since they can be used to remove most of the impurities that are present in the unpurified fish oil (Huang & Sathivel, 2010). This is due to the fact that the activated clays are able to adsorb (apart from oxidation products and pigments) trace metals and remains of phospholipids and soaps, which improves to a great extent the oxidation parameters and sensory quality of the oil (Proctor & Toro-Vazquez, 1996; Sathivel & Prinyawiwatkul, 2004). In the view of the above, the study of the bleaching process is an important issue to be considered in order to produce more efficiently fish oil for human consumption.

The purpose of the present work was to optimize the operational conditions of the bleaching process for sardine oil. Such a systematic study is not present in the current scientific literature. The influence of temperature, clay amount and contact time on key oxidation (free fatty acids, peroxide, p-anisidine and totox value, and induction period) and

color (CIELAB color coordinates, hue-angle and chroma) parameters was evaluated. Finally, multi-objective optimization was performed since optimum values of oxidation and color parameteres were simultaneously pursued.

2. MATERIALS AND METHODS

2.1 Materials

Degummed and neutralized sardine oil (DNSO) was employed in this work. It was purchased from Industrias Afines, S.L. (Vigo, Spain) and kept in plastic bottles covered with aluminum foil at 4 ºC under nitrogen atmosphere. Acid-activated clay, Tonsil 278, was utilized in the bleaching procedure. Acid-activated bleaching clays are preferably employed than natural bleaching earths due to their demonstrated better efficiency during the adsorption process (Rossi et al., 2003; Sathivel, 2010). The bleaching clay was kindly provided by Süd-Chemie España, S.L. (Toledo, Spain).

2.2 Bleaching procedure

DNSO was subjected to bleaching under partial vacuum (70 mmHg vacuum pressure). Sixty grams of DNSO were introduced with a given clay amount into a three-neck round-bottom flask. The samples were stirred at 300 rpm and kept at the desired temperature using an oil bath controlled by a thermo-regulator. The three experimental factors considered for this study were temperature, clay amount and contact time. A factorial experimental design comprising 27 runs was executed, in which each input variable was set at three levels: 90, 110 and 130 ºC for temperature; 1, 3 and 5 % for clay amount; and 20, 40 and 60 min for contact time. After the contact time was completed for each run, the adsorbent was removed from the oil by centrifugation. The oil samples were stored under nitrogen at 4 ºC in 50 mL Falcon tubes covered with aluminum foil until analysis.

2.3 Determination of oxidation parameters

2.3.1 Free fatty acids, peroxide, p-anisidine and totox

The free fatty acids (FFA), peroxide value (PV) and p-anisidine value (AV) were calculated as described in the Materials and Methods section of chapter IV.

Totox is a comprehensive oxidation index calculated from a weighted sum of peroxide value (PV) and p-anisidine value (AV) by applying Eq. 8:

$$Totox = 2 \cdot PV + AV \quad (8)$$

2.3.2 Oxidative stability

The induction period (IP in h) of the oil samples was calculated as described in the material and section of chapter IV. The heating block was set at 100 ºC.

2.4 Color measurements

Spectroradiometry was employed for color measurements. Compared to spectrophotometry, this method is preferred for the analysis of samples presenting some degree of turbidity. Furthermore, in contrast to the widely extended Lovibond Tintometer, spectroradiometry yields non-subjective measurements (Smedley, 1995; Melgosa et al., 2009).

The color measurements of the oil samples were carried out in duplicate placing the cuvettes (10 mm x 10 mm x 45 mm) containing the oil in the center of a SpectraLight III light booth equipped with a daylight source simulating the D65 illuminant and a non-fluorescent white background. D65 has long been the daylight source specifically recommended by the CIE for most of applications (Roa et al., 2006). Because of the translucency of the samples, fixed background was positioned behind the oil samples. Spectral radiant power was measured using a spectroradiometer CS-2000 (Konica Minolta Sensing Inc., Tokyo, Japan), which measures the spectrum from 380 to 780 nm at 1 nm steps.

From the spectral measurements, CIELAB color coordinates were computed assuming CIE 1964 Supplementary Standard Observer (Billmeyer & Saltzman, 2000). For computations of these coordinates, a standard white was used (Konica Minolta Sensing Inc., Tokyo, Japan) (CIE, 2004). Within the CIELAB color space, two color coordinates a* and b*, and a psychometric index of lightness, L*, are defined. a* takes positive values for reddish colors and negative values for the greenish ones, whereas b* takes positive values for yellowish colors and negative values for the bluish ones. L* is an estimation of the relative luminosity, and according to this parameter any given color can be regarded as equivalent to a member of a grey scale, between black (L* = 0) and white (L* = 100). From a* and

b*, the psychometric parameters hue-angle (h_{ab}) and chroma (C^*_{ab}) were calculated as Eq. 9 and Eq. 10, respectively:

$$h_{ab} = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (9)$$

$$C^*_{ab} = \sqrt{\left(a^*\right)^2 + \left(b^*\right)^2} \quad (10)$$

The hue-angle is the attribute according to which colors have been traditionally defined as reddish, greenish and others (Wyszecki & Stiles, 1967). The chroma value is related to the quantitative attribute of colorfulness and allows to determine (for each hue-angle) the degree of difference when compared to a grey color with the same lightness.

2.5 Statistical analysis

The Statgraphics software (version 5.1) was used to generate the statistical analysis and the regression models. Firstly, the output variables (Y: FFA, PV, AV, totox, IP, a*, b*, L*, chroma and hue-angle) were related to the input variables (X: temperature, clay amount and contact time) by second degree polynomials as follows, Eq. 11:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j}^{3} b_{ij} X_i X_j \quad (11)$$

where the coefficients bi and bii are related to the linear and quadratic effects, respectively, of each input factor on the output variable and the cross-product coefficients b_{ij} represent the interactions between two input variables.

Secondly, the analysis of variance (ANOVA) was carried out. The significance of all terms in the models was judged statistically by computing the p-value at a confidence level of 95%. The regression coefficients were then used to generate contour maps and to find the optimal bleaching conditions which maximize the quality of the oil in terms of color and oxidation parameters, following the response surface method (Myers & Montgomery, 2002).

2.6 Multi-objective optimization

A problem of multi-objective optimization arises when several objectives, possibly conflicting, must be satisfied. In our case, it is desired to obtain minimum values for totox and chroma while maximizing hue-angle in the final bleached oil.

The concept of Pareto Front, related to the identification of an adequate solution, consists of a set of non inferior solutions, which are defined as those in which an improvement in one objective requires a degradation of another (Halsall-Whitney & Thibault, 2006). In this work, the weighted-sum method (Kim, 2004) was employed to generate the Pareto Front. This method consists in expressing a comprehensive objective function (OBJ) as a linear combination of the individual objectives (totox, hue-angle and chroma), by means of weight factors (w_i), which quantifies the relative importance given to the accomplishment of each individual objective, Eq. 12:

$$OBJ = w_1 \cdot (Totox) + w_2 \cdot (-Hue\text{-}angle) + w_3 \cdot (Chroma) \qquad (12)$$

where $w_1+w_2+w_3=1$ and $0 \leq w_i \leq 1$

Note that the contribution of hue-angle is negative in order to pose the problem as a minimization of the objective function. The Solver Tool, included in the MS Excel software, was chosen to carry out all the calculations required for the multi-objective optimization.

3. RESULTS AND DISCUSSION

3.1 Characterization of bleached fish oils

Table 17 presents the experimental values of free fatty acid content (FFA), peroxide (PV), p-anisidine (AV), totox, induction period (IP), CIELAB color coordinates (L*, a* y b*), chroma and hue-angle of the DNSO and of the 27 bleached oils produced.

Table 17. Experimental design and measured values for the response variables

Exp#	Temp. (ºC)	Clay amount (%w)	Time (min)	FFA (% oleic)	PV (meq/kg oil)	AV	Totox	IP (h)	Color			Chroma	Hue angle (deg)
									L*	a*	b*		
DNSO	-	-	-	0.17	2.36	77.00	81.72	0.59	71.46	16.39	96.75	98.13	80.39
1	90	1	20	0.19	2.79	57.48	63.06	0.35	74.61	15.90	98.60	99.88	80.84
2	90	1	40	0.19	3.33	57.21	63.87	0.30	75.66	14.56	98.13	99.20	81.56
3	90	1	60	0.17	2.42	48.81	53.65	0.43	76.22	16.68	99.56	100.95	80.49
4	90	3	20	0.18	2.46	48.78	53.70	0.37	81.82	10.98	99.02	99.63	83.67
5	90	3	40	0.22	2.66	38.87	44.19	0.47	81.80	9.70	94.68	95.18	84.15
6	90	3	60	0.21	1.92	46.07	49.91	0.50	81.57	11.72	95.67	96.39	83.02
7	90	5	20	0.17	1.31	41.21	43.83	0.32	85.05	5.94	92.73	92.92	86.33
8	90	5	40	0.15	1.97	33.94	37.88	0.43	87.09	2.59	78.97	79.01	88.12
9	90	5	60	0.13	1.00	35.37	37.37	0.72	86.51	1.80	87.76	87.78	88.82
10	110	1	20	0.14	2.67	53.66	59.00	0.49	74.64	16.56	93.62	95.07	79.97
11	110	1	40	0.23	2.92	45.90	51.74	0.40	75.19	17.48	94.58	96.18	79.53
12	110	1	60	0.15	3.08	49.47	55.63	0.28	75.00	17.17	93.67	95.23	79.62
13	110	3	20	0.15	2.42	44.44	49.28	0.55	78.73	8.22	90.36	90.73	84.80
14	110	3	40	0.21	1.77	36.85	40.39	0.62	81.22	9.92	92.96	93.49	83.91
15	110	3	60	0.16	1.45	40.32	43.22	0.73	80.53	8.57	91.61	92.01	84.66
16	110	5	20	0.12	0.98	42.43	44.39	0.72	82.48	0.87	83.45	83.45	89.41
17	110	5	40	0.12	1.00	35.06	37.06	0.78	83.61	0.33	83.21	83.21	89.77
18	110	5	60	0.10	0.83	25.88	27.54	0.83	83.56	-1.08	80.85	80.85	90.76
19	130	1	20	0.21	1.72	45.99	49.43	0.45	71.63	16.69	95.96	97.41	80.13
20	130	1	40	0.21	2.02	47.46	51.50	0.58	73.05	14.26	95.45	96.51	81.50
21	130	1	60	0.18	1.56	46.62	49.74	0.76	72.16	14.90	95.05	96.21	81.09
22	130	3	20	0.19	0.75	42.99	44.49	0.77	74.50	12.51	93.64	94.47	82.39
23	130	3	40	0.14	0.87	31.80	33.54	0.95	75.08	10.34	91.66	92.24	83.56
24	130	3	60	0.15	0.00	33.39	33.39	0.57	74.58	12.13	93.19	93.98	82.58
25	130	5	20	0.14	0.00	29.05	29.05	0.78	76.63	8.16	88.32	88.70	84.72
26	130	5	40	0.12	0.00	23.37	23.37	0.95	77.04	7.05	86.74	87.03	85.35
27	130	5	60	0.18	0.00	21.76	21.76	0.95	77.44	6.96	86.51	86.79	85.40

The FFA content of the initial, non-bleached DNSO was of 0.17 % oleic, greatly lower than the acceptable level in refined fish oil (1.8 %) reported by Sathivel (2003). It is observed, in Table 17, that the acidity of the DNSO was reduced in most cases during the bleaching process when a clay amount of 5 wt% was employed. On the contrary, by utilizing adsorbent concentrations of 1 and 3 wt%, the FFA percentage suffered a general increase. Exposure of the oil to heat in the presence of traces of water caused the hydrolysis of triglycerides, which lead to the formation of free fatty acids that could not be adsorbed by the activated clay when the clay concentration used was lower than 5 wt%. Possible explanations include the presence of other impurities such as minerals and protein in the fish oil, or the high viscosity of the oil which slows down mass transfer and hence, the final removal of FFA (Huang & Sathivel, 2010).

In terms of oxidation products, the peroxide value was determined in order to quantify the primary oxidation products presented in the oil. Moreover, the p-anisidine analysis was carried out to measure the concentration of secondary oxidation products such as aldehydes, ketones and alcohols produced by decomposition of hydroperoxides. Then, totox value was computed to evaluate the rancidity level of the oils, reflecting total oxidation to date. In Table 17, DNSO presented an initial PV of 2.36 meq/kg and an AV of 77, resulting in a totox of 81.72. Although the initial oil presented an acceptable PV, PV < 5 meq/kg for refined fish oil (Ackman, 2005), the high AV obtained, AV > 20 (Ackman, 2005), denotes an advanced state of rancidity.

In Table 17, the effective removal of primary oxidation products during the bleaching process is shown. For some bleached oils, processed at the highest clay amount and temperature (5 wt% and 130 ºC), no significant value of PV was observed. Nevertheless, carrying out the bleaching process using low concentrations of clay, 1-3 wt%, at low process temperatures, 90 – 110 ºC, did not reduce the PV of the oil; on the contrary these conditions even enhanced the formation of peroxides due to the exposure of the oil to high temperatures. Considering the secondary oxidation products, Table 17 shows an AV reduction in all the bleached oils. This fact denotes the capacity of the acid-activated earths to adsorb these types of compounds (Rossi, 2003; Sathivel, 2010). From the AV results shown in Table 17, it could be clearly observed, within the experimental conditions assayed, that the adsorption of secondary oxidation products is more effective at higher temperatures and concentration of activated earth. Although the values of the Rancimat induction period, IP, did not follow a clear trend, it was noticed that the oils bleached at

130 ºC, employing a clay amount of 5 wt% and a long time of contact (40-60 min) showed a higher oxidative stability (IP=0.95 h).

On the other hand, a direct correspondence between the Rancimat induction period, IP, and any of the oxidation indices, PV and AV, measured in the bleached oil samples was not clearly observed (Table 17).

The initial L*, a*, and b* values of the DNSO were, respectively, 71.46, 16.39, and 96.75 (Table 17). These CIELAB coordinates denote a dark brown color, attributable to the advanced state of rancidity of the oil and to the presence of pigments such as carotenoids (Indrasena & Barrow, 2010). The bleaching process increased the lightness of the oil, reaching L* values up to 83.61. Besides, the activated earth adsorption process effectively reduced a* value indicating that a decrease in red color occurred. As a consequence, the oil samples became lighter and slightly more transparent. Nevertheless, the b* value of the bleached oils generally suffered a minor reduction taking values in the range of 78.97 – 99.56, which denotes yellowish color. Chroma values of the bleached oils, which represent the intensity of color, were reduced, while hue-angle value increased with the concentration of the activated earth. The hue-angle values were in the range of 79.53-90.76 which implies yellow-orange color for the bleached oils.

3.2 Statistical modeling

The experimental data of each measured variable were fitted to a complete quadratic model. The polynomial coefficients for the surface response model were calculated by multiple regressions, and the results are expressed in Table 18 for the oxidation and in Table 19 for the color parameters. A p-value of associated probability was also calculated for each term of the regression model. Similar statistical procedures have already been employed to optimize numerous processes involved in the up-grading of fish by-products such as extraction of fish oil from herring by-products (Aidos et al., 2003), enzymatic hydrolysis of sardine wastes (Dumay, 2006) and hydraulic pressing of sardine discards (Pérez-Gálvez et al., 2009).

Table 18 and Table 19 show that all the measured variables (FFA, PV, AV, totox, IP, a*, b*, L*, chroma and hue-angle) are highly dependent on the linear effect of clay percentage, with an associated probability $p < 0.0001$. Regarding temperature, its linear effect was statistically significant for FFA, PV, AV, totox, IP and L*, with $p < 0.001$. On the other

hand, the time was the input variable having the lowest influence on the measured variables, with linear effects being statistically significant ($p < 0.005$) in the cases of FFA, AV, totox and L*. Quadratic effects were found to be significant only for clay percentage in the cases of L*, b* and chroma; and time in the cases of FFA, PV, AV and L*. The p-values for the remaining effects indicated that the interaction between experimental factors was not statistically significant ($p > 0.05$).

In Table 18 and Table 19, it is also observed that the proposed quadratic models explain the variability of the data to a large extent, with coefficients of determination, R^2, being around 0.90 for most of the output variables except for FFA.

For the optimization of the bleaching process, totox value was chosen as measured variable to indicate the total oxidation of the oils, while hue-angle and chroma were selected as output variables indicating the final color of oil samples.

Firstly, the goodness of the fit for these three variables was proved. Figure 14 plots the measured values against the predicted ones for totox (Figure 14a), hue-angle (Figure 14b) and chroma (Figure 14c). The data are correlated by means of a regression line whose equation is inserted in each figure. Also shown in each figure are the dotted lines representing a deviation of ±10% between experimental and model values.

Secondly, by means of the second-order models obtained above and employing response surface methodology, contour maps were generated, where the totox, hue-angle and chroma were plotted against temperature (ºC) and time (min), as shown in Figures 15a to 15c. Adsorbent concentration was set at the maximum level assayed, 5 wt%. It was due to the fact that, within the experimental range, higher clay amount resulted in bleached oils of superior quality. In Figure 15, it was observed that the hue-angle and chroma followed a pronounced curved surface. It is due to the significance of clay percentage quadratic effect in the case of hue-angle and to the significance of temperature and clay amount quadratic effects in the case of chroma. By optimizing the quadratic models, optimum values for totox, hue-angle and chroma were found, marked as circles in the contour plots (Figure 15).

Table 18. Polynomial coefficients and p-values for the oxidation parameters.

	FFA (% oleic)		PV (meq/kg oil)		AV		Totox		IP (h)	
	Coefficient	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*
Constant	7.33E-01	-	-6.57E+00	-	5.64E+01	-	4.33E+01	-	-7.94E-01	-
A: Temperature, ºC	-1.12E-02	<0.0001	1.96E-01	<0.0001	3.81E-01	<0.0001	7.73E-01	<0.0001	1.22E-02	<0.0001
B: Clay Amount, %w	2.65E-02	<0.0001	-2.45E-01	<0.0001	-1.30E+00	<0.0001	-1.79E+00	<0.0001	-4.35E-02	<0.0001
C: Time, min	2.60E-03	0.0007	5.07E-02	0.0501	-6.62E-01	0.0007	-5.60E-01	0.0003	1.15E-02	0.0682
AA	5.14E-05	0.4771	-1.04E-03	0.0053	-2.48E-03	0.4771	-4.55E-03	0.1989	-2.08E-05	0.8640
AB	-1.25E-04	0.2802	-2.17E-03	0.3576	-2.69E-02	0.2802	-3.12E-02	0.2119	1.04E-03	0.2356
AC	-2.65E-20	0.9349	6.46E-05	0.7814	2.00E-04	0.9349	3.29E-04	0.8929	-6.87E-05	0.4283
BB	-4.44E-03	0.5855	1.39E-02	0.6736	1.90E-01	0.5855	2.17E-01	0.5317	-7.50E-03	0.5396
BC	4.17E-05	0.1500	-7.08E-04	0.7610	-3.64E-02	0.1500	-3.78E-02	0.1351	1.04E-03	0.2356
CC	-3.61E-05	0.0462	-7.94E-04	0.0253	7.33E-03	0.0462	5.74E-03	0.1098	-5.42E-05	0.6569
R^2	0.5417		0.9352		0.9205		0.9431		0.7935	

Table 19. Polynomial coefficients and p-values for the color parameteres

	Colour						Chroma		Hue angle (deg)	
	L*		a*		b*					
	Coefficient	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*
Constant	2.77E+01	-	8.45E+01	-	2.20E+02	-	2.23E+02	-	4.23E+01	-
A: Temperature, ºC	8.01E-01	<0.0001	-1.16E+00	0.1312	-2.09E+00	0.0865	-2.11E+00	0.1085	6.62E-01	0.0754
B: Clay Amount, %w	7.01E+00	<0.0001	-4.58E+00	<0.0001	-2.46E-01	<0.0001	-9.19E-01	<0.0001	2.51E+00	<0.0001
C: Time, min	1.78E-01	0.0045	-9.51E-02	0.4109	-3.67E-01	0.2631	-3.73E-01	0.2614	4.61E-02	0.4519
AA	-3.82E-03	<0.0001	5.12E-03	0.0201	8.75E-03	0.0024	8.86E-03	0.0025	-2.93E-03	0.0381
AB	-3.73E-02	<0.0001	2.73E-02	0.0688	2.49E-02	0.1706	2.65E-02	0.1524	-1.59E-02	0.1034
AC	-2.90E-04	0.4638	-1.54E-04	0.9130	8.71E-04	0.6221	7.73E-04	0.6674	7.08E-05	0.9398
BB	-1.68E-01	0.0072	-1.58E-01	0.4391	-7.28E-01	0.0088	-6.93E-01	0.0131	1.10E-01	0.4108
BC	1.81E-03	0.6548	-1.43E-02	0.3224	-1.98E-02	0.2717	-2.02E-02	0.2697	8.89E-03	0.3500
CC	-1.64E-03	0.0084	1.70E-03	0.4059	3.73E-03	0.1483	3.94E-03	0.1336	-8.62E-04	0.5185
R^2	0.9906		0.9208		0.8763		0.8894		0.9000	

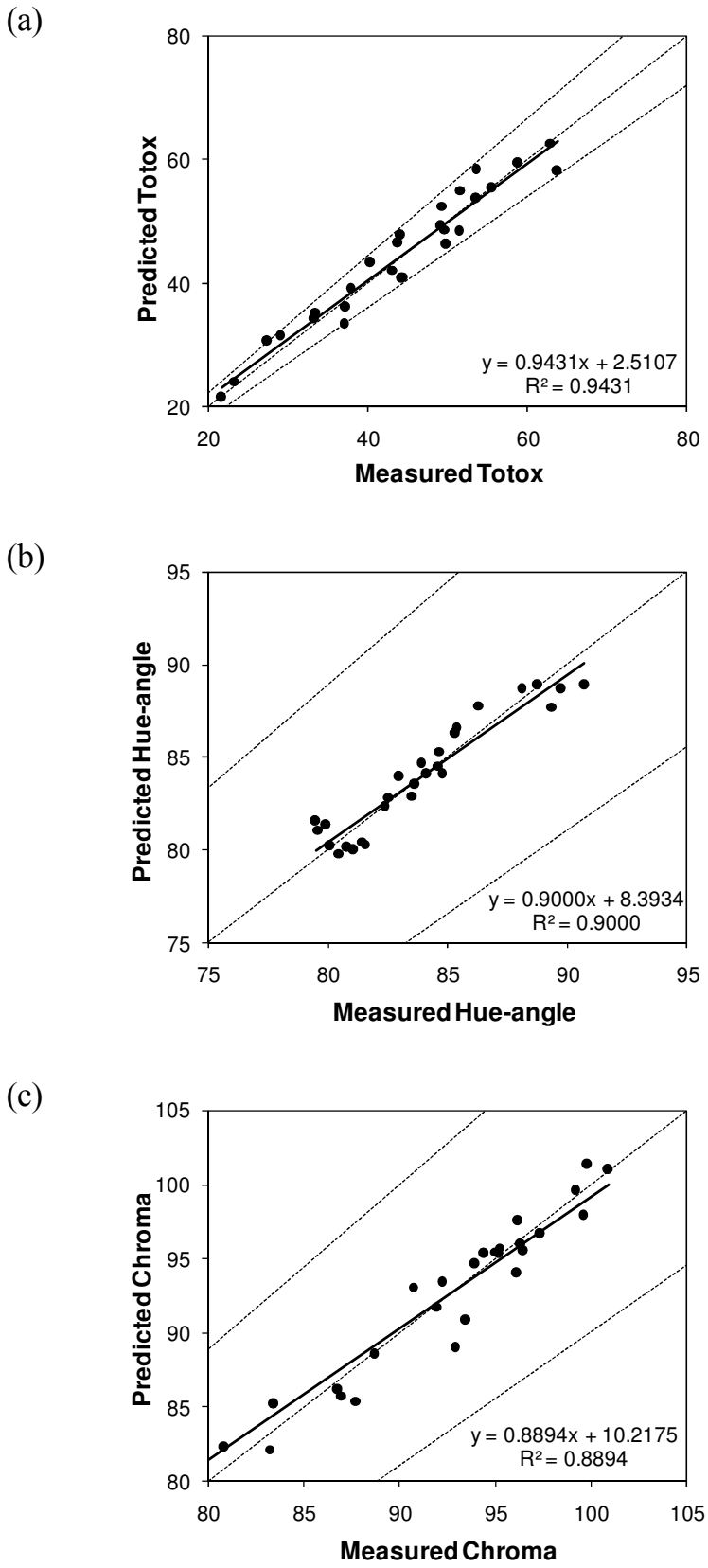

Figure 14. Correlation between predicted and measured values of chroma (a), hue-angle (b) and chroma (c)

In order to optimize the quality of the bleached oils in terms of oxidation products, a minimum totox value is required. In Figure 15a, the optimum value for the totox, 21.38, was located at the maximum levels assayed for the three input parameters, 130 ºC, 60 min and 5 wt%. According to Sathivel (2010), during the bleaching process, peroxides are broken down to aldehydes and ketones as a consequence of the high temperatures employed. Subsequently, these secondary oxidation products are adsorbed onto the activated clay surface. It is therefore reasonable to conclude that the optimum bleaching conditions for totox reduction were: a) the maximum temperature evaluated, 130 ºC, which allowed the highest rate for peroxides decomposition maintaining a proper activation of the adsorbent; b) the highest clay amount, 5 wt%, and c) the highest contact time, 60 min, which maximized the adsorption of secondary oxidation products.

Additionally, optimizing the color quality in oil samples implies maximizing hue-angle, obtaining yellowish oils, and to minimize chroma, reducing the color intensity (Huang & Sathivel, 2010). In Figure 15b, the maximum value predicted for hue-angle is observed to be 89.19. This optimum value was found at the highest adsorbent concentration, 5 wt%, and in the vicinity of the maximum level assayed for the time of 56.6 min. Nevertheless, a temperature of 99.2 ºC was obtained as the optimum temperature for the reduction in red color. Figure 15c shows the optimum value predicted for chroma, 81.76, which was within the experimental region: 49.4 min and 109.4 ºC, employing the highest level of clay (5 wt%). Moderate temperatures, in the range of 99 – 110 ºC, were required to obtain an optimum color for the bleached oil. This can be explained by the fact that working in this range of temperature the following phenomena may occur: i) a better activation, in terms of colored compounds removal, of the acid-activated earth employed (Tonsil 278), ii) a reduction in the oxidation of colorless components which cause alteration in the oil color, and iii) a less pronounced fixing of the existing color pigments (Antoniassi et al., 1998; Crexi et al., 2010).

The results stated above indicate that in order to obtain optimum values for totox, hue-angle and chroma, different process conditions such as temperature and time of contact are required. It is observed that temperature increase had a positive effect on the minimization of totox, obtaining its minimal value at 130 ºC. On the other hand, moderate temperatures, in the range of 99 – 110 ºC, were required to obtain hue-angle and chroma optimal values.

Considering time conditions, it is noticed that although each evaluated optimal point gave a different value, in general, the three output variables were optimized at long contact time conditions, close to the experimental upper bound, ranging from 49 to 60 min.

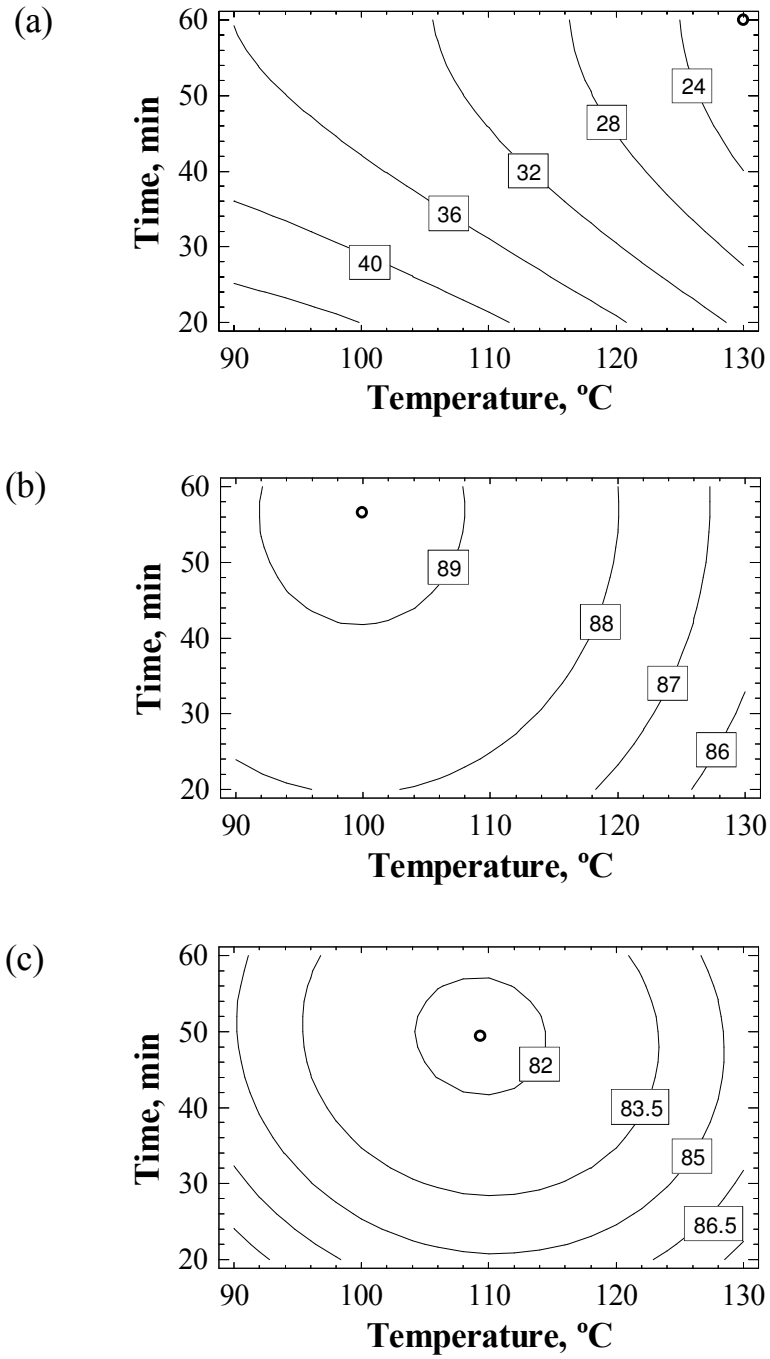

Figure 15. Contour plots for totox (a), hue-angle (b) and chroma (c)

3.3 Multi-objective optimization

The conflict in the optimization of the three key output variables (totox, hue-angle and chroma) suggested employing a multi-objective optimization technique. A Pareto Front, shown in Table 20, was generated using the weighted-sum method in order to find a set of non inferior solutions which satisfied the three objectives to an adequate degree

In rows 1-3 of Table 20, the optimization of the objective function corresponds to the optimization of a single objective, since two of the three weight factors are given a value of zero. In rows 4-30, a bi-objective optimization problem arises where just one of the weight factors takes a value of zero. In this situation, three cases are possible:

a) Bi-optimization of hue-angle and chroma where $w_1 = 0$ (Rows 4-12). It is observed that an increase of hue-angle (desired) implies an augmentation of chroma (undesired). This trend is more pronounced at values of hue-angle higher than 89.02 ($w_3<0.5$).

b) Bi-optimization of totox and chroma where $w_2 = 0$ (Rows 13-21). It is noticed that a decrease of chroma (desired) denotes an increase of totox (undesired), being this tendency more noticeable at values of chroma lower than 85.6 ($w_1 < 0.4$).

c) Bi-optimization of totox and hue-angle where $w_3 = 0$ (Rows 22-30). It is noted that an augmentation in hue-angle (desired) causes an increase in totox values (undesired). This trend is more evident at values of hue-angle greater than 86.54 ($w_1 < 0.3$).

Additionally, in rows 31-34 of Table 20, four new solutions were obtained, in which none of the weights was set to zero.

Table 20. Set of optimal solutions (Pareto front) for the multi-objective optimization problem

Row	w_1	w_2	w_3	Totox	Hue-angle (deg)	Chroma
1	1.00	0.00	0.00	21.38	86.54	86.14
2	0.00	1.00	0.00	33.87	89.19	82.70
3	0.00	0.00	1.00	31.62	88.88	81.76
4	0.00	0.10	0.90	31.71	88.90	81.77
5	0.00	0.20	0.80	31.82	88.93	81.77
6	0.00	0.30	0.70	31.94	88.96	81.78
7	0.00	0.40	0.60	32.09	88.98	81.80
8	0.00	0.50	0.50	32.26	89.02	81.82
9	0.00	0.60	0.40	32.46	89.05	81.87
10	0.00	0.70	0.30	32.70	89.09	81.94
11	0.00	0.80	0.20	33.00	89.14	82.07
12	0.00	0.90	0.10	33.38	89.17	82.29
13	0.90	0.00	0.10	21.38	86.54	86.14
14	0.80	0.00	0.20	21.39	86.54	86.09
15	0.70	0.00	0.30	21.43	86.54	85.99
16	0.60	0.00	0.40	21.48	86.54	85.89
17	0.50	0.00	0.50	21.56	86.54	85.80
18	0.40	0.00	0.60	21.83	86.58	85.60
19	0.30	0.00	0.70	26.61	87.96	82.93
20	0.20	0.00	0.80	29.04	88.50	82.10
21	0.10	0.00	0.90	30.54	88.75	81.82
22	0.90	0.10	0.00	21.38	86.54	86.14
23	0.80	0.20	0.00	21.38	86.54	86.14
24	0.70	0.30	0.00	21.38	86.54	86.14
25	0.60	0.40	0.00	21.38	86.54	86.14
26	0.50	0.50	0.00	21.38	86.54	86.14
27	0.40	0.60	0.00	21.38	86.54	86.14
28	0.30	0.70	0.00	21.38	86.54	86.14
29	0.20	0.80	0.00	26.85	88.12	83.10
30	0.10	0.90	0.00	31.84	89.07	82.20
31	0.50	0.25	0.25	21.44	86.54	85.95
32	0.25	0.25	0.50	27.32	88.16	82.65
33	0.25	0.50	0.25	25.91	87.85	83.29
34	0.33	0.33	0.33	21.54	86.54	85.81

Each solution or efficient point (totox, chroma, hue-angle) is determined by a decision vector or combination of factors inside the ranges of the independent variables (temperature, clay amount and contact time). Then, the Pareto Front can be translated to the decision space by obtaining the optimal combination of experimental factors for each selection of weight factors. Figure 16 shows the optimal temperatures and times for each efficient solution presented in rows 1-30 of Table 20. Optimum clay amount was 5 wt% in all cases. It is observed that the decision space is comprised between 99.91 and 130 ºC and between 49.45 and 60 min. The three vertexes of the decision space represent the single optima for totox (a), hue-angle (b) and chroma (c). The points placed in the line from one vertex to another correspond to the bi-objective optimization of the variables involved. The

solutions presented in rows 31-34 of Table 20 and any other solution considering non-zero weights only, would be placed inside the closed region.

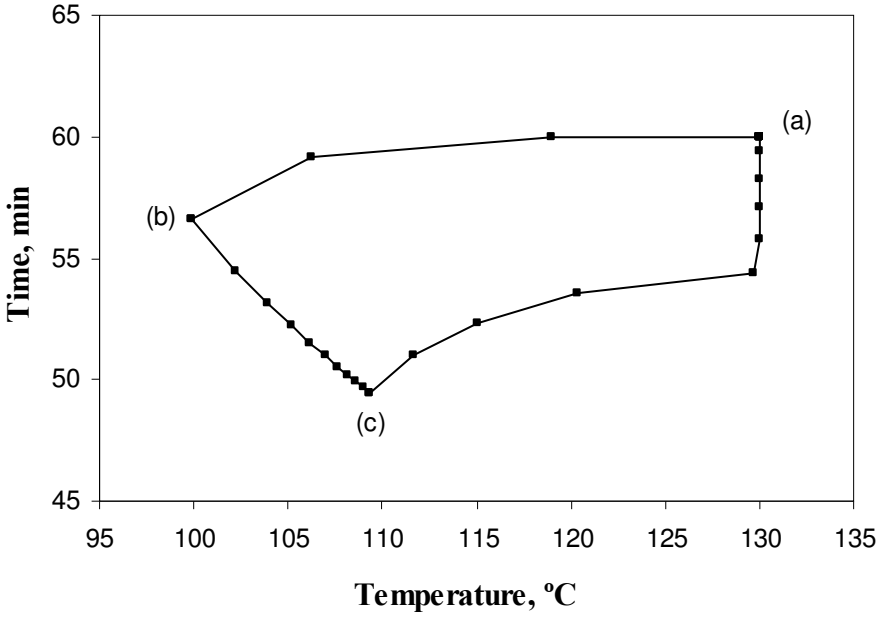

Figure 16. Decision space for the multi-objective optimization problem

Finally, the selection of a single optimal solution from the Pareto Front (optimal combination of temperature and time employing 5 wt% of clay), will depend on the required characteristics for the bleached oil in terms of oxidation and color quality. Such selection would arise from a given combination of weights proposed by the decision maker. For instance, a value of w_1<0.4 is not recommended in order to obtain a bleached oil with a good quality in terms of oxidation products (totox<21.83). It implies to operate at the following conditions: 130 ºC, clay amount of 5 wt% and process time ranging from 54 to 60 min.

4. CONCLUSIONS

Based on this study, the optimum conditions to carry out the bleaching process of DNSO in order to remove oxidation products, and thus, improving the oxidative stability of the oil, are: 130 ºC, setting the adsorbent amount at 5 % and employing long contact times (~ 60 minutes). This fact denotes that, within the experimental range, working at high temperatures, enhances the breaking down of peroxides and the subsequent adsorption of secondary oxidation products onto the surface of the activated clay. Nevertheless,

regarding color quality, an optimum bleaching process required moderate temperatures (99 - 110 ºC), high adsorbent amount (5%) and shorter contact time (49 - 57 minutes). Under these conditions, the oil suffers reduction in redness (a*) whilst maintaining high values for yellowness (b*) and lightness (L*). It may therefore be concluded that employing moderate temperatures, the clay employed (Tonsil 278) is more activated in terms of pigments adsorption.

Consequently, the conflicting behavior of the experimental factors (temperature and time) towards the accomplishment of the optimization objectives necessitates finding a compromise solution employing multi-objective optimization techniques. The weighted-sum method was chosen for this purpose, generating a set of optimal solutions (Pareto Front). Thus, the required final characteristics for the bleached oil (optimal oxidation and/or color quality) will determine the selection of a single solution inside the Pareto Front.

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VI. Optimization of Antioxidants Addition for the Stabilization of Sardine Oil*

The purpose of the present work was to optimize the addition of natural antioxidants alpha-tocopherol, ascorbyl palmitate and citric acid) for the stabilization of sardine oil rich in omega-3 PUFA. The stabilized oils were evaluated for free fatty acids, peroxide value, p-anisidine value, totox and oxidation stability (Rancimat) at short and long time of storage. The experimental results obtained for each measured variables were fitted to quadratic models by response surface methodology. The optimum values for PV (which minimizes primary oxidation products) were obtained at low concentration of alpha-tocopherol (50-207 ppm), high content of ascorbyl palmitate (450 ppm) and 50 ppm citric acid. On the other hand, optimum values for AV (which minimize secondary oxidation products) were found at medium concentration of alpha-tocopherol (478-493 ppm), high content of ascorbyl palmitate (390-450 ppm) and 50 ppm citric acid. The conflicting effect of alpha-tocopherol on the individual optimization of PV and AV at long time of storage (30 days) motivated the generation of a Pareto front (set of non inferior solutions) employing the weighted-sum multi-objective optimization technique.

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1. INTRODUCTION

Fish oils are widely known because of the beneficial role they play in human health and nutrition. These properties are attributed to the high content of polyunsaturated fatty acids (PUFA), especially to the omega-3 PUFA family and more in particular to eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids (Fedačko, 2007). During the last decades, it has been reported the potential of these acids in the prevention of diabetes, allergy and some types of cancer such as stomach, pancreatic breast, prostate and colonic (Sidhu, 2003). Furthermore, they present anti-thrombotic, anti-arrhythmic and anti-inflammatory effects (Ruxton, 2001).

However, due to their high level of unsaturation these omega-3 PUFA are extremely susceptible to oxidative spoilage. In addition, the steps required for the extraction and processing of fish oils assist hydrolytic and oxidative reactions causing the production of free fatty acids, hydroperoxides and rancidity (Donnelly & Robinson, 1995). The mechanism of lipid autooxidation consists of three main steps: initiation, propagation and termination reactions. In short, the first step takes place when an unsaturated lipid loses a hydrogen atom and produce free radicals by the presence of initiators such as heat, light or ionizing radiation or metal ions. Then, free lipidic radicals react with oxygen so as to produce peroxyl radicals and lately hydroperoxides, which are known as primary oxidation compounds. Hydroperoxides are very unstable and lead to the production of secondary oxidation compounds such as non-volatile and volatile aldehydes, alcohols, and tertiary oxidation products. These secondary oxidation products are the main responsible for undesirable odors and off-flavors (Shahidi & Zhong, 2010).

To prevent the oxidation of the omega-3 PUFA, the stabilization of fish oils with natural antioxidants is one of the most common strategies followed. In the same manner that oxidation can be driven by several mechanisms, antioxidants can act by different pathways being classified in two main groups: primary or chain-breaking and secondary or preventive antioxidants. The mechanism of chain-breaking antioxidants consists of donating hydrogen atoms or electrons to lipid radicals so as to convert radicals into stable products. Otherwise, secondary antioxidants act by a set of mechanisms such as scavenging oxygen, binding metal ions, absorbing UV radiation or deactivating singlet oxygen (Frankel, 2005). The most typical primary antioxidant is alpha-tocopherol, while

most common natural secondary antioxidants are citric acid which acts as a metal chelator and ascorbyl palmitate that can scavenge oxygen among others (Frankel, 2009). Combinations of these three natural antioxidants shown high efficiency for the stabilization of fish oils since synergic effects among them were reported (Aubourg et al., 2004; Frankel, 2005; Olsen et al., 2005). In view of the above, the study of the stabilization process is a crucial issue to be considered for the production of food-grade fish oil.

The aim of this work was to optimize the stabilization process for sardine oil under ambient conditions. Such a systematic study is not available in the literature. The influence of alpha-tocopherol and ascorbyl palmitate concentrations on oxidation parameters (free fatty acids, peroxide, p-anisidine and totox value, and induction period) was evaluated at short and long times of storage. Finally, a bi-objective optimization was performed since optimum values of peroxide and p-anisidine were simultaneously pursued.

2. MATERIALS AND METHODS

2.1 Materials

Crude sardine oil was kindly donated by AFAMSA S.A. (Vigo, Spain), having a composition of 19.7 wt% in EPA and 9.4 wt% in DHA. Citric acid, (±)-alpha-tocopherol and 6-O-Palmitoyl-L-ascorbic acid were acquired from Sigma-Aldrich Quimica SA (Madrid, Spain). In order to enhance the solubility in oil, citric acid granules were milled until achieving a size smaller than 200 μm. All other chemicals and solvents used were of analytical grade.

2.2 Preparation of fish oil matrix

The fish oil matrix was obtained from crude sardine oil by chromatographic purification on alumina-silica column. A glass chromatographic column (5 × 50 cm) was packed with 100 g of silica gel (70-230 mesh, Merck) and 100 g of basic alumina (70-230 mesh, Merck) suspended in hexane (400 mL). The oil (200 g) was dissolved in an equal mass of hexane and passed through the column. The chromatographic column was wrapped with aluminum foil to prevent light-induced oxidation during the purification process, and the purified oil dissolved in hexane was collected in an aluminum foil-wrapped flask. The hexane was evaporated in a rotary evaporator at 40 ºC under vacuum, and the oily residue was bubbled

with a nitrogen stream. This process was carried out seven times. The oil from all the batches was homogenously mixed and stored at -20 ºC until use.

2.3 Stabilization procedure

The desired amounts of antioxidants were added to the fish oil matrix prepared previously. Then, the mixture was heated up to 95 ºC and vigorously agitated for 1.5 h under high vacuum (15 mbar). The experimental factors evaluated were the concentration of alpha-tocopherol and ascorbyl palmitate, and their effects were studied at 10 and 30 days of storage. For each antioxidant the concentration was set at three levels: 50, 550 and 1050 ppm for alpha-tocopherol; 50, 250 and 450 ppm for ascorbyl palmitate. A fixed concentration of citric acid (50 ppm) was employed for each experiment. Consequently, an aliquot of each batch was taken for analysis at the desired time of storage. All samples were stored in amber bottles at 25 ºC during all the experimental process. In addition, a sample of purified oil was subjected to the same protocol in an attempt to study the behavior of the oil without any antioxidant.

2.4 Determination of oxidation parameters

2.4.1 Free fatty acids, peroxide, p-anisidine and totox values

The free fatty acids (FFA), peroxide value (PV) and p-anisidine value (AV) were calculated as described in the Materials and Methods section of chapter IV.

Totox is a comprehensive oxidation index calculated from a weighted sum of peroxide value (PV) and p-anisidine value (AV) by applying Eq. 13:

$$Totox = 2 \cdot PV + AV \tag{13}$$

2.4.2 Oxidative stability

The induction period (IP in h) of the oil samples was calculated as described in the material and section of chapter IV. The heating block was set at 90 ºC.

2.5 Statistical analysis

The statistical analysis and the regression models were generated by using the Statgraphics software (version 5.1). Firstly, the output variables (Y: FFA, PV, AV, totox and IP) were

related to the input variables (X: concentration of alpha-tocopherol, concentration of ascorbyl palmitate and time of storage) by second degree polynomials as follows, Eq. 14:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{2} b_{ii} X_i^2 + \sum_{i<j}^{3} b_{ij} X_i X_j \qquad (14)$$

where the coefficients bi and bii are related to the linear and quadratic effects, respectively, of each input factor on the output variable and the cross-product coefficients b_{ij} represent the interactions between two input variables. It should be noted that no quadratic coefficient was employed for the time of storage since it was assayed just at two levels.

Secondly, the analysis of variance (ANOVA) was carried out. The significance of all terms in the models was judged statistically by computing the p-value at a confidence level of 95%. The regression coefficients were then used to generate contour maps and to find the optimal stabilization conditions which maximize the quality of the oil in terms of oxidation parameters at 10 and 30 days of storage.

2.6 Bi-objective optimization

A problem of bi-objective optimization arises when two objectives must be satisfied. In our case, it is desired to obtain minimum values for PV and AV in order to obtain the highest quality of the stabilized oil.

The concept of Pareto Front, related to the identification of an adequate solution, consists of a set of non inferior solutions, which are defined as those in which an improvement in one objective requires a degradation of another (Halsall-Whitney & Thibault, 2006). In this work, the Pareto Front was generated employing the weighted-sum method (Kim, 2004). This method consists in expressing a comprehensive objective function (OBJ) as a linear combination of the individual objectives (PV and AV), by means of a weight factor (w), which quantifies the relative importance given to the accomplishment of each individual objective, Eq. 15:

$$OBJ = w \cdot (PV) + (1 - w) \cdot (AV) \qquad (15)$$

where $0 \leq w \leq 1$

The Solver Tool, included in the MS Excel software, was chosen to carry out all the calculations required for the bi-objective optimization.

3. RESULTS AND DISCUSSION

3.1 Characterization of the stabilized fish oils

Table 21 presents the experimental values of free fatty acid content (FFA), peroxide (PV), p-anisidine (AV), totox and induction period (IP) of the crude, purified, and control and stabilized oils produced.

It was observed that the purification process of the oil led to a significant reduction of the FFA content, varying from 6.26 to 0.14 % oleic, and of the AV which was reduced from 17.18 to 2.17. However, the peroxide value was maintained practically constant around 2.7 meq/kg oil. Although it was reported that adsorption on silica-alumina reduces the hydroperoxides of the oil, this high-purity oil also showed rapid oxidation (Lin & Hwang, 2002). Thus, it is suggested that a reduction in PV could have happened but the oil matrix may have suffered oxidation after the purification process. Regarding the fatty acid profile, no significant differences were observed between crude and purified oil (data not shown).

During the storage period, the FFA content of the stabilized oils did not increase but even suffered a slight decrease, Table 21. It denotes that hydrolysis of the stabilized oils did not occur due to their low moisture content and that FFA were possibly oxidized since they are more susceptible to oxidation than esterified fatty acids (Aidos et al., 2001).

Regarding oxidation products, PV was employed to determine the content of hydroperoxides for the stabilized oils. In Table 21, it is shown that PV increased from 2.79 meq/kg oil for the purified oil to 30.49 and to 53.78 meq/kg oil for the control samples after 10 and 30 days of storage, respectively. It was revealed that the addition of antioxidants led, in most cases, to stabilized oils with lower PV than the control samples. The PV levels of the stabilized oil samples ranged between 18.20 and 41.81 meq/kg oil after 10 days of storage at 25 ºC, while PV levels up to 69.84 meq/kg oil were obtained after 30 days of storage. For short time of storage (10 days), low-medium concentrations of alpha-tocopherol combined with maximum addition of ascorbyl palmitate (450 ppm) resulted in the lowest PV, 20.83 and 18.20 meq/kg oil, respectively. In the case of long time of storage (30 days), the highest addition of ascorbyl palmitate assayed (450 ppm) combined with a low content of alpha-tocopherol (50 ppm) resulted in the maximum prevention of hydroperoxides formation, PV of 31.55 meq/kg oil.

Table 21. Experimental design and measured values for the response variables

Exp.	Citric acid (ppm)	α-Tocopherol (ppm)	Ascorbyl palmitate (ppm)	Time (day)	FFA (% oleic)	PV (meq/kg oil)	AV	TOTOX	IP (h)
Crude oil	-	-	-	-	6.26	2.67	17.18	22.52	-
Purified oil	-	-	-	-	0.14	2.79	2.17	7.75	0.31
Control 10	0	0	0	10	0.11	30.49	19.74	80.72	0.12
1	50	50	50	10	0.08	41.81	18.33	101.94	0.15
2	50	50	250	10	0.06	30.00	14.71	74.71	0.30
3	50	50	450	10	0.06	20.83	10.59	52.25	0.47
4	50	550	50	10	0.03	28.20	10.48	66.88	1.59
5	50	550	250	10	0.06	21.50	6.27	49.27	1.80
6	50	550	450	10	0.08	18.20	4.94	41.34	2.26
7	50	1050	50	10	0.11	27.82	10.50	66.14	2.06
8	50	1050	250	10	0.10	25.66	8.82	60.13	1.95
9	50	1050	450	10	0.00	35.00	12.99	82.99	0.68
Control 30	0	0	0	30	0.11	53.78	49.38	156.94	0.02
10	50	50	50	30	0.14	52.83	47.85	153.51	0.02
11	50	50	250	30	0.11	43.28	33.96	120.51	0.02
12	50	50	450	30	0.08	31.55	18.97	82.07	0.03
13	50	550	50	30	0.06	55.09	17.94	128.12	0.85
14	50	550	250	30	0.08	53.61	15.24	122.47	0.90
15	50	550	450	30	0.08	50.73	12.87	114.33	1.06
16	50	1050	50	30	0.11	62.45	20.37	145.27	1.33
17	50	1050	250	30	0.08	69.68	19.30	158.66	1.18
18	50	1050	450	30	0.09	69.84	34.35	174.03	0.05

Table 21 also shows that at low and medium alpha-tocopherol concentrations (50 and 550 ppm), the PV decreased when increased the ascorbyl palmitate concentration. This fact can be explained by the synergic effect between ascorbyl palmitate and alpha-tocopherol. Several works reported that ascorbyl palmitate can regenerate alpha-tocopherol from its radicals derivatives enhancing its activity and thus, reducing the amount of alpha-tocopherol radical available for participation in side reactions and further oxidation (Frankel et al., 1994; Olsen et al., 2005). On the contrary, at high concentration of alpha-tocopherol (1050 ppm) a detrimental effect in the PV was observed when increasing the concentration of ascorbyl palmitate. In previous studies (Kulås & Ackman, 2001a; Zuta & Simpson, 2007), it was revealed that alpha-tocopherol can become prooxidant when used at high concentration by regenerating peroxyl radicals, Eq. 16, and by participating in the chain transfer reaction, Eq. 17.

$$LOOH + A\bullet \rightarrow LOO\bullet + AH \quad (16)$$

$$A\bullet + LH \rightarrow AH + L\bullet \quad (17)$$

Where LOO· refers to the hydroperoxyde radical, AH is alpha-tocopherol molecule, A· is alpha-tocopherol free radical and L· lipid free radicals.

Moreover, citric acid (50 ppm) was employed as metal chelator in order to reduce the metal-catalyzed decomposition of preformed hydroperoxides. Besides, it has a synergic effect with alpha-tocopherol protecting this chain-breaking antioxidant against metal-catalyzed initiation of oxidation. Additionally, when employed at low concentrations (i.e. 50 ppm), citric acid did not alter the concentration at which alpha-tocopherol became prooxidant (Kulås & Ackman, 2001a).

The content of nonvolatile secondary oxidation products produced by the decomposition of hydroperoxides was followed with the p-anisidine value (AV). It was observed an increase in the AV from 2.17 for the purified oil to 19.74 and to 49.38 for the control samples after 10 and 30 days of storage, respectively. The stabilized oils presented lower AV than the control samples at the same time of storage (Table 21). In this case, for both short and long time of storage, medium concentration of alpha-tocopherol combined with a high addition of ascorbyl palmitate (450 ppm) allowed the maximum reduction in the decomposition of hydroperoxides, AV of 4.94 and 12.87 respectively. Although previous studies reported that ascorbyl palmitate may increase hydroperoxide decomposition by reducing trace metal ions present in the systems (Olsen et al., 2005; Hamilton et al., 1998), the opposite effect

was found in this work. It might be explained due to the fact that alpha-tocopherol can induce decomposition of hydroperoxides to stable products and that a better regeneration of alpha-tocopherol radical did not allow the decomposition of lipid hydroperoxides by Eq. 16 (Frankel, 2005).

Considering the totox index of the stabilized oils, it followed the same trend than PV at short and long time of storage, Table 21. It was due to the higher weight given to PV compared with the weight of AV in its calculation, Eq. 13.

On the other hand, Rancimat induction periods did not have a direct correspondence with PV and AV, apart from the stabilized oil obtained in experiment 6 which presented the minimum PV and AV at short time of storage and also had the maximum induction period after 10 days. This fact is attributed to the high temperature (90 ºC) employed in the Rancimat test which did not allow a comparison of the data obtained from this accelerated oxidation method and experiments at room temperature (Drusch et al., 2008).

Regarding the fatty acid profile of the oils, no significant changes were observed after 10 or 30 days of storage (data not shown). Similar results were obtained by Ayree et al. (2012) for salmon oil after 45 days of storage at 25 ºC in the dark.

3.2 Statistical modeling

The experimental data obtained for each measured variable were fitted to a quadratic model. Table 22 shows the polynomial coefficients for each surface response model and the associated p-value.

The results from Table 22 reveal that PV, totox and IP are highly dependent on the linear effect of alpha-tocopherol concentration, with an associated probability $p < 0.001$. Regarding ascorbyl palmitate concentration, its linear effect was statistically significant just for PV and totox. On the other hand, the time of storage was the input variable having the highest influence on the measured variables, with its linear effect being statistically significant in the cases of PV, AV, totox and IP. Quadratic effects were found to be significant only for alpha-tocopherol concentration in the cases of PV, AV, totox and IP. The interaction between alpha-tocopherol and acorbyl palmitate concentration resulted statistically significant for most of the variables (PV, AV, totox and IP), whereas the interaction between alpha-tocopherol concentration and time of storage was found to be significant just in the cases of PV and totox. The interaction between ascorbyl palmitate

concentration and time of storage not resulted significant for any of the output variables. It should also be noted that none of the experimental factors assayed was statistically significant for FFA.

In Table 22, it is also observed that the proposed quadratic models explain the variability of the data to a large extent, with coefficients of determination, R^2, being around 0.90 or higher for most of the output variables except for FFA.

In order to optimize the stabilization process, the measured variables PV and AV were chosen as appropriate objective functions indicating the oxidative deterioration of the oil samples. Firstly, the goodness of the fit for these variables was proved by plotting the measured values against the predicted ones for PV (Fig. 17a) and AV (Fig. 17b). The data were correlated by means of a regression line whose equation is inserted in each figure. Also, dotted lines representing a deviation of ±10% between experimental and predicted values are shown.

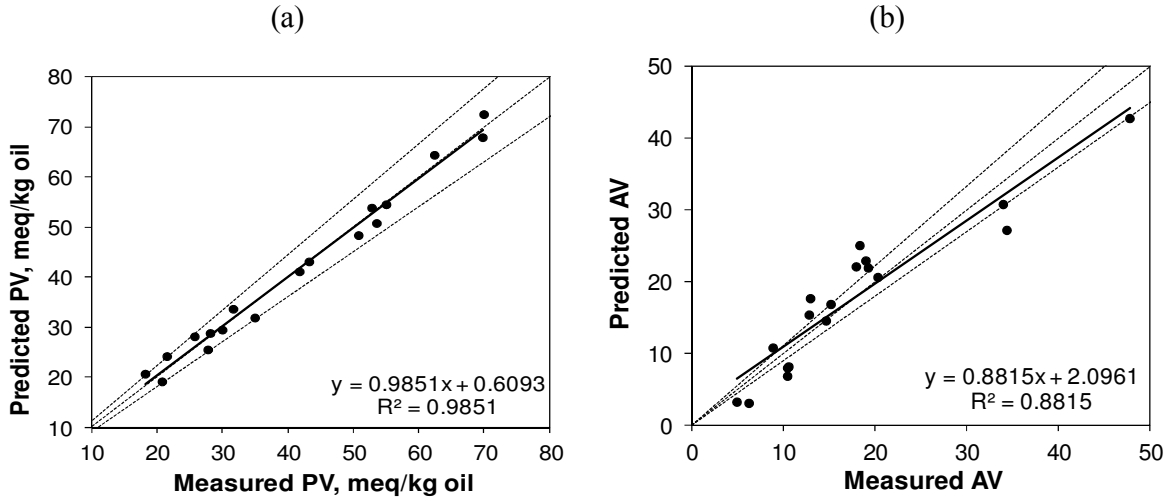

Figure 17. Correlation between predicted and measured values of PV (a) and AV (b)

Secondly, contour plot for PV and AV at short and long time of storage were generated by means of the second-order models obtained and employing response surface methodology, as shown in Fig. 18. These contour plots allowed to optimize the quality of the stabilized oils in terms of oxidation products, obtaining minimum values for PV and AV. These optimum values were marked as circles in Fig. 18.

Table 22. Polynomial coefficients and p-values for the response variables

	FFA (% oleic)		**PV** (meq/kg oil)		**AV**		**Totox**		**IP** (h)	
	Coefficient	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*
Constant	5.95E-02	-	4.06E+01	-	2.21E+01	-	1.03E+02	-	-2.58E-01	-
A: α-Tocopherol, ppm	-5.58E-05	0.7356	-5.28E-02	0.0001	-6.01E-02	0.0634	-1.66E-01	0.0008	5.57E-03	0.0009
B: ascorbyl palmitate, ppm	-2.12E-06	0.2547	-6.84E-02	0.0020	-6.62E-02	0.1216	-2.03E-01	0.0004	3.84E-03	0.2948
C: Time, day	1.80E-03	0.0977	5.57E-01	<0.0001	9.21E-01	0.0003	2.03E+00	<0.0001	-1.37E-02	0.0051
AA	8.15E-08	0.2577	1.87E-05	0.0091	3.84E-05	0.0049	7.58E-05	0.0001	-2.89E-06	0.0038
AB	-6.51E-08	0.5987	7.10E-05	0.0001	6.64E-05	0.0056	2.08E-04	<0.0001	-3.73E-06	0.0201
AC	-1.22E-06	0.5459	1.31E-03	<0.0001	-2.57E-04	0.4137	2.36E-03	0.0001	-2.13E-05	0.3486
BB	-9.69E-08	0.8234	1.44E-05	0.6934	4.91E-05	0.4688	7.79E-05	0.3265	-3.67E-06	0.4527
BC	1.36E-06	0.7869	2.31E-04	0.5850	-3.83E-04	0.6221	7.95E-05	0.9290	-2.79E-05	0.6173
R^2	0.4478		0.9851		0.8815		0.9889		0.8747	

In Fig. 18a1, the optimum value for PV after 10 days of storage, 18.56, was found at alpha-tocopherol concentration of 207 ppm and at the highest ascorbyl palmitate concentration assayed (450 ppm). For AV at short time of storage (Fig. 18b1), a minimum value, 2.64, was obtained at medium concentration of alpha-tocopherol (478 ppm) and at high concentration of ascorbyl palmitate (390 ppm). In the case of long time of storage, Fig. 18a2 shows the optimum value of PV, 33.56, which was obtained for the lowest concentration of alpha-tocopherol assayed (50 ppm) and the highest concentration of ascorbyl palmitate (450 ppm). Fig. 18b2 depicts the minimum value of AV after 30 days of storage, 15.34, which was found at medium concentration of alpha-tocopherol (493 ppm) and the highest concentration of ascorbyl (450 ppm).

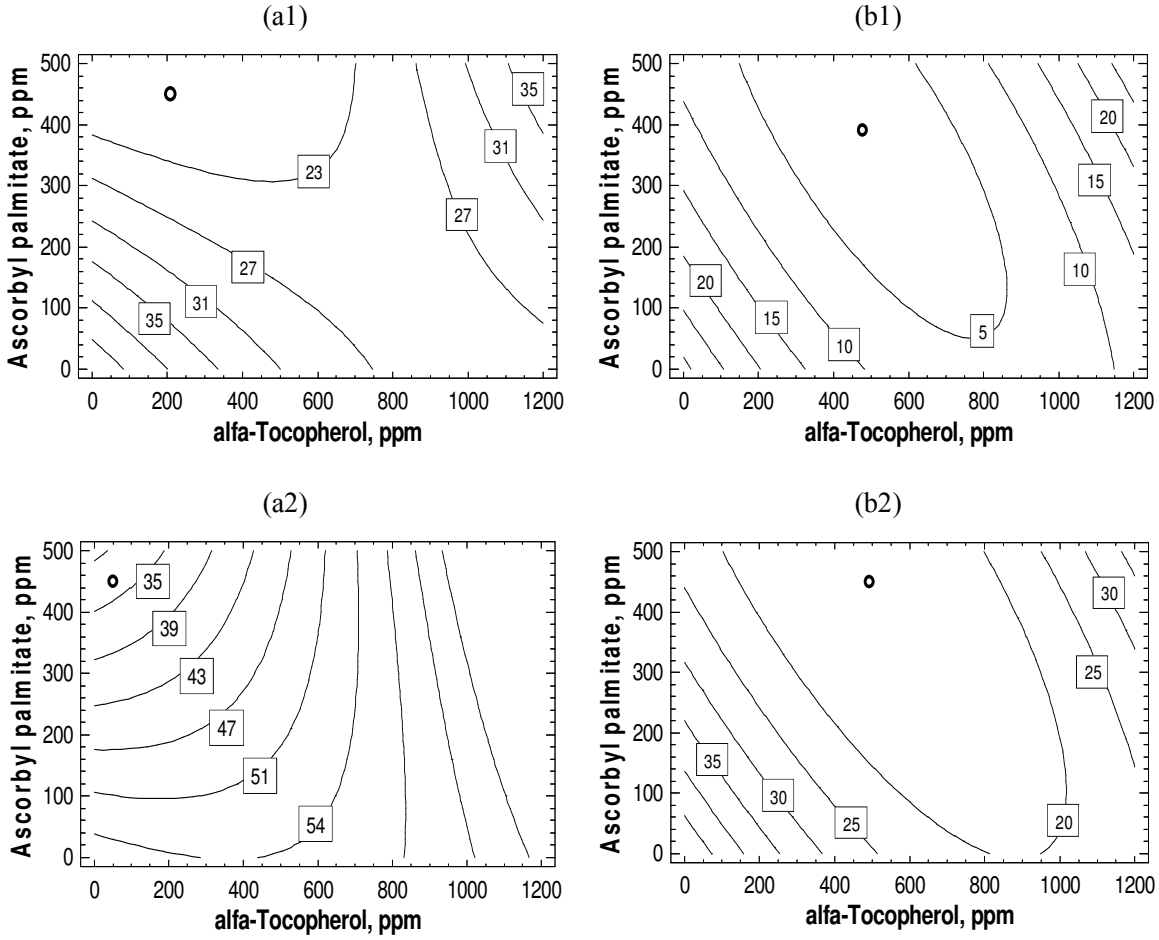

Figure 18. Contours plot for PV (a) and AV (b) at 10 days (1) and 30 days (2) of storage

The results stated above indicate that in order to minimize the formation of hydroperoxides, low concentrations of alpha-tocopherol (50 and 207 ppm) combined with

a high addition of ascorbyl palmitate (450 ppm) were required when evaluating the stabilized oil samples at short and long periods of storage. Higher concentrations of alpha-tocopherol resulted in a prooxidative effect. Similar results were found by Kulås and Ackman (2001a) who reported an optimum alpha-tocopherol content of 100 ppm for the stabilization of bulk fish oils.

On the other hand, medium alpha-tocopherol concentrations (478 and 493 ppm), also combined with 450 ppm of ascorbyl palmitate, inhibited more effectively the formation of secondary oxidation products. This is attributed to the ability of alpha-tocopherol to destroy hydroperoxides by forming either stable alcohols or inactive products by non-radicals processes, such as reduction or hydrogen donation (Kulås & Ackman, 2001b; Frankel, 2005; Olsen et al., 2005).

3.3 Bi-objective optimization

The conflict in the optimization of the two response variables selected (PV and AV) suggested the employment of a multi-objective optimization technique. Thus, a bi-objective optimization was carried out considering the data obtained for long time of storage, 30 days, which better indicates the shelf life of the stabilized oils.

A Pareto Front, Table 23, was generated using the weighted-sum method in order to find a set of non inferior solutions which satisfied both objectives to an adequate degree. The optimization of the objective function for each bound of the weight interval corresponds to the optimization of a single objective. It means, minimization of PV for w=1 (row 1) and minimization of AV for w=0 (row 12). Between these two values, each point of the non inferior solutions corresponds to a particular value of w, which varied at intervals of 0.1 (Table 23). Additionally, in row 5, a new solution was obtained, in which the weight of PV was two-fold the weight of AV, denoting the optimization of totox. Each solution (PV, AV) is determined by a combination of factors inside the ranges of the independent variables alpha-tocopherol and ascorbyl palmitate concentrations). It was observed that a decrease of AV (desired) implies an increase of PV (undesired). This trend is more pronounced at values of AV lower than 21.98 (w<0.6).

Furthermore, the decision space, also shown in Table 23, was obtained from the Pareto Front by determining the optimum combination of experimental factors alpha-tocopherol and ascorbyl palmitate concentration) for each weight factor w employed. As the weight

factor *w* decreases from *w*=1 to 0, moving from row 1 to 12, the objective of minimum AV is favored over that of minimum PV. It is important to note that varying the weight factor from 1 to 0.67, did not produce any change in the optimum concentrations of alpha-tocopherol and ascorbyl palmitate, which were maintained at the same values than the ones obtained for the individual optimum of PV, 50 and 450 ppm respectively. Nevertheless, when employing a weight factor (*w*) lower than 0.67 resulted in a higher optimum concentration of alpha-tocopherol (Table 23).

Table 23. Set of optimal solutions and decision space for the bi-objective optimization problem

Row	*w*	PV (meq/kg oil)	AV	α-Tocopherol (ppm)	Ascorbyl palmitate (ppm)
1	1.00	33.56	22.89	50	450
2	0.90	33.56	22.89	50	450
3	0.80	33.56	22.89	50	450
4	0.70	33.56	22.89	50	450
5	0.67	33.56	22.89	50	450
6	0.60	34.13	21.98	77	450
7	0.50	36.28	19.34	171	450
8	0.40	38.42	17.58	252	450
9	0.30	40.50	16.45	323	450
10	0.20	42.50	15.78	386	450
11	0.10	44.41	15.44	443	450
12	0.00	46.22	15.34	493	450

Therefore, selecting a single optimal solution from the Pareto Front (optimum concentration of alpha-tocopherol combined with 450 ppm of ascorbyl palmitate) will depend on the required characteristics of the stabilized oil. In this sense, assuming that AV is more closely related to flavor and odor deterioration than PV, medium alpha-tocopherol concentrations, that combined with ascorbyl palmitate (450 ppm) and citric acid (50 ppm), which allow the minimization of AV should be preferred.

4. CONCLUSIONS

This study revealed that low concentrations of alpha-tocopherol (50-207 ppm) optimized the reduction of hydroperoxides formation in sardine oil, when combined with sparing

synergists like ascorbyl palmitate (450 ppm) and metal chelators such as citric acid (50 ppm). Higher alpha-tocopherol content resulted in a prooxidative effect. In terms of secondary oxidation products, medium alpha-tocopherol concentrations (478-493 ppm), which improved the destroying of hydroperoxides to stable products, were required to minimize AV.

As consequence of the conflicting behavior of alpha-tocopherol concentration towards the accomplishment of the optimization objectives (minimization of PV and AV), a set of non inferior solutions (Pareto Front) which satisfied both goals was obtained by the weighted-sum method. Therefore, the final characteristics for the stabilized oil (optimal PV and/or AV) will determine the selection of a single solution inside the Pareto Front.

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VII. Influence of Casein-Phospholipids Combinations as Emulsifier on the Physical and Oxidative Stability of Omega-3 Delivery Systems*

The objective of this study was to investigate the influence, as emulsifier, of combinations of casein (0.3 wt%) and phospholipids (0.5 wt%) such as lecithin (LC), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) on the oxidative stability of 10% fish oil-in-water emulsions (pH 7). Apart from the oxidative stability, the physical stability of the emulsions was also evaluated. Antioxidant activity of each phospholipid was also determined using different *in vitro* assays. Similar DPPH scavenging activities were obtained, 95.0 % inhibition for solutions 1 wt% phospholipid, but the alpha-tocopherol content of LC was considerably lower. Besides, no significant reducing power was found for any of them, while the highest metal chelating activity was found for PC, 97.8 % at 1 wt%. The emulsion with LC showed the best physical stability in terms of zeta potential and mean droplet size. In addition, this emulsion was also the least oxidized in terms of peroxide value and concentration of the volatile oxidation product, 1-penten-3-ol. The results suggested that the combination of casein and lecithin could result in a favorable structure and thickness of the interfacial layer which may reduce lipid oxidation.

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1. INTRODUCTION

As a consequence of the numerous benefits of omega-3 polyunsaturated fatty acids (PUFA) on human health (Lees & Karel, 1990; Uauy & Valenzuela, 2000; Ward & Singh, 2005) as well as the low intake of fish and fish products in the typical Western diet, functional foods containing omega-3 lipids have resulted in one of the fastest-growing food product categories in the USA and Europe (Sloan, 2006). Nevertheless, to successfully develop foods enriched with omega-3 PUFA, lipid oxidation of these highly unsaturated fatty acids must be prevented in order to avoid both the loss of nutritional value and the formation of unpleasant off-flavors.

In this sense, one of the strategies developed to protect these oxidatively unstable lipids when incorporating them into food is the application of omega-3 PUFA delivery systems such as fish oil-in-water emulsions. Although the success of this approach has shown to be food system dependent, it resulted in increased oxidative stabilities when employed in milk (Let et al., 2007), cheese (Ye et al., 2009) and energy bars (Nielsen & Jacobsen, 2009).

In emulsions, the interface between the oil and the aqueous phase is the place of contact between lipids and prooxidative components (McClements & Decker, 2000). Therefore, the selection of the emulsifier, which determines the structure and thickness of the interfacial layer, has a significant influence on the initiation of the lipid oxidation in fish oil delivery systems (Hunt & Dalgleish, 1994).

Common emulsifiers employed by the food industry are milk proteins such as caseins. They present good emulsifying properties due to the existence of more or less hydrophobic regions in their amino acid structure (Krog, 2004), and good physically stabilizing properties because of the flexibility of their conformational structure (Creamer, 2003). In addition, caseins contain several phosphorylated serine residues that have been suggested to exhibit metal chelating activity (Diaz et al., 2003).

As caseins, phospholipids are also important natural stabilizers employed in food-processing applications. This fact is a consequence of their unique molecular structure containing both a lipophilic part in the form of fatty acid groups and a hydrophilic group in the form of phosphoric based esters. Besides, antioxidant properties of phospholipids from different sources such as milk (Chen & Nawar, 1991), egg yolk (Sugino et al., 1997), marine species (Cho et al., 2001) and soybean (Judde et al., 2003) have been reported.

Although the antioxidant mechanism of phospholipids is not completely elucidated, the following functions have been attributed to them so far: a) degradation of hydroperoxides inhibiting their accumulation (Lee et al., 1981; Saito & Ishihara, 1997), b) chelating activity for phosphatidylinositol (PI) (Pokorný, 1987), c) synergism with alpha-tocopherol regenerating the tocopheroxyl radical to tocopherol by hydrogen transfer (primarily by phosphatidylethanolamine) (Oshima et al., 1993; Bandarra et al., 1999; Lee & Choe, 2011), and d) formation of Maillard-type products, such as pyrroles, between amino phospholipids and oxidation products which have protective effect against oxidation (Hidalgo et al., 2005; Lu et al., 2012a).

Interestingly, improved emulsions stabilities were reported by Fang and Dalgleish (1993) when employing selected casein-phospholipids combinations. The authors attributed this fact to the creation of protein-phospholipid complexes which produced a better coverage of the oil droplet surface increasing the thickness of the interfacial layer. Besides, as phospholipids were expected to supplement the caseins at the interface, the caseins could protrude more into the aqueous phase and stretch less over the surface. Although several authors have evaluated the synergistic effects of casein and phospholipids as emulsifiers (Courthaudon et al., 1991; Dickinson & Ivenson, 1993; Fang & Dalgleish, 1993), there are only a few studies on the utilization of casein-phospholipids combinations in fish oil-in-water emulsions (Horn et al., 2011; Horn et al., 2012).

Therefore, the objectives of this work were: i) to evaluate the antioxidant properties of three common phospholipids (soy lecithin, phosphatidylcholine and phosphatidylethanolamine) in order to obtain a more comprehensive understanding of the antioxidant mechanisms of phospholipids; and ii) to investigate the synergistic effects of each of these three phospholipids and casein as emulsifiers on the physical and oxidative stabilities of fish oil-in-water emulsions at pH 7.

2. MATERIALS AND METHODS

2.1 Materials

Commercial cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway) and stored at -40 ºC until use. Sodium caseinate, CAS, (Miprodan® 30) was kindly donated by Arla Foods Ingredients amba (Viby J, Denmark). Arla reported a protein

content of 93.5 wt% in sodium caseinate for Miprodan® 30. Fat free soybean lecithin, LC, (LIPOID P 20); phosphatidylcholine from soybean, PC, (LIPOID S 100) and soy phosphatidylethanolamine, PE, (LIPOID S PE) were kindly donated by Lipoid (Steinhausen, Switzerland). Data sheets from Lipoid reported the following compositions: for LIPOID P 20, 25 wt% phosphatidylcholine, 20 wt% phosphatidylethanolamine, 10 wt% phosphatidylinositol, 5 wt% phosphatidic acid and 40 wt% others (mostly glycolipids); a content of 97.3 wt% phosphatidylcholine for LIPOID S 100 and a concentration of 99.6 wt% phosphatidylethanolamine for LIPOID S PE. All other chemicals and solvents used were of analytical grade.

2.2 Characterization of the fish oil and the phospholipids

Fatty acid compositions of the fish oil and the three phospholipids were determined by fatty acid methylation (AOCS, 1998a), followed by separation through GC (Agilent 7890A, Agilent Technologies, Palo Alto, CA, USA; Column: DBWAX, 10 m × 0.10 mm × 0.1 μm, Agilent Technologies, Palo Alto, CA, USA). Methyl esters were identified by comparison of the retention times of authentic standards and fatty acid compositions were expressed as area percentage of total fatty acids (AOCS, 1998b). The quality of the three PL-based emulsifiers and the fish oil was determined by their peroxide value (PV), employing the colorimetric ferric-thiocyanate method at 500 nm as described by Shantha and Decker (1994). Furthermore, their content of tocopherol was also determined (AOCS, 1998c).

2.3 Antioxidant activity assays

2.3.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The scavenging effect on DPPH free radical was measured by the method of Shimada et al. (1992) with some modifications. Solutions 1 wt% of the three phospholipids in ethanol were prepared and then diluted according to different dilution factors. In brief, 150 μL of each phospholipid solution was mixed with 150 μL of 0.1 mM ethanolic solution of DPPH. The mixture was then kept at room temperature in the dark for 30 minutes, and the absorbance was measured at 515 nm (Synergy 2 Multi-Mode, BioTek Instruments, Inc.). For the blank, 150 μL of ethanol was used instead of the sample and sample control was also made for each fraction by mixing 150 μL of sample with 150 μL of 95 wt% ethanol.

Triplicate measurements were performed. A BHT solution, 0.02 wt%, was employed as positive control. Radical scavenging capacity was calculated as follows, Eq. 18:

$$DPPH\ inhibition\ (\%) = \left(1 - \frac{(A_{sample} - A_{sample\_control})}{A_{blank}}\right) \times 100 \qquad (18)$$

2.3.2 Iron (Fe^{2+}) chelating activity

The iron chelating activity of the extracts was estimated by the modified method of Dinis et al. (1994). Phospholipids were dissolved in ethanol obtaining different solutions, 10 wt% for LC, 1 wt% for PC and 5 wt% for PE, which were then diluted according to several dilution factors. Firstly, 270 µL of distilled water were added to 200 µL of each phospholipid solution. Then, 10 µL of ferrous chloride 2 mM were added and after 3 min the reaction was inhibited by the addition of 20 µL of ferrozine solution 5 mM. The mixture was shaken vigorously and left at room temperature for 10 min, thereafter the absorbance was measured at 562 nm (Synergy 2 Multi-Mode, BioTek Instruments, Inc.). A blank was run in the same way by using ethanol instead of sample. Sample control was made for each sample without adding ferrozine. Triplicate measurements were carried out. An EDTA solution, 0.006 wt%, was employed as positive control. The chelating capacity was calculated as follows, Eq. 19:

$$Fe^{2+}\ chelating\ activity\ (\%) = \left(1 - \frac{(A_{sample} - A_{sample\_control})}{A_{blank}}\right) \times 100 \qquad (19)$$

2.3.3 Reducing power

The reducing power was measured according to the method of Oyaizu (1986) with some modifications. Phospholipids were dissolved in ethanol obtaining different solutions 1 wt% which were then diluted according to several dilution factors. To 200 µL of each phospholipid solution was added 200 µL 0.2 M phosphate buffer (pH 6.6) and 200 µL of 1 wt% potassium ferricyanide. The mixture was incubated for 20 min at 50 ºC. Then, 200 µL of 10 wt% trichloroacetic acid solution were added and the mixture was shaken vigorously. Finally, 144 µL of this solution were mixed with 342 µL of distilled water and 46 µL 0.1 wt% $FeCl_3$. After 10 minutes at room temperature, the absorbance was measured at 700 nm (Synergy 2 Multi-Mode, BioTek Instruments, Inc.). Increased absorbance (A_{700}) of the reaction mixture indicated increased reducing power. Triplicate measurements were performed. An ascorbic acid solution, 0.009 wt%, was employed as positive control.

2.4 Preparation of emulsions and sampling

Four emulsions were prepared with 10 wt% fish oil and emulsifier concentrations as follows: i) emulsion 0.3CAS stabilized with 0.3 wt% of CAS, ii) emulsion 0.3CAS+0.5LC stabilized with 0.3 wt% of CAS and 0.5 wt% of LC, iii) emulsion 0.3CAS+0.5PC stabilized with 0.3 wt% of CAS and 0.5 wt% of PC, and iv) emulsion 0.3CAS+0.5PE stabilized with 0.3 wt% of CAS and 0.5 wt% of PE. The buffer used was a 10 mM sodium acetate imidazole (pH 7.0). CAS, LC, PC and PE were dispersed in the buffer overnight at approximately 5 °C. Primary homogenization was done by adding the fish oil slowly to the buffer during mixing at 16,000 rpm (Ystral mixer, Ballrechten-Dottingen, Germany). The fish oil was added during the first minute of mixing, and the total mixing time was 3 minutes. Secondary homogenization was done on a microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75μm). Emulsions were homogenized at a pressure of 9,000 psi, running 3 passes. Emulsions were added 100 μM $FeSO_4$ to accelerate lipid oxidation and 0.05 wt% sodium azide to prevent microbial growth. Emulsions were stored in 100 mL bluecap bottles at room temperature (19-20 °C) in the dark for 14 days. Samples were taken at day 0, 4, 9 and 14 for lipid oxidation measurements. Measurements of pH, viscosity, and droplet size distributions were carried out at day 1 and 14, whereas zeta potential was determined at day 2. Samples for protein content in the aqueous phase were taken at day 3. Moreover, two emulsions, 0.3CAS and 0.3CAS+0.5PC, were prepared at exactly the same conditions but employing buffer at pH 3.5. Some results of these two emulsions are also presented.

2.5 Characterization of the emulsions

2.5.1 pH and zeta potential

The pH values of emulsions were measured at room temperature directly in the sample during stirring (pH meter, 827 pH Lab, Methrom Nordic ApS, Glostrup, Denmark). Measurements were done in duplicate.

The zeta potential was measured in a Zetasizer (Zetasizer Nano ZS, Malvern instruments Ltd., Worcestershire, UK) with a DTS-1060C cell at 25 ºC. Before analysis, the emulsions were diluted 2:1000 in 10 mM sodium acetate imidazole buffer (pH 7). The zeta potential

range was set to -100 to +50 mV and the samples were analyzed with 100 runs. Measurements were done in triplicate.

2.5.2 Droplet size

Droplet sizes were measured by laser diffraction using two different equipments: i) Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). Emulsion was diluted in recirculating water (3,000 rpm), until it reached an obscuration of 12–15%. Results are given in surface area mean diameter: $D_{3,2}=\Sigma d^3/\Sigma d^2$. Measurements were made in duplicate. ii) Zetasizer (Zetasizer Nano ZS, Malvern instruments Ltd., Worcestershire, UK) with a DTS-1060C cell at 25 ºC. The samples were prepared as described for the zeta potential measurement. The results are given as Z-average diameter. For both types of equipment the refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Measurements were made in duplicate.

2.5.3 Protein concentration in the aqueous phase

Samples (approximately 35g) were centrifuged at 45,000g for 50 min at 10 °C (Sorvall RC-6 PLUS, Thermo Fisher Scientific, Osterode, Germany). The lower aqueous phase was extracted with a syringe and needle, and centrifuged once more at 70,000g for 60 min at 15 °C (Beckman ultracentrifuge L8-60M, Fullerton, CA, USA). Once more the lower aqueous phase was extracted and then filtered (Minisart NML 0.20µm filter, Sartorius, Hannover, Germany). The protein content in the aqueous phase was determined with a BCA Protein Assay Reagent Kit (Pierce, Thermo Scientific, Rockford, IL, USA). Prior to analysis samples were diluted 1:9 in 10 mM sodium acetate imidazole buffer (pH 7). Measurements were performed in duplicate.

2.5.4 Viscosity

Viscosities of the emulsions (15 mL) were measured using a stress controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) equipped with a CC25 standard bob cup system in a temperature vessel. Measurements were done at 20 °C over a shear stress range from 0.0125 to 1.64 Pa. Viscosities are given as the average viscosity of the linear part of the plot of shear stress vs. viscosity expressed in cP. Viscosities were measured twice on each emulsion.

2.6 Measurements of lipid oxidation

2.6.1 Determination of peroxide value and tocopherol content

Lipids were extracted from the dispersions according to the Bligh and Dyer method using a reduced amount of the chloroform/methanol (1:1 wt%) solvent (Bligh & Dyer, 1959). Two extractions were made from each sample, and PV measurement was performed in duplicate. PV was measured according to the method described before. For tocopherol determination, lipid extracts (1−2 g) from Bligh and Dyer were weighted off and evaporated under nitrogen and re-dissolved in hexane prior to analysis by using the same method as mentioned previously.

2.6.2 Secondary oxidation products – SPME GC-MS

Approximately 4 g of emulsion, 30 mg internal standard (4-methyl-1-pentanol, 30 µg/g water) and 1 mL antifoam (Synperonic 800 µL/L water) was weighted out in a 100 mL purge bottle. The bottle was heated in a water bath at 45°C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile secondary oxidation products were trapped on Tenax GR tubes. The volatiles were desorbed again by heat (200°C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (-30°C), released again (220°C), and led to a gas chromatograph (HP 5890IIA, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 µm; J&W Scientific, CA, USA). The oven program had an initial temperature of 45°C for 5 min, increasing with 1.5°C/min until 55°C, with 2.5°C/min until 90°C, and with 12.0°C/min until 220°C, where the temperature was kept for 4 min. The individual compounds were analyzed by mass-spectrometry (HP 5972 mass-selective detector, Agilent Technologies, USA; electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250). The individual compounds were identified by both MS-library searches (Weley 138 K, John Wiley and Sons, Hewlett-Packard) and by authentic external standards. The external standards employed were 1-penten-3-one, pentanal, 1-penten-3-ol, (*E*)-2-pentenal, 1-pentanol, hexanal, (*E*)-2-pentenol, (*E*)-2-hexenal, heptanal, (*E*)-4-heptenal, (*E,E*)-2,4-heptadienal, nonanal, (*E,Z*)-2,6-nonadienal, 2-ethylfuran and 2-pentylfuran. These compounds were dissolved in 96 wt% ethanol and diluted to concentrations of approximately 0.5, 1, 5, 10, 25 and 50 µg/g. Two calibrations curves were made by injecting these solutions (30 mg) on 4 g of emulsion (0.3CAS and 0.3CAS+0.5LC emulsions). Measurements were made in triplicate in each sample.

2.7 Statistical analysis

The Statgraphics software (version 5.1) was used to carry out one-way analysis of variance (ANOVA) on the data. The Tukey's test was employed for that purpose and differences between means were considered significant at $p \leq 0.05$. Furthermore, oxidation data were subjected to multivariate data analysis (The Unscrambler X, version 10.2, CAMO Software AS, Oslo, Norway). A principal component analysis (PCA) was carried out with the four different emulsions as objects and peroxide values and volatiles data as variables, with one variable representing the average of a given peroxide value or volatile at a specific sampling time point. Data were autoscaled in order to make the variables contribute equally to the model, and the PCA model was validated systematically segmented, according to replicates of the emulsions.

3. RESULTS AND DISCUSSION

3.1 Composition of fish oil and phospholipids

The quality of the raw materials can have a great influence on the physical and oxidative stabilities of the final emulsions. For this reason, the fish oil and the three soybean phospholipids employed as emulsifiers were characterized in terms of peroxide value, fatty acid composition and tocopherol content (Table 24). It was observed that the commercial cod liver oil utilized presented a very low PV (0.28 meq O_2/kg), a considerable content of monounsaturated fatty acids (16.3 wt% of C18:1n-9 and 12.6 wt% of C21:1n-9) and a percentage of omega-3 PUFA higher than 20 wt% (mainly EPA, C20:5n-3, and DHA, C22:6n-3). Regarding the tocopherol composition, it contained mainly alpha-tocopherol (200 μg/g) and gamma-tocopherol (96 μg/g).

The three phospholipids had considerably higher PV than the fish oil, ranging from 1.48 meq/kg oil for PC to 4.01 meq/kg oil for PE. In terms of fatty acid composition, PC and PE presented almost the same content for each fatty acid, mainly C16:0 (~13 wt%), C18:1n-9 (~9 wt%), C18:2n-6 (~65 wt%) and C18:3n-3 (~6 wt%). This finding is explained by the fact that, according to the manufacturer, PE was produced from purified PC by conversion of the head group. Likewise, LC was composed by the same major fatty acids as PC and PE. However, it presented a higher content of saturated (21.5 wt% of C16:0) and monounsaturated fatty acids (13.6 wt% of C18:1n-9) and a lower percentage of

polyunsaturated fatty acids (53.3 wt% of C18:2n-6 and 4.7 wt% of C18:3n-3) than the two other phospholipids. As far as their tocopherol composition is concerned (Table 24), PC contained almost twice as much alpha-tocopherol as PE, whereas alpha-tocopherol was not detected in LC. This may be due to the addition of alpha-tocopherol as antioxidant in the production process of PC and PE. No other homologues of tocopherol was found in PE and low percentages of gamma and delta-tocopherol were obtained for LC and PC.

Table 24. Characterization of the fish oil and the phospholipids

	Fish oil	LC	PC	PE
PV, meq O_2/kg	0.28 ± 0.03	< 5[a]	1.48 ± 0.07	4.01 ± 0.32
Fatty acid, wt%[b]				
C16:0	9.5	21.5	12.7	12.9
C16:1n-7	8.7	0.2	0.2	0.2
C18:0	2.0	3.6	3.7	3.6
C18:1n-9	16.3	13.6	10.1	8.6
C18:1n-7	4.8	1.5	1.6	1.6
C18:2n-6	1.8	53.3	64.9	65.9
C18:3n-3	2.6	4.7	5.9	6.4
C21:1n-9	12.6	nd	nd	nd
C20:5n-3	9.2	nd	nd	nd
C22:1n-11	6.0	0.5	0.2	0.1
C22:6n-3	11.4	0.3	nd	nd
Others	15.1	0.8	0.7	0.7
Total	100	100	100	100
Tocopherol, μg/g				
Alpha	200 ± 10.2	nd	1976 ± 166.2	1006 ± 15.6
Beta	5 ± 0.2	nd	nd	nd
Gamma	96 ± 2.6	37 ± 0.1	100 ± 7.5	nd
Delta	42 ± 0.7	14 ± 0.2	40 ± 2.8	nd

[a] As reported on the data sheet provided by the manufacturers.
[b] Data are means of triplicate determinations. SD< 2 %.
nd: not detected.

3.2 Antioxidant activity of phospholipids

Phospholipids, as the rest of natural antioxidants, may act by different mechanisms. As a consequence, their antioxidant properties were evaluated by different methods such as DPPH, Fe^{2+} chelating and reducing power (Fig. 19). To the best of the authors' knowledge, such a systematic study on the antioxidant properties of phospholipids has not previously been reported.

3.2.1 DPPH radical scavenging activity

Fig. 19a shows the DPPH radical scavenging activity of the three phospholipids assayed (LC, PC and PE). Although they presented different alpha-tocopherol content (Table 24), they exhibited similar DPPH inhibition, approximately 95% at a concentration of 1 wt%. It was also observed that for LC, PC and PE, the DPPH radical scavenging activity decreased when decreasing the concentration until being practically constant at concentrations below 0.11 wt%, dilution factor of 9. This radical scavenging activity may be related, similarly to the regeneration of alpha-tocopherol by phospholipids, to the proton donating capacity of the amino group of phospholipids, particularly for PE and PC (Bandarra et al., 1999). Nevertheless, it should be mentioned that the three phospholipids tested were found to be less effective DPPH scavengers than BHT. Espín et al. (2000) reported DPPH radical scavenging activity for phospholipids presented in the lipid fraction of several vegetable oils, but it was considerably lower when compared with DPPH inhibition of alpha-tocopherol. Additionally, Reiss et al. (1997) also stated free radical scavenging activity for plasmalogen phospholipids (plasmenylethanolamine and plasmenylcholine). The authors mainly attributed this fact to interactions of enol ether double bond with initiating peroxyl radical which result in products of enol ether oxidation that apparently do not propagate the oxidation of polyunsaturated fatty acids. Furthermore, peroxyl radicals can also react with phosphatidylcholine to yield stable molecules such as trimethylammonium oxides (Cardenia et al., 2011).

Additionally, phospholipids have been proven to considerably enhance the antioxidant activity of chain breaking antioxidants such as alpha-tocopherol, both in bulk oils (Oshima et al., 1993; Bandarra et al., 1999; Lee & Choe, 2011) and in oil-in-water emulsions (Lu et al., 2012a). A Synergic effect of phospholipids with trolox, another phenolic antioxidant, was also described by Chen et al. (2011).

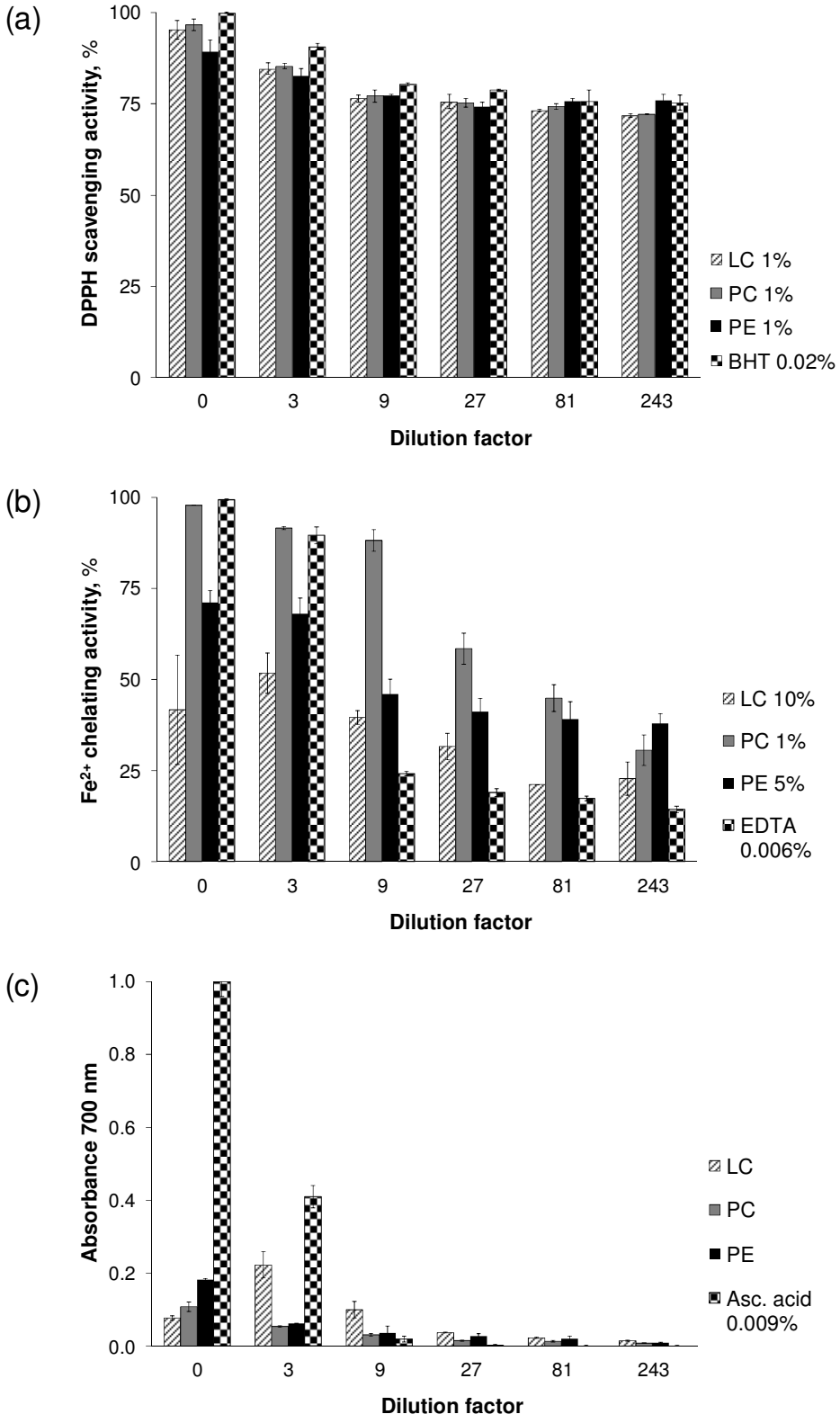

Figure 19. Antioxidant capacity of the phospholipids: (a) DPPH radical scavenging activity, (b) Fe^{2+} chelating activity and (c) reducing power. Results are average of triplicate determination ± standard deviation.

3.2.2 Iron (Fe^{2+}) chelating activity

Transition metal ions, mainly Fe^{2+} and Cu^{2+}, highly contribute to lipid autoxidation in real food emulsions. Thus, antioxidants which behave as metal chelators are considered to play an important role on the improvement of the oxidative stability of emulsions (Mancusso et al., 1999). Phospholipids have been reported to exhibit chelating activity, but contradictory results were described in the literature. Pokorný et al. (1987) stated that phosphatidylinositol and other acidic phospholipids form inactive complexes with metal ions while phosphatidylcholine and phosphatidylethanolamine do not possess such metal-chelating properties. On the contrary, Yoon and Min (1987) found that phospholipids, including phosphatidylcholine, improved the oxidative stability of purified soybean oil when iron was added. Zambiazi et al. (1998) attributed the metal-chelating properties to the amino group of PC, PE and PS and to the sugar moiety of PI.

In the present study it was observed that PC presented the highest capacity to bind metal ions followed by PE, whereas LC exhibited the lowest metal-chelating activity (Fig. 19b). For all three phospholipids, Fe^{2+} chelating activity was concentration dependent, increasing with concentration. Although all the phospholipids were considerably less effective than EDTA in binding metal ions, they may represent a unique type of chelator in case they can act in the lipid phase (Cardenia et al., 2011).

The superior Fe^{2+} chelating activity of PC may explain the results obtained by Cardenia et al. (2011). These authors found that phosphatidylcholine with either oleic or palmitic acid aliphatic side chain were more effective than 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine at inhibiting lipid oxidation in 1 wt% stripped soybean oil-in-water emulsions at pH 7. Bandarra et al. (1999) also described that the addition of phosphatidylcholine to refined sardine oil resulted in a higher protection against lipid oxidation when compared to the addition of phosphoethanolamine, cardiolipin and alpha-tocopherol.

3.2.3 Reducing power

The reducing power of phospholipids was evaluated in order to determine their capacity to donate electrons. In that way, they may convert free radicals to more stable products and terminate the radical chain reactions (Yen & Chen, 1995). However, compounds exhibiting reducing power may also have a prooxidative effect, especially when Fe^{3+} is reduced to Fe^{2+} in the presence of already existing hydroperoxides (Sørensen et al., 2011).

Fig. 19c shows the reducing power for LC, PC and PE. It was observed that they exhibited an insignificant reducing power when compared with ascorbic acid. For the three cases, absorbances at 700 nm lower than 0.2 were obtained for solutions 0.1 wt%. The higher reducing power of PE may be explained due to the fact that ascorbyl palmitate was added during the manufacturing process for its stabilization. Ascorbyl palmitate was reported in previous studies to exhibit reducing power (Mäkinen et al., 2001; Sørensen et al., 2011).

3.3 Characterization of the emulsions

3.3.1 pH and zeta potential

In Table 2, it is shown that the actual pH of the emulsions was exactly the pH aimed at (pH 7). Regarding the surface charge of the droplets, negative zeta potentials, ranging from -22.8 to -32.9 mV, were obtained for all the emulsions (Table 25). This finding can be explained by the fact that casein is negatively charged at pH values above its isoelectric point (Hu et al., 2003a). In addition, PC and PE, which are zwitterionic ions, do not carry net charge at neutral conditions. Likewise, in the case of lecithin, PI, which is an anionic ion, is also negatively charged at pH 7 (Wang & Wang, 2008). In Table 25, it was observed that the use of a combination of casein and lecithin made the zeta potential slightly more negative compared to the use of casein alone, whereas less negative zeta potential values were obtained for the combinations of casein with PC and PE.

Considering the emulsions prepared at pH 3, 0.3CAS and 0.3CAS+0.5PC, positive values of zeta potential were obtained, 22.4 and 20.5 mV, respectively (data not shown). As for the emulsions at high pH, these results also revealed that the addition of PC did not make the zeta potential value more positive. Nevertheless, having a positive surface charge of the oil droplets may imply a repulsion of the cationic transition metal ions causing a decrease in lipid oxidation (Hu et al., 2003b).

Table 25. Values of pH, zeta potential, droplet size, concentration of protein in the aqueous phase and viscosity of the emulsions

Emulsion	pH	Zeta potential[a] (mV)	Droplet size				Protein[a] (mg/mL)	Viscosity (cP)	
			$D_{3,2}$ (nm)		Z-average diameter (nm)				
			Day 1[a]	Day 14[a,b]	Day 1[a]	Day 14[a,b]		Day 1[a]	Day 14[a,b]
0.3CAS	7.1±0.0	-29.5±0.1^{w}	255.5±2.1^{w}	307.5±4.9^{w,*}	330.3±0.3^{w}	352.1±12.7^{w,*}	0.69±0.02^{w}	1.61±0.08^{w}	1.81±0.04^{w,*}
0.3CAS+0.5LC	7.0±0.0	-32.9±0.2^{x}	210.0±5.6^{x}	203.5±3.5^{x,ns}	270.5±0.7^{x}	266.9±1.7^{x,*}	1.62±0.26^{x}	1.45±0.16^{wx}	1.85±0.08^{w,*}
0.3CAS+0.5PC	7.0±0.0	-22.8±1.3^{y}	212.0±1.4^{x}	195.0±2.8^{x,*}	265.9±0.4^{y}	262.7±2.1^{x,*}	1.90±0.15^{x}	1.43±0.05^{x}	2.04±0.27^{w,*}
0.3CAS+0.5PE	7.1±0.0	-23.8±0.4^{y}	306.0±1.4^{y}	369.0±8.5^{y,*}	332.3±0.8^{z}	357.8±4.5^{w,*}	1.65±0.20^{x}	1.37±0.01^{x}	1.68±0.01^{w,*}

[a] For each column letters w–z indicate significant differences between samples ($p < 0.05$).
[b] Significant differences between day 1 and 14 are indicated by either *: significantly different ($p < 0.05$), or ns: not significantly different.

3.3.2 Particle size distribution, droplet size, casein content in the aqueous phase and viscosity

As far as the particle size distribution (PSD) of the emulsions is concerned, the results obtained from the analysis with the Mastersizer 2000 revealed that the emulsion stabilized only with casein had a monomodal PSD with a peak at 450 nm. In contrast, the three emulsions where combinations of casein and phospholipids were employed presented bimodal distributions, with a larger population of smaller droplets (mean diameter at peak 200-400 nm) and a smaller population of larger droplets (mean diameter at peak 1,000-1,200 nm). Although large unilamellar vesicles could present diameters >100 nm (Lu et al., 2012b), the peak found between 200 and 400 nm may be more likely due to the existence of small droplets covered by a phospholipid monolayer, protein-phospholipid or protein alone. Similar bimodal distributions were reported by Fang and Dagleish (1996) for 20 wt% soybean oil-in-water emulsion stabilized with 0.7 wt% casein and 0.5 wt% dioleoylphosphatidylcholine and by Lu et al. (2012) for 9.5 wt% fish oil-in-water emulsions stabilized with 0.5 wt% of lecithin obtained from fish by-products.

Likewise, the Zetasizer analysis gave similar monomodal and biomodal PSD for the emulsions stabilized with casein alone and with combinations of casein and phospholipids, respectively. Due to the fact that the PSD of the emulsions with casein and phospholipids did not present any peak around 4 nm, it was assumed that micelles were not formed from monolayers of phospholipids molecules (Thompson et al., 2006).

Regarding the mean droplet sizes of the emulsions, the results obtained from the Mastersizer 2000 and from the Zetasizer analyses were consistent with respect to rank order of the samples, and droplet sizes were also relatively similar (Table 25). At day 0, the mean droplet sizes of the four emulsions increased in the order 0.3CAS+0.5LC ≈ 0.3CAS+0.5PC < 0.3CAS < 0.3CAS+0.5PE. Smaller droplet sizes are preferred in order to increase the physical stability of the emulsions. Nevertheless, it implies larger surface areas, where prooxidative transition metal ions can more easily interact with lipid hydroperoxides. This fact could lead to less oxidatively stable emulsions (Jacobsen et al., 2000; Lethuat et al., 2002).

As shown in Table 25, the addition of LC and PC resulted in lower mean droplet sizes when compared to the use of only casein. Fang and Dalgleish (1996) also reported a reduction on the initial droplet size when incorporating dioleoylphosphatidylcholine to

emulsions stabilized with casein. Table 25 also shows that the addition of phospholipids similarly increased the protein concentration in the aqueous phase for the emulsions with LC, PC and PE. This finding suggested that part of casein at the interface was substituted by phospholipids. This was in agreement with the suggestion by Fang and Dalgleish (1993).

Although emulsions with LC and PC exhibited good physical stability, emulsions with only casein and casein plus PE increased their mean droplet sizes and their size distributions moved to larger sizes after 14 days of storage. This denotes that only 0.3 wt% of casein was not enough amount of emulsifier to stabilize 10 wt% fish oil-in-water emulsions during 14 days. From more recent studies, it was revealed that utilizing 0.5 wt% CAS decreased the increment in the droplet size at day 14. And, up to 1 wt% CAS was required in order to avoid coalescence or flocculation in 10 wt% fish oil-in water emulsions prepared at pH 7 and stored during 2 weeks (not published results). In the case of the emulsion 0.3CAS+0.5PE, the physical instability could be due to the lower hydrophilic-lipophilic balance (HLB) of PE at neutral pH, which makes it less appropriate to stabilize water-continuous emulsions (Carlsson, 2008). Despite the increase in droplet sizes observed for 0.3 CAS and 0.3 CAS+PE during storage, no creaming occurred in any of the emulsions during the 14 days. Considering the emulsions prepared at pH 3 (0.3CAS and 0.3CAS+0.5PC), higher mean droplet sizes at day 0 and more physically unstable emulsions were obtained (results not shown). Creaming was also observed during storage, particularly for the emulsion with PC. This behavior could perhaps be related to the swelling of PC which becomes more hydratable at acidic pH, diminishing its capacity as emulsifier (Comas et al., 2005).

The rheology of the emulsion can determine the diffusion of pro-oxidants in the system (Waraho et al., 2011). The viscosities at day 1 of the four emulsions ranged from 1.37 to 1.61 cP (Table 2). It was observed that the addition of phospholipids as secondary emulsifiers produced a significant and almost identical reduction in the viscosity of 0.3CAS+0.5LC, 0.3CAS+0.5PC and 0.3CAS+0.5PE emulsions compared to the value obtained for the 0.3CAS emulsion. This decrease in viscosity is not only caused by a reduction in the mean droplet size, as was obtained when employing LC and PC, but it may also be related to other properties of the emulsions such as the composition of the interfacial layer and the concentration of protein in the aqueous phase (Floury et al., 2000). This may suggest that molecular interactions between casein and phospholipids took place,

but this needs further investigation. As shown in Table 25, the viscosities of the four emulsions increased significantly after 14 days of storage.

3.3.3 Tocopherol concentration

The tocopherol concentration of the emulsions was also determined. The amount of alpha-tocopherol significantly varied during storage (Table 26), whereas the opposite was obtained for beta-, gamma- and delta-tocopherol which were maintained practically constant at 4, 90 and 37 μg/g lipid, respectively. In Table 26, it is shown that the highest content of alpha-tocopherol was presented for the 0.3CAS+0.5PC emulsion followed by the emulsion 0.3CAS+0.5PE due to the higher alpha-tocopherol content of these two phospholipids. It was also observed that the consumption of alpha-tocopherol followed the order 0.3CAS+0.5LC > 0.3CAS+0.5PE > 0.3CAS > 0.3CAS+0.5PC.

Table 26. Tocopherol content for the emulsions

Emulsion	**α-Tocopherol** (μg/g lipid)	
	Day 0[a]	Day 14[a,b]
0.3CAS	181.7±6.1^{w,*}	157.1±2.4^{w,*}
0.3CAS+0.5LC	195.1±2.1^{x,*}	158.8±1.1^{w,*}
0.3CAS+0.5PC	253.9±9.6^{y,*}	231.9±0.9^{x,*}
0.3CAS+0.5PE	226.5±4.7^{z,*}	197.8±5.2^{y,*}

[a] For each column letters w–z indicate significant differences between samples ($p < 0.05$).[b] Significant differences between day 1 and 14 are indicated by either *: significantly different, $p < 0.05$, or ns: not significantly different.

3.4 Lipid oxidation in emulsions

A PCA model was calculated to get a visual overview of all the variables, and to interpret correlations between the variables. In the PCA, the first principal component (PC1) explained 46 % of the variance and the second principal component (PC2) explained 31 % (Fig. 20a). The scores plot showed that replicates for each type of emulsion were located together in the plot. Furthermore, the plot showed that the LC emulsion was located in the 1st quadrant, the PC emulsion in the 2nd quadrant, the PE emulsion in the 3rd quadrant and the CAS emulsion in the 4th quadrant. Hence, the different emulsions had different values both on PC1 and PC2.

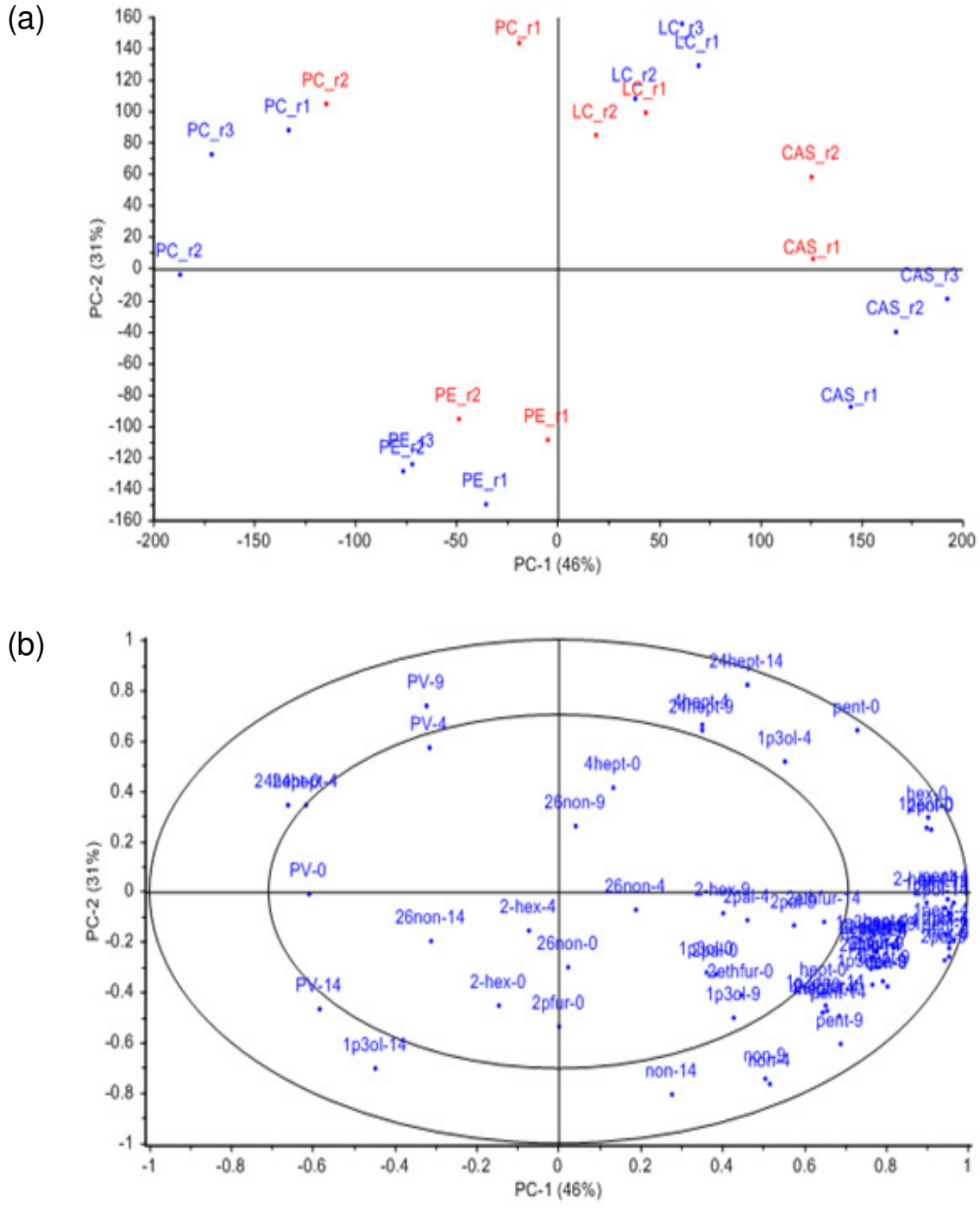

Figure 20. PCA plot: (a) scores plot with all sample replicates (r_1, r_2 y r_3); (b) loadings plot (0–14 indicate sampling days 0–14)

3.4.1 Peroxide value

The PCA loadings plot showed that peroxide values were located to the left in the plot, in the 2nd and 3rd quadrants, indicating that PE and especially PC emulsions had higher PVs than the other two emulsions (Fig. 20b). PV-0 has a value of approximately 0 in PC2, indicating less contribution from this variable than the other PV variables. The raw data confirmed this interpretation of the model, as it was observed that all the emulsions had a similar initial PV (~6 meq O_2/kg oil) and all of them suffered an increase in PV over time of storage. The emulsion with PC had the highest concentration of hydroperoxides after 14 days (24.7 meq O_2/kg oil), followed by the emulsion with PE (21.1 meq O_2/kg oil) and then the emulsion stabilized with only casein (19.9 meq O_2/kg oil) (Fig. 21). Although the emulsion with LC presented the shortest lag phase until day 9, this emulsion correlated negatively with PV-14 in the PCA, and was also shown to have the lowest PV at day 14 (12.9 meq O_2/kg oil) (Fig. 21).

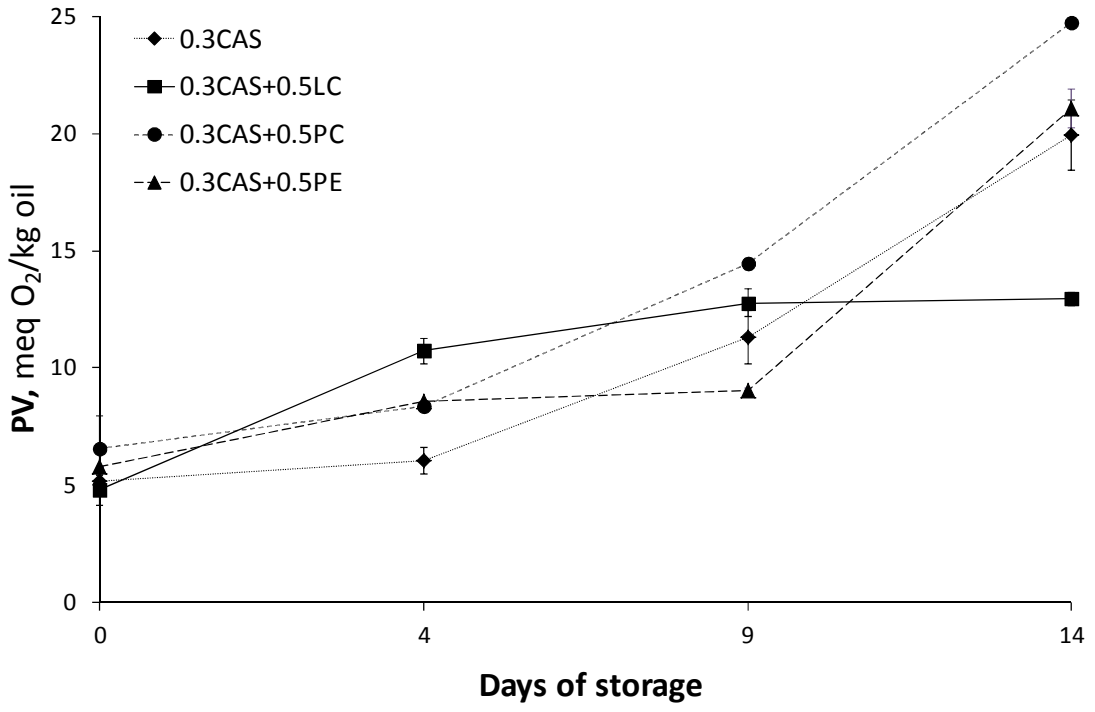

Figure 21. Peroxide value of the emulsions during storage. Results are average of duplicate determination ± standard deviation.

3.4.2 Secondary volatile oxidation products

The PCA loadings plot showed that the most volatiles were located in the 4th quadrant, indicating a higher concentration of volatiles in the CAS emulsion than the other emulsions (Fig. 20b). This was confirmed by the raw data since CAS emulsion suffered a higher increase from day 0 to day 14 in the concentration of the following volatiles: 2-pentylfuran (Fig. 4a), 2-ethylfuran, (*E*)-4-heptenal, heptanal and (*E*)-2-hexenal (data not shown). Fig. 2b also suggests that the emulsion with PE presented the highest content of 1-penten-3-ol and (*E,Z*)-2,6-nonadienal at day 14. The raw data confirmed this, as shown in Fig. 22b for 1-penten-3-ol, and indicated that this emulsion also had the maximum concentration in other volatiles such as 1-penten-3-one (Fig. 22c), 1-pentanol, pentanal, hexanal, (*E*)-2-pentenol and nonanal (data not shown). The emulsion with PC was the third most oxidized with a high increase in the content of 1-penten-3-ol (Fig. 22b), 1-penten-3-one (Fig. 22c), (*E,E*)-2,4-heptadienal (Fig. 4d) and (*E,Z*)-2,6-nonadienal (data not shown). It may result less oxidized in terms of volatiles products than the emulsion with PE due to the superior chelating properties showed by PC (Fig. 19b) and to its higher protein content in the aqueous phase (Table 25), which also could contribute to the chelation of metal ions (Diaz et al., 2003).

Regarding the emulsion with LC, it suffered the highest increase in the content of (*E,E*)-2,4-heptadienal (Fig. 22d), as was also indicated by the PCA analysis. However, this emulsion showed the lowest increase from day 0 to day 14 for the following volatiles: 2-pentylfuran, 1.7 ng/g (Fig. 22a); 1-penten-3-ol, 123.0 ng/g (Fig. 22b); and 1-penten-3-one, 1.8 ng/g (Fig. 22c). These findings, together with the lowest PV determined at day 14 (12.9 meq O_2/kg oil) and the minimum consumption of alpha-tocopherol during storage observed for this emulsion when compared with the others, indicated that the emulsion stabilized with the combination of casein and LC was the most oxidative stable.

Nevertheless, the higher oxidative stability of the emulsion with LC cannot be explained by the physical data obtained for this emulsion (Table 25). The emulsion with LC had the most negative zeta potential (-32.9 mV), which would increase the attraction of transition metals ions to the interface, and the lowest mean droplet size ($D_{3,2}$=210 nm) which implies larger surface for the contact between metal ions and hydroperoxides. Both facts highly contribute to lipid oxidation since metal ions catalyze the initiation of free radicals as well as the decomposition of hydroperoxides to secondary oxidation products (Frankel, 2005).

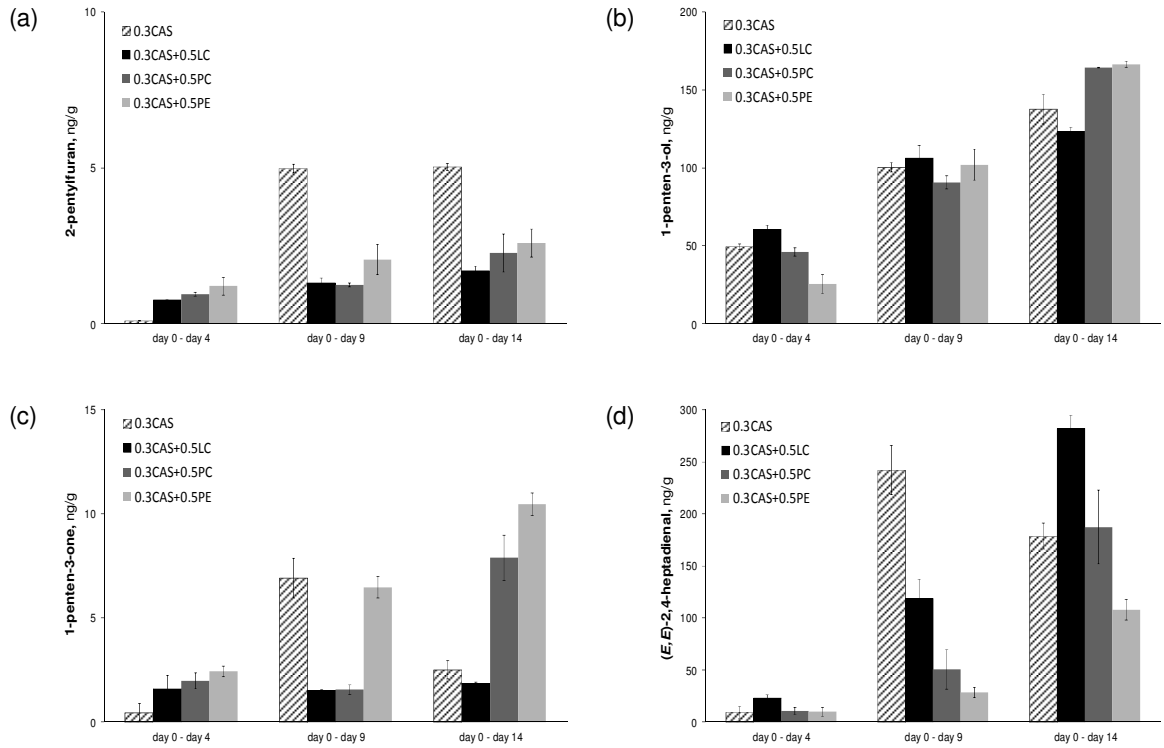

Figure 22. Increase of the concentration of secondary oxidation products in the emulsions: (a) 2-pentylfuran, (b) 1-penten-3-ol, (c) 1-penten-3-one and (d) (E,E)-2,4-heptadienal. Results are average of triplicate determination ± standard deviation.

In addition, the protein content in the aqueous phase of this emulsion was practically identical to the other emulsions with phospholipids (Table 25), and LC also did not show better metal chelating activity than the other phospholipids (Fig. 1b). Although the DPPH scavenging activity of LC was higher than the rest of phospholipids (Fig. 19a), since it leads to similar percentages of inhibition with a low alpha-tocopherol content (Table 24), it was considerably less effective than BHT.

Therefore, these results suggested that the improved oxidative stability of the LC emulsion may be related with other factors such as the characteristics of the interface. In this regard, previous studies stated that a change in the thickness and structure of the interfacial layer was obtained when adding lecithin to emulsions stabilized with low concentration of casein (0.3 wt%) (Fang & Dalgleish, 1993; Fang & Dalgleish 1996). According to these authors, the surface concentration of casein is not the only important factor influencing the adsorbed casein layer thickness. They concluded that a thicker interfacial layer is produced due to the fact that the added phospholipids take up space at the interface leading casein to adopt a conformation that projects further into the aqueous phase. The increase observed for the protein concentration in the aqueous phase for the emulsions with phospholipids

when compared with the CAS emulsion (Table 25), suggested the incorporation of phospholipids at the interface which could lead to the commented modification of the interface. In the case of LC, its content in charged phospholipids such as PI and PA, which are more soluble in water and are then more easily adsorbed at the oil-water interface (Rydhag & Wilton, 1981), may cause the suggested differences in the physical structure and thickness of the LC emulsion. However, this requires further research in order to be confirmed.

Comparing the emulsions at pH 3 and 7, it was observed that emulsions at pH 3 presented a lower content of hydroperoxides but their volatiles content was considerably higher. Although the positive zeta potential of these emulsions may result in a repulsion of metal ions causing a decrease of lipid oxidation (Hu et al., 2003b), at low pH the solubility of iron increases accelerating in this way the decomposition of hydroperoxides to volatiles (Horn et al., 2012).

4. CONCLUSIONS

The three phospholipids evaluated in this work showed similar DPPH scavenging properties, but it should be noted that LC presented a considerably lower content of alpha-tocopherol. Moreover, any of them exhibited reducing power. In terms of chelating activity, they rank in the order PC>PE>LC. This finding may be attributed to the chelating properties of the amino group in PC and PE.

The emulsion with LC resulted in the most physically stable having the most negative zeta potential (-32.9 mV) and the lowest mean droplet size ($D_{3,2}$=210 nm). It may be due to better emulsifying properties of LC as consequence of the different phospholipids (PC, PE, PI and PA) present in its composition. Besides, this emulsion was also the least oxidized, as confirmed by its lower PV at day 14 and by the lowest increase in volatiles oxidation products such as 1-penten-3-ol, 2-pentylfuran and 1-penten-3-one. However, this finding cannot be attributable to its antioxidant properties nor to its physical data such as protein concentration in the aqueous phase since they were similar to the ones presented by the other emulsions. Thus, it may be consequence of other factors such as the interaction of LC and casein at the interface, which could lead to an improved structure and thickness of the interfacial layer. This is supported by the increase observed for the concentration of protein

in the aqueous phase, which suggests that phospholipids were incorporated at the interface. Nevertheless, this statement needs further research in order to be confirmed.

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VIII. Optimization of Biodiesel Production from Fish Oil*

The present study deals with the production of biodiesel using poor quality fish oil. Following a factorial experimental design, it was investigated the effect of transesterification parameters such as temperature (40-60 ºC), molar ratio methanol to oil (3:1-9:1) and reaction time (30-90 min) on the yield and biodiesel properties. The experimental results were fitted to complete quadratic models and optimized by response surface methodology. All the biodiesel samples presented a FAME content higher than 93 wt% with a maximum, 95.39 wt%, at 60 ºC, 9:1 of methanol to oil ratio and 90 min. On the other hand, a maximum biodiesel yield was found at the same methanol to oil ratio and reaction time conditions but at lower temperature, 40 ºC, which reduced the saponification of triglycerides by the alkaline catalyst employed. Adequate values of kinematic viscosity were obtained, with a minimum of 6.30 mm^2/s obtained at 60 ºC, 5.15:1 of methanol to oil ratio and 55.52 min. However, the oxidative stability of the biodiesels produced must be further improved by adding antioxidants since low values of IP, below 2.22 h, were obtained. Finally, satisfactory values of completion of melt onset temperature, ranging from 3.31 ºC to 3.83 ºC, were measured.

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1. INTRODUCTION

The fish industry still generates large quantities of by-products, mainly due to fish processing and discards (Rustad, 2006). Although these wastes are generally employed to obtain low value products such as fish silage or fertilizer, highly valued commodities such as fish oil can be also produced from them (Aidos et al., 2003; Wu & Bechtel, 2008). Fish oil containing a high content of omega-3 PUFA (>20 wt%) has gained an increasing interest in the pharmaceutical and functional food industries, due to the recognized health benefits of eicosapentaenoic (C20:5n-3) and docosahexaenoic acids (C22:6n-3) (Uauy & Valenzuela, 2000; Ward & Singh, 2005). However, fish oil having a lower amount of EPA and DHA leads to very low yield of omega-3 concentrates which considerably reduces its applications on both fields (Bimbo, 2007). In addition, fish oil extracted from fish wastes may not meet the quality criteria required for edible purposes (El-Mashad et al., 2008).

In this framework, alternative uses for poor quality fish oil, for example as a fuel, need to be investigated. Although crude fish oil can be directly employed as combustible in boilers or furnaces, it may results in large particulate emissions and carbon deposits on the fuel injectors Ma & Hanna, 1999). As a solution, crude fish oil can be converted into biodiesel which is a renewable diesel fuel with a reduced viscosity and a low emissions profile (Helwani et al., 2009). Furthermore, using fish waste oil as feedstock for biodiesel has the potential to reduce the production costs drastically (Chiou et al., 2008). Biodiesel is a mixture of fatty acid alkyl esters mostly produced from the transesterification of fats and oils. In this process, the triglycerides contained in the oil react with an alcohol, commonly methanol, and a catalyst, usually sodium hydroxide (NaOH), to yield fatty acid methyl esters (FAME) (Gerpen, 2005). This reaction is highly influenced by temperature, molar ratio of methanol to oil, reaction time, catalyst and free fatty acids content of the oil (Meher et al., 2006). Although considerable research has been conducted on the optimization of biodiesel production from vegetable oils by this method, only a few studies have been carried out when employing fish oil as raw material.

In light of the above, the aims of this work were: a) to evaluate the influence of temperature, methanol to oil ratio and reaction time on the yield and fuel characteristics of biodiesel produced from crude fish oil via transesterification using NaOH with methanol, and b) to find, by using response surface methodology, the processing conditions which

lead to optimum values for yield, kinematic viscosity, oxidative stability and cold flow properties of the biodiesel obtained.

2. MATERIALS AND METHODS

2.1 Materials

The fish oil employed contained 90 wt% of salmon oil and the remaining 10 wt% as a mixture of other fish oil in season. It was purchased from Industrias Afines, S.L. (Vigo, Spain) and presented the following properties: acid value of 6.6 mg KOH/g oil, peroxide value of 6 and a composition of 5.7 wt% in EPA and 7.6 wt% in DHA. All the other reagents used were of analytical grade.

2.2. Production process

Due to fact that the free fatty acids (FFA) content of the crude fish oil is higher than 2 mg KOH/g oil, which would negatively affect to the biodiesel yield due to soap formation, a two-step process was performed as suggested by El-Mashad et al. (2008). The first step consisted of an acid-catalyzed pre-treatment to esterify the FFA which allowed to reduce the acid value below 2 mg KOH/g oil before the alkaline transesterification. In brief, 200 g of salmon oil were introduced with methanol (molar ratio methanol to oil of 6:1) and 1 wt% sulphuric acid as catalyst into a flat-bottomed flask. The samples were stirred at 300 rpm and kept at 60 ºC for 60 min using a hot plate controlled by a thermo-regulator. After completion of the reaction, the mixture was transferred to a separating funnel to settle for one hour and the bottom layer containing the esterified oil was separated.

In the second step, the fish oil from the first step was subjected to alkaline transesterification. In short, 200 g of oil were introduced with a given methanol amount and 1 wt% sodium hydroxide as catalyst into a flat-bottomed flask. The samples were stirred at 300 rpm and kept at the selected temperature for a desired time. The three process parameters considered for this study were temperature, molar ratio methanol to oil and reaction time. A factorial experimental design comprising 27 runs was carried out, in which each input variable was set at three levels; 40, 50, 60 ºC for temperature; 3:1, 6:1 and 9:1 for methanol to oil molar ratio; and 30, 60, 90 min for reaction time. After the reaction was completed, the content of the reactor was poured to a funnel to settle for one

hour. The alkyl esters were separated and purified by washing two times with distilled water at 50 ºC to remove unreacted catalyst, free glycerol and the remaining methanol. Then, the methyl esters were vacuum filtered through sodium sulphate to eliminate residual moisture. The biodiesel samples were stored under nitrogen at 4 ºC in amber bottles until analysis.

2.3 FAME content and yield

The FAME content of the biodiesel samples was determined according to the European standard EN 14103 with some modifications. The methyl esters of biodiesel samples were analyzed according to the method described by Camacho-Paez et al. (2002) with an Agilent 7890A gas chromatograph (Agilent Technologies, S.A., Santa Clara, California, USA) connected to a capillary column of fused silica Omegawax (0:25 mm × 30 m, 0:25 μm standard film; Supelco, Bellefonte, PA) and a flame-ionization detector. Nitrogen was used as the carrier gas and the total column flow was 44 ml/min. The oven temperature program was initially set at 150°C for 3 min, then increased at a rate of 10 °C/min until 240°C and maintained at this temperature for 12 min. Matreya (Pleasant Gap, PA) n-3 PUFAs standard (catalog number 1177) was used for the qualitative fatty acid determination and methyl heptadecanoate was employed as internal standard for quantitative determination.

Once the FAME of the biodiesel samples were identified, the peak areas were employed to determine the FAME content of each sample by Eq. 20:

$$C=\frac{(\sum A)-A_{EI}}{A_{EI}}\times\frac{C_{EI}\times V_{EI}}{m}\times 100 \qquad (20)$$

where: C = fatty acid methyl ester content (%); ΣA = total peaks area; A_{EI} = area of the peak corresponding to methyl heptadecanoate; C_{EI} = concentration of methyl heptadecanoate solution in heptane (mg/ml); V_{EI} = volume of methyl heptadecanoate solution (ml); m = mass of biodiesel sample (mg).

The yield of the produced biodiesel was expressed as follows, Eq. 21:

$$Yield=\frac{M_{Biodiesel}\times C}{M_{Oil}}\times 100 \qquad (21)$$

where: $M_{Biodiesel}$ is the mass of purified methyl esters obtained, M_{Oil} is the mass of oil employed and C is the fatty acid methyl ester content determined as described above.

2.4 Determination of biodiesel characteristics

2.4.1 Kinematic viscosity

Viscosity of the biodiesel samples were measured using a rotational viscometer Haake model VT500 and NV sensor system (Fisher Scientific, Aalst, Belgium). Each sample was placed between the two coaxial cylinders and set at 30 ºC employing a water bath. Shear stress (τ) was measured at varying shear rates (γ) from 0 to 3000 s^{-1}. Then, the dynamic viscosity (μ, Pa·s) of each sample was determined as the slope of the straight line resulting when plotting shear stress (τ, Pa) versus the shear rate (γ, s^{-1}). Measurements were performed in duplicate.

The density determinations involve heating the biodiesel sample at 30 ºC and then simply weighing the sample, at that specific temperature, to determine the mass of the sample with a known volume. Measurements were carried out in duplicate.

Once the dynamic viscosity and the density were measured, they were used to determine the kinematic viscosity at 30 ºC for each sample. It was expressed in mm^2/s.

2.4.2 Oxidative stability

The Rancimat test was employed to determine the oxidative stability of the biodiesel samples according to EN 14112. A Metrohm Rancimat model 743 (Methrom Instruments, Herisau, Switzerland) was utilized. A stream of filtered, cleaned and dried air at a rate of 10 L/h was bubbled into 7.5 g of biodiesel samples contained in reaction vessels. These vessels were placed in an electric heating block, which was set at 110 ºC. Effluent air containing volatile organic acids from the oil samples were collected in a measuring vessel with 60 mL of distilled water. The conductivity of the water was continuously recorded and the induction period (IP) was automatically determined using the apparatus. The measurements were carried out in duplicate. Rancimat IP was expressed as resistance time (in hours) of the oil to oxidation.

2.4.3 Differential scanning calorimetry

DSC curves are commonly employed to obtain information about the cold flow properties of biodiesel (Dunn, 1999). In this work, DSC heating curves were obtained for each biodiesel sample in order to determine their completion of melt onset temperature (COM).

DSC analyses were conducted using a differential scanning calorimeter Metler Toledo DSC 1 (Mettler Toledo, Barcelona, Spain). For each scan, approximately 10 mg of sample were hermetically sealed in an aluminium pan and tested against an identical empty pan. To determine the phase transition of the biodiesel samples, a ramp rate of 5 ºC/min was used under nitrogen atmosphere (50 ml/min). Samples were cooled and held isothermally at -80 ºC for 10 min, then heated to 100 ºC.

2.4.4 Acid value

The acidity of the biodiesel samples was determined according to the European standard EN 14104. The method used is based on titration of the sample, diluted with ethanol-diethyl ether mixed solvent, with potassium hydroxide solution in ethanol employing phenolphthalein as indicator to detect the end point. The results are expressed in milligrams of potassium hydroxide per gram of sample (mg KOH/g oil). The samples were measured in duplicate.

2.5 Statistical analysis

The Statgraphics software (version 5.1) was used to relate each output variable (FAME content, yield, viscosity, IP and COM) to the experimental factors (temperature, methanol to oil ratio and reaction time) by means of a quadratic model as follows, Eq. 22:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j}^{3} b_{ij} X_i X_j \qquad (22)$$

where the coefficients b_i and b_{ii} are related to the linear and quadratic effects, respectively, of each input factor on the output variable and the cross-product coefficients b_{ij} represent the interactions between two input variables.

Secondly, the analysis of variance (ANOVA) was carried out. The significance of all terms in the models was judged statistically by computing the p-value at a confidence level of 95%. The regression coefficients were then used to generate contour maps and to find the processing conditions which lead to an optimum (maximum or minimum) value for a given

response variable. The optimum solution should be obtained by a combination of the experimental factors inside their ranges of application.

3 RESULTS AND DISCUSSION

3.1 Characterization of biodiesel samples

Table 27 shows the experimental values of FAME content, yield, kinematic viscosity, IP and COM for the biodiesels produced.

Table 27. Experimental design and measured values for the response variables

Exp.	Temp. (ºC)	Methanol:Oil Ratio	Time (min)	FAME (wt.%)	Yield (%)	Viscosity (mm^2/s)	IP (h)	COM (ºC)
1	40	3	30	93.12	74.49	6.66	1.29	3.70
2	40	3	60	93.26	76.14	6.45	1.43	3.71
3	40	3	90	93.72	72.16	6.41	1.29	3.72
4	40	6	30	93.98	78.94	6.48	1.21	3.70
5	40	6	60	93.65	80.16	6.45	1.30	3.75
6	40	6	90	93.76	80.02	6.57	1.27	3.83
7	40	9	30	93.58	83.06	6.51	1.10	3.76
8	40	9	60	93.35	77.71	6.35	1.31	3.79
9	40	9	90	93.53	82.40	6.56	1.36	3.82
10	50	3	30	93.08	71.67	6.61	1.80	3.70
11	50	3	60	93.27	72.47	6.38	1.05	3.73
12	50	3	90	93.43	76.19	6.46	1.25	3.55
13	50	6	30	93.87	76.50	6.33	1.40	3.69
14	50	6	60	93.91	73.25	6.40	1.21	3.75
15	50	6	90	94.19	80.77	6.42	1.38	3.48
16	50	9	30	93.60	74.51	6.37	1.53	3.73
17	50	9	60	93.59	78.24	6.43	1.44	3.82
18	50	9	90	95.14	81.01	6.43	1.27	3.58
19	60	3	30	94.19	79.31	6.37	1.78	3.72
20	60	3	60	93.95	76.43	6.39	1.86	3.74
21	60	3	90	94.65	72.31	6.40	1.21	3.44
22	60	6	30	94.05	77.83	6.45	1.83	3.54
23	60	6	60	94.10	71.19	6.05	1.16	3.74
24	60	6	90	93.56	72.97	6.51	1.20	3.37
25	60	9	30	93.15	79.92	6.40	2.22	3.31
26	60	9	60	94.78	79.95	6.49	1.50	3.69
27	60	9	90	95.39	75.22	6.62	1.23	3.77

3.1.1 FAME content and yield

The biodiesel purity was assayed by determining its FAME content. A good conversion of triglycerides to FAME was obtained for all the samples since their FAME content were higher than 93 wt% (Table 27). Although the influence of the processing conditions was not clear, it was seen that when maintaining constant the methanol to oil ratio at 9:1 and the reaction time at 90 min, the FAME content increased with temperature (from 93.53 wt% at 40 ºC to 95.39 wt% at 60 ºC). Besides, the FAME content improved at larger reaction time when employing a methanol to oil ratio of 9:1 at 50 ºC and 60 ºC but not at 40 ºC. Hence, the maximum conversion, 95.39 wt%, was obtained at 60 ºC with a 9:1 methanol to oil ratio and a reaction time of 90 min. Nevertheless, it should be mentioned that this value is slightly lower than the minimum required by the European standard (EN 14214) for use as 100 % diesel fuel (96.5 wt%).

The fatty acids of the biodiesel samples were composed primarily of 28.7 wt% oleic acid (C18:1n-9), 11.8 wt% palmitic acid (C16:0), 10.2 wt% linoleic acid (C18:2n-6), 7.6 wt% DHA (C22:6n-3), 5.7 wt% EPA (C20:5n-3) and 5.0 wt% palmitoleic acid (C16:1n-7). The content of (SFA) and polyunsaturated fatty acids (PUFA) amounted to 17.7 wt% and 33.2 wt%, respectively. This was in agreement with the SFA content of waste cooking oil biodiesel (19.8 wt%) and with the PUFA content of marine oil biodiesel (37.3 wt%) (Lin & Li, 2009).

The biodiesel yields obtained ranged from 71.19 to 83.06 % (Table 27). It was observed that the highest percentage yields were found at low temperature (40 ºC) and high methanol to oil ratio (9:1). Although the reaction time had a positive effect on yield at 50 ºC, since at constant methanol to oil ratio the yield increases with longer contact time, this trend was not seen at 40 ºC or at 60 ºC. These yield values are in line with those reported for biodiesel produced with oil extracted from fish canning industry wastes, 73.9 % using 1 wt% of acid catalyst (Costa et al., 2013) and with menhaden oil, 86.2 % employing 2 wt% of alkaline catalyst (Hong et al., 2013). However, they were considerably lower than those found for biodiesel obtained with salmon oil, 99 % (El-Mashad et al., 2008) and with heckel fish oil, 96 % (Fadhil & Ali, 2013). It may be due to the fact that the latter two studies employed a lower concentration of alkaline catalyst, 0.5 wt%. According to Fadhil and Ali (2013), an alkaline catalyst (NaOH or KOH) concentration beyond 0.5 wt% enhanced the formation of soaps by triglyceride saponification. Owing to their polarity,

soaps are dissolved into the glycerol phase increasing also the methyl ester solubility in the glycerol, which is a cause of yield loss (Vicente et al., 2004).

3.1.2 Viscosity

Viscosity is an important fuel property since it affects the injection and atomization of a fuel. The higher the viscosity, the greater the tendency of a fuel to cause operational problems such as engine deposits (Knothe, 2005). The kinematic viscosity of the biodiesel samples varied between 6.05 and 6.66 mm^2/s at 30 ºC (Table 27). Thus, the transesterification process was effective at reducing the viscosity of the crude fish oil, which was 45.34 mm^2/s at 30 ºC. According to Knothe (2005), biodiesel viscosity increases with chain length and degree of saturation of fatty acids. As a result, the biodiesels produced in this work (17.7 wt% SFA and 33.2 wt% PUFA) showed a lower viscosity than biodiesel from a different marine fish oil (37.06 wt% SFA and 37.3 wt% PUFA) which exhibited a kinematic viscosity of 7.2 mm^2/s at 40 ºC (Lin & Li, 2009). The biodiesels obtained were close to meet the European standard (3.5-5 mm^2/s at 40 ºC) since reduced viscosities would have been obtained at 40 ºC.

3.1.3 Oxidative stability

Oxidative stability is a key fuel characteristic determining the shelf life of biodiesel. Biodiesel oxidation leads to an increase in viscosity due to the formation of high-molecular-weight products from polymerization reactions. This may results in the production of insoluble substances which can clog fuel lines and pumps (Knothe et al., 2005). The fatty acid composition of biodiesel is the main factor influencing the oxidative stability. In this sense, fish oil biodiesel is highly susceptible to oxidation due to its high content of PUFA containing more allylic methylene positions (Knothe, 2007). In this study, the Rancimat IP of the biodiesels samples was determined as a measure of biodiesel resistance to oxidation. In Table 27, it was observed that the IP values varied between 1.05 to 2.22 h. These results are significantly higher than the IP (0.1 h) reported for biodiesel produced using oil from fish canning industry wastes (Costa et al., 2013), but considerably lower than those found for high oleic sunflower oil biodiesel (IP of 5.1h) and palm oil biodiesel (IP of 8.1 h) (Serrano et al., 2013). This fact is attributed to the superior PUFA content of fish oil when compared with vegetable oils. Therefore, the use of antioxidants (Lin & Lee, 2010) or the mixture of fish oil with more stable oils before transesterification

(Costa et al., 2013) are required in order to obtain oxidative stabilities which satisfy the European standard (minimum IP of 6 h).

3.1.4 Cold flow properties

One of the main disadvantages of biodiesel is its poor cold flow properties. As ambient temperature decreases, the long-chain saturated fatty acids start to form small crystals nuclei in biodiesel fuels which can become large enough to plug fuel lines (Knothe et al., 2005). Thermal analytical methods such as DSC have been previously used to determine melting characteristics of biodiesel (Chiou et al., 2008; Dunn et al., 1999; Garcia-Perez et al., 2010). In this work, similar DSC heating curves were obtained for the 27 biodiesel samples, with three distinct endothermic peaks (Fig. 23). A first small peak was found between -73.60 ºC and -60 ºC. It was not very sharp and may be associated to the presence of impurities such as phospholipids and pigments contained in the crude fish oil (Sathivel et al., 2008). A larger peak was observed in the range of -55 ºC to -30 ºC, which is attributed to the presence of methyl esters of unsaturated fatty acids. The last visible peak was obtained between -20 ºC to 3.83 ºC, which may mainly consist of methyl esters of SFA. Hence, the melting point of the biodiesel samples ranged from -73.60 ºC to 3.83 ºC. Similar endothermic peaks were found by Garcia-Pérez et al. (2010) for biodiesel produced from poultry fat, with a melting point between -69.5 ºC to 14.2 ºC. Subsequently, DSC curves were analyzed to determine the COM for all the biodiesel samples (Table 27). COM is defined as the temperature at which the melting phenomenon finishes and it ranged from 3.31 ºC to 3.83 ºC. This small variation is due to the fact that transesterification does not alter the fatty acid composition of the feedstocks and thus, it is the fatty acid profile of the raw oil which determines the melting properties of the biodiesel (Knothe et al., 2005). A similar value was found by Chiou et al. (2008) for salmon oil methyl esters, 3.1 ºC; but a lower one was reported by the same authors for corn oil methyl esters, 1.7 ºC, which is explained by its lower saturated fatty acids content.

3.1.5 Acidity

The acid value of biodiesel is considered as a quality control parameter since it determines its corrosiveness and long-term stability (Fan et al., 2010). In this study, all the biodiesel samples showed an acid value below 0.31 mg KOH/g oil after the transesterification step, which is in accordance with the European standard (<0.5 mg KOH/g oil).

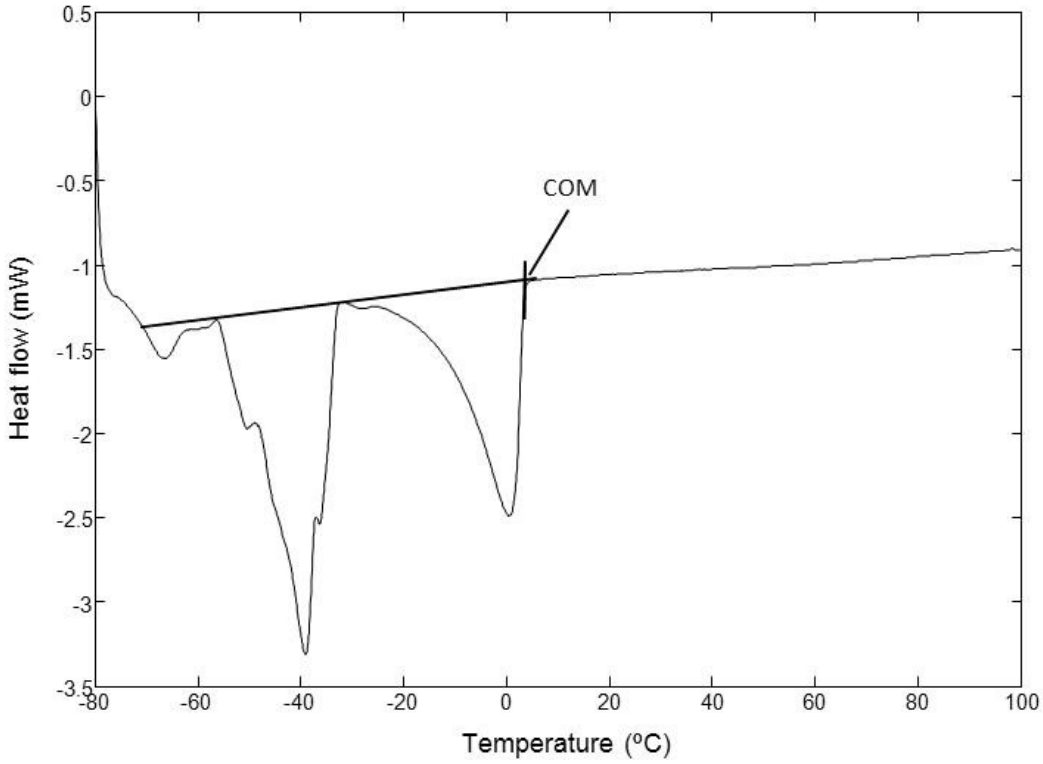

Figure 23. DSC heating curve of biodiesel (Exp. 15)

3.2 Statistical modeling and optimization

The experimental data for each output variable were fitted to a complete second order model. Table 28 shows the polynomial coefficients calculated by multiple regressions for each response variable and their associated p-value. It was observed that the linear effect of temperature was statistically significant for FAME content, IP and COM. The linear effect of reaction time was also significant for FAME content and IP. On the other hand, the linear effect of methanol to oil ratio was found to be statistically significant only for yield. Quadratic effects were significant only for reaction time in the case of viscosity and COM. The p-values for the remaining effects indicated that only the interaction between temperature and reaction time was statistically significant in the case of IP. Nevertheless, it should be mentioned that the proposed quadratic models seems to not explain the variability of the data to a large extent. The highest coefficient of determination was obtained for IP, R^2 = 0.7076, whereas values above 0.5 were found for the rest of the output variables (Table 28).

Table 28. Polynomial coefficients and p-values for the response variables

	FAME (wt.%)		Yield (%)		Viscosity (mm^2/s)		IP (h)		COM (ºC)	
	Coefficient	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*
Constant	9.61E+01	-	8.86E+01	-	8.65	-	9.86E-01	-	3.65	-
A: Temperature, ºC	-8.88E-02	0.0139	-8.10E-01	0.1371	-4.20E-02	0.0784	2.53E-03	0.0061	-8.33E-04	0.0072
B: Methanol:Oil ratio	1.39E-02	0.1249	2.28	0.0053	-1.72E-01	0.9442	-2.02E-01	1.0000	-3.01E-02	0.5938
C: Time, min	-4.70E-02	0.0415	2.97E-02	0.8066	-2.23E-02	0.6527	2.09E-02	0.0029	1.29E-02	0.5523
AA	8.78E-04	0.6760	1.17E-02	0.3565	2.33E-04	0.5692	4.69E-04	0.5172	2.22E-05	0.9621
AB	4.26E-04	0.9310	-3.70E-02	0.2185	1.24E-03	0.2067	9.44E-04	0.5989	-1.03E-03	0.3567
AC	5.19E-04	0.3007	-4.07E-03	0.1785	1.16E-04	0.2370	-6.94E-04	0.0010	-5.56E-05	0.6152
BB	-8.33E-03	0.7207	-4.80E-04	0.9975	5.96E-03	0.1985	1.23E-02	0.1555	3.58E-03	0.4933
BC	2.15E-03	0.2025	5.51E-03	0.5762	6.43E-04	0.0569	1.11E-04	0.8401	7.22E-04	0.0621
CC	1.41E-04	0.5478	1.12E-03	0.4226	1.08E-04	0.0268	6.79E-05	0.4250	-1.25E-04	0.0254
R^2	0.5221		0.5171		0.5294		0.7076		0.5599	

For the optimization of the biodiesel production, the yield was chosen as measured variable showing the efficiency of the process, whereas viscosity, IP and COM were selected as variables indicating the fuel quality of the biodiesel produced. The goodness of the fit for these variables was also proved by plotting the measured values against the predicted ones for yield (Fig. 24a), viscosity (Fig. 24b), IP (Fig. 24c) and COM (Fig. 24d). The data were correlated by means of a regression line whose equation was inserted in each figure. Although a low coincidence between the regression line and the diagonal was observed for the four variables, most of the experimental points were placed inside the region delimited by the dotted lines at both sides of the diagonal, which represent a deviation of ±10% between predicted and measured values. Subsequently, by means of the second-order models obtained above and using response surface methodology, contour maps were generated where the yield, viscosity, IP and COM were plotted against time of reaction and methanol to oil molar ratio, as shown in Fig 25a to 25d. Temperature was set at the optimum value obtained for each output variable: 40 ºC for yield and 60 ºC for viscosity, IP and COM.

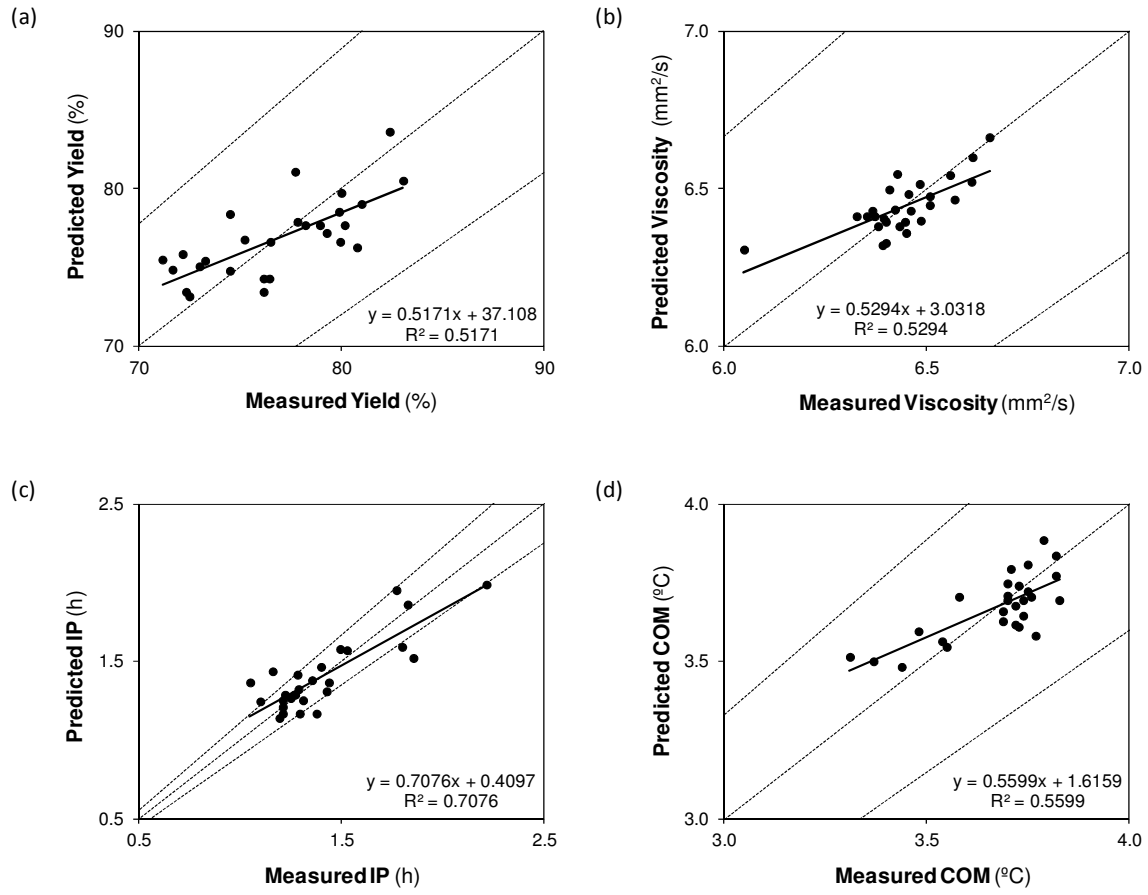

Figure 24. Correlation between predicted and measured values of (a) Yield, (b) Viscosity, (c) IP and (d) COM

These contours plot allowed to find optimum values for each objective variable, which were marked as circles in Fig. 25. In Fig. 25a, the maximum value for yield, 83.6 %, was located at the lowest temperature assayed, 40 ºC, and the highest methanol to oil ratio and reaction time, 9:1 and 90 min respectively. Due to the high amount of catalyst employed, 1 wt%, the saponification of triglycerides by NaOH before alcoholysis is reduced at low temperature, which results in a higher FAME yield (Ramadhas et al., 2005; Fadhil & Ali, 2013). Although the stoichiometric methanol to oil ratio is 3:1, increasing this ratio permits to drive the transesterification reaction at a faster rate. Similarly to our result, Ramadhas et al. (2005) also obtained a maximum of biodiesel yield produced from rubber seed oil at a methanol to oil molar ratio of 9:1. These authors also stated that increasing the mole ratio beyond 9:1 did not result in a significant increase of yield. Regarding reaction time, it was found to have a positive influence on the biodiesel yield since a higher reaction time allows longer for the completion of transesterification of triglycerides.

Optimizing the fuel properties of the biodiesels obtained implies to minimize viscosity, in order to improve the biodiesel flow, and COM, to reduce the crystallization temperature of the fuel; while maximizing IP, which implies a longer biodiesel shelf life.

The minimum value for kinematic viscosity, 6.30 mm^2/s, was obtained at the highest value of temperature assayed, 60 ºC, and at medium values of methanol to oil ratio and reaction time, 5.15:1 and 55.52 min respectively (Fig. 25b). For a same feedstock, biodiesel viscosity is mainly related to its purity (Costa et al., 2013). These authors attributed higher biodiesel viscosities to a lower FAME content. Thus, the higher temperature assayed, 60 ºC, which resulted in the maximum FAME content was also preferred to obtain reduced viscosities. The optimum for methanol to oil ratio and reaction time at medium values may be due to the fact that not relevant differences in FAME content (less than 1.3 wt%) were found when using higher values for these variables.

Fig. 25c shows the maximum value obtained for IP, 1.99 h, which was found at 60 ºC, methanol to oil ratio of 9:1 and 30 min for reaction time. Although increasing temperature favors the oxidation of FAME, it may also enhance the mixture of the produced FAME and excess of methanol which could reduce the oxidative deterioration of the FAME during the production process. Thus, the highest ratio of methanol to oil was also preferred. Considering reaction time, the lowest value of time assayed was found as optimum value since it implies a shorter contact time of FAME with oxygen at the processing temperature.

Finally, in Fig. 25d it is observed the minimum for COM, 3.48 ºC, which was obtained at 60 ºC, 3.74:1 methanol to oil ratio and 90 min. The COM value of biodiesel mainly depends on the SFA composition of the fuel (Knothe, 2005). In this regard, no significant differences were found among the SFA content of the 27 samples obtained (less than 0.42 wt%). Therefore, it cannot explain the location of the optimum at these processing conditions values.

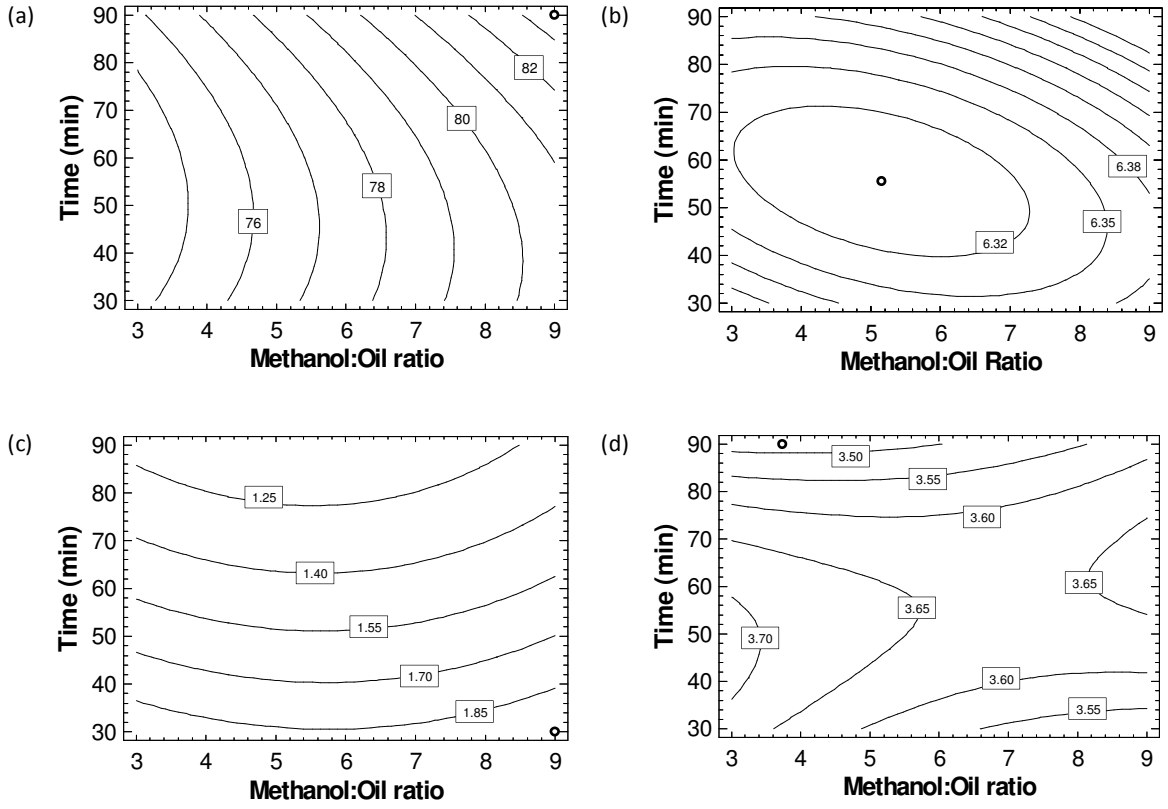

Figure 25. Contour plots for (a) Yield at 40 ºC, (b) Viscosity, (c) IP and (d) COM at 60 ºC

4. CONCLUSIONS

Oil obtained from fish waste, having a low content of omega-3 PUFA, was proven to be a promising raw material for the production of biodiesel. Due to the high FFA content of the crude fish oil, 6.6 mg KOH/g oil, an acid-catalyzed esterification was performed before the transesterification with methanol and 1 wt% sodium hydroxide as catalyst. Then, it was evaluated the influence of the transesterification processing conditions such as temperature, methanol to oil ratio and reaction time on yield and fuel properties of the

biodiesel produced. It was found that all the biodiesels samples presented a FAME content higher than 93 wt%, with a maximum of 95.39 wt% at 60 ºC, 9:1 of methanol to oil ratio and 90 min. By response surface methodology optimization, a maximum yield, 83.60 %, was obtained at 9:1 methanol to oil ratio, 90 min of reaction time and low temperature, 40 ºC, at which the saponification of triglycerides by NaOH before alcoholysis is reduced. Acceptable values of kinematic viscosity were found, with a minimum of 6.30 mm^2/s obtained at 60 ºC, 5.15:1 of methanol to oil ratio and 55.52 min. The oxidative stability of the biodiesels produced was low due to their high PUFA content, with a maximum IP of 2.22 h. Thus, it must be further improved through the use of antioxidants. Finally, satisfactory melting points, from -73.60 ºC to 3.83 ºC, with COM values ranging from 3.31 ºC to 3.83 ºC, were provided for the fish waste oil biodiesels.

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IX. Lipid Characterization and Properties of Protein Hydrolysates Obtained from Discarded Mediterranean Fish Species*

Discards are an important fraction of the by-products produced by the fishing industry. As consequence of their low commercial acceptance, it is required to provide an added value to these underutilized materials. In this study, it was carried out the characterization of the lipid fraction of three fish discarded species in the west Mediterranean Sea, sardine (*Sardine pilchardus*), mackerel (*Scomber colias*) and horse mackerel (*Trachurus trachurus*), as well as the evaluation of both angiotensin I-converting enzyme (ACE)-inhibitory and antioxidative activities of their protein hydrolysates. The processing of these biomaterials led to oils with a high content of omega-3 PUFA, ranging from 22.05 wt% for horse mackerel to 30.60 wt% for sardine. Regarding the protein fraction, most of the hydrolysates presented ACE inhibition values higher than 60 %, which corresponds to IC_{50} values varying from 345 μg protein/mL for mackerel to 400 μg protein/mL for sardine. Moreover, most of the hydrolysates exhibited an acceptable antioxidative activity, 35-45 % of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) inhibition. Therefore, this study suggests that the three discarded species evaluated are valuable raw material for the production of bioactive ingredients such as omega-3 PUFA and protein hydrolysates exhibiting antihypertensive and antioxidant activities.

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1. INTRODUCTION

Discards are an important fraction of the by-products produced by the fishing industry. They represent an underutilization of the marine resources and they also produce a significant ecological impact on the marine organism's food chain because most of discards are dumped at sea dead or dying (Kelleher, 2005). To this regard, the EU Commission has lately considered several regulations avoiding progressively discard practices in the EU fishing fleet with the aim of adopting a policy of zero-discard in EU fisheries as a part of a reformed Common Fisheries Policy in 2013 (EU, 2011).

Spanish fishing vessels in the west Mediterranean Sea (FAO fishing area 37.1.1.) employ trawling and purse seine as main fishing gears, which generate average discard rates of 23% and 5-10%, respectively (FROM, 2008). Discards in west Mediterranean Sea comprise non-target species such as mackerel, which appears as by-catch species in trawling fisheries. Other species, such as sardine and horse mackerel, are mostly discarded due to quota restriction or minimum landing sizes (11 cm for sardine and 12 cm for horse mackerel). Horse mackerel is the commercial species presenting the largest discard rate (5-10%) for the Spanish Mediterranean fisheries employing purse seine (FROM, 2008). In the case of sardine, although the average discard rate is estimated at only 0.1 – 0.2% of the total catches in Mediterranean Spanish fisheries, the total mass of the sardine discarded yearly is significant (Carbonell et al., 1997).

Given the low commercial acceptance of discards, it is necessary to provide an added value to these underutilized materials. In this sense, the development of new extraction technologies and research has permitted the identification and isolation of an increasing number of bioactive compounds from remaining fish muscle proteins, collagen and gelatine, fish oil, fish bone, internal organs and shellfish and crustacean shells (Kim & Mendis, 2006). These bioactive compounds have recently received much attention in the biotechnological, nutraceutical and pharmaceutical fields.

Considering the protein fraction, many scientific studies have demonstrated that some peptides present in fish muscle tissue exhibit a number of biological activities such as antihypertensive, antithrombotic, immunomodulatory and antioxidative activities, among others (Je et al., 2009; Kim & Wijesekara, 2010).

Antihypertensive activity of peptides lies in their capacity to inhibit the action of the angiotensin converting enzyme (ACE, EC 3.4.15.1), a dipeptidil carboxipeptidase able to convert the plasma peptide angiotensin I into angiotensin II, which possesses a strong vasoconstrictor activity. Furthermore, this enzyme is responsible for the degradation of bradykinin, an endogenous vasodilator nonapeptide (Li et al., 2004). Consequently, the administration of ACE inhibitor peptides can reduce blood pressure, which is one of the major risk factors for cardiovascular diseases. To this regard, hydrolysates from marine origin have been widely reported to exhibit ACE-inhibitory activity (Li et al., 2012), some of them belonging to target species in Mediterranean fisheries such as sardinelle (Bougatef et al., 2008) or cuttlefish (Balti et al., 2010).

Another biological activity of interest is the antioxidative capacity. Oxidative damage is caused by natural occurring radicals, such as superoxide anion ($O_2 \cdot^-$) or hydroxyl radical ($OH\cdot$), which are unavoidably generated by cell respiration. These radicals, known as reactive oxygen substances (ROS) can act as initiators or propagators of chain reactions, which result in the damage of lipid membranes, structural proteins, enzymes and DNA structure (Lee et al., 2004). Many studies have related the accumulative oxidative damage with the occurrence of several chronic diseases such as cancer, diabetes, inflammatory and neurodegenerative diseases (Butterfield et al., 2002). In the field of food processing, lipid oxidation reactions are responsible for the appearance of undesirable odors and flavors due to the formation of secondary oxidation products (Frankel, 2005). Antioxidant compounds can delay or interrupt chain oxidation reactions by scavenging free radicals or acting as chain terminators. The current regulations restraining the use of synthetic antioxidants as food additives, due to their potential hazardous effects, has paved the way to the research of new natural antioxidants. To this regard, antioxidant potency has been reported in protein hydrolysates from several marine species (Pires et al., 2012), some of them present in the Mediterranean Sea such as sardinelle (Bougatef et al., 2010).

Besides the protein content, fish oil is another important fraction which can be extracted from fish materials of high availability such as non-commercial species, fish by-products or wastes from fish processing. Its incorporation into feedstuffs for both animal and human consumption is justified by its unique content in polyunsaturated fatty acids belonging to the omega-3 family, such as eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA). Both have been reported to promote several benefits on human health, such as prevention of atherosclerosis, reduction of blood pressure and protection

against arrhythmias (Lees & Karel, 1990); improvement of the anti-inflammatory response (Uauy & Valenzuela, 2000)); and development of brain and eye retina in infants (Ward & Singh, 2005).

Therefore, the aim of this research work was: i) to study the characterization of fish oil, and ii) to evaluate the ACE-inhibitory and antioxidative activities of protein hydrolysates, both produced from three discarded fish species in the west Mediterranean Sea, sardine (*Sardina pilchardus*), mackerel (*Scomber colias*) and horse mackerel (*Trachurus trachurus*).

2. MATERIALS AND METHODS

2.1 Raw material

Sardine (*Sardina pilchadus*), mackerel (*Scomber colias*) and horse mackerel (*Trachurus trachurus*) were chosen for this study as species discarded by the Spanish fleet in the Mediterranean Sea. The raw material was provided by the fishing harbour of Motril (Spain) in May 2011. It was kept in ice during the transportation and pressed in the same day.

2.2 Proximate chemical composition

The proximate chemical composition of the species studied was determined as described in the Materials and Methods section of chapter II.

2.3 Separation of protein and oily fractions

The oil extraction was carried out by hydraulic pressing as described in the Materials and Methods section of chapter II. The cakes resulting from the pressing operation were frozen at -20ºC prior to their use as substrate for protein hydrolysis. It should be noted that the pressing stage also allowed a reduction in the moisture content and in the volume of the raw material. Thus, it allows a better preservation of these materials from microbial spoilage prior to their up-grading and also reduces the handling and isolation costs.

2.4 Fatty acid profile of the oils

The fatty acid profile of the oils was determined as described in the Materials and Methods section of chapter II.

2.5 Composition of the lipid fraction

The composition of the lipid fraction was determined as described in the Materials and Methods section of chapter IV.

2.6 Enzymes and hydrolysis procedure

The press cakes were processed in order to obtain hydrolysates displaying ACE inhibitory and antioxidative activities. For this purpose, the cakes were hydrolysed employing two serine endoprotease enzymes, one of bacterial origin (subtilisin, EC 3.4.21.62) and other from an animal source (trypsin, EC 3.4.21.4), both provided by Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S, respectively. All the experiments were conducted at pH 8 and 50ºC, while enzyme-substrate ratio was set at 3 wt% for both enzymes. Protein was considered as substrate.

For each experiment, the press cake was grinded in a cutter SK-3 (Sammic, Spain). A given mass of grinded press cake was then homogenised with demineralised water until reaching a final volume of 200 mL. This suspension was then transferred into a jacketed reactor of volume capacity 250 mL. Three reaction patterns were studied: (a) 2-hour hydrolysis with subtilisin followed by addition of trypsin until completing 4 h of reaction; (b) 2-hour reaction with trypsin followed by subtilisin and; (c) 4-hour hydrolysis with simultaneous addition of both enzymes. The degree of hydrolysis, defined as the percentage ratio of the number of peptide bonds cleaved to the total number of peptide bonds in the substrate studied, was calculated as a function of the base consumption throughout the reaction (Camacho et al., 2001), employing an automatic titrator 718 Stat Titrino (Metrohm, Switzerland). According to this method, the degree of hydrolysis (DH) can be related to the amount of base consumed to keep the pH constant during the reaction as follows (Eq. 23):

$$DH = B \cdot N_b / (\alpha \cdot m_P \cdot h_{TOT}) \times 100 \qquad (23)$$

where B (mL) is the amount of base consumed, N_b (eq/L) is the normality of the base, α is the average degree of dissociation of the α-NH_2 amino groups released during the hydrolysis, which is dependent on the temperature and the pH, m_P (g) is the mass of protein in the substrate and h_{TOT} (meq/g) is the number of equivalents of peptide bonds per gram of protein. At pH 8 and temperature of 50ºC, the 88.5% of the amino groups are dissociated, while h_{TOT} was assumed to be 8.6 milliequivalents of peptide bonds per gram of protein, as reported in literature (Adler-Nissen, 1986).

A set of 150 hydrolysates samples, originated from the three species and three enzymatic treatments studied and drawn at different times of reaction, were evaluated in this research work. For this purpose, the samples were heated at 100ºC for 15 min to deactivate the enzyme and were then centrifuged in order to remove the remained solids. They were kept under refrigerated conditions until performing the analysis.

2.7 Determination of the ACE inhibitory activity

The ACE inhibitory activity of the hydrolysates was determined *in vitro* by means of the assay described by Shalaby et al. (2006). This method is based in the hydrolysis of the tripeptide N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG, Sigma F7131) with the Angiotensin converting enzyme (ACE) from rabbit lung (Sigma A6778). The assay was carried out in 96-well microplate at 37ºC. Each well contained 10 µL of enzyme solution (0.25 U/mL), 10 µL of sample, and 150 µL of 0.88 mM of FAPGG in buffer Tris-HCl 50 mM, pH 7.5 and 0.3 M of NaCl. The wavelength was set at 340 nm and the absorbance was monitored during 30 minutes by means of a Multiskan FC microplate photometer (Thermo Scientific, Finland). Each sample was analysed in triplicate.

The absorbance decreases linearly with time as ACE hydrolyses the substrate FAPGG. The slope of this descent is commonly used as a measurement of the enzyme activity. Indeed, the numerical value of inhibitory activity of each hydrolysate can be calculated by Eq. 24:

$$ACE\ inhibition\ (\%) = \left(1 - \frac{\rho_i}{\rho_0}\right) \times 100 \qquad (24)$$

where ρ_i was the slope in the presence of inhibitor (hydrolysate) and ρ_o the slope obtained in the absence of inhibitor (pure water). These slopes were calculated from the values obtained within the interval of 10 to 25 minutes, where a better linearity was observed.

The ACE inhibitory activity of a given hydrolysate is widely reported in literature by the IC_{50} value. This value is defined as the concentration of hydrolysate (μg protein/mL) needed to inhibit ACE activity by half. In this work, the IC_{50} values were calculated for the samples presenting the highest percentage of ACE inhibition. Each IC_{50} value was reported to the content of soluble protein in the hydrolysates, which was determined using the Micro-Lowry assay (Sigma Aldrich, USA).

2.8 Determination of antioxidative activity

The antioxidative activity of the hydrolysates was estimated by determination of their DPPH radical scavenging activity according to Brand-Williams et al. (1995). An aliquot of each sample (50 μL) was mixed with 100 μL of Tris buffer solution (50 mM, pH 7.4) and with 850 μL of a daily-prepared solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) at 0.1 mM concentration in methanol. The mixture was then kept at room temperature in the dark for 30 minutes, and the reduction of DPPH radical was measured at 515 nm. A blank was run in the same way by using distilled water instead of sample, and sample control was also made for each sample by adding methanol instead of DPPH solution. Then, DPPH radical scavenging activity was calculated according to Eq. 25:

$$DPPH\ inhibition\ (\%) = \left(1 - \frac{(A_{sample} - A_{sample\_control})}{A_{blank}}\right) \times 100 \qquad (25)$$

Then, the antioxidative capacity of the hydrolysates presenting the highest activity were expressed as Trolox Equivalent Antioxidant Capacity value (TEAC), which is determined by interpolating the DPPH inhibition on a calibrating curve using different Trolox (Sigma-Aldrich, USA) concentrations (Samaniego-Sanchez et al., 2007).

2.9 Statistical analysis

The Statgraphics software (version 5.1) was used to generate the statistical analysis. Differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Proximate composition of fish species

Table 29 shows the proximate composition of the fish discarded species studied. The three species presented similar protein content, ranging from 16.9 wt% for horse mackerel to 18.4 wt% for mackerel. On the other hand, the lipid content varied much more from one species to another. The horse mackerel exhibited the highest lipid content (7.8 wt%) and mackerel the lowest (2.5 wt%). Regarding moisture content, it was found to correlate inversely with the lipid content. The ash content ranged from 2.8 wt% for mackerel to 4.0 wt% for horse mackerel. Nevertheless, it should be taken into account that, although proximate composition of fish is specific for each species, it varies according to the stage of maturity, the sex, the spawning cycle, the environment, the season, and the nutritional condition of the animal (Børresen, 1992).

Table 29. Proximate composition of sardine, mackerel and horse mackerel

Composition (wt%)	**Sardine**	**Mackerel**	**Horse mackerel**
Moisture	71.8	74.0	70.4
Protein	17.6	18.4	16.9
Lipid	4.4	2.5	7.8
Ash	3.8	2.8	4.0

3.2 Lipid fraction

Table 30 shows the fatty acid profile of sardine, mackerel and horse mackerel oils. The major fatty acids identified for the three oils were: (i) saturated fatty acids (SFA), palmitic (C16:0), myristic (C14:0) and stearic (C18:0); (ii) monounsaturated fatty acids (MUFA), oleic (C18:1n-9), palmitoleic (C16:1n-7), erucic (C22:1n-9), vaccenic (C18:1n-7) and gadoleic (C20:1n-9); and (iii) polyunsaturated fatty acids (PUFA), docosahexaenoic (C22:6n-3), eicosapentaenoic (C20:5n-3), stearidonic (C18:4n-3), docosapentaenoic (C22:5n-3), linoleic (C18:2n-6) and linolenic (C18:3n-3).

The SFA composition of the oils ranged from 25.90 wt% for mackerel to 29.63 wt% for sardine, being the palmitic acid the primary SFA for the three species and contributing to

16.47-19.78 wt% of the total content of lipids. The MUFA content was significantly different ($p<0.05$) for the three species and it varied from 23.17 wt% for sardine to 36.53 wt% for horse mackerel. As a result, the MUFA composition of the oils was found to be higher than the SFA for mackerel and horse mackerel oils but not for sardine. Oleic acid was the most represented of the MUFA for the three species, accounting for 29.0-40.0 wt% of the total MUFA. Similarly, significant differences in the PUFA composition were found among the three species, ranging from 33.06 wt% for horse mackerel to 43.97 wt% for sardine. The PUFA were the predominant in the oils except for horse mackerel where MUFA were the major lipids. These results are in line with previous studies on the fatty acids profile of the same species (Celik, 2008; Orban et al., 2011).

The ratio of n-3/n-6 ranged from 13.55 for mackerel to 14.08 for sardine. This fact strongly supports that the three discarded fish species studied are important sources of omega-3 PUFA. Especially relevant is the content of EPA and DHA, which together account for 30.60 wt% of the total fatty acids for sardine oil, followed by mackerel oil (27.92 wt%) and horse mackerel oil (22.05 wt%). Among the three fish oils, sardine presented the highest content of both EPA (12.96 wt%) and DHA (17.63 wt%).

The lipid fraction of the oils was mostly composed by triacylglycerols (TAG), ranging from 97.3 wt% for mackerel to 99.8 wt% for sardine. On the other hand, the free fatty acids content (FFA) was not significant for the three species when compared to the triacylglycerols content, varying from 0.2 wt% for sardine to 2.7 wt% for mackerel. However, although these oils could also present cholesterol and phospholipids (García-Moreno et al., 2013), they were not detected by the TLC analysis carried out.

As a consequence of these results, the oils obtained from the three discarded fish species exhibited a high quality due to the fact that they presented most of the omega-3 PUFA as TAG form which are more resistant to oxidation (Wijesundera et al., 2008) and which are also more bioavailable for the human digestive system (Lawson & Hughes, 1998).

Table 30. Fatty acid profile of sardine, mackerel and horse mackerel oils

Fatty acids (wt%)	**Sardine**	**Mackerel**	**Horse mackerel**
14:0	5.88[a]	4.37[b]	6.79[c]
16:0	19.78[a]	17.53[b]	16.47[c]
18:0	3.97[a]	4.06[b]	3.15[a]
Σ SFA	29.63[a]	25.90[b]	26.41[b]
16:1n-7	6.42[a]	5.07[b]	5.36[c]
18:1n-9	8.69[a]	13.28[b]	10.64[c]
18:1n-7	2.90[a]	2.94[b]	1.46[b]
20:1n-9	2.71[a]	4.82[b]	7.25[c]
22:1n-9	2.45[a]	7.07[b]	11.81[c]
Σ MUFA	23.17[a]	33.10[b]	36.53[c]
16:2n-4	1.29[a]	1.14[b]	1.22[ab]
16:3n-4	0.59[a]	0.65[b]	50.8[a]
16:4n-1	0.51[a]	0.09[b]	0.67[a]
18:2n-6	1.72[a]	1.66[a]	1.27[b]
18:3n-3	1.82[a]	1.21[b]	1.16[c]
18:4n-3	3.42[a]	2.62[b]	2.76[c]
20:4n-6	1.04[a]	0.90[b]	0.80[c]
20:4n-3	1.04[a]	0.97[a]	0.86[b]
20:5n-3	12.96[a]	10.34[b]	10.78[c]
22:5n-3	1.94[a]	1.89[a]	1.69[b]
22:6n-3	17.63[a]	16.97[b]	11.27[c]
Σ PUFA	43.97[a]	39.02[b]	33.06[c]
Others	32.3[a]	1.91[a]	4.00[a]
Σ n-6	2.76[a]	2.55[ab]	2.07[b]
Σ n-3	38.82[a]	34.58[b]	28.52[c]
n-3/n-6	14.08[a]	13.55[a]	13.79[a]
EPA + DHA	30.60[a]	27.92[b]	22.05[c]

Data are means of triplicate determinations. SD< 2 %. Mean values within a row followed by different letter mean significant differences ($p<0.05$).

3.3 Protein fraction

3.3.1 Hydrolysis curves

In figure 26, the hydrolysis curves are shown for the three fish species studied. All the curves presented a high rate of hydrolysis after the addition of the corresponding enzyme. This rate decreased throughout the reaction until reaching a steady-state phase when no apparent hydrolysis took place.

The DH reached after 4 hours of reaction was different depending on the species and treatment used. In general, the lowest values of DH were obtained for mackerel, while horse mackerel protein presented the best degradability to the enzymes used. Regarding the enzymatic treatments, the sequential enzymatic treatment subtilisin plus trypsin led to the highest degree of hydrolysis (DH) for sardine (18%), as shown in Figure 26a. In the case of mackerel, Figure 26b, both the two-step reaction subtilisin plus trypsin and the simultaneous addition of both enzymes led to the maximum DH (14%), while the maximal extent of the hydrolysis (21%) was obtained for horse mackerel after the simultaneous addition of subtilisin plus trypsin (Fig. 26c). In the three cases, the increase of DH after the addition of trypsin was less marked than that obtained when subtilisin was added in the second step. This fact was attributed to the specificity of trypsin, which only cleaves peptidic bonds involving Arginine and Lysine, while subtilisin is an endoprotease of broad spectrum (Adler-Nissen, 1986).

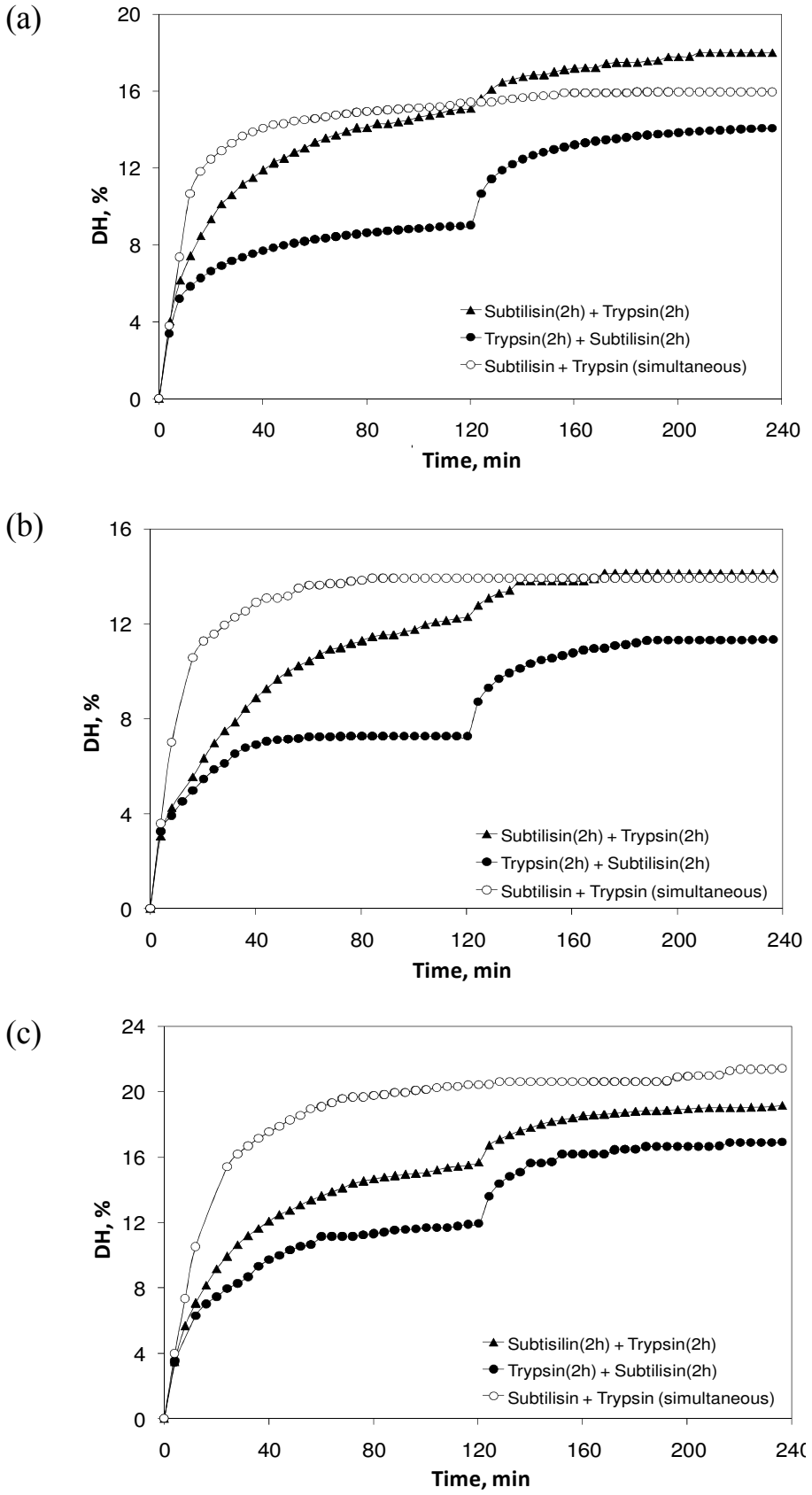

Figure 26. Hydrolysis curves for (a) sardine, (b) mackerel and (c) horse mackerel protein

3.3.2 Antihypertensive activity

For all the combinations between species and enzymatic treatments the ACE-inhibitory activity increased sharply within the first 30 – 60 min of hydrolysis, attaining a maximum value of ACE inhibition at DH between 5 – 15%. This period corresponds to the higher reaction rates observed in the hydrolysis curves. Above this point, ACE inhibition values remained constant or presented a slight decreasing trend. As an example, Fig. 27a shows the ACE inhibition of sardine hydrolysates versus the time of hydrolysis for each enzymatic treatment, and Fig. 27b depicts the ACE-inhibitory activity of the hydrolysates produced with simultaneous addition of subtilisin plus trypsin for the three species studied.

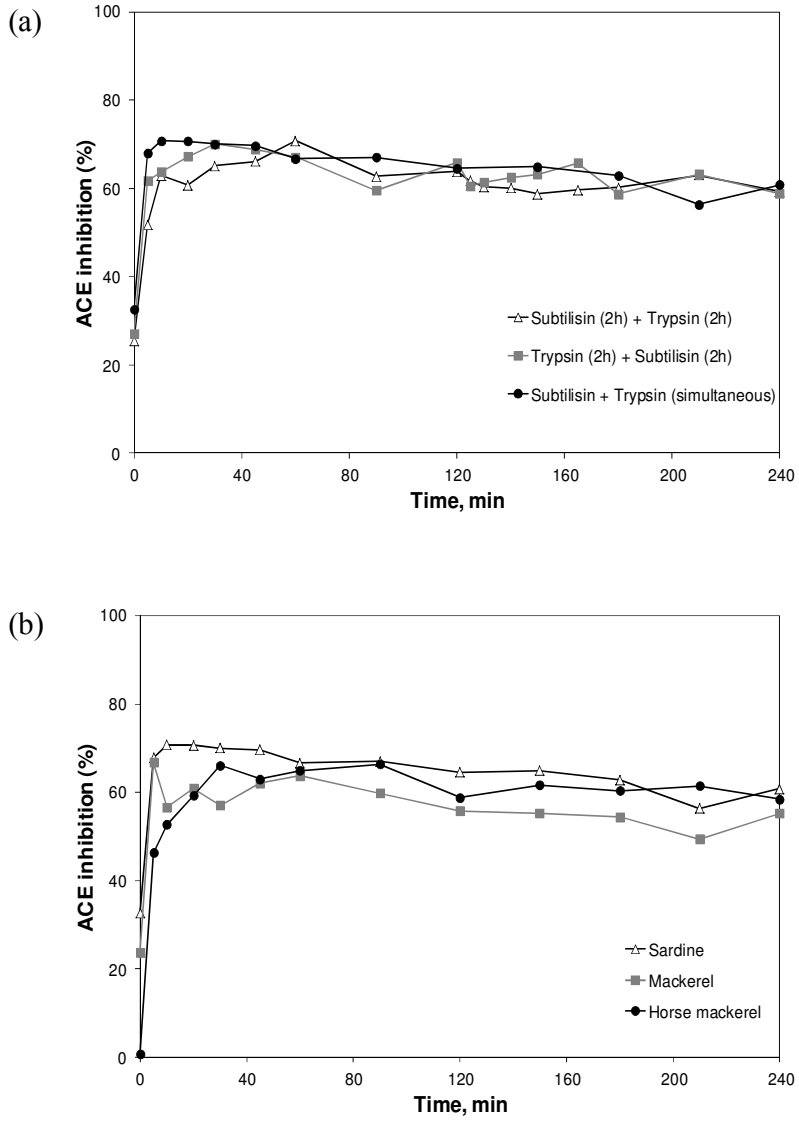

Figure 27. (a) ACE inhibition of sardine hydrolysates at different times of hydrolysis, (b) ACE inhibition of the hydrolysates obtained with simultaneous addition of subtilisin plus trypsin

A similar relationship between DH and ACE inhibitory activity was reported in previous works (Cinq-Mars & Li-Chan, 2007; Bougatef et al., 2008). This phenomenon may be explained due to the fact that the hydrolysate was constituted by a mixture of proteins and peptides where antihypertensive peptides would be continuously destroyed by proteolytic action while new active sequences might be released to the medium. The addition of the second enzyme slightly increased the ACE-inhibitory activity (Fig. 27a), but this effect was not significant. Therefore, it can be concluded that there is a positive correlation between the degree of hydrolysis and the ACE-inhibitory activity of the hydrolysates at the beginning of the reaction; but, after reaching a maximum of ACE inhibition, it would remain constant or slightly decrease.

Although most of the active peptides identified to date are di- and tripeptides, active peptides larger than 20 amino acid residues have been reported (Ryan & Ross, 2011). The ACE inhibition potency of a given peptide is thought to be linked to its structure and amino acid sequence, specially the tripeptide sequence at the C-terminal end. Indeed, it has been reported that the presence of hydrophobic amino acid residues in this region seems to favor the ACE inhibitory activity of the peptides, while hydrophilic peptides display weak or no affinity for the ACE active sites (Li et al., 2004; Hong et al., 2008).

Considering sardine hydrolysates, the enzymatic treatments assayed which produced the highest ACE-inhibitory activity were the simultaneous addition of subtilisin plus trypsin and the sequential enzymatic reaction with subtilisin (2h) followed by trypsin (2h), Fig 27a. ACE inhibition values up to 70% were obtained when reaching DH values of 13% (Table 31). An estimation of the average peptide chain length (PCL) of the hydrolysates can be made from the percentage degree of hydrolysis (DH), according to Eq. 26 (Adler-Nissen, 1986):

$$PCL = 100 / DH \qquad (26)$$

This means that the hydrolysate at DH 13% presented a peptide length distribution around an average value of 8 amino acid residues. Some of these peptides are responsible for the ACE inhibitory activity (70%). The IC_{50} values for these hydrolysates were 430 and 400 μg protein/mL, respectively (Table 31), which was of the same order of magnitude than that reported by Matsui et al. (1993) for the crude hydrolysates (i.e. without further fractionation or purification) of sardine muscle, IC_{50} = 260 μg protein/mL. These authors employed an alkaline protease from *Bacillus licheniformis*, at pH 9, 50ºC and 17 hours of

hydrolysis. This value is slightly better than that obtained in our work at the expense of larger times of hydrolysis (17 h).

ACE inhibition values up to 65% were obtained for mackerel hydrolysates when employing the sequential treatment subtilisin plus trypsin and the simultaneous addition of subtilisin and trypsin (Table 31). Degree of hydrolysis around 14%, corresponding to PCL of 7 amino acids, maximized the ACE-inhibition activity. IC_{50} values of 345 and 360 μg protein/mL were obtained for these hydrolysates (Table 31).

Regarding horse mackerel hydrolysates, Table 31, it was noticed that there is no difference on the ACE-inhibitory activity when employing the sequential reaction subtilisin plus trypsin or the simultaneous treatment with subtilisin and trypsin. ACE inhibition values up to 66.5% were obtained for those enzymatic patterns when DH values of 16% were reached, corresponding to PCL of 6 amino acids. IC_{50} values of 395 and 364 μg protein/mL were obtained for these hydrolysates, respectively.

Table 31. ACE inhibitory activity for sardine, mackerel and horse mackerel hydrolysates

Species	Maximum ACE inhibition (%)	Enzymatic treatment	Reaction time (min)	DH (%)	PCL	IC_{50} (μg/mL)
Sardine	70.0	Subtilisin + Trypsin (simultaneous)	30	13.0	≈ 8	430
		Subtilisin + Trypsin	60			400
Mackerel	65.0	Subtilisin + Trypsin (simultaneous)	60	14.0	≈ 7	345
		Subtilisin + Trypsin	150			360
Horse mackerel	66.5	Subtilisin + Trypsin (simultaneous)	30	16.0	≈ 6	364
		Subtilisin + Trypsin	150			395

ACE Inhibition data are means of triplicate determinations.

To the authors' knowledge, there is no previous reference on the IC_{50} of crude hydrolysates from mackerel and horse mackerel. It can be concluded that the enzymatic treatment performed in this work favored the release of ACE inhibitory peptides. Nevertheless, further fractionation and purification of the hydrolysates is required, in order to obtain peptidic fractions with enhanced antihypertensive potency or even identify the peptide or peptides responsible for the ACE inhibition.

3.3.3 Antioxidative activity

The antioxidative activity of fish protein hydrolysates could be related to the degree of hydrolysis and the enzyme specificity. Besides, the amino acid composition and sequence of the different peptides released during the hydrolysis reaction play a significant role in the antioxidant capacity of the final hydrolysate (Chabeaud et al., 2009). In this sense, it is believed that the presence of cationic and hydrophobic peptides enhance the capacity of the hydrolysate to inhibit oxidation (Rajapakse et al., 2005).

In this work, it was generally observed that, for the different enzymatic patterns used to hydrolyse the three cakes obtained, the DPPH inhibition increased with the degree of hydrolysis, reaching the following maxima values after 90 minutes of reaction: 40 % for sardine, 35 % for mackerel and 45 % for horse mackerel. These values of DPPH inhibition remained practically constant until completion of the reaction. For instance, Fig. 28a depicts the DPPH inhibition of horse mackerel hydrolysates versus the time of hydrolysis for each enzymatic treatment, and Fig. 28b shows the DPPH scavenging activity of the hydrolysates produced with simultaneous addition of subtilisin plus trypsin for the three species evaluated. Those results are in agreement with the study of Wu et al. (2003) who reported that the DPPH scavenging capacity of fish hydrolysates improves with the degree of hydrolysis and then, tended to a plateau.

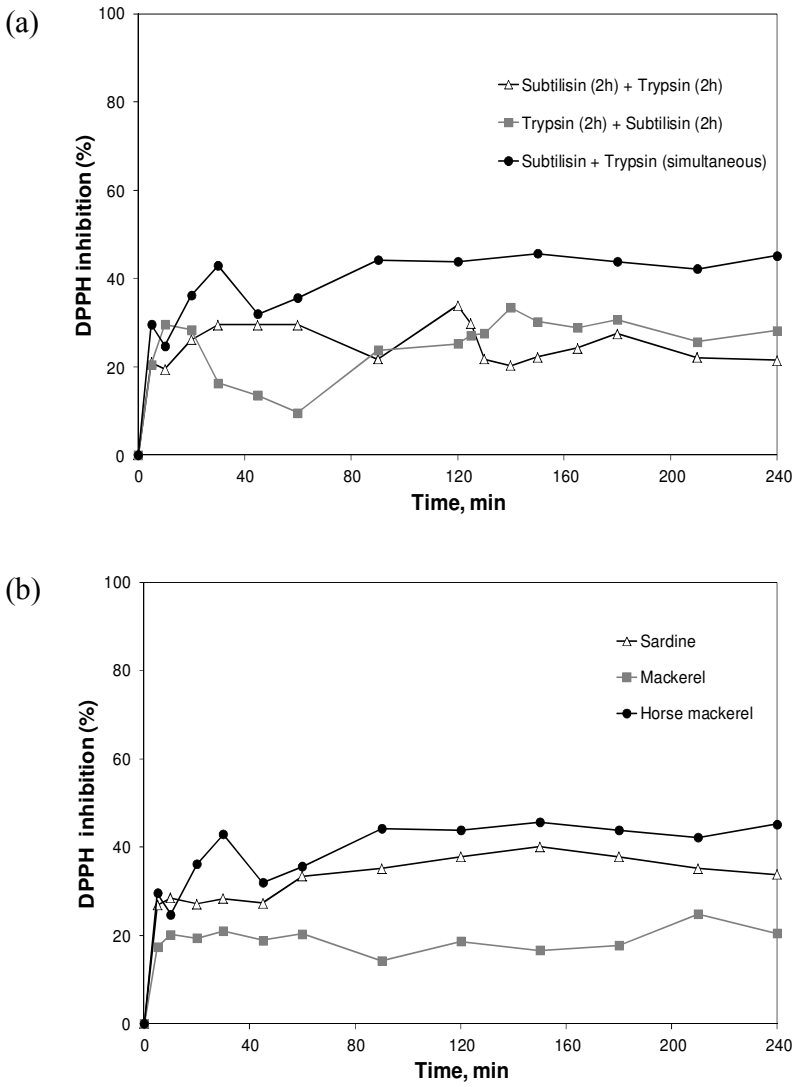

Figure 28. (a) DPPH inhibition of horse mackerel hydrolysates at different times of hydrolysis, (b) DPPH inhibition of the hydrolysates obtained with simultaneous addition of subtilisin plus trypsin.

Regarding sardine hydrolysates (Table 32), it was observed that the two-step reaction subtilisin plus trypsin at 90 minutes of reaction, and the simultaneous addition of both enzymes at 150 minutes, produced the hydrolysates presenting the highest antioxidative activity, being 40% inhibition of DPPH. It corresponded to a DH of 15 % and a PCL of 7 amino acids with TEAC values of 0.033 and 0.030 μmol Trolox/mg protein, respectively. On the contrary, hydrolysates presenting a lower DH and, as consequence, a longer peptide chain length exhibited lower DPPH inhibition capacity (Fig. 28). These results are in accordance with the study of Bougatef et al. (2010) where sardinelle hydrolysates presenting a DPPH radical-scavenging activity ranging from 15-55% were obtained. In addition, these authors revealed that peptides presenting shorter chain length exhibited higher DPPH inhibition, which is in line with the results obtained in this research work.

Considering the mackerel hydrolysates, the three enzymatic patterns assayed produced considerable differences in the DPPH radical-scavenging activity. It is shown in Table 32 that the sequential addition of subtilisin plus trypsin was the most appropriate treatment, obtaining hydrolysates with a PCL of 7 amino acids, which presented the highest DPPH inhibition (35 %). It corresponded to a TEAC value of 0.025 μmol Trolox/mg protein. In this line, Wu et al. (2003) produced mackerel hydrolysates employing Protease N with a DPPH radical-scavenging activity up to 80 % after 10 hours of hydrolysis. This fact suggests that, for mackerel, longer hydrolysis and different enzymatic treatments may result in hydrolysates presenting a higher antioxidative activity.

Table 32. Antioxidative activity for sardine, mackerel and horse mackerel hydrolysates

Species	Maximum DPPH inhibition (%)	Enzymatic treatment	Reaction time (min)	DH (%)	PCL
Sardine	40	Subtilisin + Trypsin (simultaneous)	150	15.0	≈ 7
		Subtilisin + Trypsin	90		
Mackerel	35	Subtilisin + Trypsin	150	14.0	≈ 7
Horse mackerel	45	Subtilisin + Trypsin (simultaneous)	150	20.0	≈ 5

DPPH Inhibition data are means of duplicate determinations.

Regarding horse mackerel hydrolysates, it was observed that the different enzymatic treatments produced significant differences in the DPPH inhibition (Fig. 3a). It was revealed that the most suitable combination of enzymes was subtilisin and trypsin simultaneously, producing hydrolysates with a PCL of 5 amino acids, which exhibited a DPPH radical-scavenging activity up to 45 %. This corresponds to a TEAC value of 0.030 μmol Trolox/mg protein.

The results commented above stated that the produced hydrolysates of sardine, mackerel and horse mackerel were significant DPPH radical scavengers. Nevertheless, further fractionation and characterization of the hydrolysates is required in order to obtain peptidic fractions with enhanced antioxidative activity.

4. CONCLUSIONS

The three discarded species considered, sardine, mackerel and horse mackerel, were found to have a potential as raw material for the production of bioactive compounds. The oils recovered from their lipid fraction exhibited a high content of omega-3 PUFA, presenting the oil of sardine the highest content in EPA (12.96 wt%) and DHA (17.63 wt%). Moreover, the three oils were mostly composed by triacylglicerols (97.3 – 99.8 wt%) which means higher quality in terms of oxidation stability and bio-availability. Regarding the protein fraction, most of the hydrolysates produced, exhibited ACE inhibition values higher than 60 %, mackerel presented the lowest IC_{50} value (345 μg protein/mL) and sardine the highest one (400 μg protein/mL). In addition, most of the hydrolysates presented an acceptable antioxidative activity. The highest DPPH inhibition was obtained for horse mackerel (45 %), followed by sardine (40%), whereas mackerel gave the lowest antioxidative activity (DPPH inhibition of 35 %).

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X. Antioxidant Activity of Protein Hydrolysates Obtained from Discarded Mediterranean Fish Species*

The production of bioactive fish protein hydrolysates is a convenient technical solution to successfully upgrade fish discards. In this study, five discarded species in the Mediterranean Sea, namely sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), axillary seabream (*Pagellus acarne*), bogue (*Boops boops*) and small-spotted catshark (*Scyliorhinus canicula*), were evaluated as raw material for obtaining fish protein hydrolysates exhibiting antioxidant activity. The DH of the hydrolysates ranged from 13.2 to 21.0 %, with a protein content varying from 60.7 to 89.5 %. The peptide profile of all hydrolysates was very similar with most of the peptides below 150 Da. Their lipid content was found to be between 4.6 and 25.3 %. The highest DPPH scavenging activity was obtained for the hydrolysates of sardine and horse mackerel with IC_{50} values varying from 0.91 to 1.78 mg protein/mL. Sardine and small-spotted catshark hydrolysates exhibited the highest ferrous chelating activity with an IC_{50} value of 0.32 mg protein/mL. Finally, sardine and bogue hydrolysates presented the highest reducing power. The antioxidant activity exhibited by the hydrolysates suggests that it is feasible to obtain added-value products such as natural antioxidants from these discarded species.

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1. INTRODUCTION

Discards are that portion of total catch which is not retained for sale and returned to the sea. It comprises non-target species with low commercial value, individuals below minimum commercial size, fish caught in excess of individual quota and damaged fish which is not worthy for fishermen to keep on board (Kelleher, 2005). In the Alboran Sea, portion of the West Mediterranean Sea lying between the Spanish southern coast and the north of Morocco, discards are mainly composed by commercial species such as sardine (*Sardina pilchardus)*, horse mackerel (*Trachurus mediterraneus*) and axillary seabream (*Pagellus acarne*) which are dumped at the sea due to high-grading practices, quota restriction and minimal commercial-size requirements. Other species such as bogue (*Boops boops*) and small-spotted catshark (*Scyliorhinus canicula*) are discarded due to their reduced commercial value (García-Moreno et al., 2013).

These practices represent an important underutilization of the marine resources. Besides, since discards are generally died or dying when returned to the sea, they also cause significant environmental problems such as alterations on marine trophic chains (Bozzano & Sardà, 2002). As a consequence and in order to ensure the sustainability of EU fisheries, the EU Commission is in the way to put into practice a reformed Common Fisheries Policy which aims to gradually implement a practice of zero-discards (EU, 2011). Nevertheless, technical measures should be also applied in order to successfully meet discards bans. In this sense, and as discards can be reduced (i.e. by improving the selectivity of the fishing gears) but cannot be completely eliminated, it seems to be of special importance the development of up-grading processes which allow to obtain added-value products from this underutilized raw material.

In this context, discarded species in the Alboran Sea are good sources of protein, with protein contents ranging from 17 to 23 % depending on the species (García-Moreno et al., 2013). Thus, enzymatic hydrolysis of their protein fraction is a convenient method for the production of bioactive compounds presenting a high demand in the nutraceutical and pharmaceutical fields. In this regard, several fish protein hydrolysates have shown numerous bioactivities such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, antimicrobial, among others (Je et al., 2009; Kim & Wijesekara, 2010).

Due to the increasing interest in finding antioxidants from natural sources which may have less potential hazard than synthetic ones, research on fish protein hydrolysates exerting antioxidant activity has gained a great interest. Antioxidants are generally employed to prevent lipid oxidation in foods which avoids the formation of toxic compounds and undesirable odors and flavors (Lin & Liang, 2002). Furthermore, antioxidants deficiency has also been involved in the occurrence of several diseases such as hypertension, cancer, diabetes, Alzheimer's and aging (Hajieva & Behl, 2006).

In the last decade, several authors have reported a strong antioxidant activity for fish protein hydrolysates obtained from different species such as black scabbardfish (*Aphanopus carbo)* (Batista et al., 2010), sardinelle (*Sardinella aurita*) (Bougatef et al., 2010), saithe (*Pollachius virens*) (Chabeaud et al., 2009), yellowfin sole (*Limanda aspera*) (Jun et al., 2004), mackerel (*Scomber austriasicus*) (Wu et al., 2003), and herring (*Clupea harengus*) (Shativel et al., 2003). However, there is little information about the production of fish protein hydrolysates with antioxidant activity from discarded species in the Alboran Sea.

The choice on the enzyme has a great impact on the release of antioxidant peptides by hydrolysis of fish protein (Laroque et al., 2008). The endoproteases subtilisin (EC 3.4.21.62) and pancreatic trypsin (EC 3.4.21.4) have previously shown good results in the production of fish protein hydrolysates exhibiting antioxidant activity (Amarowicz & Shahidi, 1997; Rajapakse et al., 2005; Thiansilakul et al., 2007). Nevertheless, only a few works have addressed the effect of a combination of these enzymes (García-Moreno et al., *In press*).

Therefore, the objective of this study was to investigate the combined effect of subtilisin and trypsin on the production of fish protein hydrolysates exhibiting antioxidant activity from discarded species in the Alboran Sea such as sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark

2. MATERIALS AND METHODS

2.1 Raw material

Raw fish, sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), bogue (*Boops boops)*, axillary seabream (*Pagellus acarne*) and small-spotted catshark

(*Scyliorhinus canicula*), was provided by the fishing harbour of Motril (Spain) in September 2011. It was kept in ice during the transportation and pressed in the same day.

2.2 Separation of protein fraction

The separation of the oil and protein fractions was carried out by hydraulic pressing as described in the Materials and Methods section of chapter II. The cakes obtained from the pressing operation were grinded in a cutter SK-3 (Sammic, Spain) and then frozen at -20ºC prior to their use as substrate for protein hydrolysis. For small-spotted catshark, the grinding and homogenization of the press cake was not possible due to the high resistance of its skin. Thus, muscle of this species was employed as substrate for protein hydrolysis. It was obtained by de-heading, de-gutting and removing the skin from the whole fish.

2.3 Hydrolysis procedure

The enzymatic hydrolysis was carried out as described in the Materials and Methods section of chapter IX. The calculation of the degree of hydrolysis (DH) is also indicated in chapter IX.

A set of 250 hydrolysates samples, originated from the five species and three enzymatic treatments studied and drawn at different times of reaction, were evaluated in order to determine the influence of DH on the DPPH scavenging activity. To this end, the samples were heated in a boiling water bath for 15 min to deactivate the enzyme and were then centrifuged in order to remove the remained solids. They were kept at -20 ºC until performing the analyses.

After completion of the hydrolysis, the final hydrolysates were also heated in a boiling water bath for 15 min to deactivate the enzymes and centrifuged in order to remove the remained solids. Then, they were lyophilized and stored at -20 ºC until performing the analyses.

2.4 Characterization of the hydrolysates

2.4.1 Protein content

The protein content of the lyophilized hydrolysates was determined using a FP-528 LECO nitrogen analyser (LECO, St Joseph, MI, USA) calibrated with ethylenediaminetetraacetic acid according to the Dumas method (Sain-Denis & Groupy, 2004).

2.4.2 Lipid content and lipid classes

The lipid content of the lyophilized hydrolysates was determined according to the method described by Folch et al. (1956). Lipid classes were determined as described in the Materials and Methods section of chapter II.

2.4.3 Molecular weight distribution of hydrolysates

The molecular weight distribution of the fish protein hydrolysates was estimated by gel filtration chromatography in a FPLC ÄKTA (Amersham Biosciences, Uppsala, Sweden) using a Superdex Peptide 10/300 GL column with a UV detector at 254 nm. The eluent was 30% acetonitrile with 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. A molecular weight calibration curve was prepared using the following standards: ribonuclease A (13700 Da), aprotinin (6500 Da), angiotensin I (1296 Da), triglycine (194 Da) and glycine (75 Da).

2.5 Determination of antioxidant activity

2.5.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity of the hydrolysates was determined by two different methods depending on the objective pursued. In order to evaluate the evolution of the DPPH scavenging activity of the hydrolysates with the degree of hydrolysis, the method reported by Brand-Williams et al. (1995) which requires a low amount of sample was employed. It is completely described in the Materials and Methods section of chapter IX.

For the determination of the IC_{50} value, which is defined as the concentration of hydrolysate (mg protein/mL) needed to inhibit DPPH activity by half, the method described by Picot et al. (2010) was used. In short, a volume of 1 mL of each protein hydrolysate having different protein concentrations (0.2–5 mg/mL) was added to 1 mL of 0.1 mM DPPH in methanol. The mixture was shaken for 1 h at 25 ºC in the dark. Then, the

absorbance of the reaction mixture was measured at 517 nm. A blank was run in the same way by using distilled water instead of sample, and sample control was also made for each sample by adding methanol instead of DPPH solution. Triplicate measurements were carried out for each sample and DPPH scavenging activity was calculated by Eq. 27:

$$DPPH\ inhibition\ (\%) = \left(1 - \frac{(A_{sample} - A_{sample\_control})}{A_{blank}}\right) \times 100 \qquad (27)$$

2.5.2 Reducing power

The reducing power of fish protein hydrolysates samples was determined according to the method of Oyaizu (1992). 2 mL of each hydrolysate at different protein concentrations (3–20 mg/mL) were added to 2 mL of 0.2 mM phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. The reaction mixture was incubated at 50 ºC for 20 min and then 2 mL of 10% TCA were added. The mixture was centrifuged at 1500×g for 10 min. A 2 ml aliquot of the supernatant was mixed with 2 mL distilled water and 0.4 mL of 0.1% ferric chloride. The absorbance of the resulting solution was recorded at 700 nm after 10 min. An equivalent volume of distilled water instead of sample was used as control. Analyses were carried out in triplicate.

2.5.3 Iron (Fe^{2+}) chelating activity

The iron chelating activity of the fish protein hydrolysates was estimated by the method described by Decker and Welch (1990). Distilled water (3.7 mL) was added to 1 mL of each fish protein hydrolysate solution at different concentrations (0.2–1 mg/mL). Then, 100 µL of ferrous chloride 2 mM were added and after 3 min the reaction was inhibited by the addition of 200 µL of ferrozine solution 5 mM. The mixture was shaken vigorously and left at room temperature for 10 min, thereafter the absorbance was measured at 562 nm. A blank was run in the same way by using distilled water instead of sample. Sample control was made for each sample without adding ferrozine. Triplicate measurements were carried out. The chelating capacity was calculated as follows (Eq. 28):

$$Fe^{2+}\ chelating\ activity\ (\%) = \left(1 - \frac{(A_{sample} - A_{sample\_control})}{A_{blank}}\right) \times 100 \qquad (28)$$

Then, the IC_{50} value was calculated for each hydrolysate produced. This value is defined as the concentration of hydrolysate (mg protein/mL) needed to have a chelating activity of 50 %.

2.6 Statistical analysis

The Statgraphics software (version 5.1) was used to carry out a one-way analysis of variance (ANOVA) on the data. The Tukey's test was employed for that purpose and differences between means were considered significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Hydrolysis of protein fraction

Similar hydrolysis curves were obtained for the five press cakes evaluated (Fig. 29). High rates of hydrolysis were observed after the addition of each protease or mixture of proteases, which decreased along the reaction time until reaching a plateau. Subtilisin, which cleaves a broad spectrum of peptide bonds, resulted in higher increases of DH when compared with the addition of trypsin, which only cleaves peptidic bonds involving arginine and lysine (Adler-Nissen, 1986). Besides, an increase in the DH took place immediately after the addition of the second enzyme in the processes where the enzymes were added in sequence. This fact was also observed by Guerard et al. (2002) when intermediate addition of fresh enzyme was carried to study the deactivation of the protease during the course of the hydrolysis.

Table 33 shows the values of DH obtained for the 15 hydrolysates produced after 4 hours of hydrolysis. It was noticed that the hydrolysates of horse mackerel presented the highest DH, ranging from 18.2 to 21.0 %. They were even higher than the final DH obtained from the muscle of small-spotted catshark, 17.3 – 19.2 %. The high degradability of horse mackerel protein was also observed by García-Moreno et al. (*In press*). DH varying from 16.0 to 17.2 were obtained for the hydrolysates of axillary seabream. Similar results were observed for the hydrolysates of bogue, 15.3 – 17.6 %, while the lowest DH were found for sardine hydrolysates, ranging from 13.2 to 14.9 %. These DH were in the range of values mentioned by other authors (Batista et al., 2009; Pires et al., 2013).

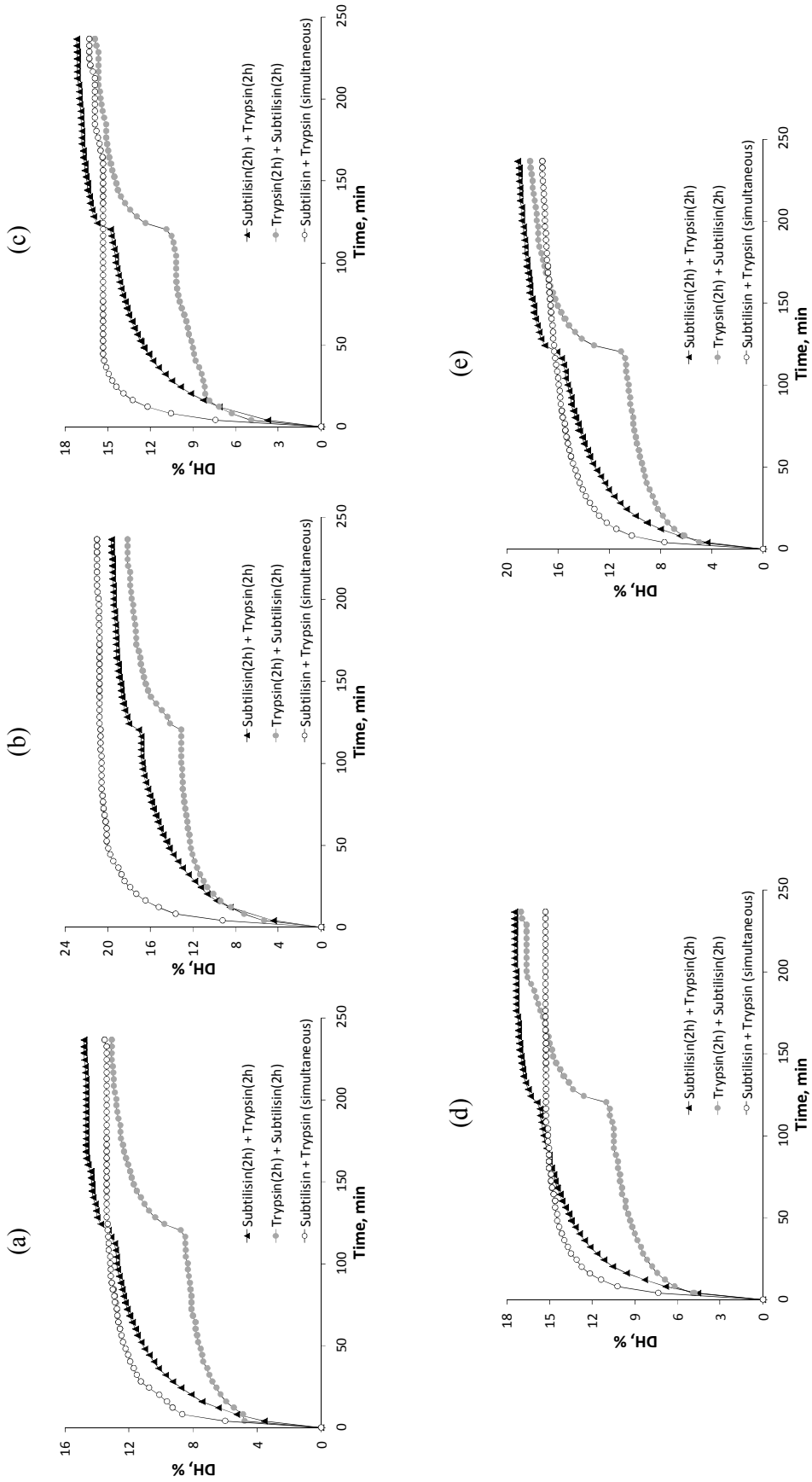

Figure 29. Hydrolysis curves for (a) sardine, (b) horse mackerel, (c) axillary seabream, (d) bogue and (e) small-spotted catshark

Different DH values were also obtained after 4 h of hydrolysis depending on the enzymatic treatment employed (Fig. 29 and Table 33). It was observed that the sequential enzymatic treatment subtlisin and trypsin led to the highest DH for small-spotted catshark (19.2 %), bogue (17.6 %), axillary seabream (17.2 %) and sardine (14.9 %). On the other hand, the simultaneous addition of subtilisin and trypsin resulted in the highest DH for horse mackerel (21.0 %).

Table 33. Degree of hydrolysis and protein and lipid content of the final hydrolysates

	Hydrolysate	DH (%)	Protein (%)	Lipid (%)
Sardine	Subtilisin(2h)+Trypsin(2h)	14.9	61.5 ± 0.4^{a}	22.3 ± 0.2^{a}
	Trypsin(2h)+Subtilisin(2h)	13.2	60.7 ± 0.4^{b}	25.3 ± 0.0^{a}
	Subtilisin+Trypsin (simultaneous)	13.7	66.4 ± 0.4^{c}	19.7 ± 0.9^{b}
Horse mackerel	Subtilisin(2h)+Trypsin(2h)	19.7	67.8 ± 0.2^{d}	17.4 ± 0.3^{c}
	Trypsin(2h)+Subtilisin(2h)	18.2	67.1 ± 0.2^{e}	17.6 ± 0.3^{c}
	Subtilisin+Trypsin (simultaneous)	21.0	62.5 ± 0.2^{f}	21.0 ± 0.1^{ab}
Axillary seabream	Subtilisin(2h)+Trypsin(2h)	17.2	73.0 ± 0.3^{g}	8.8 ± 0.9^{d}
	Trypsin(2h)+Subtilisin(2h)	16.0	73.5 ± 0.3^{g}	8.0 ± 0.6^{d}
	Subtilisin+Trypsin (simultaneous)	16.3	74.9 ± 0.2^{h}	8.4 ± 0.9^{d}
Bogue	Subtilisin(2h)+Trypsin(2h)	17.6	75.7 ± 0.7^{i}	8.3 ± 0.1^{d}
	Trypsin(2h)+Subtilisin(2h)	17.0	76.8 ± 0.2^{j}	8.1 ± 0.0^{d}
	Subtilisin+Trypsin (simultaneous)	15.3	76.4 ± 0.2^{j}	8.4 ± 0.2^{d}
Small-spotted catshark	Subtilisin(2h)+Trypsin(2h)	19.2	87.0 ± 0.2^{k}	6.5 ± 1.9^{e}
	Trypsin(2h)+Subtilisin(2h)	18.3	89.5 ± 0.2^{l}	4.6 ± 1.3^{f}
	Subtilisin+Trypsin (simultaneous)	17.3	88.7 ± 0.5^{m}	4.8 ± 1.8^{f}

Protein and lipid data are means of triplicate determinations ± standard deviation. Mean values within a column followed by different letter mean significant differences ($p<0.05$).

3.2 Influence of DH on DPPH scavenging activity

It has been reported that apart from the properties of the matrix hydrolysed, the choice of the enzymatic treatment and the processing conditions (temperature, pH and enzyme/substrate ratio), the extent of the hydrolysis reaction also has an important significance on the release of antioxidant peptides (Laroque et al., 2008).

In this sense, the DPPH method was chosen for the evaluation of the antioxidant activity of the hydrolysates with different DH. This method is commonly employed for the determination of the scavenging potency of protein hydrolysates due to its reliability and reproducibility (Laroque et al., 2008). Nevertheless, it needs to be said that some peptides released in the course on the enzymatic treatment may also exhibit antioxidant activity by other mechanisms such as chelating of transition metals and participating in the redox reactions involved in the oxidation process (Frankel & Meyer, 2000).

Fig. 30 shows the evolution of the DPPH scavenging activity of the hydrolysates with DH. Comparing the results from the three enzymatic treatments assayed, differences were found among species. For instance, for horse mackerel it was observed that the sequential treatment subtilisin (2h) plus trypsin (2h) and the enzymatic treatment of trypsin (2h) plus subtilisin (2h) produced hydrolysates with a higher DPPH scavenging activity, around 45%, than the one resulted from the simultaneous addition of both enzymes, 35 % (Fig. 30a). On the contrary, the sequential treatment subtilisin (2h) plus trypsin (2h) led to the hydrolysate with the lowest DPPH inhibition for bogue. In the case of sardine and small-spotted catshark, the simultaneous addition of subtilisin and trypsin resulted in the hydrolysates exhibiting the lowest scavenging of DPPH radical; whereas for axillary seabream no differences were obtained by the three enzymatic patterns employed (data not shown).

Fig. 30b depicts the evolution of DPPH inhibition with the time of hydrolysis for all species. This figure shows that when employing the enzymatic pattern subtilisin (2h) plus trypsin (2h), the hydrolysates obtained from horse mackerel and sardine exhibited higher DPPH inhibition (40-45 %) than the hydrolysates produced from the other species (around 15 %). This trend was also observed for the other two enzymatic treatments employed (data not shown). It was also observed that a maximum of DPPH inhibition was reached in the first hydrolysis period with subtilisin for sardine (at 30 min), bogue (at 30 min) and small-spotted catshark (at 10 min) (Fig. 30b). It may be due to the fact that antiradical peptides were released until the maximum and then they were hydrolyzed. On the other hand, for horse mackerel and axillary seabream a gradual increase of peptides exhibiting DPPH scavenging activity was obtained with DH in this period. The addition of trypsin also led to an increase in the antiradical activity of the hydrolysates, which was more notorious in the case of sardine and horse mackerel hydrolysates (Fig. 30b). A maximum of activity was also found in this period particularly for sardine (at 130 min) and small-

spotted catshark (at 180 min). Likewise, maximum values of DPPH inhibition were obtained for most of the hydrolysates when adding trypsin as first enzyme (data not shown). In this line, Jao and Ko (2002) also reported a maximum of DPPH inhibition at 2.5 hours for hydrolysates obtained from tuna cooking juice. Then, the scavenging activity of the hydrolysates decreased until completion of the reaction. In contrast, different results were reported by Wu et al. (2003) which found that the DPPH inhibition of mackerel hydrolysates increased with DH and then tended to a plateau after 5 hours of reaction.

As a consequence of the results commented above, the hydrolysates obtained after 4 hours of reaction, which presented an important DPPH inhibition and the highest DH, were chosen as the best candidates to exhibit antioxidant activity. Hydrolysates with the highest DH were preferred in order to ensure the presence of small peptides which have been reported to show potent antioxidant activities (Jeon et al., 2000; Kim et al., 2001). Moreover, peptides with low molecular weights have less chance to be modified during digestion and, thus can be absorbed intact from the gastrointestinal tract and produce specific biological actions (Roberts et al., 1999).

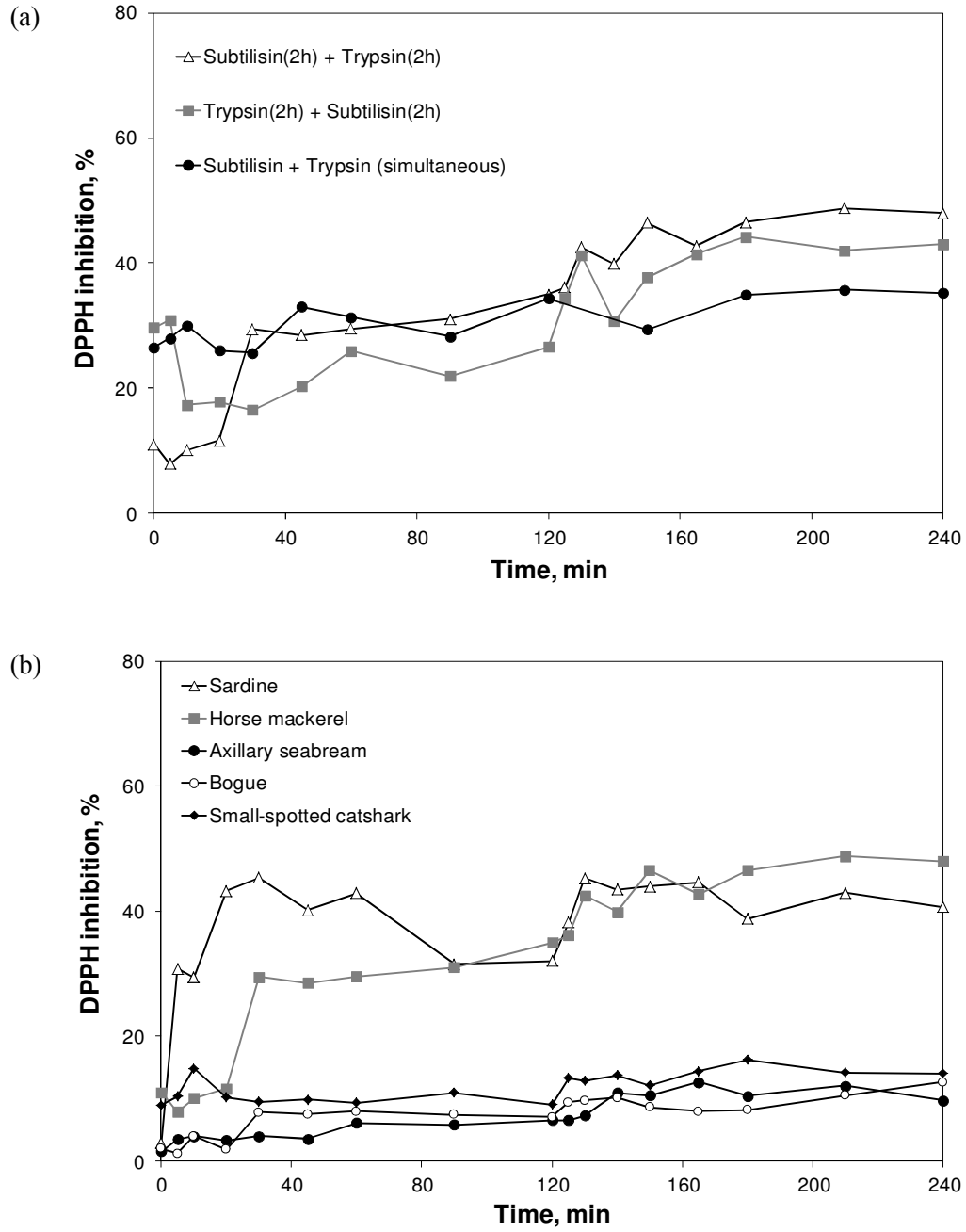

Figure 30. DPPH inhibition of horse mackerel hydrolysates at different times of hydrolysis (a), DPPH inhibition of the hydrolysates obtained with the enzymatic pattern subtilisin(2h) + trypsin(2h) for the five species studied (b).

3.3 Characterization of final lyophilized hydrolysates

The protein and lipid contents of the final lyophilized hydrolysates are presented in Table 33. The highest protein content was found in the hydrolysates produced from the muscle of small-spotted catshark, ranging from 87.0 to 89.5 %. Axillary seabream and bogue hydrolysates presented lower protein content, varying from 73.0 to 76.8 %, whereas the

lowest protein amount was found in sardine and horse mackerel hydrolysates, 60.7-67.8 %. These results are in line with previous studies which reported fish protein hydrolysates with a protein content varying from 60 to 92 % (Šližyte et al., 2005; Ovissipour et al., 2013). These results also confirmed the study carried out by Šližyte et al (2005) which stated that the raw material to be hydrolysed containing the highest amount of lipids such as pelagic species gave the hydrolysates with the lowest content of protein.

Regarding the lipid content, it was found to correlate inversely with the protein content (Table 33). It was observed that the lyophilized hydrolysates of sardine and horse mackerel presented the highest content, 17.4-25.3 %, axillary seabream and bogue hydrolysates had lower values, 8.0-8.8 %, whereas small-spotted catshark hydrolysates presented the lowest lipid amount, 4.8-6.5 %. These results are explained due to the lipid content reported for these species in autumn, which was higher for sardine (11.3 %) and horse mackerel (5.0 %), followed by axillary seabream (3.7 %), small-spotted catshark (2.5 %) and bogue (0.8 %) (García-Moreno et al., 2013). Chalamaiah et al. (2013) obtained similar fat content for rohu egg protein hydrolysates which was attributed to fat globules present in the supernatant after removing undigested material by centrifugation. However, it should be mentioned that hydrolysates with low lipid content are preferred in order to obtain final products with improved oxidative stability (Raghavan et al., 2008; Khantaphant et al., 2011).

Table 34 shows the lipid classes presented in the lyophilized hydrolysates obtained with the sequential treatment subtilisin (2h) plus trypsin (2h). For the hydrolysates produced from press cakes, it was found that triacylglycerols represented the major lipid class. They were around 75.0 % for sardine and horse mackerel and 45.0 % for axillary seabream and bogue. In the hydrolysates from these species free fatty acids were the second most important lipids. Its content was species dependent, but it was considerably higher in axillary seabream and bogue hydrolysates, approximately 35.0 %. Considering cholesterol content, it ranged from 3.9 % for sardine to 14.7 % for bogue. Phospholipids content of these hydrolysates was practically constant and it was around 9.0 %. On the other hand, phospholipids were the most abundant lipids for small-spotted catshark muscle hydrolysate, 54.4 %, followed by cholesterol, 35.2 %. This result may due to the fact than in lean fish, phospholipids make up most of the lipids of the cell (Liang & Hultin, 2005). Conversely, triacylglycerols and free fatty acids contents of this hydrolysate were practically negligible, 5.0 and 5.4 % respectively. Similarly, Daukšas et al. (2005) reported

that triacylglycerols content of cod hydrolysates depended on the raw material and enzyme employed, varying from 37 to 88 %. These authors also reported phospholipid content for cod hydrolysates of 59 %, which is in the range of the content found in small-spotted catshark hydrolysate.

Table 34. Lipid classes presented in the final hydrolysates produced by the enzymatic treatment subtilisin(2h) + trypsin(2h)

Lipid class (wt%)	**Sardine**	**Horse mackerel**	**Axillary seabream**	**Bogue**	**Small-spotted catshark**
Triacylglycerols	74.9 ± 6.6^{a}	73.9 ± 3.7^{a}	45.0 ± 0.5^{b}	43.8 ± 3.5^{b}	5.0 ± 1.1^{c}
Free fatty acids	12.2 ± 2.3^{a}	6.5 ± 0.9^{b}	36.2 ± 0.4^{c}	33.8 ± 3.7^{c}	5.4 ± 1.0^{b}
Cholesterol	3.9 ± 2.3^{a}	9.7 ± 1.0^{b}	10.2 ± 0.1^{b}	14.7 ± 0.1^{c}	35.2 ± 1.7^{d}
Phospholipids	9.0 ± 2.0^{a}	9.9 ± 1.9^{a}	8.6 ± 0.2^{a}	7.7 ± 0.3^{a}	54.4 ± 1.9^{b}

Data are means of triplicate determinations ± standard deviation. Mean values within a row followed by different letter mean significant differences ($p<0.05$).

The molecular weight size distribution of the hydrolysates obtained from the press cakes by the three enzymatic treatments was very similar. Fig. 31 shows the average values of the hydrolysates for each species. The gel filtration profile of the hydrolysate samples indicated hydrolysis of the fish proteins into small molecular weight peptides and free amino acids. A low proportion of these peptides, less than 10 %, have a molecular size higher than 450 Da. Also, a percentage below 10 % of the peptides were in the range of 450 - 150 Da. Higher proportions were obtained in the range of 150 - 50 Da, varying from 35 % for bogue to 50 % for sardine; and below 50 Da, varying from 33 % for sardine to 54 % for bogue. The small-spotted catshark hydrolysates showed a rather different peptide profile from the hydrolysates prepared from the press cake of the bony fish. It presented lower percentages of peptides higher than 450 Da (2 %) and 50-150 Da (16 %) but higher percentages of peptides in the range of 150-450 Da (21 %).

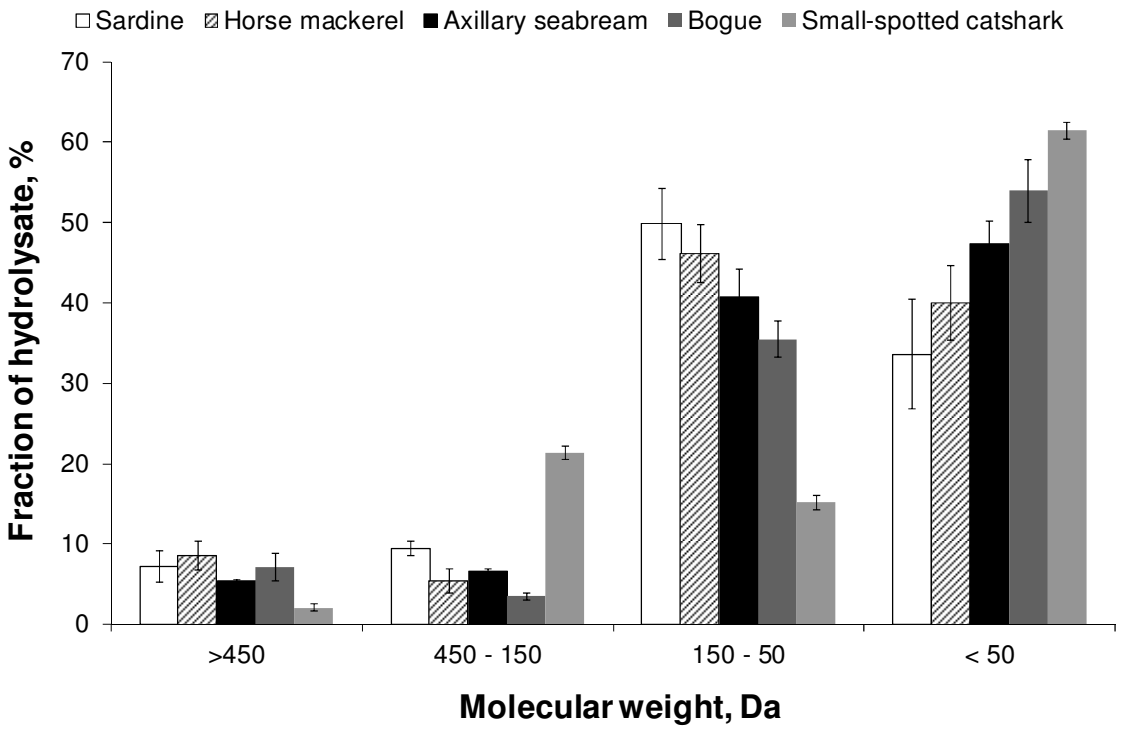

Figure 31. Fractions of the final hydrolysates according to their molecular-weight range. Results are average of triplicate determination ± standard deviation.

3.4 Antioxidant activity of final hydrolysates

It is widely known that antioxidants can act by different mechanisms. Therefore, the use of various methods for the evaluation of the antioxidant activity is recommended (Frankel & Meyer, 2000).

The DPPH radical scavenging and the Fe^{2+} chelating activities of the 15 final hydrolysates were measured as a function of the protein concentration in order to determine their IC_{50} values (Table 35). It was observed that sardine and horse mackerel hydrolysates presented the highest DPPH scavenging activity with IC_{50} values ranging from 0.91 to 1.78 mg protein/mL. They were followed by axillary seabream and bogue hydrolysates with IC_{50} values varying from 1.94 to 2.91 mg protein/mL, whereas small-spotted catshark hydrolysates presented the lowest DPPH scavenging-activity with IC_{50} values in the range of 3.82 - 4.45 mg protein/mL. These results are in line with the IC_{50} value reported for rohu egg protein hydrolysates, 1.5 mg hydrolysate/mL, (Chalamaiah et al., 2013), but are considerable lower than the IC_{50} value found for protein hydrolysates from toothed ponyfish muscle, 25 mg hydrolysate/mL, (Klomklao et al., *In press*) and for hake protein

hydrolysates when concentrations up to 30 mg hydrolysate/mL did not allow to obtain a DPPH inhibition of 50 % (Pires et al., 2013).

Table 35. IC_{50} values of the final hydrolysates

Hydrolysate		IC_{50} (mg protein/mL)	
		DPPH radical scavenging	Fe^{2+} Chelating activity
Sardine	Subtilisin(2h)+Trypsin(2h)	1.30 ± 0.12^{a}	0.32 ± 0.01^{a}
	Trypsin(2h)+Subtilisin(2h)	0.91 ± 0.02^{b}	0.32 ± 0.01^{a}
	Subtilisin+Trypsin (simultaneous)	1.75 ± 0.05^{c}	0.32 ± 0.01^{a}
Horse mackerel	Subtilisin(2h)+Trypsin(2h)	1.63 ± 0.03^{d}	0.42 ± 0.03^{b}
	Trypsin(2h)+Subtilisin(2h)	1.47 ± 0.01^{e}	0.49 ± 0.01^{c}
	Subtilisin+Trypsin (simultaneous)	1.78 ± 0.08^{c}	0.46 ± 0.01^{d}
Axillary seabream	Subtilisin(2h)+Trypsin(2h)	2.56 ± 0.02^{f}	0.45 ± 0.01^{d}
	Trypsin(2h)+Subtilisin(2h)	2.34 ± 0.05^{g}	0.5 ± 0.03^{c}
	Subtilisin+Trypsin (simultaneous)	2.44 ± 0.03^{g}	0.51 ± 0.01^{c}
Bogue	Subtilisin(2h)+Trypsin(2h)	2.91 ± 0.06^{h}	0.51 ± 0.01^{c}
	Trypsin(2h)+Subtilisin(2h)	2.84 ± 0.05^{h}	0.50 ± 0.01^{c}
	Subtilisin+Trypsin (simultaneous)	1.94 ± 0.02^{i}	0.63 ± 0.03^{e}
Small-spotted catshark	Subtilisin(2h)+Trypsin(2h)	4.45 ± 0.06^{j}	0.32 ± 0.02^{a}
	Trypsin(2h)+Subtilisin(2h)	3.82 ± 0.06^{k}	0.32 ± 0.02^{a}
	Subtilisin+Trypsin (simultaneous)	4.35 ± 0.11^{l}	0.51 ± 0.01^{c}

Data are means of triplicate determinations ± standard deviation. Mean values within a column followed by different letter mean significant differences ($p<0.05$).

In most of the cases, significant differences were found in the DPPH scavenging activity of the hydrolysates when employing different enzymatic treatments (Table 35). As general trend, it was observed that the sequential addition of trypsin (2h) plus subtilisin (2h) resulted in the hydrolysates with the lowest IC_{50} values. It may be due to the fact that adding subtilisin as second enzyme may favor the cleavage at the C-terminal of hydrophobic residues which can contribute to the DPPH inhibitory activity (Je et al., 2009).

As low molecular weight peptides, <300 Da, were reported to exhibit a higher antioxidant activity (Jeon et al., 2000), the lower proportion of peptides for small-spotted catshark hydrolysates in the range of 150 - 50 Da (Fig. 3) may be the reason for their higher IC_{50} values. Additionally, the antioxidant activity of the hydrolysates also depends on their amino acid composition and the sequence of the peptides.

The capacity to bind transition metals is also a useful indication of antioxidant activity. It is due to the fact that transition metal ions, Fe^{2+} and Cu^{2+}, catalyze the generation of reactive oxygen species such as hydroxyl radical ($OH\cdot$) which initiates lipid peroxidation (Stohs & Bagchi, 1995). Sardine and small-spotted catshark hydrolysates exhibited the highest ferrous-chelating activity with IC_{50} values of 0.32 mg protein/mL (Table 35). The hydrolysates prepared from horse mackerel, axillary seabream and bogue presented slightly higher IC_{50} values ranging from 0.42 to 0.63 mg protein/mL. They showed higher ferrous binding capacity than those of silver carp hydrolysates produced with Flavourzyme, which exhibited only 60 % at a concentration of 5 mg hydrolysate/mL (Dong et al., 2008). Nonetheless, they were in the line of IC_{50} values obtained by Ktari et al. (2012) for zebra blenny protein hydrolysates, which ranged from 0.15 to 0.25 mg hydrolysate/mL depending on the enzymatic treatment used.

The metal binding capacity of protein hydrolysates is generally attributed to their content in effective sites capable of chelating metal ions (Ovissipour et al., 2013). In this sense, histidine containing peptides have been reported to exhibit metal chelating activity through their imidazole ring (Bougatef et al., 2009). Considering the different enzymatic treatments assayed, it was revealed that for axillary seabream, bogue and small-spotted catshark, the simultaneous addition of subtilisin and trypsin led to the hydrolysates with the lowest metal chelating activity (Table 35). This fact may be caused by differences in the structure of the peptides in these hydrolysates (Thiansilakul et al., 2007). However, further investigations are required in order to obtain more information about the amino acid sequences of the active peptides which can confirm it.

The reducing power assay is another common test employed to determine the antioxidant activity of fish protein hydrolysates. Particularly, this method evaluates the capacity of fish protein hydrolysates to act as a reducing agent (Batista et al., 2010). As observed in Fig. 32, the reducing power of all the hydrolysates increased with the concentration of protein. A similar trend was reported in previous studies (Ktari et al., 2012; Pires et al., 2013). From Fig. 32, it was observed that sardine and bogue hydrolysates exhibited the highest

reducing power independently of the enzymatic treatment, whereas small-spotted catshark hydrolysates presented the lowest. This fact may be probably due to the differences observed in the molecular weight profile of these hydrolysates (Fig. 31), as well as the amino acid composition of their peptides (Theodore et al., 2008). It should be also mentioned that the simultaneous addition of subtilisin plus trypsin was the least appropriate enzymatic treatment for the production of sardine protein hydrolysates with a high reducing power, whereas the sequential treatment with subtilisin (2h) and trypsin (2h) led to the bogue hydrolysate with the lowest reducing power (Fig. 32). The results obtained in this work were similar to that reported for black scabbardfish (Batista et al., 2010), higher than those found for hake by products (Pires et al., 2013), but considerably lower than those obtained for zebra blenny (Ktari et al., 2012) and sardinelle (Bougatef et al., 2010).

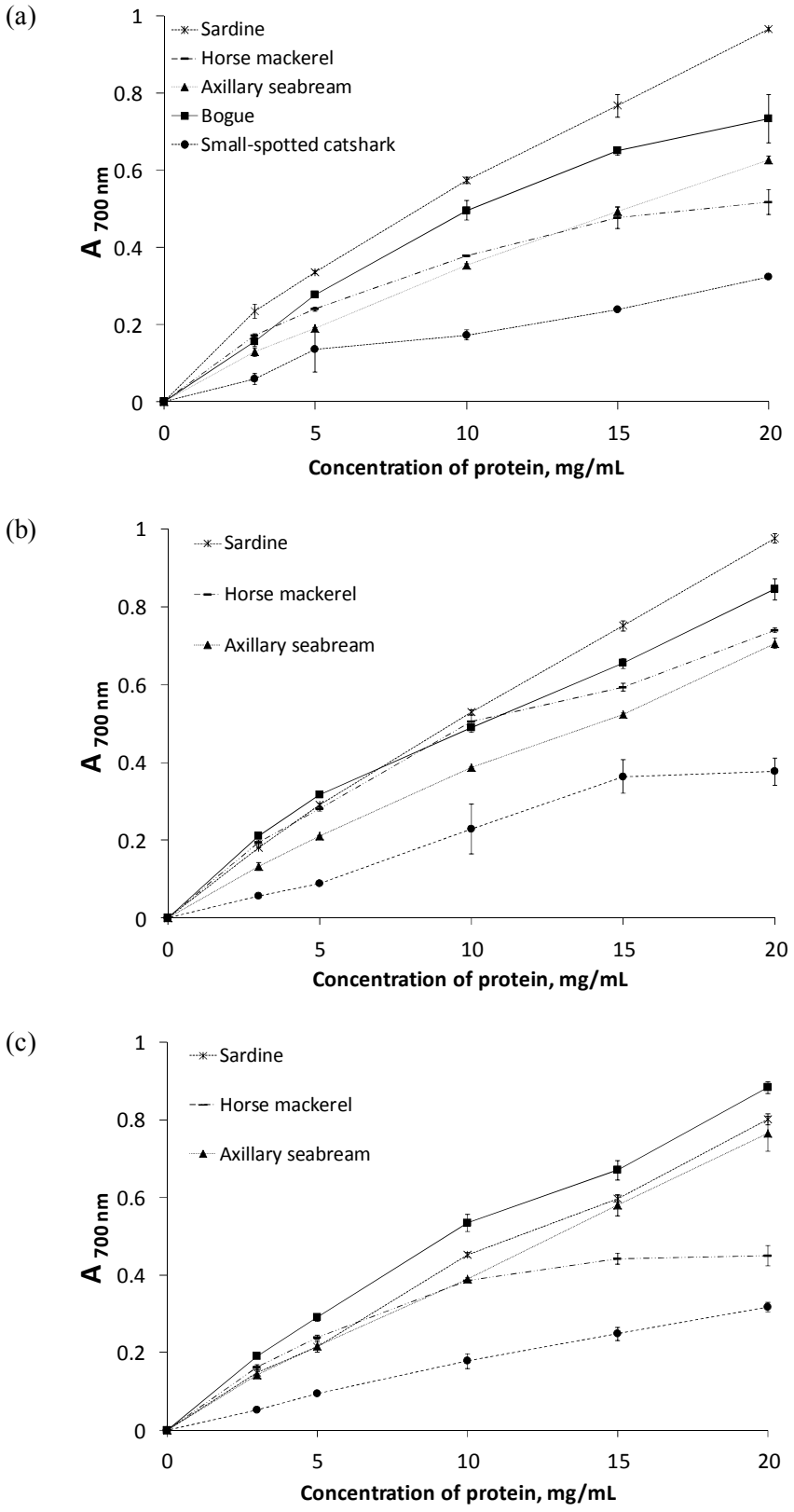

Figure 32. Reducing power of the final hydrolysates produced by: (a) subtilisin(2h) + trypsin(2h), (b) trypsin(2h) + subtilisin(2h) and (c) subtilisin+trypsin (simultaneous). Results are average of triplicate determination ± standard deviation.

4. CONCLUSIONS

This work denoted that it is feasible to produce fish protein hydrolysates exhibiting strong antioxidant activity when employing press cakes of discarded species as raw material. The hydrolysates presented a varying protein (60.7-89.5 wt%) and lipid (4.6-25.3 wt%) content depending on the species. The molecular weight profiles indicated that the hydrolysates were mainly constituted of small peptides, below 450 Da, which have been reported to contribute to their antioxidant activity. The highest DPPH scavenging activity was found for the hydrolysate of sardine produced by the enzymatic treatment trypsin (2h) plus subtilisin (2h), with an IC_{50} value of 0.91 mg protein/mL. Sardine and small-spotted catshark hydrolysates exhibited the highest ferrous chelating activity, with IC_{50} value of 0.32 mg protein/mL, except for the small-spotted catshark hydrolysate obtained by the simultaneous addition of subtilisin and trypsin. In terms of reducing power, sardine and bogue hydrolysates presented the highest electron donating capacity, with an absorbance higher than 0.8 at a concentration of 20 mg protein/mL. Therefore, it can be concluded that, among the five species evaluated, sardine is the discarded fish species with the highest potential for the production of fish protein hydrolysates with antioxidant activity. Nevertheless, further research should be carried out in order to purify and identify the bioactive peptides.

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XI. Angiotensin I-Converting Enzyme Inhibitory Activity of Protein Hydrolysates Obtained from Discarded Mediterranean Fish Species*

This work studies the production of protein hydrolysates exhibiting ACE-inhibitory activity from fish discards species in the Alboran Sea. For that purpose, three different combinations of subtilisin and trypsin were employed as enzymatic treatments. The evolution of the ACE-inhibitory activity of the hydrolysates with DH was also evaluated. The highest antihypertensive activities were found for the final hydrolysates of horse mackerel (IC_{50}=279 μg/mL) and small-spotted catshark (IC_{50}=302 μg/mL), both obtained with the simultaneous addition of subtilisin and trypsin. This high inhibitory activity may be related with the good degradability shown by the horse mackerel protein, as indicated by the DH values, and with the high proline content of the small-spotted catshark protein. The elution profile of horse mackerel hydrolysate presented 7 fractions, with fraction B (130-2,350 Da) exhibiting the highest ACE-inhibitory activity (IC_{50}=85 μg/mL). In the case of small-spotted catshark hydrolysate, five fractions were identified, being fraction C (470-1210 Da) and fraction D (58-470 Da) the ones presenting the highest ACE-inhibitory activity, IC_{50} of 72 and 27 μg/mL respectively.

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1. INTRODUCTION

Among the numerous bioactivities shown by fish protein hydrolysates (Je et al., 2009; Kim & Wijesekara, 2010), antihypertensive peptides, which do not exhibit known side effects (Ishida et al., 2011), have been comprehensively studied as natural compounds for the treatment of hypertension (Jung et al., 2006). To this regard, the *in vitro* measurement of angiotensin converting enzyme (ACE, EC 3.4.15.1) inhibitory activity is a common first approach to identify antihypertensive peptides. This is due to the fact that ACE, a zinc metallopeptidase, plays a crucial role in the regulation of blood pressure. In the renin-angiotensin system, ACE transforms the inactive decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) into the potent vasoconstrictor octapeptide, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Besides, in the kallikrein-kinin system, ACE catalyzes the degradation of bradykinin, a vasodilator nonapeptide (Li et al., 2004). Thus, the inhibition of ACE would originate a reduction in blood pressure.

A number of previous studies have described the ACE-inhibitory activity of fish protein hydrolysates produced from different species such as yellowfin sole (*Limanda aspera*) (Jung et al., 2006), pacific hake (*Merluccius productus*) (Cinq-Mars & Li-Chan, 2007), sardinelle (*Sardinella aurita*) (Bougatef et al., 2008), cuttlefish (*Sepia officinalis*) (Balti et al., 2012) and loach (*Migurnus anguillicaudatus*) (Li et al., 2012). However, to the best of the authors' knowledge, apart from sardine (*Sardina pilchardus*) which has been extensively studied (Matsui et al., 1993, Bordenave et al., 2002; García-Moreno et al., *In press*), there is no previous work on the production of fish protein hydrolysates with ACE-inhibitory activity from the other discarded species in the Alboran Sea.

In the light of the above, the aim of this work was to investigate the potential of five discarded species in the Alboran Sea (*Sardina pilchardus, Trachurus mediterraneus, Pagellus acarne, Boops boops and Scyliorhinus canicula*) as raw material for the production of fish protein hydrolysates exhibiting ACE-inhibitory activity. Firstly, the evolution of ACE inhibition with the degree of hydrolysis was studied. Then, selected hydrolysates were purified by size exclusion chromatography in order to find the size range with the highest ACE-inhibitory activity.

2. MATERIALS AND METHODS

2.1 Raw material and separation of protein fraction

The raw material described in the Materials and Methods section of chapter X was also employed in this work. The press cakes and the muscle of small-spotted catshark were also obtained as indicated in chapter X.

2.2 Enzymes and hydrolysis procedure

The endoproteases subtilisin (EC 3.4.21.62) and pancreatic trypsin (EC 3.4.21.4) have been previously reported to yield fish protein hydrolysates exhibiting ACE-inhibitory activity (Matsui et al., 1993; Bougatef et al., 2008). However, there is a limited knowledge about the production of ACE-inhibitory hydrolysates from fish protein by combinations of these two enzymes (García-Moreno et al., *In press*). Hence, the enzymatic patterns described chapter IX were also evaluated in this work. The conditions of the hydrolysis reaction and the calculation of the degree of hydrolysis (DH) were also indicated in chapter IX.

A set of 250 hydrolysates samples, originated from the five species and the three enzymatic treatments studied and drawn at different times of reaction, were evaluated in order to determine the influence of DH on the ACE-inhibitory activity. Each sample was immersed in boiling water for 15 min to deactivate the enzyme and was then centrifuged in order to remove the remained solids. They were kept at -20 ºC until performing the analyses.

After 4 h, the final hydrolysates were also immersed in boiling water for 15 min to deactivate the enzymes and centrifuged in order to remove the remained solids. Then, they were lyophilized and stored at -20 ºC until performing the analyses.

2.3 Fractionation by size-exclusion chromatography

Selected lyophilized hydrolysates were re-dissolved in distilled water (15 grams of protein/mL) and were then fractionated by size exclusion chromatography (SEC) using an FPLC system (AKTA purifier UPC 100, GE Healthcare, Uppsala, Sweden) mounted with a Superdex Peptide 10/300 GL column (GE Healthcare, Uppsala, Sweden). Five hundred

microliters of the sample solution were injected and eluted with ultrapure water at a flow rate of 0.5 mL/min. The effluent was monitored at 280 nm and the fractions were automatically collected according to slope changes. The area of each fraction was integrated using Unicorn 5.1 software (GE Healthcare, Uppsala, Sweden). Up to five injections were performed for each selected hydrolysate in order to collect enough amount of protein for each fraction. Then, each fraction was concentrated by freeze-drying for the subsequent ACE-inhibitory activity determination.

Four standards with different molecular weights (Ribonuclease A, Vitamin B_{12}, Gly-Gly-Gly and Gly; Sigma-Aldrich, St. Louis MO, USA) were analyzed to set a calibration curve which allowed to relate the elution volume with the peptide size.

2.4 Protein determination

The protein content of the final lyophilized hydrolysates was determined using a FP-528 LECO nitrogen analyser (LECO, St Joseph, MI, USA) calibrated with ethylenediaminetetraacetic acid according to the Dumas method (Saint-Denis and Goupy, 2004). The protein concentration of the SEC fractions was evaluated using a BCA protein assay kit acquired from Sigma-Aldrich Quimica SA (Madrid, Spain). Triplicate measurements were performed.

2.5 Determination of ACE-inhibitory activity

The ACE inhibitory activity of the hydrolysates and of the SEC fractions was determined *in vitro* as described in the Materials and Methods section of chapter IX. For the final hydrolysates and for the selected SEC fractions, the IC_{50} value was also determined.

2.6 Statistical analysis

The Statgraphics software (version 5.1) was used to carry out a one-way analysis of variance (ANOVA) on the data. The Tukey's test was employed for that purpose and differences between means were considered significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Influence of DH on ACE-inhibitory activity

The DH of a hydrolysate, which indicates the extent of the protein degradation, plays an important role on its ACE-inhibitory activity together with the characteristics of the raw material hydrolysed, the specificity of the enzymes chosen and the reaction conditions employed (pH, temperature and enzyme/substrate ratio) (Balti et al., 2010).

The two enzymes used in this work exhibit appropriated specificities to release peptides with ACE-inhibitory activity. Subtilisin preferentially cleaves at the C-terminal of hydrophobic residues, whereas trypsin allows to release peptides with basic amino acids in the C-terminal (Espejo-Carpio et al., 2013). Both hydrophobicity and basicity in the C-terminal are desired characteristics for ACE-inhibitory peptides (Li et al., 2004). Furthermore, the operational conditions employed for the hydrolysis were adequate for both enzymes (Espejo-Carpio et al., 2013). Thus, the effect of DH on the evolution of ACE-inhibitory activity could be evaluated for the five press cakes and the three enzymatic treatments assayed. The curves of hydrolysis obtained were previously commented in chapter X.

Fig. 33a shows that undigested press cakes from bogue and small-spotted catshark did not present ACE-inhibitory activity, whereas medium ACE inhibition values were found for press cakes from axillary seabream and horse mackerel, 22 and 28 % respectively. In the case of sardine, a higher ACE-inhibitory activity, 48 %, was observed for the no hydrolysed press cake. According to Kristinsson (2006), intact fish proteins may also exhibit high ACE inactivation activity. However, intact proteins would not play a role directly in ACE regulation *in vivo*, as they would be hydrolysed in the digestive system. Moreover, the endogenous enzymes presented in fish viscera could have produced some hydrolysis, liberating active peptides before adding the exogenous enzymes in the case of axillary seabream, horse mackerel and sardine.

Fig. 33a also depicts that the ACE-inhibitory activity of the hydrolysates increased sharply within the first 20-45 min of reaction. In overall, higher ACE-inhibitory activity (70-80 % ACE inhibition) was obtained for the hydrolysates of horse mackerel, sardine and small-spotted catshark when compared to axillary seabream and bogue hydrolysates (~60 % ACE inhibition). A maximum of ACE inhibition was observed in the first hydrolysis period with

subtilisin at 45 min for sardine (DH = 11 %), horse mackerel (DH = 14 %) and small-spotted catshark (DH = 13 %), at 10 min for axillary seabream (DH = 6.5 %) and at 90 min for bogue (DH =15 %). Then, the ACE-inhibitory activity slightly decreased. It may be due to the fact that ACE-inhibitory peptides generated during this period were then cleaved at sites that do not facilitate ACE inhibition. Similar results were obtained by Cinq-Mars and Li-Chan (2007) which reported a maximum of ACE inhibition at 120-150 min for hydrolysates of hake fillets obtained with Protamex.

The later addition of trypsin led to a slight increase in the ACE inhibition of the hydrolysates, apart from axillary seabream, being more notorious for horse mackerel and small-spotted catshark hydrolysates (Fig. 33a). A maximum of ACE inhibition was also obtained in this second period of hydrolysis at 140 min for sardine (DH = 14.2 %), horse mackerel (DH = 18.6 %) and bogue (DH = 17 %), at 165 min for small-spotted catshark (DH= 18.3 %) and at 180 min for axillary seabream (DH = 16.8 %). Then, as the reaction advanced, the ACE-inhibitory activity tended to decrease but in the cases of horse mackerel and bogue the ACE inhibition also have a final increase after 165 min (Fig. 1a). Likewise, maximum values of ACE inhibition were obtained for all the hydrolysates when adding trypsin as first enzyme. As an example, Fig. 33b shows a maximum for small-spotted catshark hydrolysate in the period with trypsin at 20 min (DH = 8 %), and in the period with subtilisin at 140 min (DH = 15.5 %). However, it should be mentioned that all the maximum commented above do not exhibit an ACE-inhibitory activity considerably higher than the other points, particularly for those obtained after the addition of the second enzyme (Fig. 33).

On the other hand, a different trend was observed in the ACE-inhibitory activity of small-spotted catshark hydrolysate when it was obtained with the simultaneous treatment subtilisin and trypsin. In this case, a maximum of ACE inhibition was reached at 30 min (DH = 14 %), and then the ACE-inhibitory activity remained practically constant until completion of the hydrolysis. These results are in line with those obtained by Bougatef et al. (2008). These authors reported that the ACE-inhibitory activity of sardinelle proteins hydrolyzed with crude enzyme extract of sardine viscera increased with DH but digestion above DH of 6 % did not result in an increase in the ACE inhibition. In this line, Geirsdottir et al. (2011) also found that ACE-inhibitory activity of blue whiting hydrolysate obtained with Alcalse 2.4L increased with DH and then tended to a plateau above DH of 10 %.

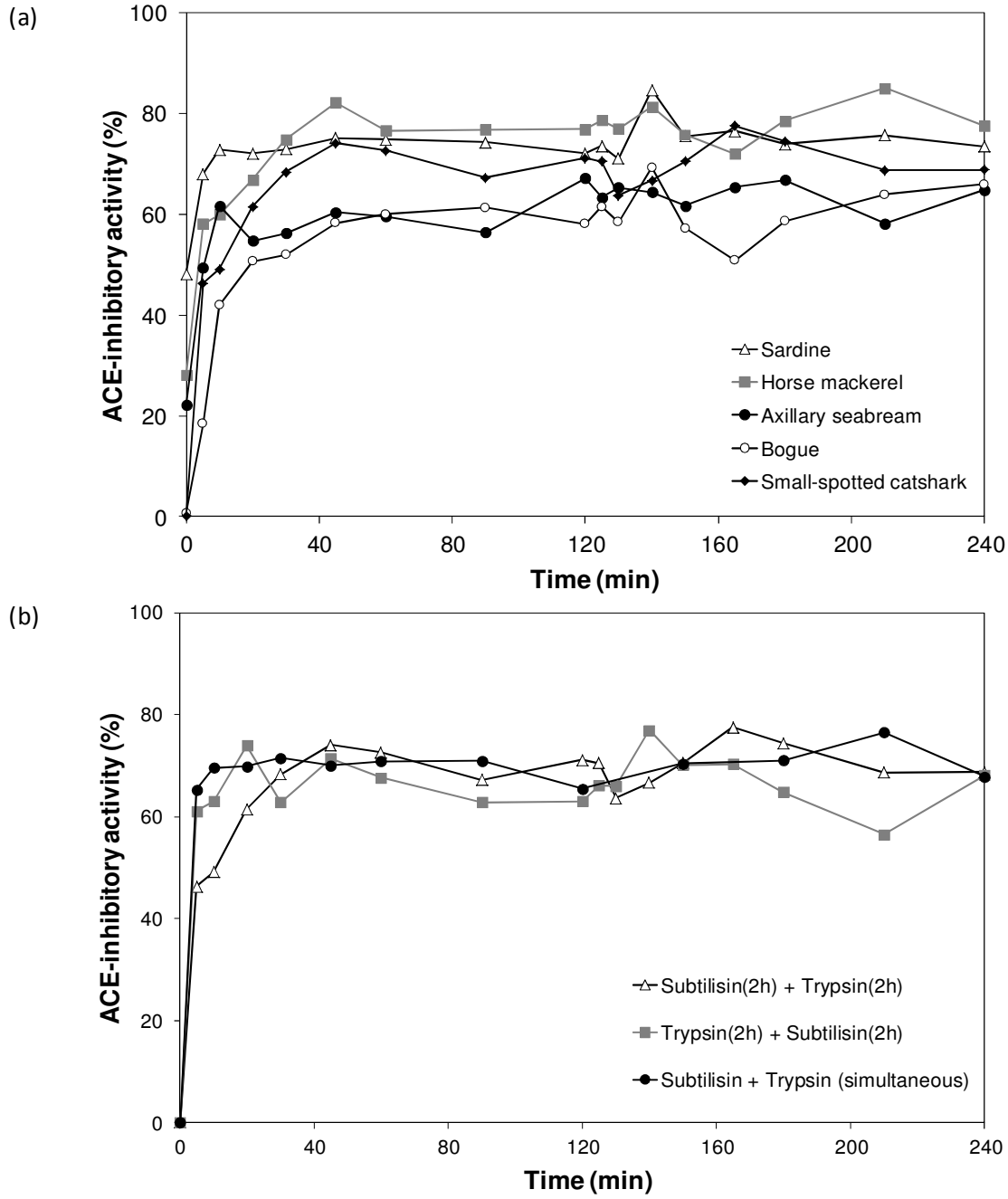

Figure 33. (a) ACE-inhibitory activity of the hydrolysates obtained with the enzymatic pattern subtilisin (2h)+trypsin (2h) for the five species studied, (b) ACE-inhibitory activity of small-spotted catshark hydrolysates at different time of hydrolysis.

3.2 ACE-inhibitory activity of final hydrolysates

ACE-inhibitory peptides generally contain 2-12 amino acids (Li et al., 2003). Hence, hydrolysates with a high DH are desired in order to obtain a product mixture rich in peptidic fractions with low molecular weight. In addition, the shorter is the peptide the

higher is its potential of reducing blood pressure *in vivo*. It is attributed to the fact that short peptides can be intactly absorbed from the gastrointestinal tract (Roberts et al., 1999).

As a consequence, the hydrolysates obtained after 4 hours of reaction, which exhibited the highest DH and a significant ACE inhibition, were chosen for the calculation of their IC_{50} values which allow further comparison among species and enzymatic treatments (Table 36). The complete characterization of these final hydrolysates was carried out in chapter X.

From Table 36, it was revealed that small-spotted catshark and horse mackerel hydrolysates exhibited the highest ACE-inhibitory activity with IC_{50} values ranging from 279 to 398 μg protein/mL. They were followed by axillary seabream and sardine hydrolysates with IC_{50} values varying from 375 to 489 μg protein/mL, whereas bogue hydrolysates presented the lowest ACE-inhibitory activity with IC_{50} values in the range of 637 – 768 μg protein/mL. These IC_{50} values were lower than those of hydrolysates from yellowfin sole with IC_{50} = 883 μg/mL (Jung et al., 2006), sardinelle with IC_{50} = 1.2 mg/mL (Bougatef et al., 2008) and cuttlefish with IC_{50} = 1 mg/mL (Balti et al., 2010), whereas they were higher than those of hydrolysates from bonito with IC_{50} = 29 μg/mL (Yokoyama et al., 1992) and from salmon with IC_{50} = 38 μg/mL (Ono et al., 2006). Regarding axillary seabream hydrolysates, the IC_{50} values obtained in this work (Table 1) were slightly lower than those reported by Fahmi et al. (2004) for the hydrolysate of seabream scales (IC_{50} = 570 μg/mL). Conversely, sardine hydrolysates exhibiting a higher ACE-inhibitory activity (IC_{50} = 260 μg/mL) were described in the scientific literature (Matsui et al., 1993). To the best of our knowledge no previous IC_{50} values were reported on the ACE-inhibitory activity of hydrolysates from *T. Mediterraneus*, *B. Boops* and *S. Canicula*.

Table 36. Degree of hydrolysis and IC_{50} value of the final hydrolysates

	Hydrolysate	DH (%)	IC_{50} (μg/mL)
Sardine	Subtilisin(2h)+Trypsin(2h)	14.9	439 ± 16^{a,b}
	Trypsin(2h)+Subtilisin(2h)	13.2	442 ± 25^{a,b}
	Subtilisin+Trypsin (simultaneous)	13.7	489 ± 22^{b}
Horse mackerel	Subtilisin(2h)+Trypsin(2h)	19.7	364 ± 38^{c}
	Trypsin(2h)+Subtilisin(2h)	18.2	398 ± 36^{a,c}
	Subtilisin+Trypsin (simultaneous)	21.0	279 ± 29^{d}
Axillary seabream	Subtilisin(2h)+Trypsin(2h)	17.2	375 ± 15^{c}
	Trypsin(2h)+Subtilisin(2h)	16.0	390 ± 16^{a,c}
	Subtilisin+Trypsin (simultaneous)	16.3	472 ± 44^{b}
Bogue	Subtilisin(2h)+Trypsin(2h)	17.6	637 ± 67^{e}
	Trypsin(2h)+Subtilisin(2h)	17.0	698 ± 70^{f}
	Subtilisin+Trypsin (simultaneous)	15.3	768 ± 2^{g}
Small-spotted catshark	Subtilisin(2h)+Trypsin(2h)	19.2	350 ± 20^{c,h}
	Trypsin(2h)+Subtilisin(2h)	18.3	281 ± 20^{d}
	Subtilisin+Trypsin (simultaneous)	17.3	302 ± 8^{d,h}

IC_{50} values are means of triplicate determinations ± standard deviation. Mean values within a column followed by different letter mean significant differences ($p<0.05$).

The superior ACE-inhibitory activity showed by small-spotted catshark may be due to the higher collagen content reported for elasmobranch, up to 10 % (Harnedy & FitzGerald, 2012). Collagen and its hydrolysed form, gelatin, are rich in non-polar amino acids such as proline which plays a significant role in the inhibition of ACE (Byun & Kim, 2001). Besides, small peptides having proline residue at the C-terminal are resistant to degradation by digestive enzymes and thus could be adsorbed intact (Li et al., 2003). In the case of horse mackerel, the good degradability of its protein by the enzymes employed, as confirmed by the DH values obtained for this species (Table 36), may be the reason for the high ACE-inhibitory activity exhibited by its hydrolysates. Higher DH implies larger quantities of low molecular weight peptides which are mainly responsible for ACE inhibition (Je et al., 2004; Li et al., 2012).

Additionally, Table 36 shows significant differences in the ACE-inhibitory activity of the hydrolysates from the same species when employing different enzymatic treatments. For

the hydrolysates of sardine, axillary seabream and bogue, the sequential addition of subtilisin (2h) and trypsin (2h) resulted in the hydrolysates with the lowest IC_{50} values. This finding may be attributed to the fact that also the highest DH was obtained for the hydrolysates of these species when using this enzymatic pattern (Table 36). In this sense, Matsui et al. (1993) also reported that the ACE-inhibitory activity exerted by an alkaline protease hydrolysate derived from sardine muscle considerably increased with increasing proteolysis. On the contrary, the simultaneous addition of subtilisin and trypsin led to the hydrolysates for these species with the lowest ACE-inhibitory activity. In the case of bogue, it may be due to the fact that the lowest DH was also obtained by this combination of enzymes. Nevertheless, this was not the same situation for sardine and axillary seabream. Thus, the lowest ACE-inhibitory activity showed by these hydrolysates might be related to different reasons than DH (Theodore & Kristinsson, 2007).

In accordance with a higher DH, the horse mackerel hydrolysates obtained by the simultaneous addition of subtilisin and trypsin gave rise to significantly higher ACE-inhibitory activity than the other hydrolysates of this species (Table 36). In the same line, small-spotted catshark hydrolysate obtained by the simultaneous addition of both enzymes also present the lowest IC_{50} value together with the hydrolysate produced by the sequential addition of trypsin (2h) and subtilisin (2h) (no significant differences were found between these two enzymatic treatments, Table 36). Nevertheless, this higher ACE-inhibitory activity of these hydrolysates cannot be explained with the DH values (Table 36). Thus, it must be attributed to other factors such as the amino acids composition of the peptides released during the hydrolysis. These findings are in agreement with previous works which found the simultaneous addition of subtilisin and trypsin as the most appropriate enzymatic treatment for the production of hydrolysates exhibiting ACE-inhibitory activity derived from goat milk (Espejo-Carpio et al., 2013) and mackerel and horse mackerel protein (García-Moreno et al., In press).

Consequently, the hydrolysates of horse mackerel and small-spotted catshark obtained by the simultaneous addition of subtilisin and trypsin, which exhibited the highest ACE-inhibitory activity, were selected for further fractionation.

3.3 SEC fractionation and ACE-inhibition of fractions

The elution profiles obtained by SEC fractionation of the two selected hydrolysates are shown in Fig. 34. Both profiles had a peak at short elution volume (5-10 mL) and they also

showed that most of their peptides eluted between 15 and 20 mL. However, as suggested by the lower DH of small-spotted catshark, the horse mackerel hydrolysate contained more small peptides which eluted after 20 mL (Fig. 34a). Seven fractions, designed as A-G, were collected from the horse mackerel hydrolysate and five, designed as A-E, from the small-spotted catshark hydrolysate. They are shown delimited by vertical lines in Fig. 34a and Fig. 34b, respectively.

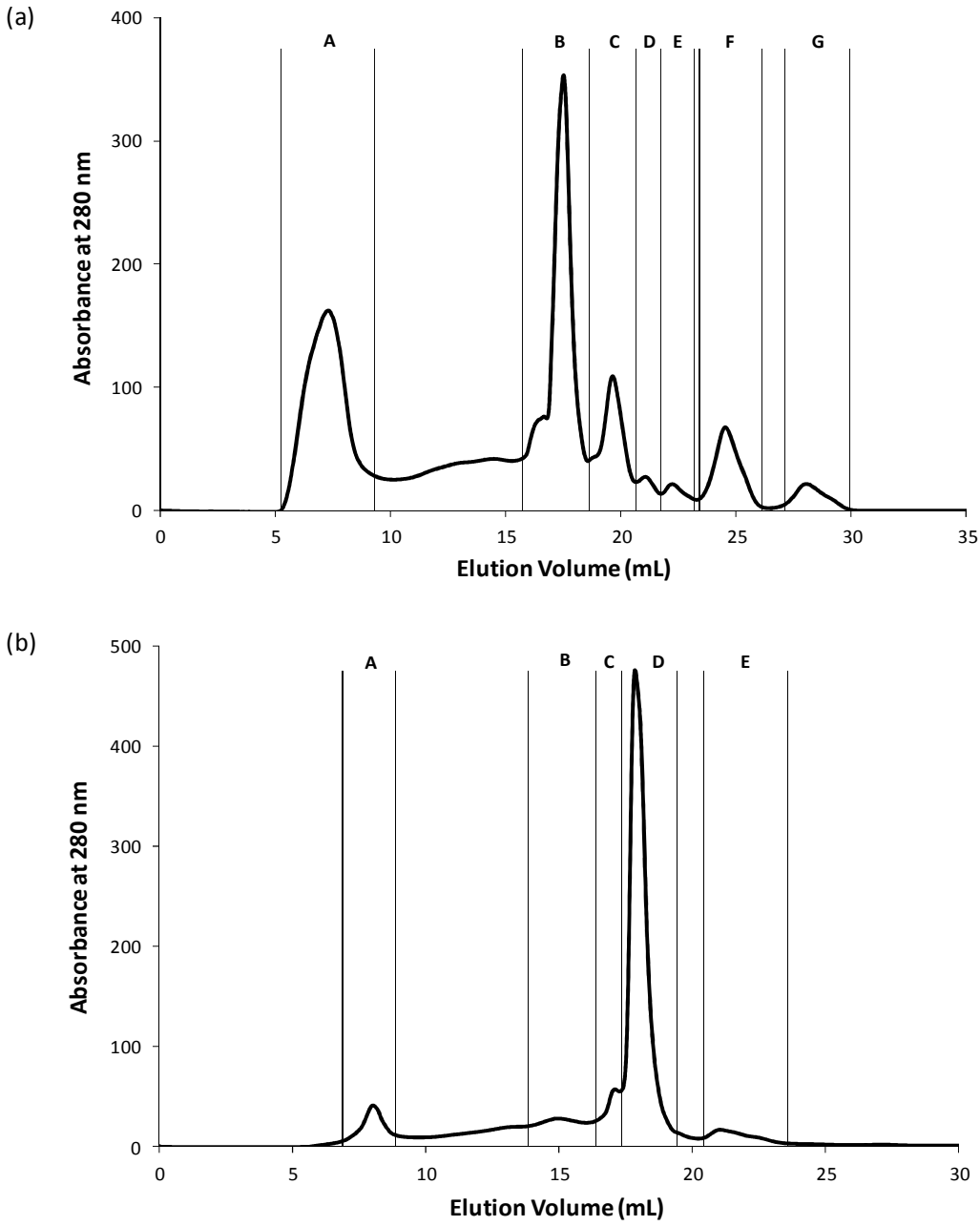

Figure 34. SEC profile of hydrolysates obtained with the enzymatic pattern subtilisin(2h)+trypsin(2h): (a) horse mackerel, (b) small-spotted catshark.

Table 37 shows the approximate size range of peptides, the protein content as well as the ACE-inhibitory activity of each fraction. Fractions D, E, F and G of horse mackerel and E of small-spotted catshark eluted after the standard Gly suggesting that the peptides and/or amino acids contained in these fractions interacted with the stationary phase. Thus, their size range could not be determined. It should also be mentioned that these results are different than those reported in chapter X for the same two hydrolysates. It may be due to the fact that different eluents and UV detectors were employed.

Table 37. Properties of the SEC fractions for the selected hydrolysates

Hydrolysate	Fraction	Molecular weight (Da)	Protein concentration[a] (μg/mL)	ACE inhibition[a] (%)	IC_{50} (μg/mL)
Horse mackerel	A	>15000	192	0	-
	B	130 - 2350	934	40	85 ± 7
	C	17 - 130	645	0	-
	D	ip	256	6	nm
	E	ip	56	3	-
	F	ip	127	1	-
	G	ip	30	5	-
Small-spotted catshark	A	>15000	90	0	-
	B	1210 - 15000	789	15	-
	C	470 - 1210	1076	26	72 ± 1
	D	58-470	1037	63	27 ± 2
	E	ip	259	9	-

ip: possible interactions of peptides with the stationary phase.
[a] Determinations were carried out in a ten-fold concentrated of the original fraction.
nm: this fraction did not show ACE inhibition and the IC_{50} value could not be measured.

For horse mackerel, it was observed that the fraction B, with a molecular weight range of 130 – 2,350 Da, exhibited the highest ACE-inhibitory activity (40 %). It was followed by the fraction D which inhibited ACE to 6 %. The fraction C, despite of its high protein content (645 μg/mL), did not exert ACE-inhibitory activity (Table 37). In order to compare the ACE inhibition capacity of the active fractions (B and D), their IC_{50} values were intended to be determined (Table 37). However, it was no possible for fraction D due to its low ACE-inhibitory activity. In the case of fraction B, an IC_{50} value of 85 μg/mL was obtained, a 3.3-fold increase of activity compared with that of horse mackerel hydrolysate.

This value is in the range of the one reported by Li et al. (2012) for a purified fraction of loach hydrolysate (IC_{50} = 89.6 μg/mL).

Regarding small-spotted catshark hydrolysate, fraction D, with a molecular weight range of 58-470, showed the highest ACE-inhibitory activity (63 %). It was followed by fraction C (26 %) having a size range of 470-1,210 Da, fraction B (15 %) with a molecular weight range of 1,210-1,5000 Da and fraction E (9 %). These results are in accordance with those reported by Je et al. (2004) which indicated an increase in the ACE-inhibitory activity exhibited by the fractions of a hydrolysate when decreasing their molecular weight. However, the lower ACE inhibition exerted by fraction E, despite of is theoretically lower size range, revealed that other properties different than molecular weight are also responsible for ACE-inhibitory activity. In this regard, Raghavan and Kristinsson (2009) reported that synergistic action amongst peptides present in the whole hydrolysate may enhance the ACE-inhibitory activity of the hydrolysate with respect to a purified fraction. Determining the IC_{50} values (Table 37), fraction C showed a lower IC_{50} value than the whole hydrolysate (IC_{50} = 72 μg/mL), resulting in a 3.2-fold increase of activity. Nevertheless, fraction D was confirmed as the fraction containing the most active peptides, with an IC_{50} value of 27 μg/mL (11.2 purification-fold). This value is similar than the IC_{50} value exhibited by a purified peptide from yellowfin sole frame (IC_{50} = 29 μg/mL) (Jung et al., 2006).

4. CONCLUSIONS

The evolution of the ACE-inhibitory activity with DH was dependent on the material hydrolyzed and the enzymatic treatment employed. In overall, the antihypertensive activity of the hydrolysates reached maximum values and then decreased slightly in the sequential periods of hydrolysis with subtilisin and trypsin. In the case of the simultaneous treatment, also a maximum value was observed at the first moments of the hydrolysis; then, the ACE-inhibitory activity was maintained practically constant or decreased slightly. The IC_{50} values of the hydrolysates ranged from 279 to 768 μg/mL. The highest ACE-inhibitory activity was found for the hydrolysates of horse mackerel (IC_{50}=279 μg/mL) and small-spotted catshark (IC_{50}=302 μg/mL), both obtained with the simultaneous enzymatic treatment. For the horse mackerel hydrolysate, 7 fractions were identified by size-exclusion chromatography, with fraction B (130-2,350 Da) exhibiting the highest ACE-

inhibitory activity (IC_{50}=85 μg/mL). In the case of small-spotted catshark hydrolysate, five fractions were identified, being fraction C (470-1,210 Da) and fraction D (58-470 Da) the ones presenting the highest ACE-inhibitory activity, IC_{50} of 72 and 27 μg/mL respectively. This high inhibitory activity of small-spotted catshark fractions may be related to the presence of small peptides containing proline at the C-terminal; however, it must be confirmed by determining the amino acid sequence of the active peptides.

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Final Conclusions

Based on the research carried out, the following conclusions were drawn:

1. Among the five discards species studied, sardine and horse mackerel exhibited the highest lipid content, 13.6 wt% and 6.2 wt% respectively, presenting also the highest amount of EPA+DHA, 3000 and 1300 mg/100 g fish, respectively. Besides, their oils had a composition of EPA+DHA higher than 23 wt%. On the other hand, axillary seabream and small-spotted catshark, in spite of their lower lipid content (5.1 y 2.7 wt% respectively) and their lower amount of EPA+DHA (960 and 650 mg/100 g fish respectively), also represent good raw materials for the production of fish oil with a composition of EPA and DHA higher than 20 wt%. The oil extracted from small-spotted catshark presented the highest content of DHA (up to 20 wt%).

2. The temperature and the airflow rate were statistically significant on the determination of the OSI of cod liver oil. Although the sample weight resulted to be not significant, the minimum obtained for the OSI may indicate an optimum relation of sample weight (6.91 g) and airflow rate (25 L/h), which guarantees an adequate saturation level in the reaction vessel. Moreover, the lineal model revealed that the temperature was the most influential factor affecting the OSI, with a temperature coefficient of -3.29×10^{-2} ºC^{-1}.

3. The highest pretreatment temperature assayed (55 ºC) improved the yield of oil obtained. It is due to a better tissue disruption and protein coagulation which facilitate the release of the oil. Moreover, pretreating the whole fish at 55 ºC may denature the endogenous lipases of the fish, which minimized the FFA content of the oil. However, the PV of the oil extracted was reduced when operating at the lowest pretreatment temperature (5 ºC). It was also revealed that the minimum pressure (60 bar) and one pressing stage minimized the oxidative stress in the oil and the residence time of the oil in the press which enhanced its quality in terms of acidity and oxidation parameters. Regarding efficiency of the extraction process, pressing at 60 bar using two pressing stages maximized the yield of oil. Finally, the multi-objective optimization of FFA, PV and yield generated a set of non inferior solutions (Pareto Front) which satisfied the three objectives to an adequate degree.

4. The optimum conditions to carry out the bleaching process of sardine oil with acid-activated earth (Tonsil 278), in terms of oxidation products removal, were: 130 ºC, clay amount of 5 wt% and 60 min. The highest temperature favored the

decomposition of hydroperoxides to secondary oxidation products which were adsorbed onto the activated clay surface. Nevertheless, the clay Tonsil 278 was more activated in terms of pigments and colored compounds adsorption at moderate temperature. A maximum for hue-angle (89.19) was obtained at 99.2 ºC, 5 wt% and 56.6 min; and a minimum for chroma (81.76) at 109.4 ºC, 5 wt% and 49.4 min. The simultaneous optimization of totox, hue-angle and chroma allowed to generate a decision space, which indicates the optimum values for the operating conditions depending on the grade of satisfaction desired for each individual variable.

5. Low concentrations of alpha-tocopherol (50-207 ppm) combined with sparing synergists like ascorbyl palmitate (450 ppm) and metal chelators such as citric acid (50 ppm), minimized the formation of hydroperoxides in the sardine oil studied. On the other hand, medium concentrations of alpha-tocopherol (478-493 ppm), which improved the destroying of hydroperoxides to stable products, reduced the formation of secondary oxidation products. The bi-objective optimization of PV and AV indicated the optimum concentration of alpha-tocopherol to be employed, together with ascorbyl palmitate (450 ppm) and citric acid (50 ppm), in order to minimize to an adequate degree the formation of hydroperoxides and their decomposition to secondary oxidation products.

6. The phospholipids evaluated (lecithin, phosphatidylcholine and phosphatidylethanolmine) showed similar DPPH scavenging activity, whereas the phosphatidylcholine exhibited the highest chelating activity. Moreover, no significant reducing power was observed for any of them. The emulsion with LC resulted in the most physically stable having the most negative zeta potential and the lowest mean droplet size. Besides, this emulsion was also the least oxidized, as confirmed by its lower PV at day 14 and by the lowest increase in volatiles oxidation products such as 1-penten-3-ol. It may be due to the incorporation of lecithin at the interface which allows the casein to adopt a different conformation. This fact may improve the thickness and structure of the interface, thus preventing lipid oxidation.

7. In order to obtain a high FAME content (95.39 wt%) for the biodiesel produced by alkaline transesterification (1 wt% NaOH) of fish oil, high temperature (60 ºC), high methanol to oil molar ratio (9:1) and long reaction time (90 min) were

required. However, with the high concentration of catalyst employed (1 wt%), the lowest temperature (40 ºC) maximized the yield of biodiesel. (83.6 %). This fact is due to a reduction in the saponification of triglycerides by NaOH at this low temperature. Acceptable values of kinematic viscosity were found (6.05-6.66 mm^2/s, measured at 30 ºC) for the biodiesel samples, with melting points ranging from -73.60 to 3.83 ºC. Finally, the biodiesel obtained showed a low oxidative stability (IP<2.22 h), which should be improved (i.e. by the addition of antioxidants).

8. The oils extracted from sardine, horse mackerel (*T. trachurus*) and mackerel had a high content of omega-3 PUFA (>22 wt%), highlighting the EPA and DHA content of sardine oil (13 and 17.6 wt%, respectively). The enzymatic hydrolysates obtained by their protein fraction exhibited a strong ACE-inhibitory activity, especially the hydrolysates obtained by the simultaneous enzymatic treatment for mackerel and horse mackerel (with IC_{50} values of 345 and 364 μg protein/mL, respectively). Moreover, the hydrolysates obtained showed an acceptable DPPH scavenging activity, with inhibition values of 45 % for horse mackerel, 40 % for sardine and 35 % for mackerel.

9. The DH had a different effect on the DPPH scavenging activity of the hydrolysates, which depended on the raw material hydrolysed and on the enzymatic treatment employed. Moreover, the hydrolysates obtained for the five species studied (*S. pilchardus*, *T. mediterraneus*, *P. Acarne*, *B. Boops* and *S. canicula)* had a protein content varying from 60.7 to 89.5 wt%, whereas the lipid content ranged from 4.6 to 25.3 wt%. The sardine hydrolysates showed the best antioxidant activity: a) the highest DPPH scavenging activity (IC_{50}=0.91 mg protein/mL, found for the hydrolysate obtained by the sequential treatment trypsin (2h) and subtilisin (2h); b) the highest Fe^{2+} chelating activity (IC_{50}=0.32 mg protein/mL); and c) the highest reducing power. Other hydrolysates also exhibited strong antioxidant activity, highlighting the DPPH scavenging activity of the horse mackerel (*T. mediterraneus*) hydrolysates (IC_{50}=1.47 mg protein/mL), the chelating activity of the small-spotted catshark hydrolysates (IC_{50}=0.32 mg protein/mL) and the reducing power of the bogue hydrolysates.

10. The influence of DH on the ACE-inhibitory activity of the hydrolysates also depended on the raw material to be hydrolyzed and on the enzymatic treatment

employed. The simultaneous addition of subtilisine and trypsine led to the final hydrolysates with the highest ACE-inhibitory activity, horse mackerel hydrolysate (IC_{50}=279 μg/mL) and small-spotted catshark hydrolysate (IC_{50}=302 μg/mL). For the horse mackerel hydrolysate, a fraction containing peptides in the range 130-2,350 Da exhibited de highest antihypertensive activity (IC_{50}=85 μg/mL). For the small-spotted catshark hydrolysate, a purified fraction (58-470 Da) showed the highest ACE-inhibitory activity with an IC_{50} of 27 μg/mL. The high inhibitory activity of this fraction may be related to the presence of small peptides containing proline at the C-terminal.

Conclusiones Finales

De la investigación desarrollada se extraen las siguientes conclusiones:

1. De entre las cinco especies de descarte estudiadas, sardina y jurel son las que presentaron el mayor contenido en grasa, 13.6 y 6.2 wt% en verano, con un alto contenido en EPA+DHA (3000 y 1300 mg/100 g pez, respectivamente). Además, los aceites extraídos de estas especies mediante prensado presentaron un contenido en EPA+DHA mayor del 23 wt%. Por otra parte, el aligote y la pintarroja, a pesar de poseer un menor contenido en lípidos (5.1 y 2.7 wt% respectivamente) y en EPA+DHA (960 y 650 mg/100 pez respectivamente), los aceites extraídos también mostraron un porcentaje en EPA+DHA mayor del 20 wt%. Destaca el aceite de pintarroja, con un contenido en DHA de hasta el 20 wt%.

2. Tanto la temperatura como el caudal de aire resultaron estadísticamente significativos en la determinación del índice de estabilidad oxidativa (OSI) de aceite de hígado de bacalao. Aunque la masa de muestra no resultó significativa en la determinación del OSI, el mínimo de OSI obtenido para el modelo cuadrático indica que podría existir una relación de caudal de aire (25 L/h) y masa de muestra (6.91 g) que optimizaría las condiciones de saturación del aceite en el recipiente de reacción. Por otra parte, el ajuste lineal puso de manifiesto la alta influencia de la temperatura con un coeficiente de -3.29×10^{-2} ºC^{-1}.

3. La mayor temperatura de pretratamiento ensayada (55 ºC) mejoró el rendimiento de extracción de aceite, debido a una mayor ruptura del tejido adiposo y una posible coagulación de proteínas. Además, la acidez del aceite fue mínima cuando se precalentó a 55 ºC, como consecuencia de una posible desnaturalización de lipasas endógenas. No obstante, la formación de hidroperóxidos se minimizó a 5 ºC. El prensado con una etapa y 60 bar minimizó el FFA y PV del aceite, ya que ello conlleva un menor tiempo de operación y someter al aceite a menos esfuerzos mecánicos en la prensa. Sin embargo, utilizar dos etapas de prensado a 60 bar maximizó el rendimiento de aceite obtenido. En último lugar, la optimización simultánea de FFA, PV y rendimiento reveló el valor de las condiciones de proceso necesarias para obtener un aceite que satisfaga las condiciones de calidad y rendimiento en el grado requerido.

4. Las condiciones óptimas para la decolorización del aceite de sardina empleado con tierras Tonsil 278, en términos de productos de oxidación, resultaron ser: 130 ºC, 5

wt% de tierras y 60 min. La temperatura más alta evaluada mejora la descomposición de hidroperóxidos a compuestos secundarios de oxidación, los cuales son posteriormente adsorbidos por las tierras activadas. No obstante, las tierras Tonsil 278 se activaron de forma más adecuada para la adsorción de pigmentos y compuestos coloreados a temperaturas medias (99-110 ºC). Un máximo para el tono (89.19) fue obtenido a 99.2 ºC, 5 wt% y 56.6 min; y un mínimo para el croma (81.76) a 109.4 ºC, 5 wt% y 49.4 min. La optimización conjunta de totox, tono y croma permitió generar un espacio de decisión a partir del cual decidir las condiciones de operación dependiendo del grado de satisfacción deseado para cada variable.

5. Bajas concentraciones de alfa-tocoferol (50-207 ppm) combinadas con la adicción de antioxidantes secundarios como palmitato de ascorbilo (450 ppm) y ácido cítrico (50 ppm), minimizaron la formación de hidroperóxidos en el aceite de sardina estudiado. Sin embargo, concentraciones medias de alfa-tocoferol (478-493 ppm), las cuales podrían mejorar la conversión de hidroperóxidos a productos estables, redujeron el nivel de productos secundarios de oxidación. La optimización simultánea de PV y AV determinó la concentración de alfa-tocoferol a utilizar, junto con palmitato de ascorbilo (450 ppm) y ácido cítrico (50 ppm), para minimizar la formación de peróxidos y productos secundarios de oxidación en el nivel deseado.

6. Los fosfolípidos analizados presentaron similar actividad inhibidora de DPPH, mientras que la fosfatidilcolina presentó una mayor actividad quelante seguida de la fosfatidiletanolamina. Por otra parte, su poder reductor fue insignificante. La emulsión estabilizada con una combinación de caseína y lecitina resultó en la más estable físicamente, con el potencial zeta más negativo y el menor tamaño de gota. Además, esta emulsión fue la más estable oxidativamente, tanto en términos de peróxidos como productos secundarios de oxidación tales como 1-penten-3-ol. Esto puede deberse principalmente a una reestructuración de la proteína en la interfase como consecuencia de la incorporación de la lecitina, dando lugar a un grosor y estructura mejorada de la capa interfacial de la emulsión.

7. En la transesterificación alcalina (1 wt% NaOH) con metanol de aceite de pescado, altas temperatura (60 ºC) junto con una alta relación molar metanol a aceite (9:1) y un tiempo de reacción largo (90 min) son requeridos para conseguir una alta pureza

en FAME (95.39 wt%). No obstante, empleando una alta concentración de catalizador (1 wt% NaOH), la mínima temperatura (40 ºC) mejora el rendimiento en biodiesel (83.6 %), ya que se reduce la formación de jabones por saponificación de triglicéridos. Valores aceptables de viscosidad fueron obtenidos (6.05-6.66 mm^2/s medidos a 30 ºC), con un rango de fusión de las muestras de biodiesel entre -73.60 y 3.83 ºC. Por otra parte, el biodiesel obtenido presentó un baja estabilidad oxidativa (IP<2.22 h) que deberá ser mejorada (e.g. adicionando antioxidantes).

8. Los aceites de sardina, jurel (*T. trachurus*) y caballa presentaron un alto contenido en omega-3 PUFA (>22 wt%), destacando el contenido en EPA (13 wt%) y DHA (17.6 wt%) del aceite de sardina. Los hidrolizados procedentes de su fracción proteica exhibieron una buena inhibición de ECA, destacando los hidrolizados de caballa (345 μg proteína/mL) y jurel (364 μg proteína/mL), obtenidos con el tratamiento simultáneo de subtilisina y tripsina. Además, los hidrolizados producidos a partir de estas especies presentaron buena actividad antioxidante, con valores de inhibición de DPPH de 45 % para el jurel, 40 % para la sardina y 35 % para la caballa.

9. La influencia del DH en la actividad inhibidora de DPPH depende tanto del sustrato a hidrolizar como del tratamiento enzimático empleado. Además, en función de la especie, el contenido en proteína y lípidos de los hidrolizados finales varió en los rangos 60.7-89.5 wt% y 4.6-25.3 wt%, respectivamente. Los hidrolizados de sardina son los que poseen mejores propiedades antioxidantes: a) mayor actividad inhibidora de DPPH para el hidrolizado obtenido con tripsina (2h) y subtilisina (2h) (IC_{50}=0.91 mg proteína/mL), b) mayor actividad quelante de Fe^{2+} (IC_{50}=0.32 mg proteína/mL), y c) mayor poder reductor. Hidrolizados de otras especies también presentaron buenas propiedades antioxidantes. En este sentido, destacan los hidrolizados de jurel (*T. mediterraneus*) en la inhibición de DPPH (IC_{50}=1.47 mg proteína/mL), los de pintarroja como quelantes (IC_{50}=0.32 mg proteína/mL), y los de boga por su poder reductor.

10. Al igual que para la actividad antioxidante, la influencia del DH en la actividad inhibidora de ACE depende del material a hidrolizar y del tratamiento enzimático utilizado. La adicción simultánea de subtilisina y tripsina condujo a los hidrolizados finales con mayor propiedad antihipertensiva, jurel (IC_{50}=279 μg/mL) y pintarroja (IC_{50}=302 μg/mL). En el hidrolizado de jurel se identificó una fracción en el rango

130-2,350 Da, que exhibió la máxima inhibición de ACE (IC_{50}=85 μg/mL). En el hidrolizado de pintarroja, se llegó a obtener una fracción purificada (58-470 Da) con un IC_{50} de 27 μg/mL. La alta actividad de esta fracción puede deberse a la presencia de péptidos pequeños conteniendo prolina en el C-terminal.

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