Nuria Rubio Rodríguez

SUPERCRITICAL FLUID TECHNOLOGY FOR EXTRACTION, CONCENTRATION & FORMULATION OF OMEGA-3 RICH OILS

A NOVEL STRATEGY FOR VALORIZATION OF FISH BY-PRODUCTS

Chemical & Food Engineering Group
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PhD Thesis directed by

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UNIVERSIDAD DE BURGOS
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TESIS DOCTORAL

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Memoria para optar al grado de Doctor por la Universidad de Burgos presenta la licenciada
NURIA RUBIO RODRÍGUEZ

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La presente Tesis Doctoral queda registrada en el folio nº 10/12/10 del correspondiente Libro de Registros, con el nº 10877.

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En Burgos a 10 de Diciembre de 2010

Fdo. Sagrario Beltrán Calvo
Directora del Departamento

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Como autora de esta Tesis, me complace poder expresar mi más profundo agradecimiento a todas aquellas instituciones, profesores, compañeros, familiares y amigos que, en estos últimos años, me han ayudado y apoyado de una u otra forma haciendo posible que todo el esfuerzo y la dedicación puesta en esta aventura haya llegado a buen término.

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me as a member more. Among them, I would like especially to thank Orlando, both for his patient to explain to me how that strange "supercritical extraction pilot plant" worked and for his technical support during all these years; and also to Beti, for her help to use the analytical equipments and to develop most of the analytical methods carried out in this work.

Finally, I want to thank my family and friends for believing in my capability to face this challenge and also all the people who have been daily close to me and, those that, as my grandfather, have been a little bit further.

This Thesis is dedicated to all of them.

Nuria

There is nothing like a dream to create the future.

Victor Hugo

GENERAL INDEX

Focus of the Work & Main Targets

Summary / Resumen

SUMMARY OF THE THESIS

The present research work is focused on the study of novel production processes of lipid extracts with a high content of omega-3 fatty acids through a green technology, such as supercritical fluid technology, and using fish by-products as raw material.

Omega-3 fatty acids, especially EPA and DHA, have acquired a great importance in current society since several scientific essays have proved the positive effect of these compounds on the prevention and treatment of many diseases. In the last years, the consumption of nutritional supplements and omega-3 fortified food products has increased noticeably, hence, the search of new omega-3 natural sources, the design of competitive omega-3 production processes and the development of different strategies for omega-3 incorporation as ingredients in food products have become fields of research worth to be explored.

The most important natural source of omega-3 is fish oil, which is usually obtained by a physical process (cooking, pressing and centrifuging) from small size fish, such as herring, or from fish by-products. Due to its high level of impurities (free fatty acids, heavy metals…), the fish oil obtained by physical extraction is a crude oil no suitable for human consumption and with a low omega-3 content (less than 30 %), thus, it must be subsequently refined and concentrated. Current refining procedures are based on physical and chemical methods (neutralization, degumming, deodorization…), which often require the use of high temperatures and / or organic solvents. On the other hand, common omega-3 concentration procedures are based on separation process (distillation, urea complexation, chromatography…), able to isolate omega-3 EPA and / or DHA with a good efficiency, but as ethyl ester instead of as in their natural acylglyceride form, which is easier metabolized by humans and more stable against oxidation. Finally, due to the high tendency to oxidation of the polyunsaturated fatty acids, such as omega-3, several strategies have been designed in order to prolong the omega-3 shelf-life, being the addition of antioxidants the most common practice.

Furthermore, the use of fish oil and omega-3 concentrates as ingredients in food products and / or nutraceuticals usually requires a previous formulation as a dry powder in order to enhance the dispersion of the lipid extract within the food matrix (meat, milk…) and mask the sensorial impact of the fishy odour compound in the final food product. Among fish oil formulation processes, microencapsulation in a solid matrix by spray-drying is the most common method used nowadays at industrial scale.

As alternative to these conventional processes, this project proposes the use of different methods based on the supercritical fluid technology in order to obtain lipid extracts rich in omega-3 and formulated as dry powder from fish by-products, as it is presented schematically in Figure 1. The main objectives of the work, developed in four chapters, are summarized below:

Chapter 1 *Omega-3 fatty acids: Health implications & Current Productions Processes. A Review.*

This chapter is focused on a bibliographical search of the current situation of omega-3 fatty acids, considering both the last clinical studies about their beneficial for human health and the most recent production processes at industrial scale, either based on physical and chemical methods or based on the supercritical fluid technology.

Chapter 2 *Supercritical Fluid Extraction of Fish Oil rich in Omega-3 from Fish by-products.*

This chapter is focused on fish oil production from fish by-products, being the main targets:

- The characterisation of different fish by-products (offcuts and livers from kingklip, hake, salmon, orange roughy or jumbo squid) provided by a fish industry, Pescanova S.L., in order to establish their profitability as raw material of high quality fish oil rich in omega-3.
- The study of a fish oil extraction process from fish by-products by using

supercritical carbon dioxide, SC-CO_2, as solvent, taking into account the influence of different process parameters on both, extraction yield and oil quality, in order to establish the optimal operation conditions and carry out the modelling of the process required for its scale-up.

- The comparative study of the quality and sensorial parameters of fish oil extracted from conventional physical and chemical processes and by supercritical fluid extraction (SFE) processes in order to establish the implications of using SC-CO_2 as lipid solvent on fish oil quality.
- The study of the oxidation stability along time of the fish oil extracted by SC-CO_2, in order to establish its shelf-life and select the best storage conditions.

Chapter 3 *Fish Oil Refining & Omega-3 Concentration by Supercritical Fluids.*

This chapter is focused on the use of SC-CO_2 in fish oil refining and production of lipid extracts enriched in omega-3, and involves the followed targets:

- The development of an extraction - fractionation processes in order to obtain a deacidificated fish oil directly from fish by-products taking advantage of the tunability of the solvation powder of SC-CO_2 by changing pressure and / or temperature.
- The design of a continuous coupled extraction - enzymatic reaction - fractionation process based on the use of an immobilized sn 1,3-especific lipase and SC-CO_2 as reaction media, in order to obtain omega-3 concentrates, mainly DHA, as acylglycerides from fish oil and / or fish by-products.

Chapter 4 *Use of Supercritical Fluids in Fish Oil Stabilisation & Formulation.*

This chapter is focused on the study of a novel liquid microencapsulation process based on the use of SC-CO_2, PGSS-drying, for the production of dry powder fish oil

microencapsulated in a solid maltodextrine / chitosan coating able to prevent the omega-3 oxidation.

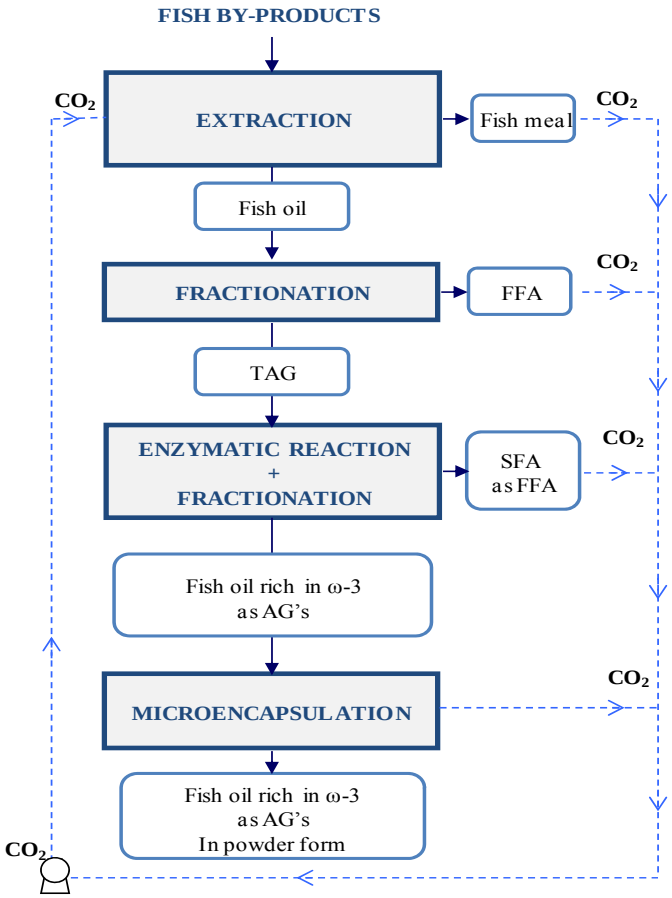

FIGURE 1

Scheme of the global process to obtain fish oil rich in omega-3 fatty acids from fish by-products using supercritical fluid technology proposed in this work. AG's: Acylglycerides. FFA: Free Fatty Acids. TAG: Triacylglycerides SFA: Saturated Fatty Acids.

RESUMEN DE LA TESIS

El presente trabajo de investigación se ha centrado en el estudio de procesos no convencionales de obtención de extractos lipídicos con un alto contenido en ácidos grasos omega-3, basados en el uso tecnologías limpias, como la tecnología de fluidos supercríticos y utilizando subproductos de la pesca como materia prima.

Los ácidos grasos omega-3, y en especial el EPA y el DHA, han adquirido una gran importancia en la sociedad actual desde que diversos estudios científicos probaran el efecto positivo de estos compuestos en la prevención y tratamiento de muchas enfermedades. En los últimos años, el consumo de suplementos nutricionales y alimentos enriquecidos en omega-3 se ha incrementado notablemente, de ahí que la búsqueda de nuevas fuentes naturales de omega-3, el diseño de métodos competitivos de producción de aceite de pescado y concentrados de omega-3 a gran escala y el desarrollo de diferentes estrategias para la incorporación de omega-3 como ingredientes en productos alimenticios se hayan convertido hoy en día en interesantes líneas de investigación.

La fuente natural más importante de omega-3 es el aceite de pescado, el cual suele obtenerse mediante un proceso físico (cocción, prensado y centrifugado) a partir de pescados de pequeño tamaño, como el arenque, o de subproductos de pescado. Dado su alto nivel de impurezas (ácidos grasos libres, metales pesados), el aceite obtenido mediante extracción física es un aceite bruto no destinado a consumo humano y con un contenido relativamente bajo en omega-3 (inferior al 30 %), razón por la cual debe ser posteriormente refinado y concentrado. Los procesos actuales de refinado se basan en métodos físico-químicos (neutralización, desgomado, deodorización…), los cuales a menudo requieren del uso de altas temperaturas y / o disolventes orgánicos. Por otro lado, los procedimientos comunes de concentración de omega-3 se basan en procesos de separación (destilación, complejación con urea, cromatografía…), capaces de aislar omega-3 EPA y / o DHA con buena eficacia, pero en forma de ésteres etílicos en lugar

de en su forma natural de acilglicéridos, la cual es más fácil de metabolizar por el organismo y más estable frente a la oxidación. Finalmente, debido a la alta tendencia a la oxidación de los ácidos grasos poliinsaturados, como los omega-3, se han diseñado diversas estrategias con el objetivo prolongar su vida útil, siendo la adición de antioxidantes la práctica más habitual hoy en día. Además, el uso de aceite de pescado y concentrados de omega-3 como ingredientes en productos alimenticios y / o nutracéuticos requiere a menudo de una formulación previa en forma de polvo seco para mejorar la dispersión del extracto lipídico dentro de la matriz del alimento (carne, leche…) y enmascarar en el producto final el impacto sensorial derivado de la adición de compuestos volátiles característicos del pescado. Entre los procesos de formulación de aceite de pescado, la microencapsulación en una matriz sólida mediante spray-drying es el método más común empleado hoy en día a escala industrial.

Como alternativa a estos métodos convencionales, en este proyecto se propone el uso de la tecnología de fluidos supercríticos para obtener extractos lipídicos ricos en omega-3 y formulados en polvo a partir de subproductos de la pesca, tal y como se muestra esquemáticamente en la Figura 1.

Los principales objetivos del trabajo, desarrollados en cuatro capítulos, se resumen a continuación.

Capítulo 1 *Ácidos grasos Omega-3: Implicaciones en la salud & procesos de producción actuales.*

Este capítulo se centra en el estudio bibliográfico llevado a cabo sobre la situación actual de los omega-3, considerando tanto los últimos estudios clínicos sobre sus beneficios para la salud humana como los procesos más recientes de producción a escala industrial, tanto basados en métodos físico-químicos como basados en la tecnología de fluidos supercríticos.

Capítulo 2 *Extracción con Fluidos Supercríticos de aceite rico en omega-3 a partir de subproductos de pescado.*

Este capítulo se centra en la producción de aceite de pescado a partir de subproductos de la pesca, siendo los principales puntos a desarrollar:

- La caracterización de diferentes subproductos de pescado (pieles y / o hígados de rosada, merluza, salmón, orange roughy o calamar gigante), suministrados por una industria pesquera, Pescanova S.L., con el objetivo de establecer su potencial como materia prima para la obtención de aceite de pescado rico en omega-3 de alta calidad.
- El estudio del proceso de extracción de aceite de pescado a partir de subproductos mediante el uso de dióxido de carbono supercrítico, CO_2-SC, como disolvente, teniendo en cuenta la influencia de diferentes parámetros del proceso tanto en el rendimiento de la extracción como en la calidad del aceite extraído, con la finalidad de establecer las condiciones óptimas de operación y llevar a cabo el modelo del proceso necesario para su cambio de escala.
- El estudio comparativo de los parámetros de calidad y sensoriales del aceite de pescado extraído mediante métodos físico-químicos y mediante la extracción con CO_2-SC, con el objetivo de establecer la implicaciones del uso del CO_2-SC como disolvente de lípidos en la calidad del aceite de pescado.
- El estudio de la estabilidad del aceite de pescado extraído con CO_2-SC frente a la oxidación a lo largo del tiempo, con la finalidad de establecer su vida útil y seleccionar las mejores condiciones de conservación.

Capítulo 3 *Refinado de aceite de pescado & concentración de omega-3 mediante la tecnología de fluidos supercríticos.*

Este capítulo se centra en el uso del SC CO2 en el refinado del aceite de pescado y la obtención de extractos lipídicos enriquecidos en omega-3, y ha sido desarrollado en los siguientes puntos:

- El estudio de un proceso de extracción-fraccionamiento para la obtención de aceite de pescado con baja acidez directamente a partir de subproductos, variando la capacidad solvente del SC-CO_2 con la presión y / o la temperatura.
- El diseño de un proceso en continuo de extracción - reacción enzimática - fraccionamiento basado en el uso de una lipasa sn 1,3-específica y del CO_2-SC como medio de reacción para la obtención de concentrados de omega-3, principalmente DHA, en forma de acilglicéridos a partir de aceite de pescado y / o subproductos de la pesca.

Capítulo 4 *Empleo de la tecnología de fluidos supercríticos en la estabilización y formulado de aceite de pescado.*

Este capítulo se centra en el estudio de un proceso novedoso de encapsulación de líquidos basado en el uso del CO_2-SC, el PGSS-drying, para la producción de aceite de pescado en polvo microencapsulado con un recubrimiento de maltodextrina / quitosan capaz de prevenir la oxidación de los ácidos grasos omega-3.

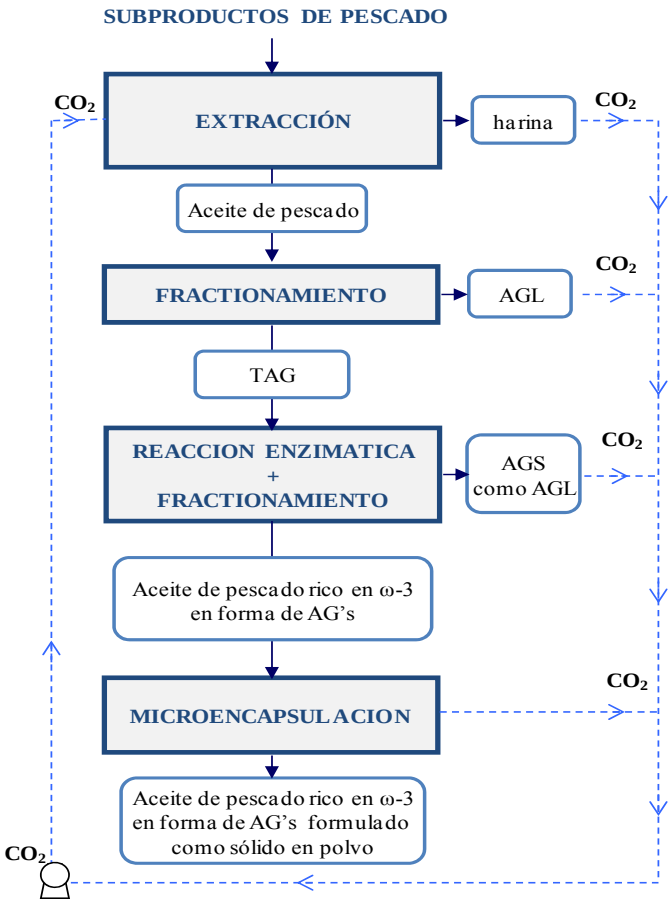

FIGURA 1

Esquema del proceso global propuesto en este trabajo para la obtención de aceite rico en omega-3 en forma de sólido mediante el uso de la tecnología de fluidos supercríticos. AGL: ácidos grasos libres. AG's: acilglicéridos. AGS: Ácidos grasos saturados. TAG: triacilglicéridos.

Chapter 1

Omega-3 fatty acids:

Health Implications & Current Production Processes

This Chapter has been partially published in the Journal of Innovative Food Science and Emerging Technologies (*Rubio-Rodríguez, et al.*, 2010)

SUMMARY

Since, at the end of the ´80s, epidemiological studies began to establish a certain relationship between a high fatty fish consumption and a low incidence of cardiovascular diseases in Nordic people, the amount clinical and biological essays about polyunsaturated fatty acids (PUFA), especially omega-3 fatty acids, has greatly increased, being voluminous the current available information related to their biochemical mechanisms of action, biological effects and health implications. Nowadays, nobody doubts about the importance of omega-3 fatty acids, especially EPA and DHA, in human diet, which recommended daily intakes have been established by several nutritionist committees all over the world. As a consequence, the demand of omega-3 enriched products and nutraceuticals has grown up noticeably, especially in those societies with a diet based a on high consumption of seed oils and meat, which has promoted the development of plenty of novel strategies and competitive methods for producing, on large scale, the high quality omega-3 concentrates requested for both food and pharmaceutical industries.

This chapter is proposed as an overview of the current situation of omega-3 fatty acids. Firstly, a brief introduction about essential fatty acids, focusing on the most relevant clinical studies published in the last 10 years about their role in human health. The last part of the chapter reviews the state-of-the-art of fish oil and omega-3 production processes on large scale, with an emphasis on the scientific articles and patents published from 2000 to 2010 and distinguishing two main groups: conventional methods based on physical and chemical processes and alternative methods based on the use of supercritical fluid technologies. In addition, a short compilation of different fish oil and omega-3 concentrates currently available in the market is presented.

RESUMEN

Desde que, a finales de los años ’80, estudios epidemiológicos empezaran a establecer una cierta relación entre el alto consumo de pescado graso y una baja incidencia de enfermedades cardiovasculares en poblaciones nórdicas, el número de ensayos clínicos y biológicos sobre los ácidos grasos poliinsaturados (AGPI), especialmente los ácidos grasos omega-3, se ha incrementado enormemente, siendo muy voluminosa la información de que se dispone actualmente sobre sus mecanismos bioquímicos de actuación, sus efectos biológicos y sus implicaciones en la salud humana. Hoy en día nadie duda de la importancia de los ácidos omega-3, especialmente EPA y DHA, en la dieta, cuyas ingestas diarias recomendadas han sido establecidas por diversos comités de nutricionistas en todo el mundo. Como consecuencia, la demanda de productos enriquecidos en omega-3 y nutracéuticos ha crecido notablemente, en especial en aquellas sociedades con una dieta basada en un elevado consumo de aceites de semillas y carne, lo cual ha impulsado un gran desarrollo de nuevas estrategias y métodos competitivos para la producción, a gran escala, de los concentrados de omega-3 de alta calidad requeridos tanto por la industria alimentaria como por la farmacéutica.

Este capítulo se propone como una revisión general del panorama actual de los ácidos grasos omega-3. En primer lugar, se presenta una breve introducción a los ácidos grasos esenciales, centrada en los estudios clínicos más relevantes publicados en los últimos 10 años acerca de su papel en la salud humana. En la última parte del capítulo, se resumen los procesos más novedosos de producción de aceite de pescado y omega-3 a gran escala, con énfasis en los artículos científicos y patentes publicados desde el año 2000 al 2010 y distinguiendo dos grupos principales: métodos convencionales basados en procesos físico-químicos y métodos alternativos basados en el uso de la tecnología de fluidos supercríticos. Así mismo, se recoge una pequeña recopilación de diferentes aceites de pescado y concentrados de omega-3 disponibles actualmente en el mercado.

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NOMENCLATURE

AA	Arachidonic Acid
ALA	Alpha-Linolenic Acid
AV	Anisidine Value
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CHD	Cardiovascular Heart Disease
CP	Critical Point
DGLA	Dihomo-Gamma Linolenic Acid
DHA-EE	Docosahexaenoic Acid (Ethyl ester)
DPA	Docosapentaenoic Acid (n-3)
EDTA	Ethylenediaminetetraacetic acid
EPA-EE	Eicosapentaenoic Acid (Ethyl ester)
FAEE	Fatty Acid Ethyl Ester
FAO	Food and Agriculture Organisation
FFA	Free Fatty Acid
GC	Gas Ghromatography
GLA	Gamma-Linolenic acid
GOED	Global Organization for EPA and DHA
HPLC	High Pressure Liquid Chromatography
ISSFAL	International Society for the Study of Fatty Acids and Lipids
LA	Linoleic Acid
LC-PUFA	Long Chain Polyunsaturated Fatty Acids
MUFA	Monounsaturated Fatty Acid
P	Pressure (MPa)
P_c	Critical Pressure (MPa)
P_r	Reduced Pressure (dimensionless)

PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-para-dioxins
PCDFs	Polychlorinated dibenzofurans
PGSS	Particles from Gas Saturated Solutions
PUFA	Polyunsaturated Fatty Acid
PV	Peroxide Value
RESS	Rapid Expansion of Supercritical Solutions
SAS	Supercritical Anti Solvent precipitation
SC-CO_2	Supercritical Carbon Dioxide
SCF	Supercritical Fluid
SFA	Saturated Fatty Acids
SFC	Supercritical Chromatography
SFE	Supercritical Fluid Extraction
SFEE	Supercritical Fluid Extraction of Emulsions
SFF	Supercritical Fluid Fractionation
SSI	Supercritical Solvent Impregnation
T	Temperature (K)
T_c	Critical Temperature (K)
T_r	Reduced Temperature (dimensionless)
TAG	Triacylglycerides
TP	Triple Point
UFA	Unsaturated Fatty Acids
WHO	World Health Organization
WHO-TEQ	World Health Organization Toxic Equivalent

Greek Letters

ρ	Density (kg / m^3)
ρ_c	Critical density (kg / m^3)
ρ_r	Reduced density (dimensionless)

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1.1. Omega-3 Fatty Acids: reasons for being interesting

Abstract

Omega-3 fatty acids have been very often the subject of scientific research in the last years, being numerous the studies developed about their biological effects and health implications.

In this Section, a description of the role of essential fatty acids in the organism and the influence of omega-3 fatty acids in human health is presented, as well as the requirements of these fatty acids on human diet.

➢ **Section Content**

1.1.1. Introduction to essential fatty acids

Fatty acids are organic compounds formed by a hydrocarbonated chain and a carboxylic group that is normally bounded with glycerol forming acylglycerides (mono-, di- or triglycerides).

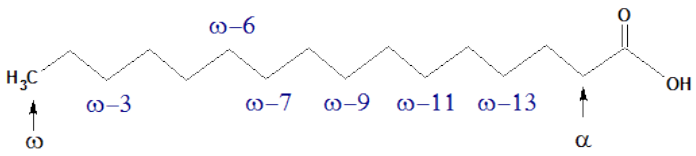

FIGURE 1.1

Common carbon positions in the hydrocarbonated chain of fatty acids.

TABLE 1.1

Common fatty acids in human diet.

Type		Common name	Carbon number	Double bounds C-C	Formula
SFA		Butyric	4	0	C4:0
		Caproic	6	0	C6:0
		Caprilic	8	0	C8:0
		Capric	10	0	C10:0
		Lauric	12	0	C12:0
		Myristic	14	0	C14:0
		Palmitic	16	0	C16:0
		Stearic	18	0	C18:0
		Araquidic	20	0	C20:0
		Behenic	22	0	C22:0
		Lignoceric	24	0	C24:0
MUFA	ω 9	Palmitoleic	16	1	C16:1n-9
		Oleic	18	1	C18:1n-9
		Gadoleic	20	1	C20:1n-9
	ω 11	Gondoic	20	1	C20:1n-11
	ω 13	Erucic	22	1	C22:1n-13
PUFA	ω 6	Linoleic	18	2	C18:2n-6
		γ-Linolenic	18	3	C18:3n-6
		Dihomo- γ-Linolenic	20	3	C20:3n-6
		Araquidonic	20	4	C20:4n-6
	ω 3	α-Linolenic	18	3	C18:3n-3
		Stearidonic	18	4	C18:4n-3
		EPA	20	5	C20:5n-3
		DPA	22	5	C22:5n-3
		DHA	22	6	C22:6n-3

The most common fatty acids in nature are reported in Table 1.1. They are classified according to the number of unsaturations (number of double bounds in the hydrocarbonated chain) as saturated (SFA), monounsaturated (MUFA) or polyunsaturated fatty acids (PUFA); or according to the position of the first C-C double bound as omega-3, -6, -7, -9, -11 or 13 fatty acids, where the term omega, expressed as "ω" or "n", is related to the carbon position further from the functional group –COOH, as shown in Figure 1.1.

The human body is able to synthesize SFA such palmitic acid (C16:0) and MUFA of the n-7 and n-9 series such as palmitoleic acid (C16:1n-7) or oleic acid (C18:1n-9). However, it cannot produce fatty acids with a C-C double bound in the position n-6 or n-3 of the hydrocarbonated chain such as linoleic acid (C18:2n-6, LA) or α-linolenic acid (C18:3n-3, ALA), which are considered essential fatty acids in human nutrition and must be taken directly for the diet.

LA and ALA are the parent of omega-6 and omega-3 fatty acids respectively and, from them, other omega-6 such as arachidonic acid (C20:4n-6; AA) or omega-3, as eicosapentaenoic acid (C20:5n-3; EPA), docosapentaenoic acid (C22:5n-3, DPA) and docosahexaenoic acid (C22:6n-3, DHA), can be synthesized in the human body by a series of enzymatic reactions based on desaturation and elongation of the hydrocarbonated chain (*see* Figure 1.2). Nevertheless, since LA and ALA compite for the same enzymes, a high level of LA in the organism, related to a high consumption of vegetable oils, means a lower conversion of ALA in

EPA and DHA, and, for that reason, these omega-3 fatty acids are considered essential fatty acids too.

1.1.2. Omega-3 fatty acids and human health

The implications of fatty acids in human health have been the subject of many scientific articles and reviews in the last years. One of the most complete compilations of scientific research on this subject can be found in the book edited by Chow (*Chow*, 2000) and one of the most recent review articles on dietary fat and cancer risk has been published by Chen et al. (*Chen, et al.*, 2007).

Specifically dealing with omega-3 fatty acids, there is also plenty of research. Their importance in human health was realized in the ´80s when several authors (*Dyerberg*, 1986, *Kromann, et al.*, 1980) published epidemiological studies showing the relation between an omega-3 enriched diet and the prevention of some diseases like myocardial infarction or bronchial asthma. Subsequently, an enormous number of epidemiological and clinical studies have deal with the effect of omega-3 PUFA, especially EPA and DHA, in human health and the mechanism by which this effect takes place.

In this section, we will only mention the latest review articles that compile most of the research done on this subject and summarise the conclusions to clarify the real effect of omega-3 PUFA in human health.

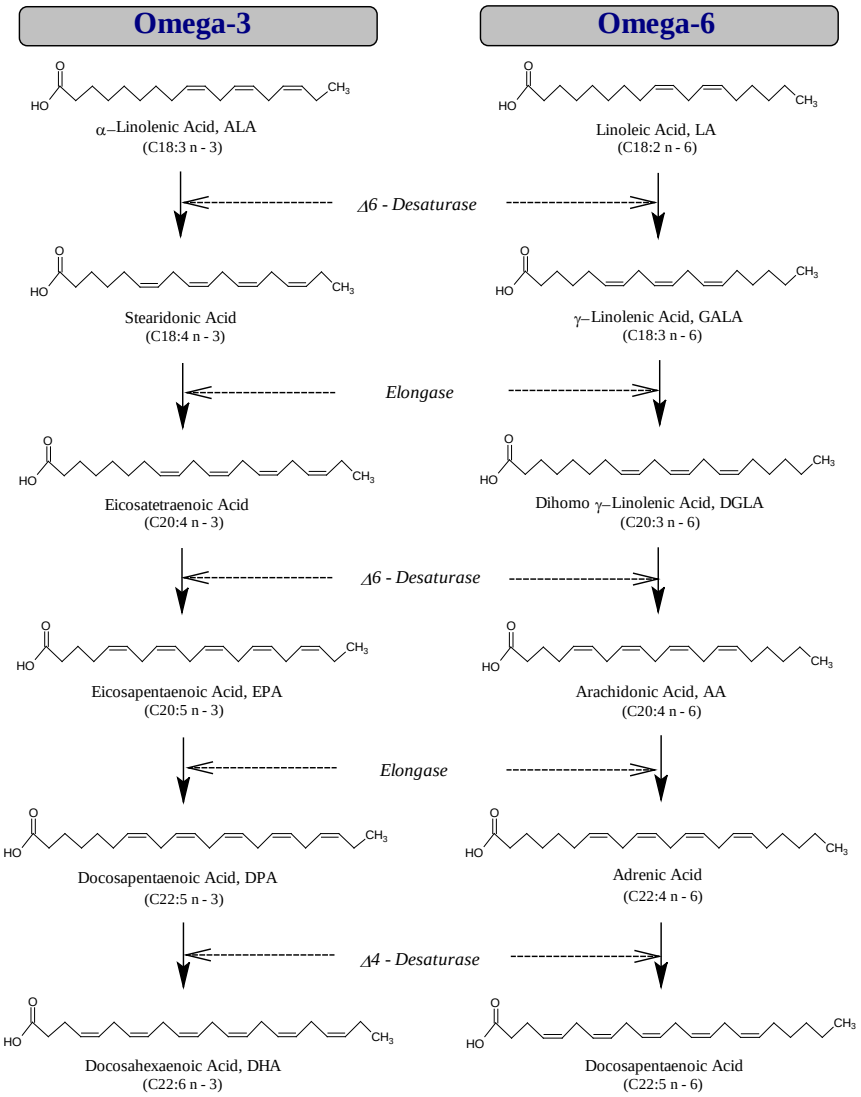

FIGURE 1.2

Metabolic pathways for the synthesis of omega-3 and omega-6 fatty acids (*adapted from* (*Simopoulos*, 1991)).

Ruxton et al. published a general review in 2005 on the impact of long chain (LC) n-3- PUFA on human health. They concluded that, there is strong evidence of the clinical benefit of this type of n-3 PUFA on cardiovascular diseases or rheumatoid arthritis, although further studies are needed to show the benefits for other inflammatory conditions as asthma, cystic fibrosis, bowel diseases or their role in mental illnesses (*Ruxton, et al.*, 2005).

The effect of LC n-3 PUFA when treating asthma has been recently reviewed by Reisman et al. (*Reisman, et al.*, 2006).

Zulfakar et al. have reviewed the works that have examined the potential use of n-3 PUFA in psoriasis (*Zulfakar, et al.*, 2007).

Several recent reviews have studied the influence of omega-3 PUFA on bowel diseases (*Diamond, et al.*, 2008, *Razack, et al.*, 2007, *Turner, et al.*, 2008).

The application of omega-3 fatty acids in the treatment and prevention of mental illnesses (*Clayton, et al.*, 2007, *Horrobin, et al.*, 1991, *Mazza, et al.*, 2007, *McNamara*, 2006, *Ross, et al.*, 2007, *Song, et al.*, 2007, *Stillwell, et al.*, 2003) and on the prevention of several types of cancer (*Calviello, et al.*, 2007 , *Chen, et al.*, 2007, *MacLean, et al.*, 2006) has also been widely reviewed.

The latest overview found about the effect of omega-3 fatty acids in rheumatoid arthritis was published in 1998 (*Ariza-Ariza, et al.*, 1998) although there is a more recent one (2006) not published in English (*Stancík, et al.*, 2006).

Several recent reviews on cardiovascular effects have also been recently published (*Biscione, et al.*, 2007, *Das*, 2008, *Tziomalos, et al.*, 2007, *von-Schacky, et al.*, 2007).Von Schacky & Harris proposed the omega-3 index (percentage of EPA + DHA of total fatty acids in red blood cells) as a risk factor for sudden cardiac death and concluded that this index should be higher than 8 % (*von-Schacky, et al.*, 2007).There are also several studies that propose the mechanisms by which the omega-3 PUFA act in humans (*Horrobin, et al.*, 1991, *Massaro, et al.*, 2007, *Shaikh, et al.*, 2008, *Stillwell, et al.*, 2003).

All this scientific activity regarding long chain omega-3 PUFA usually ends up by advising an intake of this type of PUFA, either through the natural food products that contain them or as a supplement in the diet for beneficial effects on health (*Lands*, 2008). Moreover, nutrition experts have suggested that an n-6:n-3 fatty acid ratio of 5:1 or less is desired in order to maintain a good human health (*WHO/FAO*, 1994), and other expert committees have proposed specific recommendations of EPA and DHA intakes in different population sectors such as healthy adults, pregnant and lactating women, patients with cardiovascular heart disease (CHD) or patients with high triacylglyceride levels in blood (*see* Table 1.2).

1.1.3. Omega-3 fatty acids in human diet

Fish, and especially oily fish from *Scombridae*, *Clupeidae* and *Salmonidae* families, is the most important natural source of omega-3 EPA and DHA in

the foodstuff portion (*see* Table 1.3); whereas seed oils such as sunflower oil, are the main source of omega-6 (*see* Table 1.4).

TABLE 1.2

Daily intakes recommendations for EPA and DHA.

Population sector	Recommendation	Expert Committee	Reference
Healthy adults	500 mg EPA + DHA / day	ISSFAL	(*Cunnane, et al.*, 2004)
Pregnant and lactating women,	200 mg DHA / day	Perinatal Lipid Intake Working Group	(*Koletzko, et al.*, 2007)
Patients with CHD	1 g EPA + DHA / day (*)	American Hearth Asociation	(*Kris-Etherton, et al.*, 2002)
Patients with high triglyceride level	2-4 g / day (*)	American Hearth Asociation	(*Kris-Etherton, et al.*, 2002)

(*) Under medical supervision.

Nowadays food habits in western society are characterized by a high consumption of meat, seed oils, fast food (pizzas, hamburgers…) and snack food (cakes, biscuits…), that contain a large amount of saturated fatty acids and a low proportion of omega-3 fatty acids (*Fernández-SanJuan*, 2000). Comparing the composition of fast food, consumed mostly by young people, with typical Japanese food (*Kamei, et al.*, 2002) or Mediterranean food (*Ambring, et al.*, 2006), both of them with a large amount of fish, it can be observed that the n-6:n-3 ratio in blood, in people who consume Japanese

or Mediterranean food, is close to 2:1, while in people who consume fast food, it can reach values up to 25:1, much higher than desired.

TABLE 1.3

EPA and DHA content in some species of fish. Adapted from literature (*Mataix, et al.*, 2005)

Species	Common name	g/100 g of foodstuff portion	
		C20:5n-3 (EPA)	C22:6n-3 (DHA)
Scomber scombrus	mackerel	1.10	2.56
Mullus surmuletus	red mullet	0.91	1.66
Sardina pilchardus	sardine	0.62	1.12
Salmo salar	salmon	0.50	1.00
Thunnus thinnus	ton	0.24	0.98
Engraulis encrasicolus	anchovy	0.14	0.80
Pagellus bogaraveo	sea bream	0.12	0.61
Gadus morrhua	cod	0.23	0.47
Merluccius merluccius	hake	0.10	0.54
Conger conger	conger eel	0.15	0.43
Luvarus imperialis	swordfish	0.15	0.30
Galeorhinus geleus	dogfish	0.04	0.30

In some cases, maintaining a good omega-3/omega-6 ratio through fish consumption is quite difficult, since it requires changing the nutritional habits, or even impossible when it involves an allergic reaction.

Furthermore, the consumption of marine fishes like salmon, sardine, tuna, anchovy, mackerel or hake, sometimes is questioned due to the presence of toxic compounds such heavy metals as copper or mercury, or organic pollutants as PCBs or dioxins (*Domingo, et al.*, 2007).

TABLE 1.4

Lipids (g / 100 g oil) in different kind of seed and fish oils. Adapted from Mataix et al. (*Mataix, et al.*, 2005).

Oil	SFA	MUFA	PUFA	n-3	n-6	n-6/ n-3
Sunflower	12.0	20.5	67.5	0.10	63.2	632
Corn	14.5	29.9	55.6	0.90	50.4	56
Soya	15.6	21.2	63.2	7.30	51.5	7.05
Palm	47.8	37.1	15.1	0.30	10.1	33.66
Olive	14.3	73.0	12.7	0.70	7.8	11.14
Cod liver	22.6	20.7	56.8	19.8	0.9	0.04
Herring	21.3	56.6	22.1	11.9	12	1.01
Salmon	19.9	17.0	63.1	35.3	1.06	0.03
Sardine	30.4	14.5	55.1	28.1	2.2	0.07

In the last years, many products enriched with n-3, like nutritional supplements or functional foods, have been developed in order to maintain a a good n-6:n-3 ratio in the diet with a low fish consumption. Nowadays, the market offers a wide variety of commercial food products enriched with omega-3 including bread and bakery products, milk and derivatives,

spreadable fats, eggs, juices and soft drinks, meat and poultry products, etc. (*Kolanowski, et al.*, 2006).

The increment in the production of omega-3 supplements and enriched food products involves a higher demand of omega-3 concentrates with a good quality at low cost. Therefore, the study of novel and more competitive omega-3 production processes has acquired a great interest both in the food and the pharmaceutical industries.

The most important natural sources of omega-3 PUFA are marine organisms (fish, seafood, algaes…), that are fed, directly or indirectly, from marine phytoplankton, the primary producer of omega-3 in the trophic chain. Nowadays, the majority of commercial omega-3 concentrates derive from fish or fish by-products, and its production involve several steps, i.e.: fish oil extraction, refining, concentration, stabilization and / or formulation (*see* Figure 1.3).

Conventional processes, based on physical and/or chemical methods, are the most common methods carried out in the current industrial production of omega-3. However, recent studies have shown that supercritical fluid technology could be interesting in omega-3 processing, since it allows avoiding the use of high temperatures, which prevent omega-3 oxidation, and minimising the use of organic solvents.

In next sections, omega-3 processing by both conventional methods and supercritical fluid technology are reviewed.

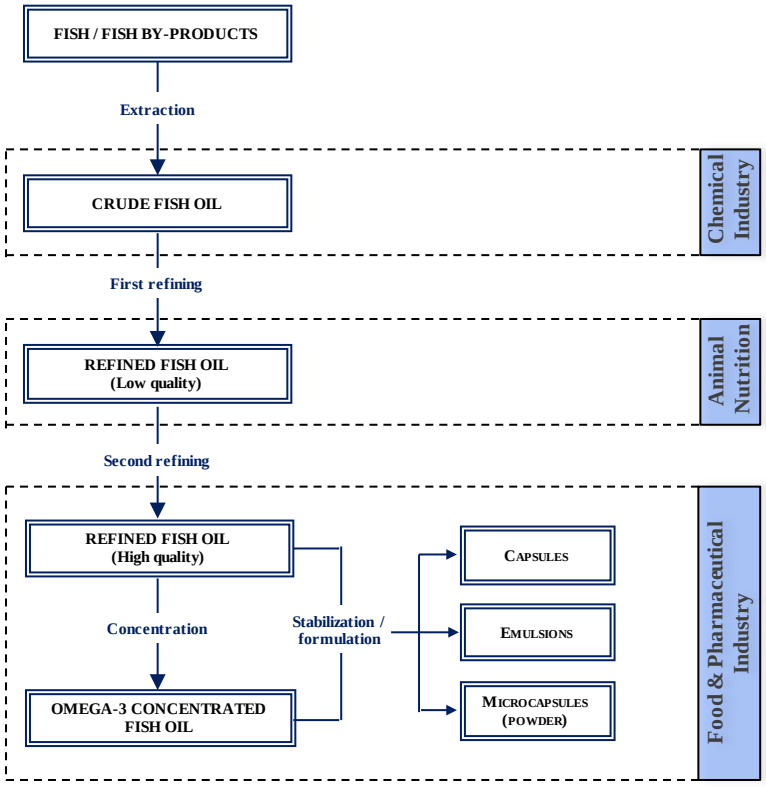

FIGURE 1.3

Scheme of fish oil production from fish and fish by-products.

1.2. Production of omega-3 fatty acids from fish oil: state-of-the art

Abstract

Due to their benefits for human health, the production of omega-3 fatty acids has acquired a great importance in the last years, especially in the food and the pharmaceutical industries.

This Section accounts for both, the current methods and the most recent advances, related to the several stages of omega-3 processing from fish and fish-by-products, i.e., extraction, refining, concentration, formulation and / or stabilization. In the first part, a review of conventional procedures based on physical and chemical methods is provided, including a compilation of commercial fish oil and omega-3 concentrates available nowadays in the market. The last part of the Section is focused on the recent studies developed about the use of supercritical fluid technologies in the production of both fish oil and omega-3 concentrates.

➢ **Section Content**

1.2.1. Conventional methods in omega-3 production

1.2.1.1. Extraction of crude fish oil

Natural fish oil, or crude fish oil, is the lowest quality fish oil obtained directly from whole fish or fish by-products. This oil is not suitable for human consumption, but is used in the chemical industry as well as raw material for high quality fish oil and omega-3 fatty acids.

Historically, crude fish oil is produced from the antiquity by Nordic towns that used it as fuel in lamps.

TABLE 1.5

Main patents about fish oil extraction published in the twenty and twenty-first centuries.

Year	Title	Inventors	Patent number
1901	Process of extracting oil from fish or other material	E.R. Edson	US689472
1930	Process of recovering oil from fish livers and other similar materials	A.W. Owe	US1742666
1932	Fish oil extraction	S. Hiller	US1840715
1933	Method and process of extracting oil and moisture from fish press cake	W.T. Conn	US1903503
1938	Improvements in and relating to the production of fish-liver	Non available	GB0486277
1939	Process and apparatus for the extraction of oil from fish-liver	Non available	GB0500645
1943	Improved method of obtaining oil form fish livers	Non available	GB0558290
1952	Production of oil and proteins from fish	Non available	GB0664827
1952	Improvements in the extraction of oil from fish-liver	Non available	GB0675661
1966	Production of pharmaceutical oil from fish-oil	E. Potter	GB1026359
1967	Method of recovering fish oil	Non available	GB1070128
1976	Process for producing improved fish meal, and fish oil by-product	D.B. Vincent	US3959518
1982	Process for recovering meal and oil from raw fish	P. Bladh	US4344976

TABLE 1.5

(*Continued*)

Year	Title	Inventors	Patent number
1987	Purified fish oil and its production	T. Wakabayashi, and S. Nagahama	JP62093234
1988	Production of fish liver oil containing high units of vitamins	M. Morimitsu	JP63290823
1989	Process for producing protein-rich fish meal and/or fish oil	Y. Shirakawa, Y. Minowa, T. Azumi, and J. Hisano	EP0301795
1990	Method for extracting oil from fish offal	F. Eriksson	WO9008179
1995	Fish oil having decreased fish odour and a method for preparing the same	H. Konishi, I. Riverside, K. Tatsumi, and N. Sato	EP0665287
1996	A method for recovering fish oil having low cholesterol content from fat fish.	A. Oterhals,	WO9608547
1998	Production of high-quality fish oil and high-quality edible extract from head of bonito	N. Koichi	JP10225275
2001	Method and plant for extracting fish oil ad resulting products	P. Barrier, and J.Y. Rousseau	US6214396
2001	Process for producing edible quality refined fish oil	J.B. Crowther, B.H. Booth, and D.D. Blackwell	WO014158
2007	A process for the production of oil and protein from fish waste	J.B. Cloughley	GB2428682
2007	Process for the separation of anchovy meat and anchovy oil	L. B. Tae and C.B. Soo	KR100718551
2008	Method from preparing high-purity edible fish oil from coarse fish oil	S. Yong	CN 101297708
2010	Method for extracting crude fish oil in leftovers of tilapia	B. Yang et al.,	CN101768507

At the beginning of the 19th century, US began to produce crude fish oil from menhaden, using a process with two steps: fish cooking and rock-weighted pressing. Around 1850, this type of press was replaced by mechanical screw presses and later by hydraulic presses. Since the beginning of the 20th century, numerous patents have been published to describe new extraction apparatuses and/or to improve the yield of the extraction, the quality of the fish oil extracted and the profitability of the process for industrial purposes, especially using fish by-products as raw material (*see* Table 1.5).

Nowadays, crude marine oil extraction is usually carried out in fish meal and oil plants by pressing or centrifuging fish or fish by-products previously cooked, although alternative methods based on the protein digestion with a protease have been also proposed in the literature in order to avoid the use of high temperatures and improve oil quality.

i) Production of fish oil in fish meal plants

The traditional process to obtain crude fish oil from whole fresh fish such capelin or mackerel at industrial scale is the wet pressing method, as described by the Food and Agriculture Organization of the United Nations (*FAO*, 1986).

This process involves the production of fish oil and fish meal through several steps, i.e. cooking of the raw material, pressing of the cooked material and final filtration or centrifugation to recover the oil from the

micelle (*see* Figure 1.4). The use of a 3-phase centrifuge can greatly simplify the separation stages after cooking.

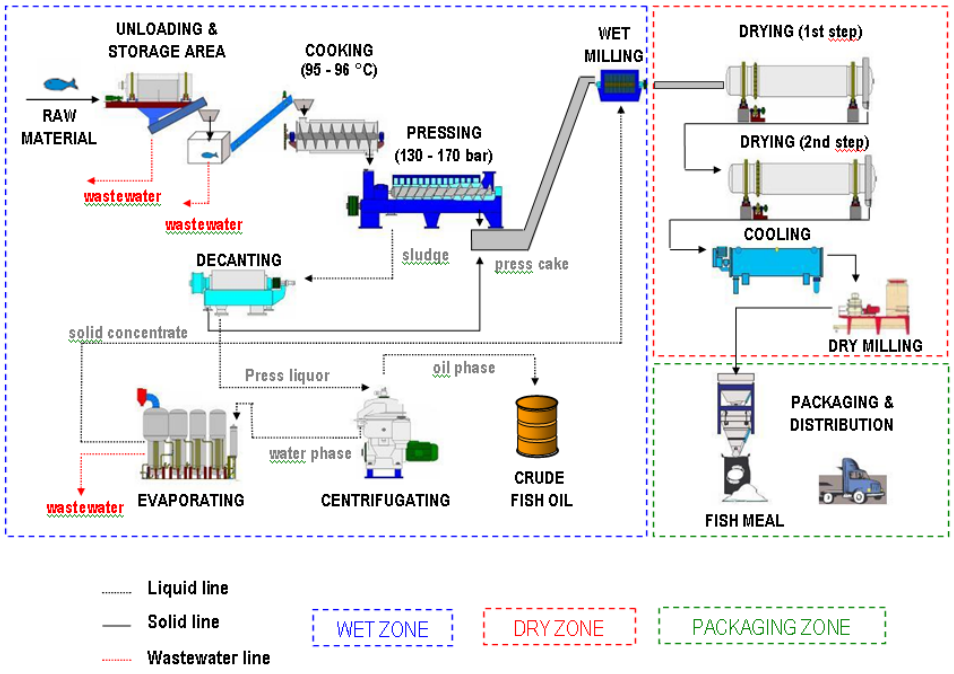

FIGURE 1.4

Industrial process for crude oil extraction in fish meal and oil plants. Adapted from www.copeinca.com.pe/.

Following this process, several companies, mainly located in Europe, South America and Asia, produce crude fish oil from different types of fish and fish by-products (*see* Table 1.6).

TABLE 1.6

Industrial plants for crude fish oil extraction in Europe.

Company	Country	Raw material
Triple Nine Fish Protein	Denmark	Sand eel, sprat, blue whiting, Norway pout, horse mackerel
Havsbrún Ltd.	Faroe Islands (Denmark)	Norwegian pout, capelin, blue whiting
SR-Mjöl HF.	Iceland	Capelin, herring, blue whiting
Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG	Germany	Pollock, rosefish, plaice, herring
Agro-Fish	Poland	Herring, sprat, spotted silver carp, mackerel, cod
ARTABRA, S.A.	Spain	Whole fish mixed with by-products of canned fish
Copeinca ASA	Peru	Anchovy
Salmonoil	Chile	Salmon and trout by-products
Pesquera Pacific Star, S.A.	Chile	Sardine, scad, anchovy, salmon by-products
Janatha Fish Meal & Oil Products	India	Sardine
Raj Fish Meal & Oil Products	India	Sardine, pink perch

In the last years, several studies have been carried out in order to improve the fish oil quality through improving the raw material freshness and avoiding or minimising the use of high temperatures during the extraction process.

Focussing on the influence of the raw material, Chantachum et al. published a study about the separation of oil from tuna heads by a wet reduction method (*Chantachum, et al.*, 2000). They studied both the yield and the quality of the oil extracted from precooked (100 ºC for 60 min) and non-precooked fish at different heating temperatures and times, and concluded that the optimum results were achieved when heating at 85 ºC for 30 min and without precooking.

Aidos et al., also reported the oil production from herring by-products using a method consisting of mincing the raw material (16 mm), cooking it in a heat exchanger (8 min at 95 ºC) and recovering the oil in a three-phase decanter (4 min at 95 ºC) with good results (*Aidos, et al.*, 2001). Moreover, they focused on the influence of by-product freshness (*Aidos, et al.*, 2003) and the different process parameters (cooking temperature, pumping speed to the heat exchanger and decanter speed) on fish oil quality (*Aidos, et al.*, 2003). They concluded that herring by-products have good storage stability and that the main variables that influence oil quality are the separation speed of the decanter and the pumping speed.

ii) Fish oil extraction by enzymatic methods

The use of enzymes in fish oil extraction is fairly recent but it has become a good alternative to traditional methods, since it can be simpler and cheaper regarding investment cost and energy expense. Besides, this technology requires neither organic solvents nor high temperatures (*Rolle*, 1998). These advantages have promoted the research on the use of enzymatic technology to release lipids from fish avoiding the use of solvents and high temperatures.

Liaset et al. studied the enzymatic hydrolysis of salmon frames with proteases and the composition of the different fractions obtained after separation by centrifugation. They reported that this process enables to obtain omega-3 enriched oil with a good recovery (about 77 %) as well as several interesting products such as peptides or essential amino acids (*Liaset, et al.*, 2003).

Linder et al. developed another enzymatic method to extract oil from ground salmon heads at middle temperature (55 ºC) using different commercial enzymes: a protease (Alcalase), an exo-peptidase (Neutrase) and an endo-peptidase (Flavourzyme). They concluded that the highest oil recovery (17.4 % after 2 h) was obtained by using Alcalase, being close to the recovery obtained by the Bligh & Dyer method (20 %) (*Linder, et al.*, 2005).

Šližyte et al., studied the enzymatic hydrolysis of mixtures of cod by-products using Flavourzyme and Neutrase respectively. They studied the

influence of the composition and state of the substrate on the yield and composition of the hydrolysis products (oil fraction, hydrolysed protein, sludge and emulsion) (*Daukšas, et al.*, 2005) and on the chemical composition of the lipid phase (*Daukšas, et al.*, 2005). The same authors, (*Daukšas, et al.*, 2005), also studied the influence of initial heating of the raw material and on the yield and the chemical composition of the oil extracted by enzymatic hydrolysis of cod by-products using other enzymes such as Alcalase and Lecitase.

Liu et al. proposed an enzymatic process based on a proteolytic extraction of oil from crude tuna heads followed by an urea complexation step, obtaining a mixture of DHA and EPA with a purity of 85.02 % and a yield of 25.10 % (*Liu, et al.*, 2006).

Gbogouri et al. studied the enzymatic hydrolysis of fresh salmon heads, with Alcalase, Neutrase and Protamex concluding that Alcalase was the most efficient enzyme regarding oil yield. They also found phospholipids to be more effective carriers of LC-PUFA (*Gbogouri, et al.*, 2006).

Most recently, Al-Sayed Mahmoud et al., have reported a similar study about the enzymatic extraction of oil from rainbow trout roe by using Alcalase, Neutrase and Protamex respectively (*Al-Sayed-Mahmoud, et al.*, 2008). They also found Alcalase to be the enzyme that leads to the highest oil recovery.

The general conclusion reached by the authors that have studied fish oil extraction by using enzyme technology is that this technology yields better oil than thermal treatments.

iii) Other physico-chemical methods for fish oil extraction

Fish oil extraction based on protein precipitation has been also proposed in the literature (*Okada, et al.*, 2007). This method replaces the cooking step by a pH adjustment to the isoelectric point of the protein of the raw material, and has shown good results for extracting oil from high-oil pelagic species such as sardine, although the main drawback is that several acids (hydrochloric, citric and tartraric acid) and salts (calcium citrate and calcium tartrate) are required.

1.2.1.2. Fish oil refining

Crude fish oil obtained directly in fish meal and oil plants are characterized by a high free fatty acid content and other impurities that limit its use in animal or human nutrition. Therefore a subsequent refining step must be carried out in order to aim the quality standards established by the British Pharmacopoeia (*BP*, 2008) or the Global Organization for EPA and DHA (*GOED*, 2006) (*see* Table 1.7).

The conventional oil refining process involves different physical and chemical methods aimed to remove impurities (free fatty acids, glycerides, phospholipids, sterols, pigments…), toxic compounds (heavy metals,

dioxins, PCBs…) and smelly compounds, depending on the final use of the refined fish oil, that is, animal feeding or human nutrition.

In the last years, the demand of health food-grade or pharmaceutical-grade aimed to human nutrition has increased noticeably, and nowadays several companies produce it all over the world (*see* Table 1.8) following the quality standards required. The most common refining methods used are reviewed below.

TABLE 1.7

Specifications for fish oil aimed to human nutrition established by GOED (*GOED*, 2006) and British Pharmacopeia (*BP*, 2008).

Parameter	Specification	Unit
Acid value	Max. 3	mg KOH / g
Peroxide value (PV)	Max. 10	mq O_2 / kg
Anisidine value (AV)	Max. 20	---
PCDDs/PCDFs	Max 2	pg (WHO-TEQ) / g
Dioxine-like PCBs	Max. 3	pg (WHO-TEQ) / g
Lead, Pb	Max 0.1	mg / kg
Cadmiun, Cd	Max 0.1	mg / kg
Mercury, Hg	Max. 0.1	mg / kg
Inorganic arsenic, As	Max. 0.1	mg / kg

TABLE 1.8

Industrial production of health-grade and pharmaceutical-grade fish oil.

Country	Company	Product
Iceland	Lysi	Cod liver oil, salmon oil, tuna oil
Norway	Denomega	Anchovy oil
United Kingdom	Seven Seas	Cod liver oil
France	Winterisation Europe	Fish oil, cod liver oil
Spain	GSN	Salmon oil
Spain	Solgar	Cod liver oil
Spain	Nutrytec	Salmon oil
Canada	Enerex	Salmon oil
USA	Lamberts	Herring oil, mackered oil, cod liver oil
USA	NewChapter	Salmon oil
New Zealand	Xtend-life	Hoki oil

i) Removing impurities and contaminants

The main procedure to remove fish oil impurities involves several steps such as degumming, to separate phospholipids; neutralization or deacidification, to clear free fatty acids and decrease oil acidity and bleaching to absorb pigments.

This process presents several drawbacks since it implies the use of chemical products (alkalis), that contaminate the environment, and some neutral oil losts, mainly in oils with high free fatty acid content.

Physical refining processes, based on the application of superheated steam under low pressure have been proposed as alternative to remove free fatty acids and volatile compounds. However, these methods require a preliminary step of chemical refining and, due to the use of high temperatures, they are not suitable for thermolabile oils such as fish oil (*Čmolík, et al.*, 2000).

Physical adsorption on activated carbon has been proposed recently to remove contaminants, such as dioxins and PCBs, from fish (*Maes, et al.*, 2005, *Oterhals, et al.*, 2007)

ii) Removing smelly compounds

Oil deodorization is also an important process stage, especially in crude fish oils, which are usually characterized by a fishy odour that reduces their sensory quality and limits their application in the food industry.

Traditional oil deodorization is based on the application of high temperatures (*Čmolík, et al.*, 2000). This method is also applied to fish oil although several studies have demonstrated that, at a temperature above 180 ºC, an important PUFA degradation, involving the formation of many undesirable compounds such as polymers, PUFA isomers, mono and di-trans and cyclic fatty acids monomers, is produced (*Fournier, et al.*, 2006).

Alternative methods based on vacuum steam distillation at low temperatures followed by a treatment in a silica gel column (*Chang, et al.*, 1989), adsorption with a resin (*Nishimoto, et al.*, 1996) or treatment with

diatomaceous earth (*Yoshikazu, et al.*, 1997), have been proposed for removing smelly compounds from fish oil.

1.2.1.3. Omega-3 concentration

Concentrated fish oil is a refined fish oil subjected to modification in order to increase the amount of omega-3 to at least 600 mg omega-3 / g oil, usually in form of ethyl esters.

The majority of the industrial processes for production of omega-3 concentrates on large scale involve a first step of marine oil extraction from marine organisms, followed by concentration and stabilization steps.

In the last years many processes have been developed to isolate, fractionate or concentrate n-3 PUFA from fish oil, although the majority of them are referred to omega-3 ethyl esters formed by the esterification or saponification of triglycerides with ethanol.

Nowadays, these methods are used by several companies to produce omega-3 concentrates both with pharmaceutical and nutraceutical quality and with different amounts of EPA and DHA (*see* Table 1.9). However, the main drawback of these procedures is that the natural triglyceride form is lost during the process.

TABLE 1.9

Commercial omega-3 concentrated fish oils.

Company	Process	Product	Chemical Form	Omega-3
Croda	PureMax Molecular Distillation Technology	Incromega DHA500TG SR	TAG	≤ 100 mg/g EPA Min 500 mg/g DHA
		Incromega EPA500TG SR	TAG	Min 500 mg/g EPA ≤ 60 mg/g DHA
		Incromega TG3322	TAG	Min 33 % EPA Min 22 % DHA
		Incromega E3322	EE	Min 33 % EPA Min 22 % DHA
		Incromega E7010	EE	Min 70 % EPA ≤ 10 % DHA
		Incromega DHA 700E SR	EE	≤ 10 % EPA Min 70 % DHA
Huatai	Multi-stages short-path distillation	Omega-3 H API	EE	Min 400 mg/g EPA Min 340 mg/g DHA
		Omega-3 H Ultra Purity EPA700	EE/TAG	Min 700 mg/g EPA
		Omega-3 H Ultra Purity DHA700	EE/TAG	Min 700 mg/g DHA
		Omega-3 H Ultra Purity	EE/TAG	Min 75 – 5 % EPA Min 5 - 75 % DHA
		Omega-3 H EPA rich	EE/TAG	Min 60 – 30 % EPA Min 15 – 30 % DHA
		Omega-3 H DHA rich	EE/TAG	Min 20 – 10 % EPA Min 40 – 50 % DHA
Pronova biopharma	Urea complexation / molecular distillation	Omacor	EE	55 % EPA 45 % DHA

Several authors, as Mishra et al. (*Mishra, et al.*, 1993) or Shahidi & Wanasundara (*Shahidi, et al.*, 1998) have reported a summary of the traditional methods to purify omega-3 fatty acids, i.e. chromatography, vacuum or molecular distillation, low-temperature crystallization, urea complexation, as well as other new processes based on the use of enzymes or supercritical fluids.

i) Traditional concentration procedures

The most common methods to concentrate omega-3 from fish oil on large scale are molecular distillation and urea complexation.

- *Molecular distillation* implies two main steps: esterification of fatty acids as ethyl esters (EE) and separation of the EE at a low temperature and vacuum according to their molecular weight.
- *Urea complexation* involves the formation of a solution of FFA and urea in a mixture of ethanol / water (95 : 5 %) at 60 °C. The solution is then cooled at room temperature in order to obtain an urea complex slurry, which includes preferentially the saturated fatty acids (SFA), and a liquid phase, formed mainly by unsaturated fatty acids (UFA). Therefore, UFA are easily separated from SFA through gravity filtration.

ii) Enzymatic processes

Enzymatic processes in omega-3 concentration are based on the use of specific enzymes, normally lipases, which are able to catalyse reactions such hydrolysis, ethanolysis or transesterification of triglycerides.

Due to the fatty acid distribution in the glycerol backbone found in several marine triglycerides (*Ando, et al.*, 1992) and the stereospecifical activity of certain lipases (*Wong*, 2003), these methods are very useful both in omega-3 concentration from fish oil and in the production of structured lipids.

In the last years, a great amount of enzymatic methods followed by a separation process such membrane filtration, urea complexation or molecular distillation have been proposed in the literature to obtain omega-3 concentrates, especially EPA and DHA, as different forms such free fatty acids, ethyl esters or 2-acylglycerides (*Oliveira-Carvalho, et al.*, 2003, *Shahidi, et al.*, 1998). Among these procedures, both enzymatic hydrolysis and enzymatic esterification or transesterification it may be distinguished.

- *Enzymatic hydrolysis*

  Enzymatic hydrolysis has been proposed by several authors as an interesting way to obtain omega-3 concentrates as free fatty acids from fish oil.

  Linder et al. developed a method to increase the amount of PUFA in salmon oil from 39.20 mol % to 43.05 mol % throughout an enzymatic hydrolysis catalyzed by Novozym® SP398, followed by a

filtration step in a flat membrane device. In addition, they proposed a final esterification step with glycerol using an immobilized 1,3-specific lipase to slightly increase the amount of PUFA up to 43.29 mol % (*Linder, et al.*, 2002).

Gámez-Meza et al. studied the concentration of omega-3 from sardine oil coupling hydrolysis reaction and urea complexation. Comparing enzymatic hydrolysis with different *Pseudomona* lipases with the chemical hydrolysis using KOH, ethanol and hexane, they concluded that the lipase from *Pseudomona cepacia* immobilized on chemically modified ceramic with a 10 % protein is the best enzymatic system to obtain the highest purity of omega-3 fatty acids (86.58 %) with an acceptable yield (78.0 %), while chemical hydrolysis enabled to obtain a higher omega-3 recovery (90.5 %) but with a lower purity (83.13 %). In addition, they pointed out that the enzymatic method is more advantageous since the separation of the products is easier and the process can be developed in continuous packed-bed reactors (*Gámez-Meza, et al.*, 2003).

Kojima et al. designed a process to concentrate alternatively DHA or EPA from a mixture of natural fish oil through an enzymatic method using HU-lipases (which show a high reactivity for any fatty acid with 20 atoms of carbon) or AK-lipases (which shows a poor reactivity for fatty acids with 20 atoms of carbon and a $\Delta 5$ unsaturated bond), followed by urea complexation to increase the

purity. They concluded that, using this method with HU-lipase, it is possible to selectively enrich EPA from 12.2 % to 43.1 %, while the amount of DHA is practically constant. On the other hand, if the same method was repeated using AK-lipase, a selective enrichment of DHA from 16.3 % to 44.6 % was obtained, while the amount of EPA was hardly altered (*Kojima, et al.*, 2006).

- *Enzymatic esterification*

  Enzymatic esterification or transesterification processes are very useful in the production of lipids derived from omega-3 PUFA that present different properties than the corresponding free fatty acids. For instance, omega3 ethyl or methyl esters are easily separated by SFF or SFC, as mentioned in section 1.2.2, triacylglycerides are more attractive in the food industry since they are well absorbed by the human organism (*Lawson, et al.*, 1988), and phospholipids (*Lyberg, et al.*, 2005) or wax esters (*Gorreta, et al.*, 2002) are important as a stabilization strategy because they are the most stable lipid forms against oxidation.

  In the last decade, several studies about enzymatic esterification and transesterification of fish oil with microbial lipases have been also reviewed in the literature (*Oliveira-Carvalho, et al.*, 2003, *Shahidi, et al.*, 1998). Recently, a method has been patented by Le Goffic et al, which combines chemical and enzymatic transesterification of fish oil with short path distillation steps to achieve a fraction rich in DHA

(more than 90 %) and poor in EPA (less than 5 %), besides to another fraction rich in EPA (more than 50 %) and poor in DHA (less than 2 %) (*LeGoffic, et al.*, 2000). However, the main drawback of this method is the use of high temperatures (above 100 ºC) during the distillation steps, which may be related with a high omega-3 oxidation.

From then until now, many scientists have continued investigating on the improvement of the omega-3 concentration processes, to achieve higher yield and purity at lower cost. Among the new trends, it is worth to noting the enzymatic processes involving the use of supercritical fluids as reaction media, which are described in section 1.2.2.5.

1.2.1.4. Fish oil formulation and stabilization

One of the major drawbacks of oils containing a high amount of omega-3 PUFA, such as fish oils, is their high susceptibility to oxidation, which involve the formation of toxic products as peroxides or volatile compounds relative to non-desirable off-flavors.

Omega-3 oxidation is carried out by a complex mechanism that depends on the type of lipids and their presentation (bulk oil, water-in-oil emulsion or oil-in-water emulsion, encapsulation or microencapsulation), and can be catalyzed by other parameters as temperature, exposure to light and oxygen or presence of trace metals (*see* Figure 1.5).

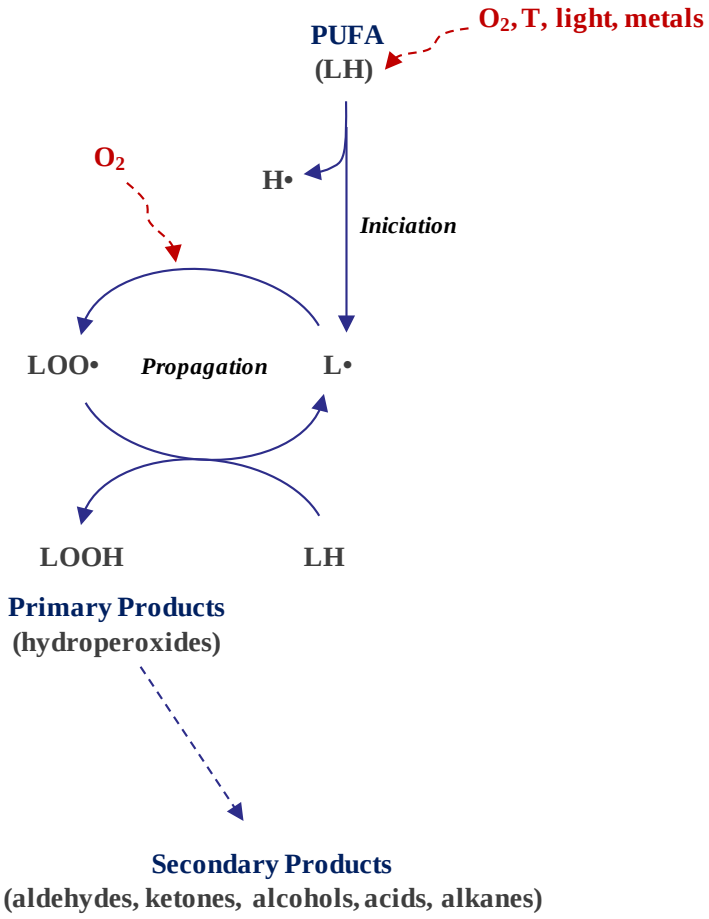

FIGURE 1.5

Mechanism of PUFA oxidation (*adapted from* (*FAO*, 1995)).

Therefore, optimum processing, storage and packaging of fish oils are essential to preserve omega-3 PUFA from oxidation. In addition, other

strategies to improve fish oil stability and prolong its shelf-life are necessary (*Kamal-Eldin, et al.*, 2002).

i) Addition of antioxidants

The use of antioxidants is one of the most common methods to prevent fish oil oxidation. A great amount of antioxidants, as BHA, BHT, EDTA, tocopherols, ascorbic acid, ascorbyl palmitate, propyl gallate, gallic acid, lactoferrines, etc., has been studied to prevent lipid oxidation both in bulk fish oil (*Kamal-Eldin, et al.*, 2002) and in fish oil-in-water emulsions (*Jacobsen, et al.*, 2008).

In the last decade, the use of natural antioxidants instead of synthetic compounds has acquired great relevance and several studies regarding the efficiency of plant extracts, as oregano, rosemary, parsley or olive mill wastewaters, on the stabilization of bulk fish oil and fish oil-in-water emulsions have been developed (*Bhale, et al.*, 2007, *Frankel, et al.*, 1996, *Jiménez-Álvarez, et al.*, 2008, *Wada, et al.*, 1992).

In addition, several studies about the effect of adding natural antioxidants in omega-3 enriched foods, as meat products (*Valencia, et al.*, 2008) or frozen surimi (*Pérez-Mateos, et al.*, 2006), have been published.

ii) Encapsulation or microencapsulation

Encapsulation or microencapsulation with a coating material has also been proposed as strategy to retard lipid auto-oxidation and enzyme hydrolysis,

improve the oil stability and control off-flavors (*Matsuno, et al.*, 1993). In addition, it has been proved that the use of fish oil microcapsules offers good results in the design of functional foods as cream to fill sandwich cookies (*Borneo, et al.*, 2007), instant foods (soups, cocoa drinks…) (*Kolanowski, et al.*, 2007a), dairy products (yoghurt, fresh cheese, butter…) (*Kolanowski, et al.*, 2007b) or pasta (*Iafelice, et al.*, 2008).

Nowadays, a great amount of physical and chemical processes using several wall materials have been developed to encapsulate and stabilize active compounds as fish oil (*Desai, et al.*, 2005) and several companies produce and commercialise powder fish oil and fish oil concentrated formulas especially designed for bakery, baby food, beverage or dairy industry (*see* Table 1.10).

Among the methods used, spray-drying is the most common and cheaper (*Gharsallaoui, et al.*, 2007), although other processes as freeze drying (*Heinzelmann, et al.*, 2000) or ultrasonic atomization (*Klaypradit, et al.*, 2008) have also been developed recently as alternative to reduce omega-3 oxidation since they avoid the use of high temperatures during the drying step.

TABLE 1.10

Commercial fish oil microencapsulated aimed to food industry.

Company	Product	Applications
Lipid Nutrition	MarinolTM Omega-3 HS MarinolTM DHA HS	Backery Baby food
The Wright Group	O-3 Complete®	Backery Baby food Beverages
NuMega	Driphorm HiDHA®	Backery Baby food Beverages
Ocean Nutrition	MEG-3®	Backery Dairy Sauces
BASF	Dry n-3® 5:25 C Dry n-3® 18:12	Dairy Baby food
DSM	ROPUFA®	Backery Dairy Baby food

1.2.2. Production processes based on supercritical fluid technology

1.2.2.1. Introduction to supercritical fluids

The use of supercritical fluids technology in the production of natural compounds has acquired an enormous interest in the last years, especially when the final products is, as occurs with omega-3 fatty acids, a high value product aimed to food and pharmaceutical industries.

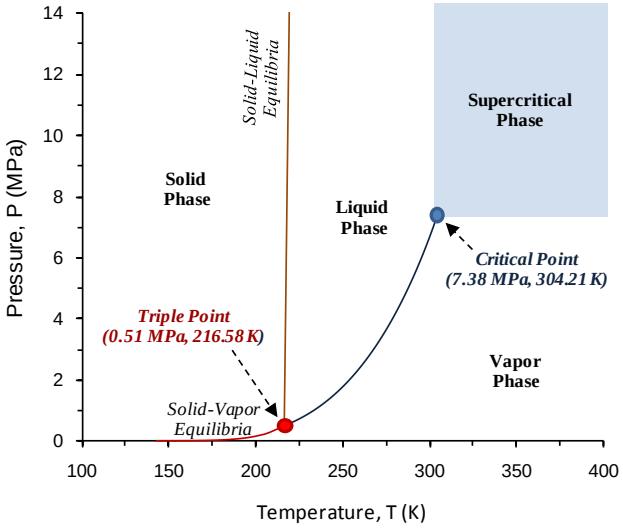

FIGURE 1.6

Phase diagram of carbon dioxide illustrating the supercritical region above the critical pressure (P_c) and temperature (T_c).

Supercritical fluids are considered to be any fluid at a reduced pressure, temperature and density, defined as (1.1), (1.2) and (1.3) respectively, above unity, pressure being below the necessary to solidify the fluid.

$$P_r = \frac{P}{P_c} \quad \textbf{(1.1)}$$

$$T_r = \frac{T}{T_c} \quad \textbf{(1.2)}$$

$$\rho_r = \frac{\rho}{\rho_c} \quad \textbf{(1.3)}$$

TABLE 1.11

Comparison of physical properties of gases, liquids and SCF. Adapted from Brunner (*Brunner*, 1994).

Physical State	Pressure & Temperature conditions [T (K) / P (MPa)]	Density (kg/m^3)	Viscosity (Pa s)	Diffusivity (m^2/s)	Surface Tension (mN/m)
Gas	[0.1 / 298]	0,5 – 2	10^{-5}	10^{-5}	0
SCF	[P_c, T_c]	200-500	$1.3 \cdot 10^{-5}$	$0.7 \cdot 10^{-7}$	0
	[$4P_c$, T_c]	40-900	$3.9 \cdot 10^{-5}$	$0.2 \cdot 10^{-7}$	0
Liquid	[0.1 / 298]	600 – 1600	10^{-3}	10^{-9}	25-80

In the region above its critical point, characterized by one only phase (*see* Figure 1.6) the fluid possesses intermediate properties between a gas and a

liquid, i.e.: liquid-like density and gas-like viscosity and diffusivity (*see* Table 1.11). Therefore supercritical fluids (SCF) have at the same time a good solvent power and good transport properties (*Brunner*, 1994).

TABLE 1.12

Critical properties of different solvents. Adapted from Cengel et al., (*Cengel, et al.*, 2002).

Solvent		T_c (K)	P_c (MPa)	ρ_c (g/cm^3)
Carbon dioxide	CO_2	304.1	7.38	0.469
Water	H_2O	647.1	22.06	0.322
Metane	CH_4	190.4	4.60	0.162
Ethane	C_2H_6	305.3	4.87	0.203
Propane	C_3H_8	369.8	4.25	0.217
Ethylene	$H_2C=CH_2$	282.4	5.04	0.215
Propylene	$CH_3CH=CH_2$	364.9	4.60	0.232
Methanol	CH_3OH	512.6	8.09	0.272
Ethanol	CH_3CH_2OH	513.9	6.14	0.276
Acetone	CH_3COCH_3	508.1	4.70	0.278

In the last decades, different SCF have been proposed (*see* Table 1.12), being supercritical carbon dioxide, SC-CO_2 the most widely used since it is considered a green solvent, non toxic, cheap and non flammable; has mild critical conditions (T_c = 304.15 K and P_c= 7.38 MPa) that allow to process thermolabile compounds, as omega-3 PUFA, and makes operation costs not

too expensive. In addition, CO_2 is gaseous under ambient conditions, which means that it is easy to separate from the processed products after processing.

1.2.2.2. Historical development of the Supercritical Fluids Technology

The first reported observations of the supercritical phase phenomenon were carried out by Baron Cagniard de la Tour in 1822, who reported that, when the temperature of some substances was increased by heating in a closed vessel, the gas-liquid boundary disappears (*Tour*, 1822). From this starting point, the critical point of a substance was defined.

Years later, authors like Hannay & Hogarth, who studied the solubility of different inorganic salts in supercritical ethanol (*Hannay, et al.*, 1879); or Buchner, who studied the solubility of non-volatile organic compounds in supercritical carbon dioxide (*Buchner*, 1906), demonstrated that the solvating power of supercritical fluids were much higher than would be expected from the vapour pressure of the solid, and, moreover, this was tunable according to the supercritical fluid pressure. During 1930 and 1940 different phases equilibria were studied, which aimed to design a deasphalting process for refining lubricants oils, although the most significant development in supercritical fluid technology was carried out from 1960 when Zosel described and patented more than 80 extraction processes using supercritical fluids, the most important related to the decaffeination of green coffee with supercritical carbon dioxide (*Zosel*, 1982).

TABLE 1.13

Current industrial processes based on supercritical fluid extraction.

Process	Manufacturer	Country
Coffee / tea decaffeination	Kaffee HAG AG Hermsen Jacobs Suchard	Germany
	SKW-Trostberg	Italy
	General Foods	USA
Hops Extraction	Pfizer Hops Extraction	Australia
	Hopfenextraktion, HVG, Barth, Raiser & Co. SKW Trostberg Barth & Co. SKW-Trostberg	Germany
	Carlton, United Breweries Pauls & White	United Kingdom
	Hops Extraction Corp. of America J.I. Haas, Inc. Pitt-Des Moines, Inc.	USA
	NORAC	Canada
	Nan Fang Flour Mill	China
	Natal Cane By-Products Ltd.	South Africa

TABLE 1.13

(Continued)

Process	Manufacturer	Country
Flavors/Aromas/Spices	Flavex GmbH Raps & Co.	Germany
	Camilli Albert & Louie,	France
	Soda Flavor Co.	Japan
	Guangxia Toothpaste Shaanxi Jia De Agriculture Eng. Co., Ltd.	China
	Mohri Oil Mills Fuji Flavor	Japan
Nicotine extraction	Philip Morris	USA
	Nippon Tobacco	Japan
Color Extraction / Red Pepper	Sumitomo Seiko Yasuma (Mitsubishi Kokoki facility) Hasegawa Koryo Takasago Foods (Mitsubishi Kokoki facility)	Japan

Since 1980, the development of supercritical fluid extraction has increased noticeably, and several extraction plants have been built all over the world to extract different natural compounds on commercial scale by using supercritical carbon dioxide as solvent (*see* Table 1.13).

Nowadays, a great amount of processes involve the use of supercritical fluids as solvents, such as supercritical fluid extraction (SFE), fractionation (SFF), chromatography (SFC); whereas other procedures are based in the use of supercritical fluid as a media for enzymatic reaction or particle formation. The main processes related to the use of supercritical fluid technologies in fish oil extraction, refining, omega-3 concentration or formulation are reviewed below.

1.2.2.3. Supercritical fluid extraction of fish oil

Fish oil is a natural mixture of different lipid compounds, liquid at room temperature, soluble in non-polar solvents, as hexane or petroleum ether, and insoluble in polar solvents as water.

SC-CO_2 has been also shown to be a good solvent of natural fish oil: Imanishi et al. measured the solubility of fish oil from sardine in SC-CO_2 in a pressure range of 20 to 35 MPa at four different temperatures (313, 333, 343 and 353 K). They observed that, at constant temperature, fish oil solubility increased significantly with pressure, whereas, at constant pressure, oil solubility decreased with temperature up to a pressure value

from which oil solubility began to increase as temperature increased (*Imanishi, et al.*, 1989).

This crossover behaviour has also been observed in other natural fish oils from sand eel (*Borch-Jensen, et al.*, 1997), orange roughy, spiny dogfish liver or cod liver (*Catchpole, et al.*, 1998). It is explained considering that, at constant pressure, a temperature increase has a double effect: the density of the solvent decreases, which would make solubility to decrease, and the vapour pressure of the solute increases, which would make the solubility to increase. At low pressures the first effect predominates and at high pressures is the solute vapour pressure effect which becomes stronger (*Brunner*, 1994). It has been also observed that fish oil solubility increases by adding polar co-solvents such as ethanol (*Catchpole, et al.*, 1998).

The experimental determination of phase equilibrium data of mixtures formed by the different components of fish oil, and its derivatives, and SC-CO_2 has acquired more relevance in the last years because phase equilibrium data are essential to design fish oil processes such as supercritical fluid extraction (SFE), fractionation (SFF) or supercritical fluid chromatography (SFC), which are technologies involved in oil refining and omega-3 concentration.

Staby & Mollerup summarized most of the published phase equilibrium data involving SC-CO_2 and fish oil components or derivatives. Their paper includes reference data for the main lipid compounds, pure or mixtures, such as glycerides, free fatty acids, fatty acid methyl or ethyl esters, and

other minority components such as cholesterol, β-carotene or α-tocopherol (*Staby, et al.*, 1993).

Güçlü-Üstündağ & Temelli published a compilation of solubility data for the main pure lipid classes such as fatty acids, fatty acid esters and mono-, di- and triglycerides (*Güçlü-Üstündağ, et al.*, 2000) and for other minor lipids as β-carotene, α-tocopherol, stigmasterol and squalene in SC-CO_2 (*Güçlü-Üstündağ, et al.*, 2004). In both reviews, they used the Chrastil's solubility model to quantify the influence on solubility, not only of the equilibrium pressure and temperature, but also of the physical properties of the solute such as molecular weight, polarity or vapour pressure.

Based on the good solubility of fish oil, and its individual components, in SC-CO_2, abundant research dealing with fish oil extraction using SC-CO_2 as solvent has been made. Raventós et al. (*Raventós, et al.*, 2002) made a general overview of the possibilities of SC-CO_2 as an extracting solvent in the food industry, including the extraction of lipids and cholesterol from fish. Staby & Mollerup, besides reviewing the solubility or phase equilibrium data of typical components of fish oil in supercritical fluids, also made an overview of the supercritical fluid extraction, fractionation and/or chromatography of these compounds, especially acylglycerides, free fatty acids, fatty acid methyl or ethyl esters, cholesterol, α-tocopherols, squalene or phospholipids (*Staby, et al.*, 1993).

Some recent studies about SC-CO_2 extraction of oil from fish, and some not so recent studies that have not been considered in previous reviews

(*Dunford, et al.*, 1998, *Dunford, et al.*, 1997, *Esquível, et al.*, 1997, *Letisse, et al.*, 2006, *Rubio-Rodríguez, et al.*, 2008) are detailed next: Esquível et al. studied the extraction of sardine oil with SC-CO_2, concluding that it is possible to find the conditions to recover most of the oil (95 %) without degradation of the omega-3 PUFA (*Esquível, et al.*, 1997). Dunford et al. studied the effect of moisture on mackerel oil extractability using SC-CO_2. They concluded that the extraction yield increased with decreasing moisture content and proposed a mathematical model to explain this extraction behaviour (*Dunford, et al.*, 1998, *Dunford, et al.*, 1997). Some fish by-products have also been considered as raw material to obtain omega-3 rich oil by using supercritical carbon dioxide: Letisse et al. looked for the optimum extraction conditions to obtain an oil rich in omega-3 PUFA from freeze dried sardine heads (*Letisse, et al.*, 2006). Rubio-Rodriguez et al. studied the oil extraction from hake by-products, specifically from hake skin, by using SC-CO_2. They studied the influence of all the important process parameters to search the most efficient extraction conditions regarding oil yield and quality (*Rubio-Rodríguez, et al.*, 2008).

Nowadays, SFE is generally considered a useful technology to replace traditional extraction processes as steam distillation or solvent extraction. Therefore, SFE is a promise technology to be scaled up to indusial scale in order to produce good quality fish oil, suitable as raw material to produce n-3 concentrates.

1.2.2.4. Fish oil refining using supercritical fluids

Supercritical fluid technology, together with membrane and enzymatic processes, are some of the most recent technologies proposed as alternative to oil refining with chemical products or high temperatures.

Several studies have been recently reported dealing with the application of these technologies, both in degumming or bleaching (*Čmolík, et al.*, 2000), and in deacidification (*Bhosle, et al.*, 2005) of different seed oils, as well as in fish oil refining.

Jakobsson et al. proposed a semi-continuous extraction process using SC-CO_2 with and without ethanol for removing dioxins and free fatty acids from cod liver oil. They found that it is difficult to lower the free fatty acid content at a level of 0.5 %-1 % and, at the same time, to remove a great amount of dioxins, due to the different polarity of both types of compounds. However, they found possible to separate dioxins from deacidified oil by using pure SC-CO_2 at low pressures (*Jakobsson, et al.*, 1991). Subsequently, the same authors (*Jakobsson, et al.*, 1994) proposed a counter-current extraction process to improve the contact between oil and SC-CO_2 and increase the amount of dioxins to be removed. Their results showed that 80 % of the dioxins can be extracted together with the first 17 % of the oil.

Catchpole et al. developed a method for removing impurities as peroxides, fatty acids or odour components, from different crude fish oils as orange roughy oil, deep sea shark liver oil, spiny dogfish oil and cod liver oil, by using SC-CO_2. They used a pilot plant with a countercurrent

fractionation column with two separation vessels. Additionally, they proved the effect of using ethanol as co-solvent in the supercritical CO_2 fractionation, concluding that a mixture of CO_2-ethanol (5 % by mass) enabled to obtain a product (raffinate) lower in acidity (aprox. 0.5 %) and with a negligible fishy odor compared to the raffinate obtained using pure SC-CO_2 under the same experimental conditions (*Catchpole, et al.*, 2000).

Yuqian et al. also proposed a method for refining crude fish oil by combining supercritical fluid extraction (SFE) with CO_2 or propane and a physical adsorption step (*Yuqian, et al.*, 2001).

Kawashima et al. proposed combining extraction with SC-CO_2 and adsorption on activated carbon to remove polychlorinated biphenyls (PCB), polychlorinated dibenzo-p-dioxins (PCDD), and polychlorinated dibenzofurans (PCDF) from fish oil. They found the SFE process to be effective for removal of PCB and the adsorption method for removal of PCDD / DF. By combining both methods they could achieve a reduction of the total toxicity of about 100 % (*Kawashima, et al.*, 2006).

As a conclusion, it can be said that traditional oil refining is being revised to explore new technologies, mainly supercritical fluid technologies that have been shown to produce high quality oil.

1.2.2.5. Supercritical fluids in omega-3 concentration

In the last years, the use of supercritical fluids in omega-3 concentration from fish oil has acquired a great interest and several processes have been

developed based on supercritical fluid fractionation (SFF), supercritical fluid chromatography (SFC) or enzymatic reaction under supercritical media.

i) Supercritical fluid fractionation (SFF)

During the 1980ties, several authors proposed some of the first methods to fractionate fish oil ethyl esters by using supercritical fluids (*Pettinello, et al.*, 2000, *Snoey-Elich*, 2001) and take advantage of their previously mentioned attractive features (*see* Section 1.2.2.1). Since then, several authors have continued exploring this field. In many cases, the research has included the study of the phase equilibrium of the mixtures involved in the processes, because it is essential for the design of an extraction or fractionation process. Nowadays, most of the fractionation studies using SCF are performed over omega-3 ethyl esters and, therefore, phase equilibria involving this type of esters has been also the most widely studied.

Fleck et al. designed an automated countercurrent column for fractionation of FAEE by using SC-CO_2. Only adding automation to the column caused a significant improvement in the separation efficiency, very important for the development of SFF processes at industrial scale (*Fleck, et al.*, 1998).

Riha & Brunner studied phase equilibria between SC-CO_2 and many fatty acid ethyl esters (FAEE) derived from fish oil, proving that it is possible to fractionate them according to their carbon number (*Riha, et al.*, 1999).

Based on these results, the same authors developed a method to separate FAEE from fish oil by countercurrent multistage extraction with CO_2 in a pilot scale column (*Riha, et al.*, 2000). They also proposed a thermodynamic model that enabled to calculate the optimal conditions to separate FAEE by this process that resulted to be 14.5 MPa and 333 K. Finally, they proved that the application of the optimized method at industrial scale produced a concentration of EPA, DHA and DPA higher than 90 %. Moreover, the use of a theoretical model also enabled to reduce the operation cost and increase the profitability of the process (*Brunner,* 2000).

Espinosa et al. studied phase equilibria between a mixture of FAEE and SC-CO_2 based in a Group Contribution Equation of State (GC-EOS). From these results, they performed a simulation of the extraction process, and optimized the experimental conditions. Finally, they proposed a method to obtain a high yield of high purity fractions of EPA and DHA respectively, using a high pressure countercurrent system with three columns, which enabled to obtain an EPA fraction with a purity of 60.46 mol % and a yield of 98.18 %; and a DHA fraction with a purity of 80.09 mol % and a yield of 80.34 %. Alternatively, to increase the purity of the product, they proposed a previous step based on urea complexation (*Espinosa, et al.*, 2002).

Perreti et al. have recently confirmed the possibility of modifying the FAEE concentration of a mixture by using SC-CO_2 and optimizing the fractionation process in terms of pressure, temperature, and flow rate of this selective solvent (*Perretti, et al.*, 2007).

Gironi & Maschietti proposed a thermodynamic model that assumed the oil to be composed of five ethyl esters (EE), each of them representing a specific acid chain length. They validated this model with some experimental data obtained in a semicontinuous single-stage fractionation process and then applied it to simulate a multistage continuous process. They demonstrated that this process enables to produce a raffinate with 95% by weight of EE-EPA and EE-DHA, together with 95% recovery of these compounds (*Gironi, et al.*, 2006). However, the scale-up of this process at industrial scale has not been reported yet.

Martin & Cocero developed a comprehensive mathematical model for the SFF of liquids with SC-CO_2 by including the differential mass and energy balances in the column, and the coupled mass and heat transfer in the interface between the two fluid phases. Phase equilibrium and volumetric and thermal properties, were described by using a consistent equation of state and transport properties and mass transfer coefficients were estimated applying suitable correlations. They validated this model for the SFF of fish oil ethyl esters mixtures and found it to be able to predict the trends of variation of the composition of extract and raffinate with the different operating parameters (*Martín, et al.*, 2007).

Antunes-Corrêa et al. focused their work towards obtaining concentrates of omega-3 PUFA in their natural form instead of their ethyl esters. They measured natural fish oil solubility by the dynamic method and determined the composition of both, the original oil and the collected fraction. These

data allowed them to calculate the distribution coefficients of several of the oil components. The distribution coefficients they found were very close to unity giving little expectation to SFF of the natural triacylglycerides to concentrate omega-3 PUFA (*Antunes-Corrêa, et al.*, 2008). Similar conclusions were reached also by other authors (*Davarnejad, et al.*, 2008).

Research on fish oil SFF is still open with the aim of achieving production at large scale. The main limitations for scaling up the process is the knowledge of phase behaviour of the mixtures involved on SFF and models to describe it. As has been detailed previously, models to describe SFF are well developed once phase equilibria can be well described.

ii) Supercritical fluid chromatography (SFC)

Because of the physical properties of supercritical fluids, they can be used as a mobile phase along with packed or capillary columns filled with a suitable stationary phase. This technique is named supercritical fluid chromatography (SFC), and is an attractive alternative to traditional chromatographic techniques as gas chromatography (GC) or high pressure liquid chromatography (HPLC) for analytical, preparative or production purposes (*Kiran, et al.*, 2000) due to the SCF properties mentioned at the beginning of this section.

SFC is especially suitable for the separation of omega-3 PUFA since it combines the high selectivity of both, the supercritical fluid and the stationary phase. Therefore, several methods based on SFC have been

developed to obtain omega-3 PUFA with a high purity and recovery not only in the laboratory but also at large scale. As for SFF, most of the research and production of omega-3 concentrates deals with the ethyl esters. The most interesting works on this subject are reviewed next.

Perrut et al. patented a method to isolate EPA and DHA from a mixture of fatty acid ethyl esters obtained from fish oil, coupling preparative supercritical chromatography and simulated countercurrent moving bed chromatography with several columns placed in series. In both cases, octadecyl silica gel is used as column stationary phase whereas SC-CO_2 is the solvent eluent. Following this method, they obtained EPA and DHA fractions with a high yield (99 %) and purity (92 % and 85 % respectively) (*Perrut, et al.*, 1998).

Brunner & Reichmann also patented a SFC method to isolate unsaturated fatty acids with at least 16 carbon atoms from a mixture of fatty acids or their derivatives using SC-CO_2 as eluent and aluminium oxide as stationary phase (*Fleck, et al.*, 1998).

Alternatively, other studies about SFC of omega-3 fatty acids have been found in the literature.

Alkio et al. reported a systematic study in order to find the optimal conditions to purify DHA-EE and EPA-EE from esterified tuna oil. They found that using SC-CO_2 at 65 ºC and 145 bar and octadecyl silane as stationary phase, it was possible to obtain simultaneously a DHA-EE production of 0.85 g/(kg stationary phase × h) with a purity of 90 %, and an

EPA-EE production of 0.23 g/(kg stationary phase × h) with a purity of 50 % (*Alkio, et al.*, 2000).

Pettinello et al. studied the enrichment on bench-scale and on pilot-scale of an ester mixture from fish oil with 68 % of EPA-EE using supercritical chromatography with SC-CO_2 as mobile phase and silica gel as stationary phase. They concluded that, on bench-scale production, using a column of 25 mm × 200 mm, the highest EPA-EE purity (90 %) and recovery (49 %) was obtained at a temperature of 70 ºC with a pressure programme between 180 and 220 bar and a SC-CO_2 flow rate of 13.5 g/min. However, on semi-industrial scale using a column of 100 mm × 520 mm, the optimal conditions involved the use of the same temperature and pressure, but a higher CO_2 flow rate (15-25 kg/h), which enabled to process a total amount of 300 g fish oil per cycle and obtain an EPA-EE purity of 93 % with a yield of 24.6 % (*Pettinello, et al.*, 2000).

A recent method patented by Snoey-Elich has been also proposed to concentrate EPA-EE from fish oil, from a purity of 50 % to more than 95 %, using an industrial column of 50-60 cm × 260-280 cm filled with a porous silicon oxide impregnated with (3-aminopropyl)- triethoxysilane as stationary phase and SC-CO_2 as mobile phase working at a pressure of 100-150 bar, a temperature of 40-60 ºC and a flow rate between 4000-7000 kg/h (*Snoey-Elich*, 2001).

Other authors, as Yang et al., have continued the researches about SFC to isolate both EPA-EE and DHA-EE from fish oil with a high purity and yield (*Yang, et al.*, 2004).

This process is already installed at production scale, and companies as KD-Pharma or Solutex, produce a combination of omega-3 PUFA in a proportion over 90 %, EPA up to a concentration of 95 % and DHA up to 80 % by applying this technology.

iii) Enzymatic reactions in supercritical fluids

Supercritical CO_2 has also been proposed as a good medium to carry on enzymatic reactions, since it is considered a green solvent, nontoxic and easily removed from the reaction products. In addition, SC-CO_2 presents interesting physical properties such density, diffusivity or viscosity, which can be changed by changing pressure or temperature. Moreover, enzymatic reactions under supercritical fluids may be easily coupled with other processes such SFE, SFF, SFC or microencapsulation procedures using SC-CO_2, which has an enormous interest in the production of microencapsulated omega-3 concentrates directly from the raw material, minimising the oil manipulation and, therefore, preventing its oxidation.

In last years, several studies have been published by Gunnlaugsdottir et al. about the influence of experimental conditions, as pressure (*Gunnlaugsdottir, et al.*, 1995)or solvent flow rate (*Gunnlaugsdottir, et al.*, 1997b) on the ethanolysis of cod liver oil with an immobilized lipase

(Novozym 435). The same authors have also studied the phase behaviour of the reaction mixture in SC-CO_2 (*Gunnlaugsdottir, et al.*, 1997a) and a coupled process with supercritical fluid extraction and enzymatic ethanolysis to produce omega-3 ethyl esters from cod liver oil in a continuous mode (*Gunnlaugsdottir, et al.*, 1998).

Another recent procedure, proposed by Lin et al., enables an omega-3 enrichment of fish oil through an enzymatic transesterification with an immobilized 1,3-specific lipase (Lipozyme IM-60) in a supercritical CO_2 medium (5 h, 323 K and 10.34 bar). They reported that under supercritical conditions, enzymatic transesterification was 40 % higher than under conventional solvents, proving that coupled processes with supercritical fluids and biotechnological methods may be an interesting strategy to produce omega-3 structured lipids from fish oil under mild conditions and with a high yield (*Lin, et al.*, 2006).

A recent review shows all the possibilities of SCF technology to perform enzymatic reactions, including oil hydrolysis and transesterification (*Knez*, 2009). As observed, enzymatic methods by themselves, do not lead to omega-3 concentrates but further concentration is needed. Pretreatments using supercritical fluids present advantages regarding the non use of chemicals and the high yields obtained.

1.2.2.6. Supercritical fluids in omega-3 formulation

Microencapsulation processes using supercritical fluids have also been proposed recently as a good alternative to protect active compounds as fish oil using mild temperatures in an inert atmosphere. Among them, encapsulation and co-precipitation processes as Rapid Expansion of Supercritical Solutions (RESS), Supercritical Solvent Impregnation (SSI), Particles from Gas Saturated Solutions (PGSS), Supercritical Anti Solvent precipitation (SAS) or Supercritical Fluid Extraction of Emulsions (SFEE) (*Cocero, et al.,* 2009). These methods are still under investigation and no studies about supercritical fluid microencapsulation of fish oil have been found in the literature.

1.3. Production of omega-3 fatty acids from alternative sources to fish oil

Abstract

As it has been previously treated (*see* Section 1.1.3), fish and fish by-products are the most important sources of omega-3 fatty acids, especially EPA and DHA. However, it is known that global fish stocks are in danger and marine fish production may decrease in the future.

Thus, searching for alternative sources of omega-3 PUFA is required, being marine microalgaes, algaes or transgenic plants some of the most recent proposal.

➢ **Content**

1.3.1. Omega-3 production from marine algae and microalgae

Marine microalgae, or phytoplankton, provide the food base that supports the entire animal population in open seas. Human application of microalgaes has been made in Asia since ancientness, although microalgae biotechnology has been developed in the last century. Nowadays, commercial applications of microalgaes are extended all over the world.

Spolaore et al. have reviewed some of the actual commercial applications of microalgaes in human and animal nutrition and in the cosmetic industry (*Spolaore, et al.*, 2006).

Cardozo et al. have also reviewed the most recent researches on microalgae production of high-value compounds having economic relevance in food

science, pharmacology or human health, as PUFA, sterols or carotenoids, among others (*Cardozo, et al.*, 2007).

Conchillo et al. have published a study comparing microalgae oil and fish oil. They concluded that both oils presents a similar amount of omega-3, although microalgae oil has the advantage of presenting neither an unpleasant odour nor a high amount of cholesterol, and contains squalene and phytosterols, which offer additional benefits to human health. In addition to this, microalgaes are easily cultivated, which avoid differences in seasonal production and enables increasing the productivity of PUFA from an industrial point of view (*Conchillo, et al.*, 2006). Several reviews have been published about the metabolism of PUFA in different types of marine microorganisms and the strategies to cultivate them (*Guschina, et al.*, 2006, *Sijtsma, et al.*, 2004, *Ward, et al.*, 2005, *Wen, et al.*, 2003).

Ratledge has also reviewed the application of the biotechnology in the production of single cell oils, using arachidonic acid (ARA) and DHA as main source of PUFA (*Ratledge*, 2004). In the last years, many researches have also been made to optimize the technical design of bioreactors for microalgae production at industrial scale (*Carvalho, et al.*, 2006).

Regarding the methods of oil extraction from algae or microalgae, Robles Medina et al, (*Robles-Medina, et al.*, 1998) examined the recovery and purification of microalgae derived PUFA. They discussed the two main techniques used at the time for obtaining highly pure PUFA: urea

fractionation and HPLC, and detailed "potentially useful techniques" such as supercritical fluid extraction and lipase-catalyzed processing.

TABLE 1.14

PUFA extraction processes from natural sources different than fish oil (since 1998).

Raw material	Technology	Product	Reference
Subtropical red seaweed (*H. charoides*)	SC-CO_2 extraction	Lipid extract	(*Cheung*, 1999)
Diatom (*P. tricornutum*) Green alga (*M. subterraneous*)	Extraction-transesterification Chromatography Decolorization	Pure EPA-EE	(*Belarbi, et al.*, 2000)
Red algae filaments	SC-CO_2 extraction	Lipid extract rich in EPA	(*Chen, et al.*, 2002)
Microalgaes	SC-CO_2 extraction SC-CO_2 + ethanol extraction	Lipid extract rich in GLA	(*Mendes, et al.*, 2003)
Microalgae (*Nannochloropsis sp.*)	SC-CO_2 extraction	Lipid extract	(*Andrich, et al.*, 2005)
Cyanobacterium (*A. Spirulina maxima*)	SC-CO_2 extraction SC-CO_2 + ethanol extraction	Lipid extract rich in GLA	(*Mendes, et al.*, 2006, *Mendes, et al.*, 2005)

From then to now, several authors have proposed new PUFA extraction methods from different microorganisms, many of them using supercritical fluid technology, similar to the fish oil extraction processes described in Section 1.2.2 (*Andrich, et al.*, 2005, *Belarbi, et al.*, 2000, *Chen, et al.*, 2002, *Cheung*, 1999, *Mendes, et al.*, 2003, *Mendes, et al.*, 2006, *Mendes, et al.*, 2005) (*see* Table 1.14).

1.3.2. Omega-3 production from transgenic plants

Transgenic plants have been also proposed as an alternative source of omega-3 fatty acids.

Napier et al. have recently published a review with the last advances in the production of enriched vegetables species through genetic modifications (*Napier*, 2006).

Robert et al., have also compiled the most recent studies about production of transgenic seed oils and their use in human and aquaculture nutrition (*Robert*, 2006).

In any case, nowadays the use of transgenic plants in agriculture is not well-accepted in many parts of the world, especially in Europe

1.4. References

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Chapter 2

Supercritical Fluid Extraction of Fish Oil rich in Omega-3 from Fish by-products

This Chapter has been partially published in the Journal of Supercritical Fluids (*Rubio-Rodríguez, et al.*, 2008), as well as presented in the 9th International Symposium on Supercritical Fluids 2009 (*Rubio-Rodríguez, et al.*, 2009)

SUMMARY

Supercritical fluid technology has become a promising method to extract high value natural compounds such as omega-3 fatty acids.

In this chapter, the supercritical fluid extraction of the omega-3 rich oil contained in by-products usually generated in fish industries has been studied. The characterisation of the different by-products provided by a Spanish fish industry, Pescanova S.L., is presented in the first section (2.1), in order to establish their profitability as raw material for fish oil production. Then, section 2.2 reports the experimental study carried out about the influence of several process parameters on fish oil extraction from hake by-products in a semi-pilot plant, focusing on both extraction yield and oil quality regarding the free fatty acid content (FFA). In this section a data modelling based on different mathematical models, both empirical and rigorous, is also included. The last part of the chapter (Section 2.3) compiles the results of the comparative study carried out about the quality of fish oil extracted from different fish by-products by different physical and chemical methods (centrifuging, wet reduction, enzymatic extraction) and by the use of SC-CO_2 as solvent, taking into account also some economical considerations of the supercritical fluid extraction technology.

RESUMEN

La tecnología de fluidos supercríticos se ha convertido en un método prometedor para extraer compuestos naturales con un alto valor añadido como los ácidos grasos omega-3.

En este Capítulo se ha estudiado la extracción con fluidos supercríticos de aceite rico en omega-3 a partir de subproductos generados en la industria pesquera. En la primera parte (Sección 2.1), se ha llevado a cabo la caracterización de diferentes subproductos suministrados por una industria pesquera española, Pescanova S.L., con el objetivo de establecer su potencial como materia prima en la producción de aceite de pescado a escala industrial. En la sección 2.2, se muestran los resultados experimentales sobre la influencia de distintos parámetros de proceso en la extracción con dióxido de carbono supercrítico en una planta semi-piloto, teniendo en cuenta tanto el rendimiento de la extracción como la calidad del aceite extraído. En esta sección se incluye además un apartado sobre el modelado del proceso a partir de modelos matemáticos tanto empíricos como rigurosos. La última parte del capítulo (sección 2.3) recoge los resultados del estudio comparativo sobre la calidad del aceite de pescado extraído a partir de diferentes subproductos mediante métodos físico-químicos y mediante el uso de dióxido de carbono supercrítico como disolvente, teniendo en cuenta además algunas consideraciones económicas sobre la extracción con fluidos supercríticos.

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NOMENCLATURE

AV	Anisidine value
C^*_A	Equilibrium concentration of solute A on solid surface (g / kg CO_2)
C_A	Concentration of solute A in bulk solid phase (g / kg solid)
$C_{A,FS}$	Concentration of solute A in bulk fluid (g / kg CO_2)
$C_{A,S}$	Concentration of solute A on solid surface (g / kg solid)
CE	Oil extraction by cold method (centrifuging)
C_L	Concentration of oil in fluid phase at reactor outlet (kg oil / kg CO_2)
C_o	Concentration of oil in fluid phase at reactor inlet (kg oil / kg CO_2)
C_s	Concentration of oil in fish by-products (kg / kg solid)
$C_{s,o}$	Initial concentration of oil in fish by-products (kg / kg solid)
D_{az}	Axial dispersion (cm^2 / min)
D_e	Equivalent diameter of solid particles (mm)
d_p	Solid particle diameter (mm)
D_r	Radial dispersion (cm^2 / min)
DHA	Docosahexanoic acid (omega-3)
EE	Oil extraction by enzymatic method
EPA	Eicosapentaenoic acid (omega-3)
F	Solvent flow rate (kg CO_2 / h)
f_1, f_2	Fraction of solute extracted in the first and second extraction stages according to Kandiah & Spiro's model (*Kandiah, et al.*, 1990)
FFA	Free Fatty Acids content (% wt. oleic acid)
h	Equilibrium parameter [(g oil / kg solid) / (g oil / kg CO_2)]
k	Empirical rate constant (min^{-1})
k_1	Empirical rate constant in the first extraction stage (min^{-1}) according to Sovová's model (*Sovová*, 2005)
k_2	Empirical rate constant in the second extraction stage (dimensionless) according to Sovová's model (*Sovová*, 2005)

k_3	Empirical rate constant in the second extraction stage (min^{-1}) according to Sovová's model (*Sovová*, 2005)
K_ga	Global mass transfer coefficient (min^{-1})
L	Length of extraction bed (cm)
$\dot{m}_A$	Mass flow of solute A (kg / h)
NL	Neutral lipids
P	Pressure (MPa)
PUFA	Polyunsaturated Fatty Acids
PV	Peroxide Value (meq O_2 / kg oil)
r	Length in radial direction (cm)
R	Radius of bed (cm)
S	Solubility of oil in SC-CO_2 (g / L)
SC	Supercritical
SFE	Supercritical Fluid Extraction
t	Extraction time (min)
T	Temperature (K)
TAG	Triacylglycerides
TOTOX	Total Oxidation Index (defined as 2 PV + AV)
t_r	Time (min) at which the extraction from particle core starts according to Sovová's model (*Sovová*, 2005)
u	Superficial velocity (cm_{fluid} / min)
WE	Oil extraction by wet reduction (cooking method)
w_p,	Slope of extraction curves estimated at the beginning of the extraction of marine oil (g oil / kg CO_2)
x	Dimensionless variable defined as (2.18)
y	Dimensionless variable defined as (2.19)
y_S	Dimensionless variable defined as (2.20)
z	Length in axial direction (cm)

Greek symbols

ε	Porosity of solid raw material
κ_1, κ_2	Rate constants (min^{-1}) in the first and second extraction stages according to Kandiah & Spiro's model (*Kandiah, et al.*, 1990)
ρ	Density of SC-CO_2 (g / cm^3)

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2.1. Characterisation of Fish by-products and their valorisation as source of omega-3 PUFA

Abstract

As discussed in Chapter 1, fish and fish by-products are the main natural source of omega-3, especially EPA and DHA.

However, in order to establish the profitability of a fish by-product as raw material in fish oil production on large scale, a previous knowledge of its chemical composition, specially the amount of oil and water, is required.

Thus, the aim of this section is to characterise different fish by-products commonly generated in Spanish fish industry in order to determine its possible application in fish oil production.

➢ **Section Content**

2.1.1. Current situation of fish by-products in the fish industry

The fish industry is a wide sector that includes several production processes such as filleting, curing, salting, smoking, canning, etc. Nowadays, it is estimated that more than 70 % of fish captures are processed, generating a large amount of solid wastes and by-products, which often represent more than 50 % of total fish weight (*Shahidi*, 2007) (*see* Figure 2.1).

Traditionally, fish by-products have been processed in fishmeal plants to produce low value products, which are used as animal food or fertilizers. However, in the last years, several researches have shown that fish by products can be used as a natural source of high value compounds with an

elevated commercial potential such as peptides, fish oil, collagen or gelatine (Kim, et al., 2006).

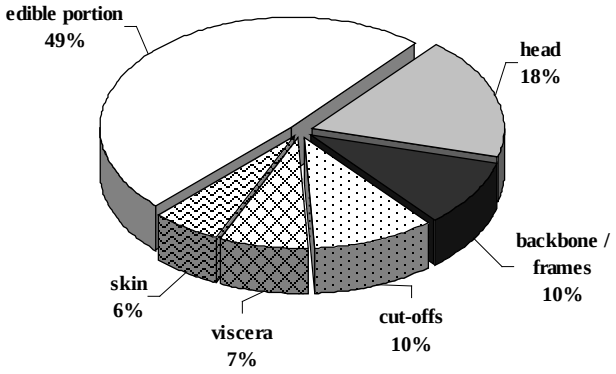


FIGURE 2.1

Average distribution of edible portion and by-products in fish

The production of high quality fish oil has acquired a great importance since it is considered one of the main natural sources of omega-3 fatty acids, which benefits in human health has been reported in Chapter 1. Furthermore, the production of fish oil rich in omega-3 has become a good opportunity in order to aim a higher valorisation of fish by-products and increase the competitiveness of fish industry.

2.1.2. Experimental section

2.1.2.1. Raw material and pretreatment

The raw materials studied were fish by-products, specifically offcuts and livers, provided by Pescanova, a Spanish fish company located in Pontevedra (Spain). These by-products were generated in the fish industry during the processing of different species such as kingklip, hake, orange roughy, salmon and jumbo squid. The main features of these species are reported in Appendix II.

The offcuts, consisted mainly of skin with some stuck muscle and were obtained by peeling fishes with a TRIO™ peeler in open seas, whereas the livers were obtained during the evisceration process. All by-products were frozen at 253 K and kept frozen until the experiments were performed. The offcuts were cut into small pieces (1-10 mm equivalent diameter, D_e), with a cutter, in order to improve the extraction rate.

2.1.2.2. Characterisation of marine by-products. Analytical methods

The marine by-products were characterised by determining their water, protein and fat content in order to establish their profitability as raw material for the oil extraction. The water and protein content were determined by the AOAC Official Methods 934.01 and 981.10 respectively (*AOAC*, 2000) The total fat content was determined by Soxhlet using petroleum ether as solvent

in a Büchi extraction system (model B-811). Further details about analytical methods are compiled in Appendix I.1.

2.1.2.3. Characterisation of lipid fractions. Analytical methods

A preliminary characterisation of the lipid fraction extracted by Soxhlet extraction with petroleum ether was carried out focused on the amount of omega-3 EPA and DHA and in the amount of impurities such as cholesterol. Omega-3 EPA and DHA content was determined by GC-FID analysis according to the method described in Appendix I.2. Cholesterol content was measured by HPLC-ELSD as also detailed in Appendix I.2.

2.1.3. Results and discussion

2.1.3.1. Total fat content

Among the different marine by-products generated in the fish industry, skin and liver have the greatest interest as potential sources of marine oil since they present fat depots in their cellular structure.

Histological studies have reported that fish skin is a solid structure composed by two cutaneous layers (epidermis and dermis) and a subcutaneous layer or hypodermis where the fat depots are located (*LeGuellec, et al.,* 2004), whereas fish liver or hepatopancreas presents a soft structure composed basically by large polyhedral cells, called

hepatocytes, the cytoplasm of which contains the majority of fat depots (*Alpini, et al.*, 1994).

TABLE 2.1

Chemical composition of the different by-products studied as potential source of marine oil.

Marine by-products		Water (%)	Fat (%)	Protein (%)	Oil / water ratio
Offcuts	Kingklip	82 ± 1	0.1 ± 0.1	18 ± 1	0.0008
	Hake	79 ± 1	4.0 ± 0.1	16 ± 1	0.1
	Orange r.	55 ± 2	32 ± 1	18 ± 1	0.6
	Salmon	57 ± 3	27 ± 5	17 ± 4	0.5
Livers	Salmon	74 ± 1	0.2 ± 0.1	25.7 ± 0.2	0.003
	Jumbo squid	70 ± 1	8 ± 3	22 ± 4	0.12

In this work, the feasibility of obtaining valuable fish oil from some by-products of common fishes (kingklip, hake, orange roughy, salmon and jumbo squid) was studied. Their chemical composition, water, fat and protein content, is summarized in Table 2.1.

It was observed that, among the different fish by-products studied, orange roughy and salmon offcuts have the highest fat content (32 % and 27 % respectively), followed by jumbo squid liver (8 %), hake offcuts (4 %) and kingklip offcuts (0.1 %). Salmon liver presents a poor fat content (0.2 %). This significant variation observed on the average lipid content in fish

offcuts and liver agrees with the classification proposed by FAO, which establishes that demersal type of fish, such as hake or kingklip, are lean fishes and store fat preferentially in their liver, whereas pelagic fish, such as orange roughy or salmon, are fatty fishes and store lipids mainly in their muscle and under the skin (*FAO*, 1986).

Therefore, taking into account their fat content, kingklip offcuts and salmon liver do not present interest as sources for oil extraction at production scale.

2.1.3.2. Amount of EPA and DHA

Since EPA and DHA are considered the most important omega-3 fatty acids due to their great implications in human health (*see* Chapter 1), their concentration in the fish oil is another feature to take into account for deciding whether a certain fish by-product is a valuable raw material for the omega-3 extraction process or not. Furthermore, the concentration of undesirable lipid compounds such as cholesterol is another factor that must be noticed.

Figure 2.2 shows the amount of both omega-3 EPA and DHA and cholesterol found in the lipid extract obtained with petroleum ether from the available fish by-products that presents total fat content higher than 1 %, that is, hake, orange roughy and salmon offcuts and jumbo squid liver.

It is observed that, the lipid extract obtained from jumbo squid liver presents a great amount of omega-3 EPA and DHA (around 13 % wt. respectively),

although a high cholesterol content is also detected, which may limit its use as raw material in production of omega-3 concentrates.

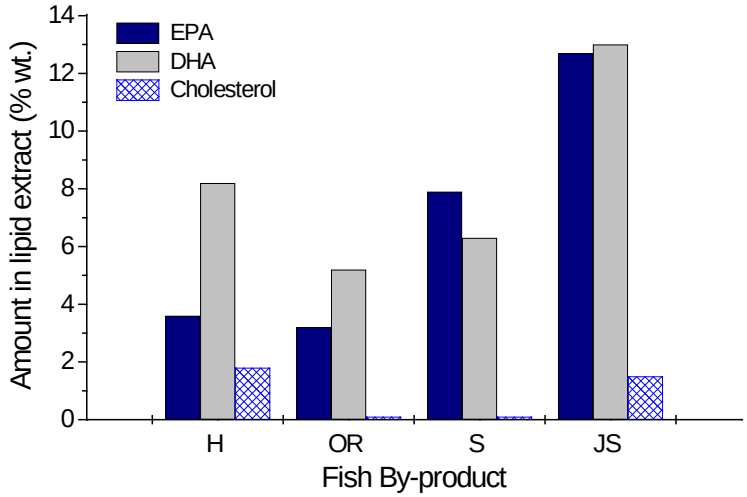


FIGURE 2.2

Amount of EPA, DHA and cholesterol found in lipid extracts obtained by soxhlet extraction from different fish by-products. H: Hake skin, OR: Orange Roughy skin, S: Salmon skin, JS: Jumbo Squid Liver.

Both salmon offcuts and hake offcuts are attractive raw materials for production of omega-3 concentrates since their lipid fractions present a low or neglected cholesterol content. Salmon offcuts lead to a valuable lipid fraction with a moderate EPA and DHA content (around 7 % respectively),

whereas hake offcuts gives a lipid fraction with a high DHA / EPA ratio (around 2), which makes them useful as raw material for producing of DHA concentrates.

Lipid fraction obtained from orange roughy offcuts, in spite of having a neglected cholesterol content, has a poor interest for producing omega-3 concentrates due to their low EPA and DHA content (< 5 % wt. respectively).

2.1.4. Conclusions

The use of different fish by-products as possible raw material for fish oil extraction on large scale has been evaluated by means of their chemical composition, i.e., fat, protein and water content.

Among the fish by-products available in this work, offcuts from hake, orange roughy and salmon, and liver jumbo squid, were considered the most suitable for having the greater fat content. On the contrary, salmon liver and kingklip offcuts were discarded due to their extremely low fat content.

Regarding the lipid composition, salmon offcuts presents the higher interest for leading to a lipid fraction with a great amount of omega-3 EPA and DHA (> 8 % respectively) and a neglected cholesterol content (< 2 % wt.). Hake offcuts, despite its total oil content, may be also a raw material worth to exploring in omega-3 production, especially DHA.

These results allow us to choose the most suitable raw material for our next extraction experiences. Nonetheless, oil quality is the subsequent factor that

must be taken into account to establish the profitability of these by-products as a source of high value fish oil for edible purposes; as well as other factors such as availability in fish industry or seasonal variation in fat depots which have a great importance from an economical point of view.

2.2. Supercritical fluid extraction of the omega-3 rich oil contained in hake (*Merluccius capensis – Merluccius paradoxus*) by-products: study of the influence of process parameters on the extraction yield and oil quality

Abstract

The supercritical fluid extraction (SFE) of the omega-3 rich oil contained in by-products of fish industry has been studied in this section. Specifically, the offcuts obtained from peeling hake (*Merluccius capensis-Merluccius paradoxus*), which have shown to have a high DHA content (around 8 % wt. lipid fraction) and a high DHA / EPA ratio (around 2), being attractive for producing DHA concentrates.

➢ **Section Content**

Previous to the extraction, hake offcuts were grounded to different sizes and freeze-dried to different moisture contents. Extraction experiments were carried out at different pressure, flow rate and flow sense in order to study the influence of such process parameters on the extraction rate and oil quality. The influence of continuous or intermittent contact solvent-subtract was also evaluated. The extraction temperature was maintained at 313 K in all cases in order to avoid thermal degradation of the polyunsaturated fatty acids contained in hake oil. The results obtained indicate that internal mass transfer controlled the rate of the process under the conditions studied in this work. The optimum extraction conditions were found to be 25 MPa, 10 kg CO_2 / h and upflow sense over freeze-dried and grounded hake. Under those conditions, more than 96 % of the total oil contained in the raw material was extracted after 3 hours with a free fatty acid content around 4.7 % oleic acid. Fish by-products such as hake offcuts can be considered a valuable source of omega-3 fatty acids, especially EPA and DHA.

2.2.1. Introduction

Polyunsaturated fatty acids (PUFA), especially omega-3 fatty acids, have been very often subject of scientific studies in the last years. There is voluminous new information available on PUFA and their presence in food and food products. There is also plenty of new knowledge on the biological effects and health implications of this type of fatty acids as it is reviewed in Chapter 1.

Fish is the most important natural source of omega-3 fatty acids in the diet, specifically fish oil, which has been extracted at production scale from the 19th century by means of traditional methods based mainly in four steps: fish cooking, pressing, decanting and centrifugation (*FAO*, 1986). Nowadays, some alternative methods are being developed, among them, methods based on supercritical fluid extraction (SFE) and methods based on enzymatic processes with proteases (*Linder, et al.*, 2005).

Supercritical fluid extraction is characterized by the use of supercritical solvents. The most used SC-solvent is carbon dioxide. CO_2 has low toxicity and economical cost, is not flammable and is environmentally friendly. In addition, CO_2 has mild critical conditions (T_c = 304.15 K, P_c = 7.38 MPa), which make it suitable for processing thermo-degradable compounds as polyunsaturated fatty acids.

In the last decade, several studies have been published about SFE of oil from different types of fish or fish by-products: Esquível et al. studied the extraction of sardine oil with SC-CO_2 (*Esquível, et al.*, 1997). Dunford et al.

studied the effect of moisture on the extraction of mackerel oil with SC-CO_2 and proposed a mathematical model to explain such effect on the extraction yield (*Dunford, et al.*, 1998). Recently, Letisse et al. studied the SFE of omega-3 rich oil from by-products of the fish industry, specifically from sardine heads (*Letisse, et al.*, 2006). However, no data about SFE of fish oil in a pilot plant and the influence of the process variables have been found in the literature. Thus, the aim of our work was to study the supercritical CO_2 extraction of oil from hake by-products in a semi-pilot plant in order to establish the influence of the main process parameters on the extraction yield.

2.2.2. Theoretical considerations about SFE from solid materials

Supercritical fluid extraction (SFE) is a separation process in which a supercritical fluid is used as solvent. This process involves two main steps: extraction, in which the supercritical fluid dissolves the solute from the solid matrix; and separation between the solvent and the solute, which is usually carried out decreasing the solubility of the dissolved compound by changing the solvation power of the fluid or by the help of adsorbent compounds or membranes (*Brunner*, 1994).

The extraction stage is highly dependent on the internal structure and complexity of the solid material that contains the solute. In the case of animal tissues, such as fish by-products, made of a complex cellular

structure, solute extraction may involve different steps, as it is described below (*see* Figure 2.3).

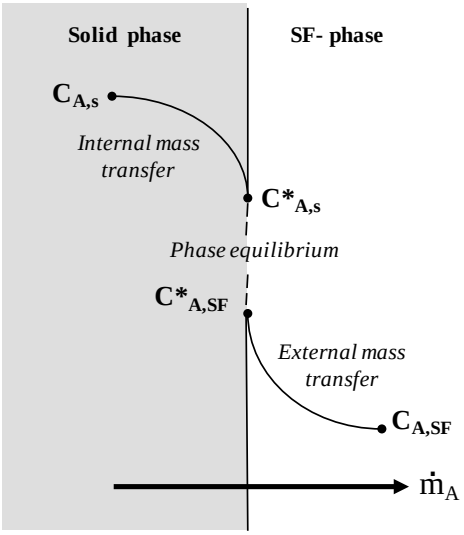


FIGURE 2.3

Diagram of the supercritical fluid extraction from a solid complex material

1) ***Internal mass transfer*****:** the supercritical fluid flows to the soli matrix and is absorbed by the solid material (animal cells) where the solute (fish oil) is located. Consequently, the solid matrix is swollen since the intercellular channels are widened reducing the internal mass transport resistance.

2) ***Phase equilibrium*****:** the fish oil is dissolved by the supercritical fluid according to its solubility equilibrium. This solubility depends on the

fish oil composition, which may include a wide range of lipids such as acylglycerides, free fatty acids, cholesterol, wax esters, squalene. depending on the origin of the fish by-product chosen as raw material.

3) ***External mass transfer*****:** the fish oil dissolved flows through the internal pores by an internal diffusion mechanism and pass through the solid surface where the phase change may take place. Then, it is carried into the bulk of the solvent and removed from the solid material.

The role of these factors in the global extraction process can be evaluated considering how the extraction yield changes when different variables related to the extractor design, the solid raw material (particle size, moisture) or the operational parameters (pressure and temperature, number of depressurization cycles, flow sense, pressure, flow rate) are changed. This study, described in next section, allows to get a better comprehension of the extraction mechanism and propose a suitable model required for the subsequent process scale-up.

2.2.3. Experimental procedure

2.2.3.1. Raw material and pretreatment

The raw material was a by-product of the Fish Industry, specifically the offcuts from hakes (*Merluccius capensis - Merluccius paradoxus*) which characterisation has been presented in 2.1. The average composition of the main components of this raw material was 4 % oil, 80 % water and 16 % protein.

Frozen hake offcuts were cut into small pieces (1-10 mm equivalent diameter, De) with a cutter (CT25, Talleres Cato S.A. Spain) in order to decrease the internal mass transfer resistance during extraction. After that, hake offcuts were dried to different moisture levels before performing the supercritical extraction experiments subject of this work. Screening of the dried raw material was carried out in order to evaluate the rate of the internal mass transport in the extraction experiments. When screening, it was found that the small particles were mostly hake muscle and the largest were mostly hake skin. Animal skin is the first animal protection to mechanical aggression, and as such, presents a larger resistance than muscle to be cut by the most usual mechanical devices in laboratories or industry (*Vaquero*, 2006).

In order to maintain the original internal cellular structure after drying and avoid that the lipid pillars of the membranes may close their pores, making the membrane impermeable to SC-CO_2 (*Brunner*, 1994), freeze-drying, carried out in FreeZone 12 Liter Console Freeze Dry System with drying chamber, Labconco, was chosen instead of conventional air drying.

2.2.3.2. SFE pilot plant

The equipment used in fish oil extraction with SC-CO_2 is a home made pilot plant designed and built to operate at a maximum specifications of: T = 373 K, P = 70 MPa and solvent flow, F = 20 kg / h. The main features of this plant has been already described in the literature (*Ganado, et al.*,

2002), although now it includes some modifications according to our requirements. The current P&I diagram is presented in Figure 2.4.

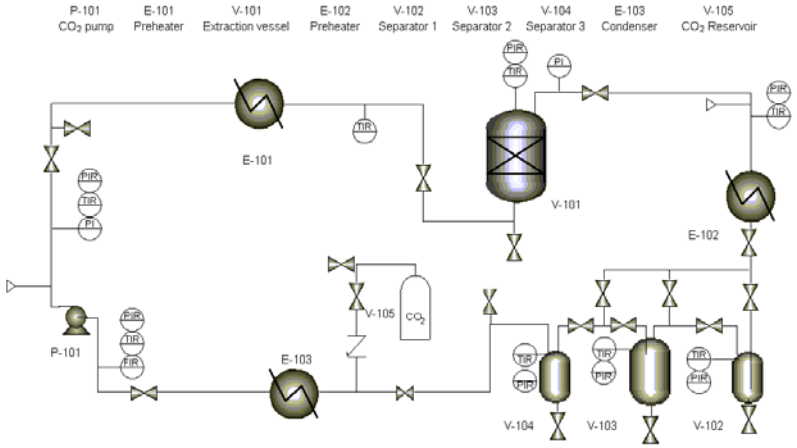


FIGURE 2.4

Flow-sheet of the SFE pilot plant with three separators.

The main elements of the extraction plant are described below.

- ***Pump*:** The pump used to pressurize the solvent up to the desired extraction pressure is a diaphragm pump (Lewa model EH-M-211V1) with metallic membranes, two hydraulic chambers (one for liquid CO_2 and another for an auxiliary fluid), a reciprocating plunger and a refrigerated head. In addition, it has an internal safety valve set at 70 MPa that protects against over-pressure.

The pumping mechanism takes places in two steps: suction stroke, in which the reciprocating plunger is moved up, the pressure in the chamber is decreased by an increment in volume, the diaphragm is moved up and the liquid CO_2 is sucked up; and discharge stroke, in which the diaphragm is moved down, the pressure in the chamber is increased by a reduction in volume, the diaphragm is subsequently moved down and the liquid CO_2, previously drawn, in is forced out. Finally, the plunger is moved up once again in order to draw the fluid into the chamber and complete the cycle (*see* Figure 2.5).

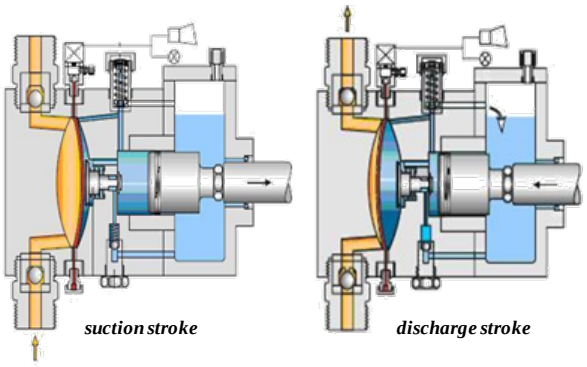


FIGURE 2.5

Working mechanism of membrane pump. Adapted from LEWA GmbH, *www.LEWA.com*.

This pump was chosen because of its efficient consumption and low maintenance costs. Additionally, the pump has not leaks, low friction leads and easily keeps a stationary way of operation.

• ***Pressure vessels:*** The plant is made up of four pressure vessels: one extractor and three separators. Their specifications are compiled in Table 2.2.

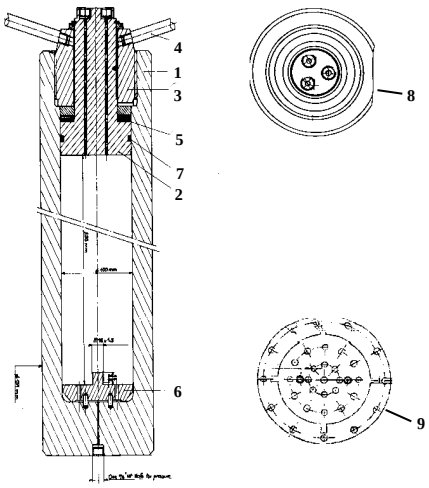
TABLE 2.2

Specification of pressure vessels.

Vessel	Volumen (L)	Dimensions (mm)			Maximun specifications		Closure system
		i.d.	i.h.	w.t.	P (MPa)	T (K)	
Extraction	2	100	255	57	70	373	O-ring
Separator 1	1	63	360	25	40	393	Bolted without gasket
Separator 2	0.75	80	150	22	20	393	Bolted without gasket
Separator 3	0.5	76	116	25	14	505	Resilient spring (zipper)

i.d.: internal diameter, i.h.: internal height, w.t.: wall thickness

The extractor (*see* Figure 2.6) is a 2-litres autoclave O-ring closure vessel, built in stainless steel AISI–316, specially designed and built by Nova Swiss for achieving the maximum specifications of 70 MPa and 373 K. A false floor, with holes on it, diffuses the SC-fluid avoiding preferential channels and minimising the dead volume, and two filters installed at the top and the bottom of the basket aim solids not to leave the autoclave. This vessel is thermally insulated to keep the processing temperature, which is measured directly inside the vessel close to the exit. Special Nova Swiss connections are installed to reduce the risk of leakage.



(1) Extractor wall
(2) Closing cover
(3) Main unit nut
(4) Handle for easy closing and opening
(5) Vent hole
(6) SCF diffusion system
(7) O-Ring
(8) Head view
(9) Diffusion system

FIGURE 2.6

Design of the extraction vessel.

The separators are installed consecutively in order to carry out a fractionation of the extract. Separator 1 is a ZipperClave® pressure vessel (Autoclave Engineering) with a zipper closure system and an O-ring seal specially designed for quick opening requirements, so it is normally used alone when only extraction is required. Separators 2 and 3 (*see* Figure 2.7) are a bolted closure pressure vessels built by a local provider according to our specifications, and are used additionally when fractionation is required. In the two, a 30 mm thick tube is welded to a clamp at the top and a conical floor at the bottom to allow the right discharge of the extracts and reducing the adhesion of extract to the wall. Both top and bottom connections are

NPT. Additionally, a heating jacket has been built to obtain the desired temperature inside the vessel.

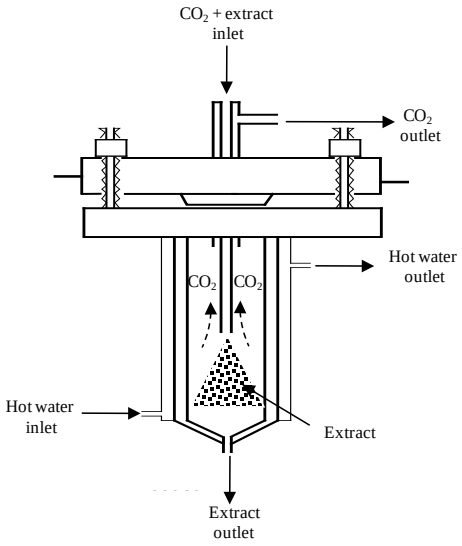


FIGURE 2.7

Design of separator vessel.

• ***Valves*****:** Three different types of valves are installed in the plant according to their function. Needle valves, with a strong variation of the flow coefficient as a function of the number of turns open, are installed to shut-off service whereas ball valves, with a low flow coefficient, are installed to flash the carbon dioxide. Check valves are also installed to force CO_2 to pass in only one way.

• ***Pipes and tubing*****:** Two types of pipes have been used depending on the zone of the plant. The tubing used in the high-pressure zone of the plant is

AISI 316, ¼" external diameter and 0.065" wall thickness. Quick connections, with a single-ferrule compression sleeve, for operating under 79 MPa are used because of their easy installation. This type of connection reduces the potential leak-paths compared to the two ferrule designs. With only three pieces to fit together, they are very difficult to misalign or mismatch. The tubing used in the low-pressure zone of the plant is 0.049" wall thickness. In this zone, pressure is always under 30 MPa. Connections with double-ferrule are installed here. The closure system is reliable at this pressure and is cheaper than the single ferrule compression sleeve.

- ***Heat exchangers*:** Heating and cooling take place in different thermostatic baths with temperature control. They allow a homogenous temperature around the tube, especially important in the heating system, in which the wall temperature must be controlled to avoid the risk of crash.

- ***Control parameters and data acquisition system*:** The plant contains several devices in order to measure mass flow, pressure and temperature. Mass flow is determined by a coriolis flow-meter (Massflo®-Danfoss) with a sensor (Mass 2100) built in Hastelloy C-22 that may operate at pressures up to 35 MPa, at a temperature range from 220 to 450 K and a fluid density from 100 to 2900 kg / m^3. A signal converter (MASS 6000) allows indication of failures and monitoring mass flow, volumetric flow, density, and total mass. Local temperature is determined by Pt-100 resistances protected against high pressure by closed tube with the right wall thickness. Local pressure is measured by several gauges to the pressure transducers

that allow monitoring. A data acquisition system is installed to monitor the process. It registers the measurements at the selected time interval, allows an easy representation of the process variables an easy control if needed.

- ***Safety*:** In order to guarantee a safe handling, the plant contains different rupture disks which are burst in case of over-pressure in a specific zone of the plant, allowing the gas to escape and avoiding the rupture of other devices. Additionally, all the pressure vessels are fixed on a metal structure and the high pressure zone is protected by a polycarbonate screen.

2.2.3.3. Experimental procedure

In a SFE experiment, approximately 100 g of freeze-dried hake by-product were placed in the extractor that was later pressurized up to the extraction pressure, p, with carbon dioxide (Carburos Metálicos, liquid $CO_2 \geq 99.9$ %).

Then, the solvent was circulated at the desired extraction temperature, T, with a certain solvent flow, F, and during a specific time, t. The solvent was continuously recycled to the extractor after removing the solute in the separator where the solvent power of CO_2 was reduced by reducing pressure down to approximately 5 MPa and keeping temperature lower than 313 K.

Twenty experiments were performed; eight of them with several extraction cycles and the rest without cycles, that is, with and without intermediate depressurizations of the extractor during the extraction experiment. When working without cycles, only the separator was depressurized to withdrawn the extract along time in order to follow the course of the extraction.

TABLE 2.3

Experimental conditions of the extraction experiments carried out in this work

Run No.	Raw material		Extraction Parameters			
	Moisture (wt %)	Particle size. (D_e equivalent diameter, mm)	P (MPa)	CO_2 Flow (kg/h)	Depressurization Type*	Flow Sense**
1	8.40	1 < De < 10	25.0	10	E + S	↑
2	17.20	1 < De < 10	25.0	10	E + S	↑
3	17.80	1 < De < 10	25.0	10	E + S	↑
4	51.50	1 < De < 10	25.0	10	E + S	↑
5	17.80	De < 5	25.0	10	E + S	↑
6	17.80	De > 5	25.0	10	E + S	↑
7	18.30	De < 5	50.0	10	E + S	↑
8	18.30	De > 5	50.0	10	E + S	↑
9	6.70	1 < De < 10	25.0	10	None	↑
10	8.40	1 < De < 10	25.0	10	S	↑
11	18.30	1 < De < 10	50.0	10	S	↑
12	8.40	1 < De < 10	25.0	10	S	↓
13	8.40	1 < De < 10	50.0	10	S	↓
14	8.30	1 < De < 10	25.0	5	S	↑
15	3.50	1 < De < 10	25.0	5	S	↓
16	9.35	1 < De < 10	57.7	5	S	↑
17	8.40	1 < De < 10	18.0	10	S	↑
18	6.70	1 < De < 10	10.0	10	S	↑
19	8.40	1 < De < 10	25.0	15	S	↑
20	7.60	1 < De < 10	25.0	20	S	↑

* E: depressurization of the extractor at times selected to follow the course of the extraction. S: depressurization of the separator at times selected to follow the course of the extraction. None: the extraction was performed without intermediate depressurizations, neither in the extractor nor in the separator.

** ↓: the extraction was performed by circulating the solvent downwards through the extractor. ↑: the extraction was performed by circulating the solvent upwards through the extractor.

All the extraction experiments were carried out at an extraction temperature of 313 ± 2 K. Also the separation temperature was kept around 313 ± 2 K. Higher temperatures were avoided along the process in order to prevent as much as possible oil degradation since high temperatures increase oil oxidation. The separator pressure was 5.2 ± 0.2 MPa in all cases. The rest of experimental conditions are reported in Table 2.3.

2.2.3.4. Analytical methods

In order to study the course of the extraction processes, moisture and fat content in the solid after oil removal were determined. Moisture was determined by the AOAC Official Methods 934.01, whereas fat content was determined by Soxhlet using petroleum ether as solvent in a Büchi extraction system (model B-811). Further details about these methods are reported in Appendix I.1.

Oil quality was evaluated by means of the free fatty acid content, FFA, which was measured according to the analytical method described in Appendix I.2.

2.2.4. Experimental results and discussion

The experimental results obtained allowed us to find the best procedure to follow the course of the extraction, to choose the contact device to be used in the extractor and to study the influence of the pre-treatment of the raw material and of the process parameters on the extraction yield

2.2.4.1. Parameters to follow the course of the extraction

In the first experiment (run 1), the course of the extraction was followed by two different procedures: by weighing the amount of extract obtained along time and by determining the remaining amount of oil in the solid.

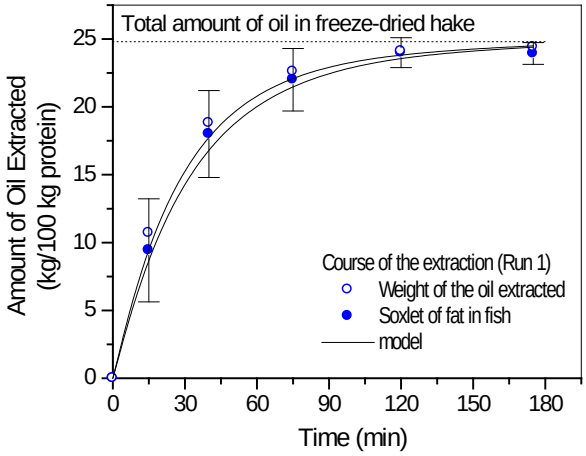


FIGURE 2.8

Extraction curves obtained for run 1 by weighing the oil extracted along time and by determining the amount of fat in the extracted hake. The continuous lines represent the correlation of the experimental data through the model proposed by Kandhia & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.5).

The results are presented in Figure 2.8, where it can be observed that both procedures gave rise to similar results. Figure 2.8 also shows the total amount of oil in the hake by-product as determined by Soxhlet using

petroleum ether. In the rest of the experiments, the course of the extraction was followed by weighing the amount of oil extracted along time.

2.2.4.2. Influence of the contact device used in the extractor

Two different devices were available to place the hake by-product inside of the extractor: a basket, where the raw material was placed as a packed bed that provided a continuous contact, and a tray contacting device that provided intermittent contact.

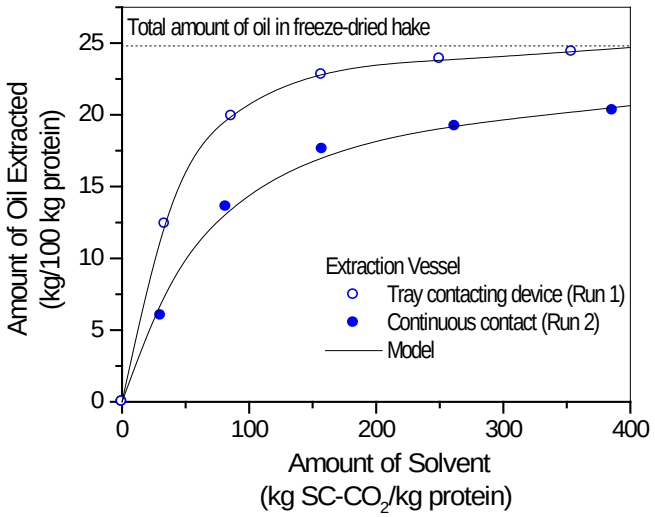


FIGURE 2.9

Influence of the contact device used in the extractor. The continuous lines represent the correlation of the experimental data through the model proposed by Kandhia & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.52.2.4.5).

Run 1 was performed using the tray contacting device and run 2 using the basket. The extraction curves obtained in both cases (*see* Figure 2.9) show that the extraction rate is higher in a tray contacting device.

This result suggests that, when using trays, the contact between solute and SC-CO_2 is better. This better contact could be due to a lower compaction of the packed bed, avoiding the formation of preferential channels, or, in general, to a better distribution of the solvent through the substrate.

Although the water content of runs 1 and 2 is different, this seems not to be the reason of the different extraction curves observed in Figure 2.9 (see influence of water content in pag. 148). Therefore, the following experiments were carried out using the tray contacting device with seven sieve trays.

2.2.4.3. Influence of the pre-treatment of the raw material on the yield of the extraction process

i) Effect of moisture on lipid extractability

Several authors have studied the influence of water on the extraction yield. King et al. found a considerable improvement of the fat extraction yield when dehydrating meat products before extraction with SC-CO_2 (*King, et al.*, 1989). Dunford et al. studied the oil extraction from Atlantic mackerel at different moisture contents and concluded that oil solubility decreased with increasing moisture level (*Dunford, et al.*, 1998). They assumed that the

solubility of oil in SC-CO_2 is influenced by the water concentration in the fluid phase and proposed a model that incorporates the negative interactions between oil and water in the supercritical phase. Although water is a polar compound, it is partially soluble in pressurized CO_2 (of the order of 10^{-3} molar fraction, at 313 K and at the range of pressures used in this work (*Gupta, et al.*, 2007)), and, in fact, can be an inhibitor of the dissolution of oil in SC-CO_2.

In order to study the effect of water on the oil extraction in this work, hake offcuts were freeze-dried to different moisture levels (8.4 %, 17.8 % and 51.5 %) and subjected to SFE (runs 1, 3 & 4). The extraction curves obtained are presented in Figure 2.10.

Figure 2.10.a shows that when moisture increases from 17.8 % to 51.1 %, the yield of the oil extraction decreases remarkably, but there is not a clear difference on the oil extraction yield when moisture decreases from 17.8 % to 8.4 %. Calvo et al. did not found either a significant difference on the extraction yield of cocoa butter with SC-CO_2 when the cocoa water content varied between 5 and 10 % (*Calvo, et al.*, 2007). Chao also found that a reduction of the moisture content of ground beef to less than 25 % did not improve much the lipid extraction from meat (*Chao*, 1996). Vaquero et al. did not found a noticeable increase of the fat extraction yield when drying pigskin in a continuously circulating air dryer from 50 % to 25 % water content (*Vaquero*, 2006). In this last case, the reason may be that air-drying

modifies the structure of the skin much more than freeze-drying that maintains the pore structure of the subtract.

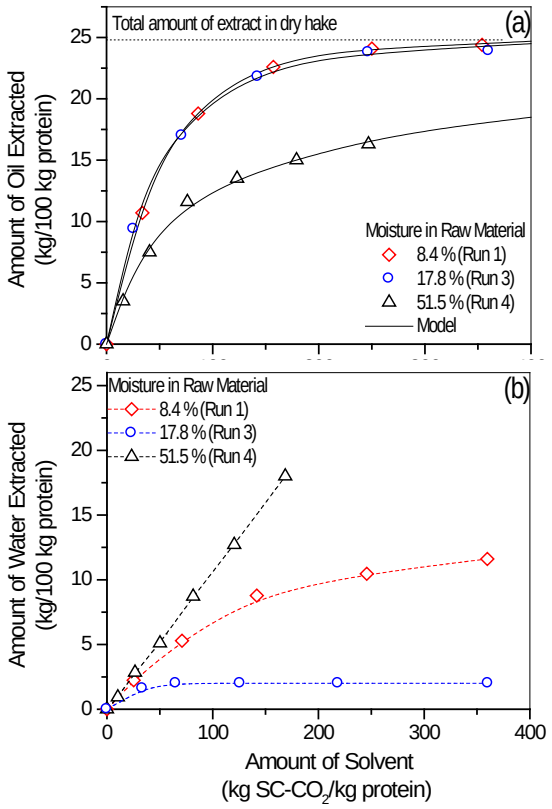


FIGURE 2.10

(a) Effect of moisture on hake oil extraction yield. The continuous lines represent the correlation of the experimental data through the model proposed by Kandhia & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.5). (b) Amount of water extracted from hake offcuts with different moisture. Dotted lines are to guide the eye.

Figure 2.10.b shows the water extraction curves at 25 MPa and 313 K as a function of the amount of SC-CO_2. The amount of water in the extract increases with initial moisture in the hake by-product; however, the molar fraction of water in SC-CO_2 at 313 K and 25 MPa, as determined from the slope of the linear part of the extraction curve, was $2.6 \cdot 10^{-3}$, whereas solubility of water in SC-CO_2 under the same experimental conditions is $6 \cdot 10^{-3}$ (*Sabirzyanov, et al.*, 2002). This result agrees with the experiments of Dumford et al., who concluded that the amount of water co-extracted in the extraction of oil from Atlantic mackerel was lower than the solubility of water in SC-CO_2 because the extraction process of water with SC-CO_2 was controlled by the intraparticle diffusion resistance (*Dunford, et al.*, 1998).

From the comments above, it seems convenient to freeze-dry the hake offcuts, reducing their water content to about 20 % or less, to increase the oil extraction yield.

ii) Effect of particle structure and size

The supercritical extraction from solids is often controlled by the transport in the solid phase. This transport (internal mass transfer) resistance can be reduced by reducing the solids size, although it has to be taken into account that very small particles may hinder the fluid flow through the particles bed and decrease external mass transfer (*Brunner*, 1994)what would reduce the final efficiency of the extraction.

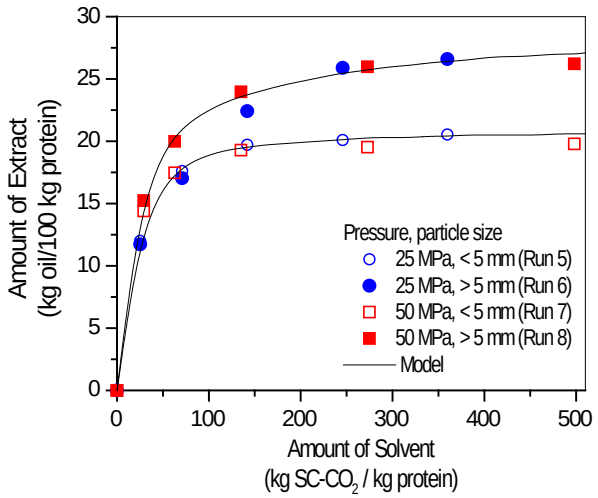


FIGURE 2.11

Influence of the particle size and composition on the amount of oil extracted. The continuous lines represent the correlation of the experimental data through the model proposed by Kandhia & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.5).

In order to study the influence of the internal mass transfer on the extraction yield, in this work, freeze-dried hake offcuts were screened with a 5 mm sieve. Particles smaller than 5 mm resulted to be mostly hake muscle with an average total fat content of 20.7 kg oil/100 kg protein while particles larger than 5 mm were mostly skin with an average total oil content of 27.7 kg oil/100 kg protein. Some experiments were carried out with the different particles obtained after screening (runs 5 to 8). Figure 2.11 shows the extraction curves obtained for particles larger than 5 mm (mainly hake

skin) and for particles smaller than 5 mm (mainly hake muscle) at 25 MPa and 50 MPa respectively.

Although differences are not very noticeable, it can be said that the initial oil extraction rate is larger in hake muscle than in hake skin. In fact, the slope of the extraction curves obtained for hake muscle at zero time (6.8 g oil/kg CO_2 at 25 MPa and 10.9 g oil/kg CO_2 at 50 MPa) are closer to the reported fish oil solubility (6.9 g oil/kg CO_2 at 25 MPa and 15.8 g oil/kg CO_2 at 50 MPa as calculated form Chrastil correlation (*see* Figure 2.15) than the slope of the extraction curves obtained for hake skin (4.7 g oil/kg CO_2 at 25 MPa and 8.8 g oil/kg CO_2 at 50 MPa). This indicates that internal mass transport is slower in hake skin than in hake muscle.

2.2.4.4. Influence of process parameters on the extraction yield

i) Effect of depressurization cycles

The influence of depressurization cycles on the extraction process was evaluated through three experiments: a continuous extraction run without depressurization neither in the extractor nor in the separator (run 9), an extraction run with five depressurizations only in the separator for sample withdrawal (run 10) and an extraction run in five cycles with total depressurization, both in the extractor and in the separator (run 1). In all cases, the experiments were carried out at 25 MPa, 313 K and 10 kg CO_2 / h during 3 hours. The results are presented in Figure 2.12, where it can be

observed that the oil extraction rate is larger when extractor and separator are depressurized than when only the separator is depressurized.

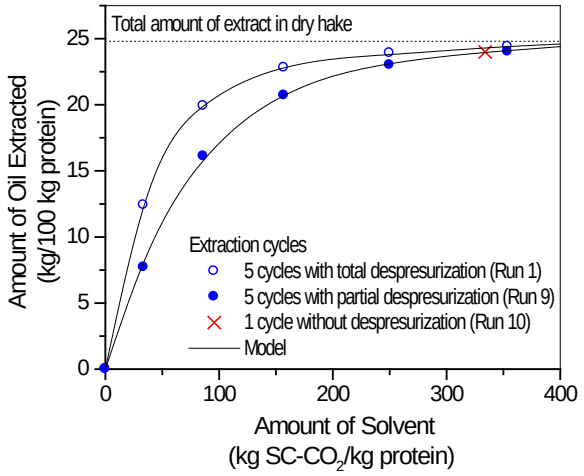


FIGURE 2.12

Effect of intermediate extractor depressurizations on the oil extraction yield. The continuous lines represent the correlation of the experimental data through the model proposed by Kandhia & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.5).

This can be considered an evidence of the control of the extraction rate by the internal mass transfer. Rapid depressurizations of the extractor may help to break the cell membranes and liberate the oil that will easier contact the solvent. Vaquero et al. reported this same behaviour when defatting pigskin (*Vaquero*, 2006). However, the total yield obtained in the

3 runs (1, 9 and 10) after 3 hours was the same. Taking into account the time and economical cost of depressurizations, a continuous extraction process is recommended. In any case, the next experiments presented in this work were performed by depressurizing the separator in order to withdraw the oil extracted and evaluate the yield of the process along time.

ii) Effect of SC-CO_2 flow sense

In the supercritical extraction of solids with a vertical extractor, flow sense is an important parameter. The extraction yield can be different if SC-CO_2 flows from the bottom to the top of the extractor (upflow) or in the opposite direction (downflow). Some authors found that downflow was more effective than upflow due to the tendency of the oil laden CO_2 to flow to the bottom of the extractor by natural convection (*Stüber, et al.*, 1996). This tendency has mainly been found in the extraction of high soluble solutes, especially at extraction pressures near critical (low viscosity), low interstitial solvent velocity and small Reynolds numbers. In the case of less soluble solutes, high pressures and high Reynolds conditions, other authors have not found natural convection to be important (*Germain, et al.*, 2005). In order to study the effect of flow sense on the extractions performed in the present work, downflow and upflow extractions were carried out at two different pressures (25 MPa and 50 MPa) (runs 10, 11, 12 & 13).

The results are presented in Figure 2.13.a where it can be observed that upflow is more effective than downflow at the two pressures studied.

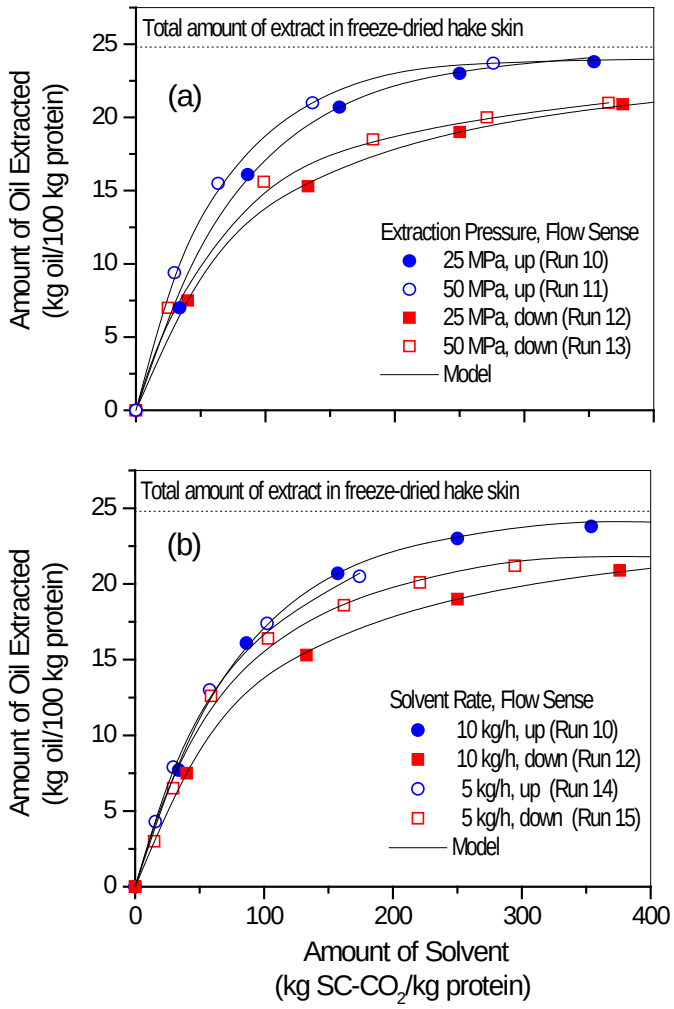


FIGURE 2.13

Influence of the solvent flow sense on the extraction process at different extraction pressures (a) and at different flow rates (b). The continuous lines represent the correlation of the experimental data through the model proposed by Kandhia & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.5).

The effect of forced convection due to impulsion seems to be more important than the effect of natural convection due to the density difference.

This behaviour could be expected since the solubility of fish oil is not high, the extraction pressure is fairly high (liquid-like viscosity) and the Reynolds number reach values up to 100. Upflow seems to prevent bed compaction, or even expand the particle bed, which contributes to a better solute-solvent contact, and therefore to higher extraction efficiency.

Further evidence of this behaviour is given in Figure 2.13.b, where experiments performed at 25 MPa and at 5 kg CO_2 / h and 10 kg CO_2 / h respectively are presented. It can be observed that the difference between working upflow and downflow is smaller for lower solvent flow. For even lower solvent flows, the tendency may invert and natural convection may be significant but our equipment does not allow working with flows lower than 5 kg / h.

iii) Effect of extraction pressure

Several extraction curves were obtained for pressures from 10 MPa to 57.7 MPa, at a fixed temperature of 313 K in order to study the effect of pressure on the extraction rate (runs 10, 11, 16, 17 & 18). The solvent flow was 10 kg / h in all runs except for run 16 where 5 kg / h were used due to limitations in the equipment.

The results are presented in Figure 2.14 where it can be observed that the slope of the different curves at zero time, that is, the initial extraction rate,

increases significantly with pressure. This result suggests that in the very first stages of the extraction, the process could be controlled by the solubility of oil in SC-CO_2. If so, it would be possible to estimate the solubility data of hake oil in SC-CO_2, from the slopes of the extraction curves at zero time. Such slopes, have been plotted in Figure 2.15 together with some solubility data found in the literature for different fish oils at 313 K (*Borch-Jensen, et al.*, 1997, *Catchpole, et al.*, 1998, *Imanishi, et al.*, 1989).

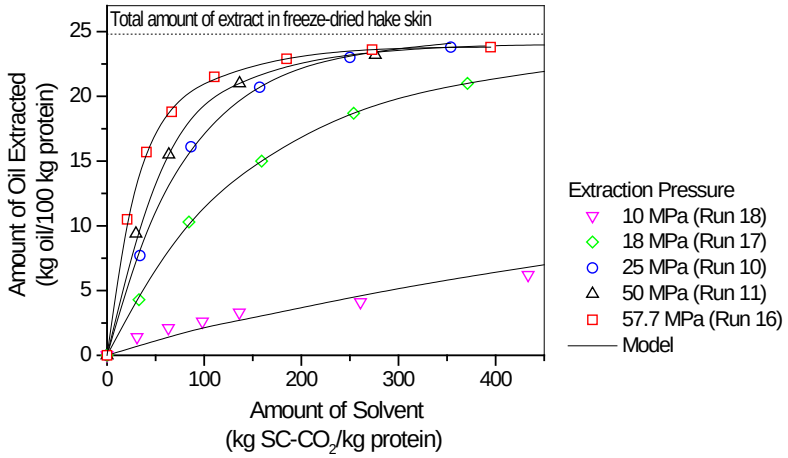


FIGURE 2.14

Influence of the extraction pressure on the extraction yield. The continuous lines represent the correlation of the experimental data through the model proposed by Kandhia & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.5). (Run 16 was performed at 5 kg CO_2 / h and runs 10, 11, 17 y 18 at 10 kg CO_2 / h).

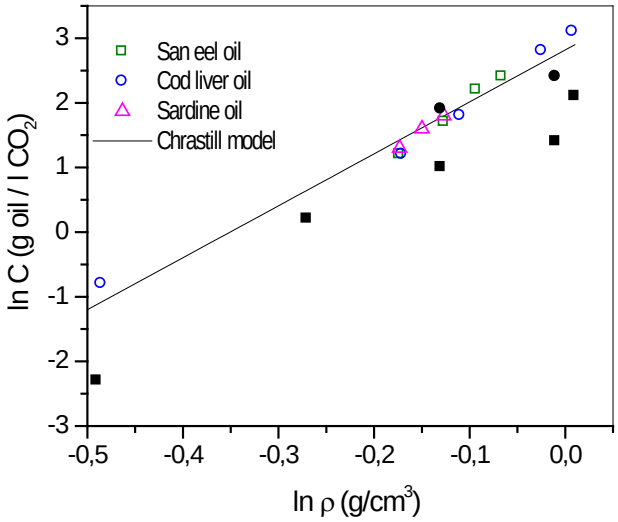


FIGURE 2.15

Solubility data of fish oil in SC-CO_2 reported in the literature. The solid line has been obtained by correlating all the reported solubility data through Chrastil model. The solid points (■) represent the slopes of the extraction curves obtained in this work (hake oil) at different extraction pressures with despresurization in the separator (runs 10, 11, 16, 17 and 18) and (●) the slopes of the extraction curves of hake muscle with despresurizations in the extractor and separator (runs 5 and 7).

Unfortunately, there are few studies on fish oil solubility in SC-CO_2 and none on hake oil. However, from Figure 2.15 it can be concluded that the slopes of the extraction curves obtained in this work are lower than the solubility of the rest of fish oils considered. The most plausible explanation is that is not solubility but mass transfer the resistance that controls the rate

of the process from the very beginning of the extraction, and either lower flows or a better contact solute-solvent would be needed to reach solvent saturation. The slopes of the extraction curves obtained in runs 5 and 7 have also been plotted in Figure 2.15. It may be observed that in these cases, where the extractions were performed over fish muscle and with intermediate depressurizations, both factors helping intraparticle mass transfer, solubility values were reached.

The solubility data sets found in the literature at 313 K have been correlated all together through the Chrastil equation (*Chrastil*, 1982) resulting the following linear relationship between the solubility of the solute, S, expressed as (g of solute)/(L of solvent), and the density of the solvent, ρ, in g/cm^3.

$$\ln S = 2.82 + 7.94 \ln \rho \qquad \textbf{(2.1)}$$

The standard error between the reported experimental solubility data and the data calculated with Eq. (2.1) was 0.18 and the linear fit presented an R-squared of 0.968. This linear fit has also been plotted in Figure 2.15 for a better observation of the deviation of the slopes of the extraction curves and the reported solubility data.

iv) Effect of the SC-CO_2 flow rate and solvent ratio

In order to study this effect, some extraction curves were obtained at different solvent flow rates and fixed pressure (25 MPa) and temperature

(313 K) (runs 10, 14, 19 & 20). The results have been plotted in Figure 2.16 (up), which shows that, for a certain extraction time, the extraction rate increased significantly when the solvent rate increased from 5 to 10 kg CO_2 / h, but decreased when the solvent rate increased from 10 kg CO_2 / h to 20 kg CO_2/h. The amount of oil extracted versus the amount of solvent used is presented in Figure 2.16 (down), which shows that the amount of oil extracted for a given amount of solvent decreases substantially when the flow rate increases above 10 kg CO_2 / h. The slope of the extraction curves obtained at 5 and 10 kg CO_2 / h at zero time are very similar, (perhaps slightly larger the slope of the curve at 5 kg CO_2 / h) and fairly different than the rest of the extraction curves. This confirms the assumption that solubility is not controlling the rate of the process under the conditions studied in this work. Lowering the solvent flow could lead to a solubility controlled process. Figure 2.16 also suggest that external mass transfer is not a significant resistance at flows larger 10 kg CO_2 / h because the extraction rate is not enhanced by increasing the solvent rate.

Figure 2.17 presents the extraction rate as a function of the solvent ratio. A maximum extraction rate is clearly observed, especially at the first stages of the process, for a solvent ratio of around 175 (kg CO_2 / h) / kg protein, which is related to a flow rate of around 13.75 kg CO_2 / h. This result may be explained by considering the residence time of the solvent in the extractor, that is presented in Table 2.4 together with the corresponding solvent flow rate, solvent ratio, extraction efficiency and extraction yield after 175 minutes of extraction.

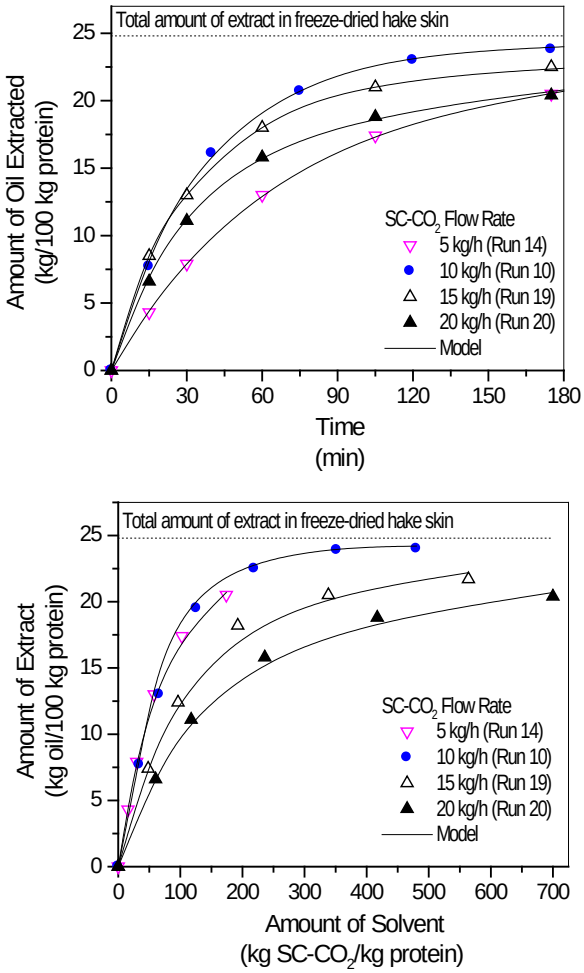


FIGURE 2.16

Amount of oil extracted vs. time at a different flow rate (up). Amount of oil extracted vs. amount of solvent at a different flow rate (down). The continuous lines represent the correlation of the experimental data through the model proposed by Kandiah & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.5).

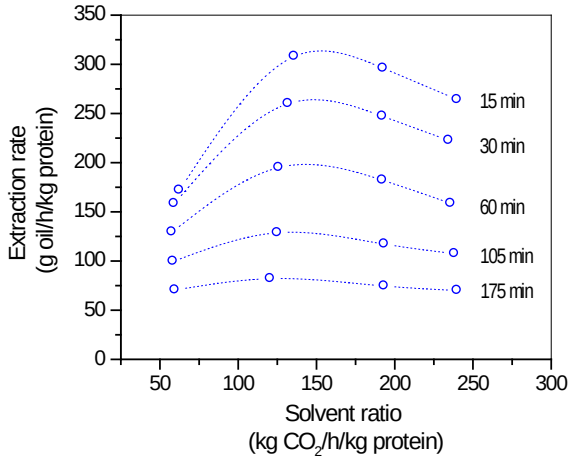


FIGURE 2.17

Influence of the solvent ratio on the extraction rate. Dotted lines are to guide the eye.

It is observed that when the solvent ratio increases, by increasing the flow rate from 5 to 10 kg CO_2 / h, the extraction efficiency decreases to nearly half, but the percentage of oil extracted increases from 82.7 % to 96.4 %. However, when solvent flow rate increases above 10 kg CO_2 / h, the extraction yield decreases significantly although solvent ratio is also incremented because the residence time is fairly low (4.1 min) and the loading of the solvent is lower than when residence time is larger. The solvent flows rapidly through the solid subtract in such a way that intraparticle diffusion (that has been shown to control the extraction) declines making the extraction yield to decrease. The extraction rate

however, increases up to a maximum at a flow of 13.75 kg CO_2 / h and decreases afterwards. Similar results have been reported before for the decaffeination of raw coffee (*Brunner*, 1994) and green tea (*Park, et al.*, 2007) and for the extraction of Amarath seed oil (*Westerman, et al.*, 2006) or GLA from cyanobacteriums (*Mendes, et al.*, 2005).

TABLE 2.4

Solvent ratio, residence time, extraction efficiency and extraction yield after 175 minutes of extraction of hake by-products, in four experiments carried at different flow rates (runs 14, 10, 19 & 20)).

SC-CO_2 flow rate (kg/h)	Solvent ratio (kg CO_2/(h kg protein))	Residence time (min)	Extraction efficiency (g oil/kg CO_2)	Extraction yield (% based on total oil content)
5	60	16.6	1.2	82.7
10	121	8.3	0.7	96.4
15	193	5.5	0.4	90.0
20	240	4.1	0.3	83.9

Taking into account the influence of the different parameters previously analysed, SFE of hake oil from hake offcuts is recommended to be performed in a 3 hours continuous extraction in a tray contacting device where freeze-dried hake, not necessarily cut in fine particles, is placed. SFE gives good yields under 25 MPa, 313 K and 13.75 kg CO_2 / h.

2.2.4.5. Influence of process parameters on oil quality

The influence of extraction parameters on oil quality was evaluated by means of the amount of free fatty acids compounds (FFA) which is mainly responsible for the acidity of the oil.

Figure 2.18 (a) shows the FFA content of oil obtained at different pressures. It is the FFA content increases noticeably as pressure decreases from 25 MPa at to 10 MPa, keeping temperature at 313 K, but remains mostly constant when pressure increases up to 50 MPa.

This behaviour may be related to the different solubility between high molecular compounds such as triacylglycerides (TAG) and low molecular compounds such as free fatty acids (FFA) and / or other volatile compounds with high vapour pressure responsible of the acidity of the oil. Thus, as it can be observed in Figure 2.18 (b) for the for oleic acid series, the differences between solubility of FFA and TAG became higher as pressure decreases from 10 MPa to 25 MPa, and therefore CO_2 densities depletes from to a density closer to the critical CO_2 density.

Furthermore, comparing the FFA content in several oil fractions, extracted at 25 MPa and 313 K (Run 10) along the time, it has been observed that there is a decrease of FFA as extraction progresses (see Table 2.5), which may be explained taking into account again that FFA solubility in SC-CO_2 is higher than triglycerides solubility; therefore, they are removed in a higher proportion at the beginning of the extraction.

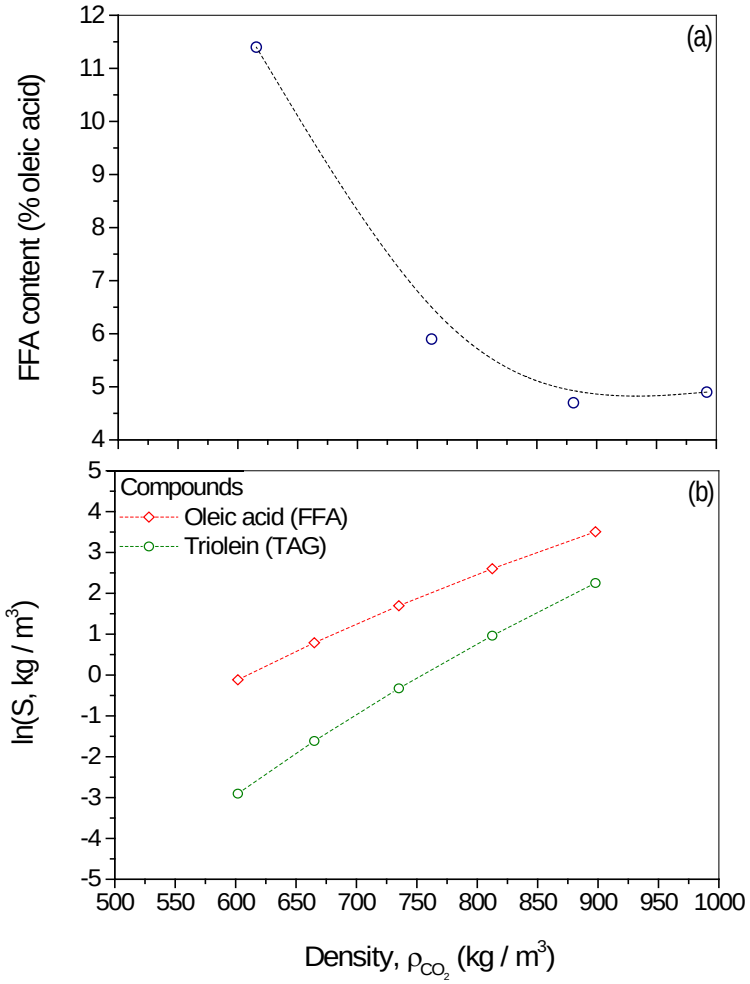


FIGURE 2.18

(a) FFA content obtained in fish oil extracted at different pressure conditions (Runs 18, 17, 10 & 11). (b) Solubility of pure FFA (oleic acid) and TAG (triolein) in SC-CO_2.

Therefore, these results suggest that 25 MPa and 313 K are the optimal pressure and temperature conditions for obtaining a low acid fish oil. Moreover, an oil fractionation can be performed to decrease the FFA content of the main extract. Some fractionation experiments to optimize oil quality and stability are reported in Section 3.1.

TABLE 2.5

FFA content and acidity of different oils extracted along time (Run 10).

Extraction time range	FFA content (% oleic acid)
From initio to 15 min	5.0
From 15 min to 40 min	5.4
From 40 min to 75 min	4.5
From 75 min to 120 min	3.5
From 120 min to 175 min	2.0

2.2.5. Data analysis and modelling

The analysis of the extraction curves and its modelling have a great importance not only for understanding the extraction mechanism itself but also for establishing the main factors required for a mathematical description and a subsequent scale-up.

In order to calculate the theoretical extraction curves, different mathematical models have been proposed in the literature, from the simplest to the more complex one. These models may be classified as empirical and rigorous

models based on solving the differential mass balances on a small section of the extractor, together with the equilibria and transport kinetic relationships.

2.2.5.1. Empirical models

Three empirical models have been considered in order to describe in an easy way the fish oil extraction process.

The first model considered (model 1) is based on the steady stationary theory and its mathematical expression is given by Eq. (2.2)

$$\frac{C_s}{C_{s,o}} = 1 - \exp(-k \cdot t) \qquad \textbf{(2.2)}$$

where $C_{s,o}$ is the initial concentration of oil in the hake offcuts (kg oil / kg protein), C_s is the oil concentration (kg oil / kg protein) after a given extraction time, t, and k is the rate constant (min^{-1}).

This is the simplest extraction model since it assumes that the extraction process occurs in one stage of diffusion controlled only by the internal mass transfer, which can be quantified by the parameter k.

The second model applied (model 2) is based on the model proposed by Sovová for extraction of essential oils from leaves and flowers, and it considers that the extraction process occurs in two different stages (*Sovová*, 2005).

In the initial stage, the internal mass transfer resistance is supposed to be negligible and the solute is extracted at a constant rate, although, because

the solute is interacting with the matrix, the maximum amount of solute extracted in this first stage is lower than the solubility value.

In the second stage, the extraction starts to take place from the particle core, and the mass transfer resistance becomes more significant.

The global model can be expressed as Eq. (2.3) and (2.4) for the first and the second stages respectively.

$$\frac{C_s}{C_{s,o}} = k_1 \cdot t \quad \text{for } t < t_r \qquad \textbf{(2.3)}$$

$$\frac{C_s}{C_{s,o}} = 1 - k_2 \exp(-k_3 \cdot t) \text{ for } t > t_r \qquad \textbf{(2.4)}$$

where k_1 is the rate constant for the initial stage, k_2 and k_3 are the rate constants for the second stage and t_r is the time at which the extraction from the particle core starts.

The third model considered (model 3) was proposed by Kandiah & Spiro and assumes that internal mass transfer resistance is significant from the beginning of the process, although there are two diffusion stages according to the following Eq (2.5) (*Kandiah, et al.*, 1990):

$$\frac{C_s}{C_{s,o}} = 1 - [f_1 \cdot \exp(-\kappa_1 \cdot t) + f_2 \cdot \exp(-\kappa_2 \cdot t)] \qquad \textbf{(2.5)}$$

where f_1 and f_2 are the fractions of solute extracted with rate constants of κ_1 and κ_2 respectively.

The experimental extraction curves obtained for the different runs performed in this work have been modelled according to the mathematical models previously reported using the non-linear regression option of the Statgraphics Plus 4.1 program.

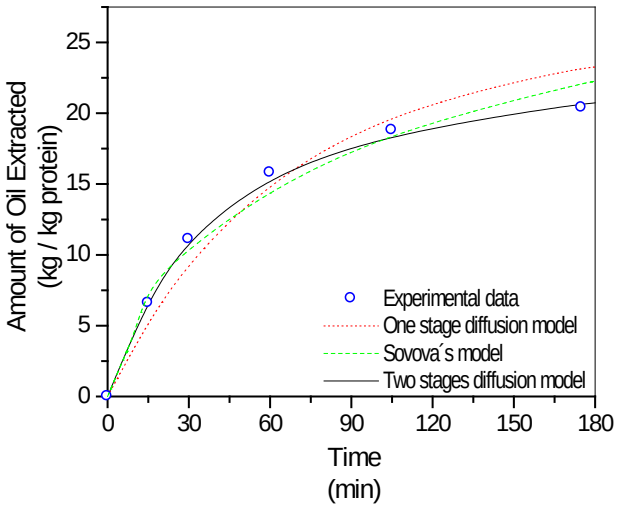


FIGURE 2.19

Comparison of the different models used for modelling the extraction curves. The experimental data correspond to run 20.

In general, the most suitable model for describing the extraction process in all cases was the third model (see Figure 2.19) which takes into account that the process is controlled by the internal mass transfer from the beginning of the extraction. In fact, this seems to be the case in the extraction studied in this work as has been proved along the analysis of the experimental results.

The first and second models fail to describe some of the extraction curves, especially those where the extraction rate is very different at the beginning and at the end of the extraction as is the case of run 20 presented in Figure 2.19.

TABLE 2.6

Adjustable parameters of model 3 for hake oil extraction at different extraction pressure (Runs 18, 17, 10, 11 & 16).

Pressure (MPa)	f_1	κ_1 (min^{-1})	f_2	κ_2 (min^{-1})	R^2	Standard error (%)
10.0	0.5	0.002	0.5	0.002	98.3	2.5
18.0	0.6	0.018	0.4	0.006	99.9	0.6
25.0	0.7	0.032	0.3	0.014	99.9	1.2
50.0	0.7	0.037	0.3	0.013	99.6	2.3
57.7	0.7	0.047	0.3	0.006	99.3	2.7

Therefore, all the extraction curves obtained in this work have been modelled through model 3 and the adjustable parameters obtained for the extraction curves obtained at different extraction pressures are reported in Table 2.6. The fractions of solute extracted f_1 and f_2 have been estimated as the inflection point of the experimental extraction curves. It can be observed that the rate constant κ_1 is higher than κ_2, what indicates that the first extraction stage is faster. The rate constant, κ_1, and the fraction of solute

extracted in the first stage, f_1, increase with pressure, but the rate constant κ_2, reaches a maximum at 25 MPa.

2.2.5.2. Mathematical models based on mass balance equations

In order to aim a better description of the mass transfer phenomena that take place during the extraction process and develop the subsequent scale-up, a mathematical model based on differential mass balances into the extractor is considered, which has been developed by Cocero et al., for seed oil extraction (*Cocero, et al.*, 2009).

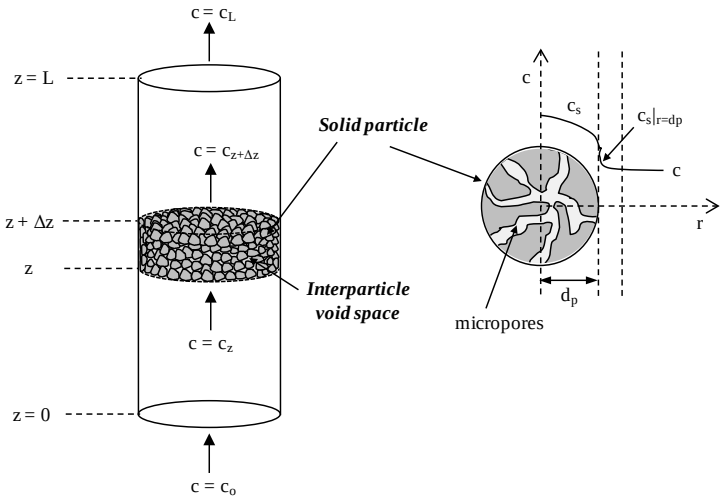


FIGURE 2.20

Scheme of the SFE extractor filled with a fixed solid porous matrix.

This model considers that the solute extraction with SC-CO_2 from a porous solid matrix takes place in different consecutive mass transfer steps as it has been described in section 2.2.2. The main model features are:

a) The extraction system is a fixed bed (*see* Figure 2.20) composed by two phases: a solid and static phase and a fluid phase. Solid phase is composed by a porous solid matrix described by a global model with time dependence. Its physical properties such as porosity, ε, are considered constant during the extraction process. Fluid phase is composed by the SC-CO_2 with the dissolved solute and is described by a distributed model with axial dispersion and time variation.

b) The solute is considered a pseudo-single compound with a global behaviour in spite of being mixture of different components as it happens with oil.

c) Solvent flow rate is considered constant as well as SC-CO_2 density and viscosity. Moreover, pressure drop, temperature gradient and heat of solution are assumed to be negligible and, therefore, no variation in superficial velocity along the fixed bed is taken into account.

d) Time from the extractor outlet to separator is assumed neglected within the global time.

General mass balance equations for both solid and fluid phase are presented below, as well as their initial and boundary conditions:

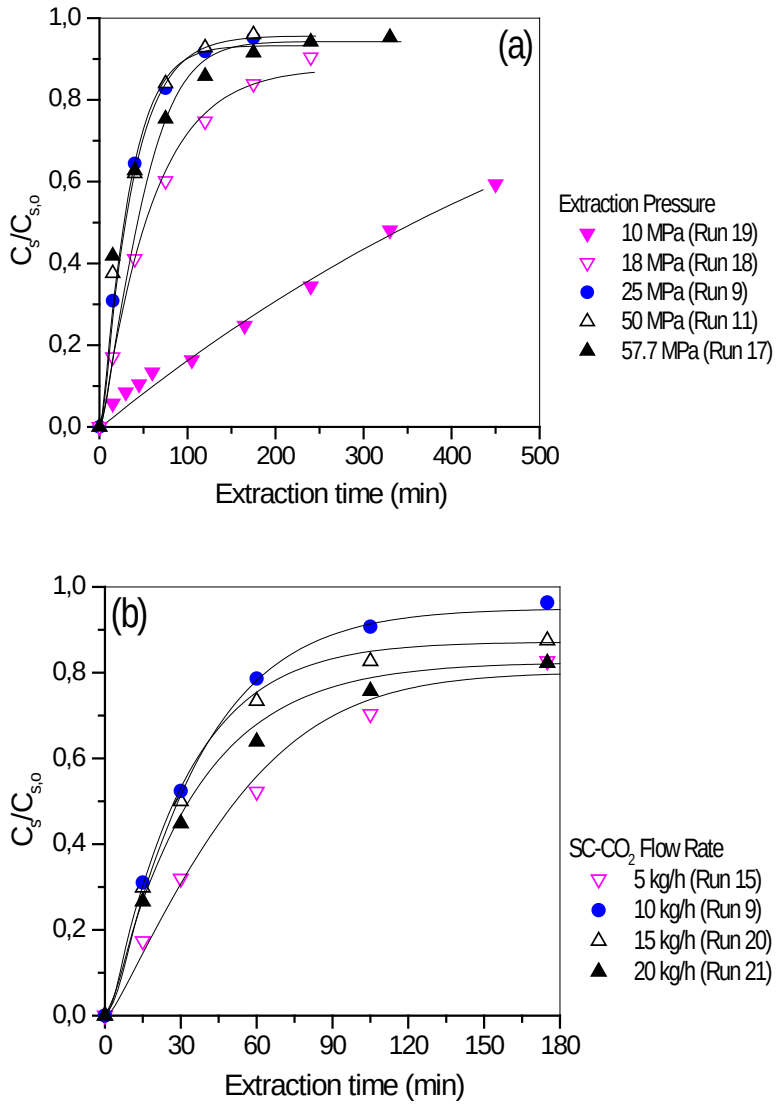


FIGURE 2.21

(a) Model curves obtained according to rigorous extraction model for experiments carried out at different pressure and (b) different flow rate.

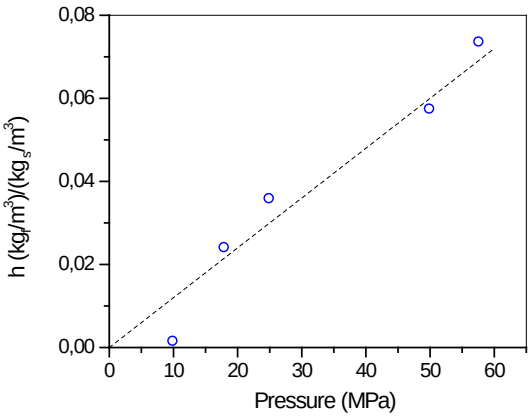


FIGURE 2.22

Dependence of the equilibrium parameter, h, with the extraction pressure.

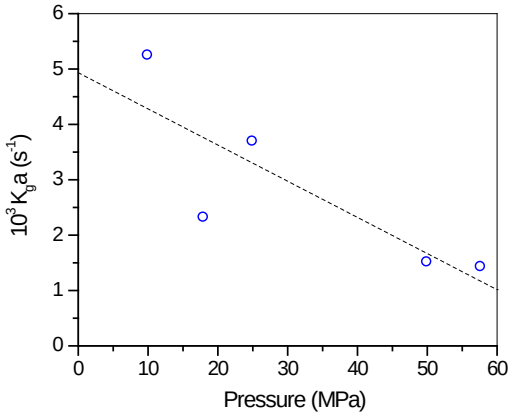


FIGURE 2.23

Dependence of the mass transfer coefficient, $K_g a$, with the extraction pressure.

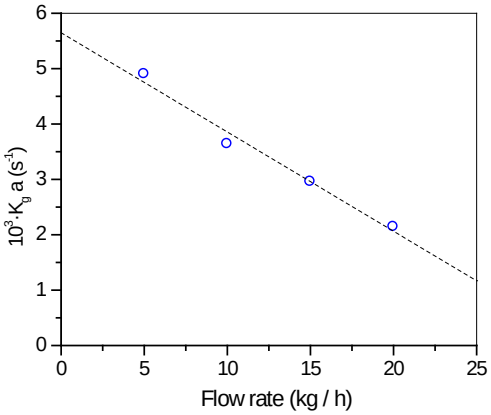


FIGURE 2.24

Dependence of the mass transfer coefficient, Kga, with the solvent flow rate.

Fitting parameter h is not affected by flow rate but increases with pressure as it is observed in Figure 2.22. This behavior was expected since parameter h is related to the solubility of oil in SC-CO_2, which is higher the higher the pressure as it was discussed in section 2.2.4.

On the contrary, as Figure 2.23 shows, the global mass transfer coefficient, $K_g a$, decreases with pressure which is explained considering that as pressure increases, the viscosity of SC-CO_2 increases making the diffusivity decreases and therefore the external mass transfer slower. Moreover, the global mass transfer coefficient, $K_g a$, decreases with flow rate (*see* Figure 2.24), which may indicate that the internal mass transfer is controlling the process.

2.2.6. Conclusions

Supercritical CO_2 extraction of the hake by-products of the fish industry permits to obtain omega-3 rich oil with a high yield (more than 96 % in 3 h). The pre-treatment of the hake by-products was found to affect the extraction yield: moisture affects negatively the yield of the process when it is larger than about 20 % and water reduction takes place by freeze-drying; particle reduction seems not to have a large influence on the extraction yield, although the type of by-product and its pre-treatment is important. Hake oil extraction was found to be faster from the muscle than from the skin. Regarding the extraction conditions, it has been found that depressurization cycles do not improve significantly the extraction yield; upwards solvent flow was found to be more effective than downflow at the extraction conditions used in this work. The yield of the extraction increased with pressure although the process seemed not to be controlled by solubility. The extraction rate was maximum for a solvent ratio of 175 kg CO_2/(h × kg protein). The extraction curves obtained under different conditions, and the type of models that best describe them, lead to the conclusion that the extraction rate was limited by the internal mass transfer from the beginning of the extraction, mainly in hake skin.

2.3. Supercritical Fluid Extraction of Fish Oil from Fish By-Products: Quality Parameters and its Comparison with Alternative Extraction Methods

Abstract

As discussed in Section 2.2, supercritical fluid extraction may be an attractive process to obtain high quality fish oil under mild temperatures and, therefore, minimize PUFA oxidation. Thus, its competiveness may be mainly related to fish oil quality.

➢ **Content**

In this section, a comparison among actual extraction processes (cold extraction, wet reduction, enzymatic extraction) and supercritical fluid extraction is presented in order to establish the advantages of SFE in production of high quality fish oil.

2.3.1. Introduction

The production of good quality fish oil has become a good opportunity to valorise fish by-products and increase the competitiveness of the fish industry. Therefore, in the last years, a great amount of by-products from fish, such as herring (*Aidos, et al.*, 2002), salmon (*Wu, et al.*, 2008), tuna (*Chantachum, et al.*, 2000) or walleye pollock (*Wu, et al.*, 2009), has been proposed as raw material for fish oil production. However, the production of high quality fish oil as source of omega-3 involves not only searching for an omega-3 rich raw material but also developing a suitable extraction procedure.

The most common method used for fish oil production is wet reduction, which involves three basic steps: cooking at high temperatures (85 – 95 ºC), pressing and centrifuging (*FAO*, 1986). This process permits obtaining high volumes of crude fish oil, but subsequent refining steps are required in order to make the crude fish oil suitable for edible purposes. Other processes such as enzymatic reaction with proteases have been also studied for obtaining oil from fish by-products (*Linder, et al.*, 2005). In the last years, supercritical fluid extraction (SFE) is being studied as a promising technology for obtaining fish oil from some by-products such as sardine heads (*Letisse, et al.*, 2006) or hake skin (*Rubio-Rodríguez, et al.*, 2008). This technology requires a raw material with a water content not higher than 20 %, well below the natural water content in fish, which makes necessary a drying stage before oil extraction. This drying stage should keep unaltered the omega-3 PUFA, and when possible, the fish structure to facilitate extraction, reason for which, freeze-drying has been mostly the procedure used before SFE in research studies. This process thus, would require high investment costs at production scale, which would make this technology only suitable to process high value oils. In this sense, a quality study of the product extracted by SFE and non-SFE methods would illustrate on the competitiveness of this technology from a commercial point of view.

The aim of this section is to compare different extraction processes (cold extraction, wet reduction, enzymatic extraction and supercritical fluid extraction) in order to obtain oil from different marine by-products taking into account, not only the extraction yield, but also the oil quality.

2.3.2. Experimental section

2.3.2.1. Raw material

In this study, different fish by-products of the Fish Industry were used as raw material for obtaining fish oil, specifically offcuts from hake, orange roughy and salmon, and livers from jumbo squid. The characterisation of all of them has been presented in Section 2.1.

2.3.2.2. Oil extraction methods

Oil extraction from marine by-products was carried out by four different methods: cold extraction (centrifuging), wet reduction, enzymatic extraction with proteases and supercritical fluid extraction. The experimental conditions used in each case are reported in Figure 2.25.

In cold extraction, wet reduction and enzymatic extraction, the fish offcuts were previously thawed at room temperature during 12 hours, and the water co-extracted together with the oil was removed by centrifuging (Centrikon T-124, Kontron Instruments). In the SFE method, the water in raw material was removed by freeze-drying (FreeZone 12 L Console Freeze Dry System with drying chamber, Labconco) before extracting the oil in order to improve the extraction yield as it was reported in a previous study (Rubio-Rodríguez, et al., 2008). SFE was performed in a semi-pilot plant whose P&I diagram has been described in Section 2.2.3.2.

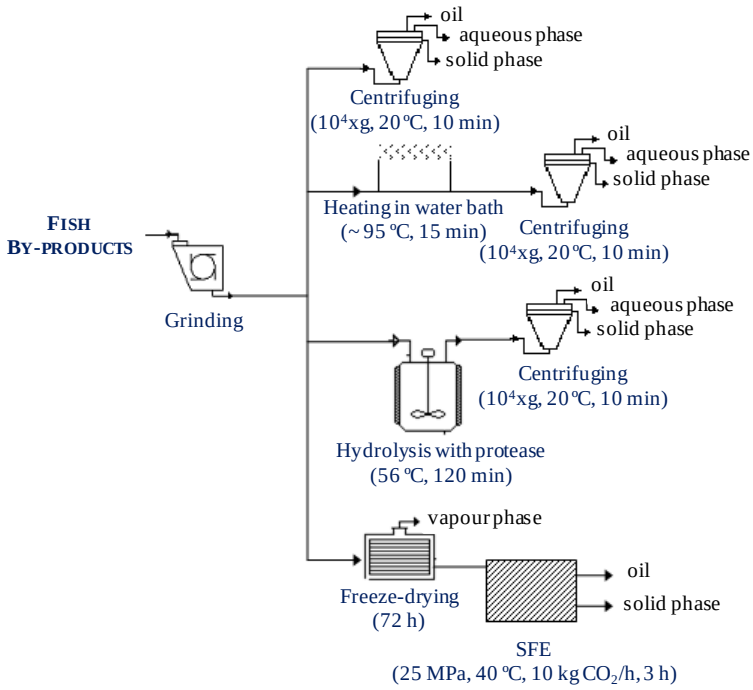


FIGURE 2.25

Scheme of the different marine oil extraction procedures studied in this work.

2.3.2.3. Analytical methods

i) Oil quality

The quality of the oils obtained by the different extraction methods was evaluated by determining several parameters, i.e.: moisture and volatile matter content, neutral lipid composition, fatty acid profile, volatile compounds profile, free fatty acids content, peroxide value, anisidine value

and trace metals. The experimental procedures for these methods are described in Appendix I.2.

ii) Oil sensorial analysis

An off-odour comparison among the oils extracted by different methods was carried out both by electronic nose and by sensorial analysis. These methods are detailed in Appendix I.2.

iii) Oil stability

Oil stability of omega-3 rich oils was evaluated by measuring the evolution, along 80 days of storage, of different parameters (free fatty acid content, peroxide value, anisidine value, TBARS and colour and odour features) in two fractions of oil obtained by SFE (25 MPa , 313 K), one kept at 4 ºC and darkness and another kept at 20 ºC and natural light. All of the analytical methods performed are detailed in Appendix I.2.

2.3.3. Results and discussion

In this section, the results on the characterization of the fish by-products used and on the oil extraction by the four methods studied are presented.

2.3.3.1. Fish oil extraction

Oil extraction from marine by-products was carried out by different extraction methods i.e.: supercritical fluid extraction (SFE), cold extraction

(CE), wet reduction (WR) and enzymatic extraction (EE), at a laboratory scale.

Extraction with SC-CO_2 has been proposed as a good method for obtaining fish oil with a high amount of omega-3 fatty acids, since it involves the use of a non-oxidant atmosphere and mild temperatures, which prevent the oxidation of the polyunsaturated fatty acids.

Previous studies (*see* Section 2.2) have concluded that hake oil can be easily extracted from hake offcuts with SC-CO_2 at a pressure of 25 MPa and a temperature of 313 K, reaching the highest yield when the by-products are previously cut and freeze-dried until a moisture content lower than 20 % in order to improve the oil-SC-CO_2 contact and to minimise the oil – water interaction in the supercritical phase. The extraction curves obtained were well fitted to the empirical model proposed by Kandiah and Spiro, which assumes that the process is controlled by two diffusion stages depending on the amount of oil accessible to the SC-CO_2. At the beginning, the amount of the most accessible oil is high, thus the internal mass transfer resistance is low and the extraction rate is large. However, when the most accessible oil is extracted, the remaining oil, less accessible, is removed more slowly due to the higher internal mass resistance (*Rubio-Rodríguez, et al.*, 2008). The rigorous model proposed by Cocero et al., (*Cocero, et al.*, 2001) also reproduces the extraction curves and allows to calculate the mass transfer coefficient as well as the distribution coefficient of the oil in the solid and fluid phases.

Oil SFE from other freeze-dried marine by-products with a fat content higher than 1 %, i.e.: orange roughy offcuts, salmon offcuts or jumbo squid liver, has been shown to be also feasible under the same experimental conditions.

Figure 2.26 shows the extraction curves where the different amounts of oil extracted along time in each species can be observed. In all cases, it was observed that at the beginning of the process the oil extracted depended linearly on the amount of SC-CO_2 that flows through the extractor, indicating either that the internal mass transfer is negligible and the process is controlled by the oil solubility in SC-CO_2, which differs depending on the type of lipids present in the different marine oils, or that the internal mass transfer is constant and the extraction rate depends on the internal structure of the solid matrix. The values of the slopes of the extraction curves estimated at the beginning (time = 0) of each experimental extraction curves are shown in Table 2.7.

TABLE 2.7

Slope of extraction curves, w_p, estimated at the beginning of the extraction (time =0) of marine oil from different by-products

	Hake skin	**Orange roughy skin**	**Salmon skin**	**Jumbo squid liver**
w_p (g oil / kg CO_2)	3.0	12.5	5.2	3.0

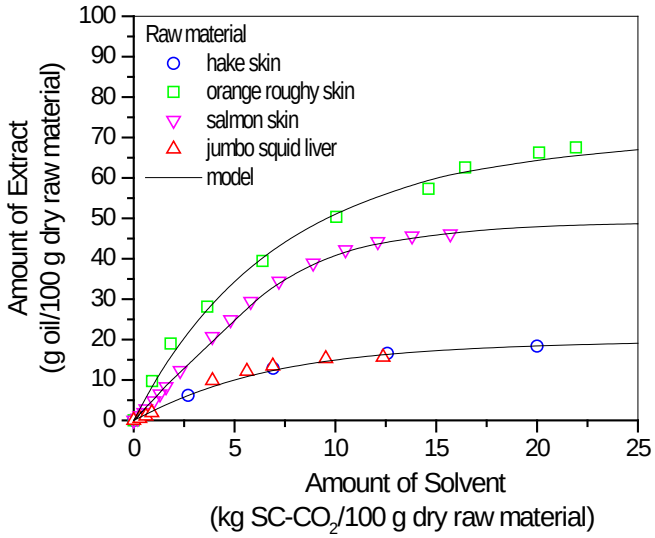


FIGURE 2.26

Extraction curves obtained for SFE of oil from different fish by-products. The continuous lines represent the correlation of the experimental data through the model proposed by Kandiah & Spiro (*Kandiah, et al.*, 1990) (*see* Section 2.2.5).

When using other methods, different than SFE, to obtain the oil, the by-products were used just after being defrost, without freeze-drying. Cold extraction is the easiest way to extract the oil since it only involves a mechanical phase separation (solid, water and oil) by centrifuging. However, by using this procedure, only the extracellular oil was removed and therefore, it only worked with fatty fishes that contain significant amounts of extracellular oil, i.e.: orange roughy (*Phleger*, 1998). Wet reduction and enzymatic extraction are also simple methods to obtain fish

oil from fish by-products, and they provide higher yields than cold extraction, through a protein denaturalisation step that takes place by the action of heat in wet reduction and by protein degradation (proteolysis) in the enzymatic extraction method. In both cases, several phases were formed besides the oil phase, i.e.: solid phase, aqueous phase or emulsion were separated, which limited the use of these methods with raw materials with a low oil / water ratio, such as hake offcuts or jumbo squid liver. In these cases, a stable oil-in-water emulsion that could not be easily broken was generated by centrifuging in the laboratory.

As a consequence, in this work, only fatty fish by-products such as orange roughy or salmon skins, were appropriate raw materials to obtain fish oil by any of the four methods proposed. Nonetheless, in these cases, the extraction method not only affects the oil extraction yield but also the quality of the fish protein or fish meal obtained, which has also a great interest as add value ingredient. Thus, as it can be observed in the mass balance presented in Figure 2.27, in the SFE method only a dry solid, rich in proteins (fish meal), is generated besides the oil, whereas in the cold extraction or in the wet reduction methods, a wet solid with a high oil content (press cake) is obtained after centrifuging, which requires a subsequent treatment in order to remove the rest of oil and water and obtain a dry protein or fish meal. When using the enzymatic method, almost the total amount of oil can be removed from orange roughy and salmon offcuts; however, the resulting protein, hydrolysed by the action of the protease, remains still together with all the initial water, and, therefore, a subsequent

drying step is required in order to obtain a dry fish protein hydrolysate (FPH).

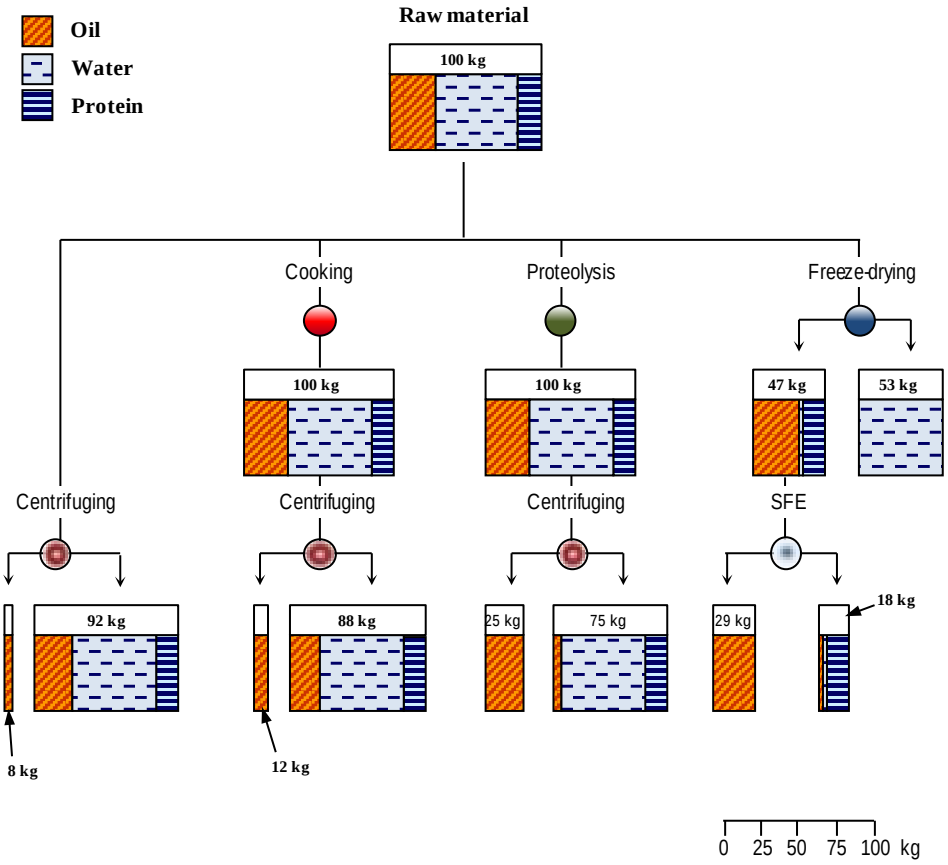


FIGURE 2.27

Mass balance estimated from fish oil production from fatty fish by-products (orange roughy and salmon skins) by different oil extraction procedures: cold extraction (centrifuging), wet reduction, enzymatic method and supercritical fluid extraction.

2.3.3.2. Influence of the raw material on the marine oil properties and composition

Marine oils are usually a mixture of different lipid compounds, mainly acylglycerides, cholesterol, wax esters and free fatty acids. In addition, volatile compounds or pigments, as carotenoids, may be present giving the oil characteristic sensorial properties such colour, odour and flavour.

This section focuses on the oil extracts obtained by SFE since it was the only method that allowed us to obtain oil from the four raw materials considered: orange roughy, salmon and hake offcuts and jumbo squid liver.

i) Physical properties

The oils extracted by SFE from the various by-products studied were liquid at ambient temperature, although a greater viscosity was observed in the case of squid liver oil. In all cases, the amount of moisture and total volatile compounds was around 0.5 %, a value slightly higher than the maximum level (0.3 %) established in fats and oils from animal sources by the Codex Alimentarius (*WHO/FAO*, 1994).

Regarding colour, hake oil presented a light yellow colour whereas orange roughy, salmon and jumbo squid oils were characterised by an intense orange colour, as it can be observed in Figure 2.28. This colour may be related to an orange pigment, asthanxine, which is a common carotenoid that has been found to be present in salmon oil (*Mendes-Pinto, et al.*, 2004).

(a) (b) (c) (d)

FIGURE 2.28

Fish oil obtained by SFE (25 MPa / 313 K / 10 kg / h) from different marine by-products. (a) Hake offcuts. (b) Orange Roughy offcuts. (c) Salmon offcuts. (d) Squid liver oil.

ii) Neutral lipid composition and fatty acid profile

Neutral lipids, especially triacylglycerides, have the highest interest in the food industry and pharmacology because they are the type of lipids easiest absorbed by humans (*Lawson, et al.*, 1988).

TABLE 2.8

Neutral lipids profile in marine oils obtained by SFE from different marine by-products.

Neutral lipids	% wt. in t			
	Oil from hake offcuts	Oil from orange roughy offcuts	Oil from salmon offcuts	Oil from jumbo squid liver
Wax esters	n.d.	> 99	n.d.	0.6
Triacylglicerides	67	n.d.	97.1	30.4
Fatty acids	3.8	n.d.	1.3	7.1
Cholesterol	1.8	n.d.	0.7	4.9

Concentration expressed taking palmityl palmitate, tripalmitine and palmitic acid as standards for the calibration curves of wax esters, triacylglycerides and fatty acids respectively. n.d. not detected.

TABLE 2.9

Fatty acid profile in marine oils obtained by SFE from different marine by-products.

Fatty acids	mg of fatty acids / g fish oil			
	Oil from hake offcuts	**Oil from orange roughy offcuts**	**Oil from salmon offcuts**	**Oil from jumbo squid liver**
C14:0 (Myristic acid)	19 ± 2	4.0 ± 0.3	40.4 ± 0.1	39 ± 3
C16:0 (palmitic acid)	129 ± 12	6.4 ± 0.4	143 ± 0.4	141 ± 12
C16:1 (palmitoleic acid)	34 ± 6	44 ± 3	59 ± 1	43 ± 4
C18:0 (stearic acid)	21 ± 2	2.5 ± 0.2	46.4 ± 0.1	43 ± 4
C18:1n-9 (oleic acid)	142 ± 13	213 ± 13	146 ± 2	42 ± 4
C18:1n-7 (vacenic acid)	22 ± 2	24 ± 2	28.9 ± 0.1	23 ± 2
C18:2n-6 (LA)	7.0 ± 0.7	4.7 ± 0.3	93 ± 1	5.8 ± 0.5
C18:3n-6 (GLA)	1.9 ± 0.2	1.4 ± 0.1	5.5 ± 0.1	3.1 ± 0.2
C18:3n-3 (ALA)	2.6 ± 0.3	n.d.	14.0 ± 0.2	2.9 ± 0.3
C18:4n-3 (stearidonic acid)	3.2 ± 0.4	n.d.	5.9 ± 0.1	2.0 ± 0.1
C20:1n-9 (gadoleic acid)	37 ± 3	50 ± 3	13.1 ± 0.4	24 ± 2
C20:3n-6 (DGLA)	0.82 ± 0.03	1.5 ± 0.1	3.2 ± 0.2	1.6 ± 0.2
C20:4n-6 (araquidónico acid)	5.5 ± 0.6	n.d.	6.7 ± 0.1	127 ± 10
C20:5n-3 (EPA)	36 ± 4	3.2 ± 0.2	79 ± 1	127 ± 10
C22:1n-11	28 ± 2	19 ± 1	n.d.	5.6 ± 0.1
C22:1n-9	4.2 ± 0.3	6.5 ± 0.4	n.d.	1.5 ± 0.1
C22:4n-6 (adrenic acid)	4 ± 2	n.d.	n.d.	5.4 ± 0.4
C22:5n-3 (DPA)	8.0 ± 0.7	n.d.	38.4 ± 0.7	22 ± 1
C22:6n-3 (DHA)	82 ± 8	5.2 ± 0.3	63 ± 1	130 ± 9
C24:1 (nervonic acid)	7.8 ± 0.6	2.9 ± 0.1	2.5 ± 0.1	2.8 ± 0.3
Total fatty acids	595 ± 53	388 ± 24	789 ± 7	691 ± 74
Saturated fatty acids	168 ± 15	13 ± 1	230 ± 1	223 ± 19
Monounsaturated fatty acids	275 ± 24	359 ± 22	250 ± 3	156 ± 32
Polyunsaturated fatty acids	151 ± 14	16 ± 1	309 ± 5	312 ± 23
ω3	132 ± 14	8 ± 1	100 ± 3	284 ± 20
ω6	19 ± 1	11 ± 1	108 ± 2	29 ± 2

Table 2.8 shows the main neutral lipids found in the oils obtained by SFE from the different by-products used in this work. It can be observed that salmon oil is the only oil composed basically by triacylglycerides, whereas hake oil and jumbo squid oil were both composed by a mixture of triacylglycerides, cholesterol and some amounts of wax esters. No triacylglycerides were detected in the oil extracted from orange roughy offcuts, which was composed basically by wax esters. This feature makes orange roughy oil suitable for industrial purposes, especially in the cosmetic or lubricant fields (*Buisson, et al.*, 1982). However, due to the low capacity of human body for wax ester absorption (*Phleger, et al.*, 1997, *Puce*, 1992), the use of orange roughy oil for human consumption is limited.

The oil fatty acid profile has also a great importance in order to know its natural omega 3 content and thus, its possible application as raw material in omega 3 concentration processes.

A total of 20 fatty acids were identified in the various marine oils extracted by SFE in this work. The composition is reported in Table 2.9.

It can be observed that palmitic acid is the main fatty acid in jumbo squid oil, hake oil and salmon oil, whereas oleic acid is the most abundant fatty acid in orange roughy oil. Regarding omega-3 concentration, jumbo squid liver oil showed the highest amount (284 mg / g oil), followed by hake offcuts oil (132 mg / g oil) and salmon offcuts oil (100 mg / g oil), whereas orange roughy skin oil showed a negligible amount of omega-3 PUFA.

Orange roughy is therefore definitely discarded as a source to obtain omega-3 concentrates.

iii) Trace metal content

Small amounts of some metals may appear in fish oils due to their natural presence in the fish by-products as organometallic compounds or due to the sea water pollution. Due to its high toxicity, arsenic, lead, cadmium, copper and mercury are considered the most important, and their upper limits have been established by the European Union (*EC*, 2006).

Table 2.10 reports the amount of the different metals found, both in the marine by-products studied and in the oil extracted by SFE.

It can observed that metals such as Fe or Zn, found in some of the fish by-products, are not found in the extracted oil or are found in a significantly lower concentration. In addition, the amount of toxic metals such as Cu, Cd, Hg or Pb, which is fairly high in some of the marine by-products considered in this work, decreases noticeable in the oils, being negligible in most cases.

Regarding the amount of arsenic, it is also observed a noticeable difference between fish by-products and the corresponding oil, although it was detectable in all cases. A high amount of arsenic compounds, both inorganic and organic, are present in sea water due to natural processes (*Smedley, et al.*, 2002) and pollution. These arsenic compounds may bio-accumulate in marine organisms, being the water soluble form, arsenobetaine, the main species found in fish (*Ackley, et al.*, 1999). However, recent studies have

also found considerable amounts (4.3 – 10.5 ppm) of non polar lipid bound arsenic compounds or arsenolipids in ten different crude fish oils (*Schemeisser, et al.*, 2005). These arsenolipids, in which arsenic is bound to a lipid, may be partially co-extracted with oil by SFE, which would explain that some arsenic was found in jumbo squid liver oil or salmon offcuts oil

TABLE 2.10

Heavy metals in marine oils obtained by SFE from different marine by-products.

Raw material		mg / kg (in oil fraction)						
		Fe	**Cu**	**Zn**	**As**	**Cd**	**Hg**	**Pb**
OR	A	5.3 ± 0.1	0.8 ± 0.3	31.3 ±0. 3	2.6 ± 0.1	n.d.	n.d.	n.d.
	B	n.d.	n.d.	1.5 ± 0.6	0.26 ± 0.03	n.d.	n.d.	n.d.
S	A	22.3 ± 0.4	1.9 ± 0.2	27.2 ± 0.1	1.5 ± 0.2	n.d.	0.5 ± 0.2	n.d.
	B	2 ± 1	0.10 ± 0.01	n.d.	0.89 ± 0.05	n.d.	n.d.	n.d.
H	A	83.4 ± 0.9	11.6 ± 0.4	114.7 ± 0.3	33.8 ± 0.1	n.d.	3 ± 1	n.d.
	B	n.d.	0.07 ± 0.04	1 ± 1	0.05 ± 0.04	n.d.	n.d.	n.d.
JS	A	> 103	> 103	726 ± 1	207 ± 1	> 103	12 ± 1	5 ± 1
	B	10.3 ± 0.2	0.48 ± 0.01	1.1 ± 0.1	6.7± 0.3	n.d.	n.d.	0.07 ± 0.01

A: in fish by-products; B: in fish oil

OR: Orange Roughy; S: Salmon; H: Hake; JS: Jumbo Squid

n.d.: not detected

2.3.3.3. Influence of the extraction procedure on oil quality

A comparison among the oils extracted from orange roughy and salmon offcuts by the four different methods considered in this work was carried out. In the case of hake offcuts and jumbo squid livers, it was only possible to obtain oil successfully when using SFE, the rest of the methods did not provide an appreciable amount of oil.

Both, orange roughy oil and salmon oil, extracted by the four different methods, presented the same colour and a similar neutral lipid composition and fatty acid profile. Therefore, the comparison was focused on other parameters such as lipid oxidation, arsenic amount or sensorial properties, in order to establish the advantages and disadvantages of the different methods.

i) Lipid oxidation

The influence of the extraction method on lipid degradation was studied taking into account the two main spoilage processes: hydrolysis and oxidation.

Hydrolysis occurs during storage and it is responsible for the production of free fatty acids (FFA), and therefore, for the acidity of the oil.

Lipid oxidation is carried out by an autocatalytic process or enzymatic action that involves two stages: primary oxidation, when hydroperoxides are formed, and secondary oxidation, when hydroperoxides are broken down to aldehydes, ketones, alcohols, acids and alkanes. Primary oxidation is

determined by the Peroxide Value (PV), whereas secondary oxidation is measured by the Anisidine Value (AV) and volatile compounds (*FAO*, 1986). The total oxidation value, TOTOX, is determined according to the expression (2 PV + AV) (*Perrin*, 1996). Figure 2.29 shows a comparison among the FFA content and TOTOX respectively in both orange roughy skin oil and in salmon skin oil extracted by different methods.

As it was expected, oil obtained from orange roughy oil offcuts, composed by wax esters and a low amount of PUFA, tends to be more stable against oxidation and, therefore, it presents a low value of PV and AV, especially when the extraction is carried out by SC-CO_2. Nonetheless, the presence of volatile compounds such as acetic acid, may affect the pH of the fish oil leading to an overestimated FFA content, as it is observed in the oil extracted by the orange roughy skin (*see* Figure 2.29). These volatile compounds, typically generated during fish oil storage by the action of anaerobic bacteria such as *S. Putrefaciens* (*Gram, et al.*, 1990), can be easily extracted with SC-CO_2 due to their high vapour pressure, remaining in the oil as it is detected in the analysis of volatile compounds (*see* Table 2.12).

Oil obtained from salmon offcuts, composed by triacylglycerides with a higher amount of PUFA, tends to hydrolyse and oxidise quite easily, showing a higher FFA content and TOTOX than the oil obtained from orange roughy offcuts. Nonetheless, both the FFA content and TOTOX found in the salmon skin oil extracted by the SFE method were noticeably lower than the same oil extracted by non-SFE methods. Those results prove

that the extraction with SC-CO_2 permits to avoid the degradation of PUFA since it not only occurs at mild temperatures but also under non-oxidising atmosphere.

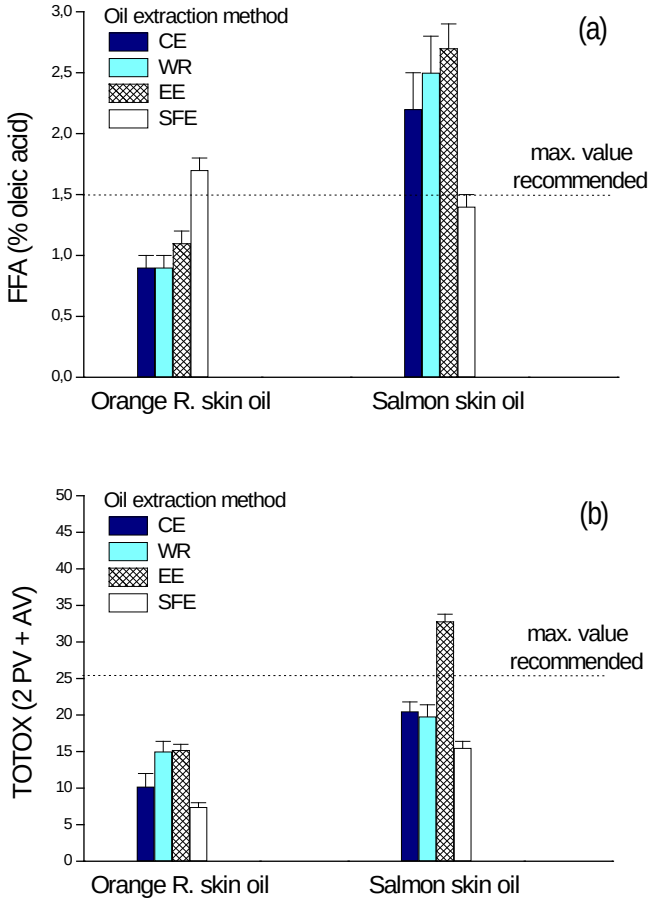


FIGURE 2.29

Free fatty acids (up) and total oxidation index (TOTOX) of the oil extracted from orange roughly and salmon offcuts by different methods.

ii) Total arsenic amount

Figure 2.30 shows a comparison among the arsenic concentration found both in orange roughy skin and salmon skin oil extracted by the four different methods proposed.

In all cases, a significant reduction in arsenic amount from the raw material to the oil can be observed. However, this reduction is higher in the oil obtained by SC-CO_2, especially in the case of orange roughy skin, since the solubility of arsenolipids in SC-CO_2 is expected to be low, which is an important advantage of the SFE over the other methods.

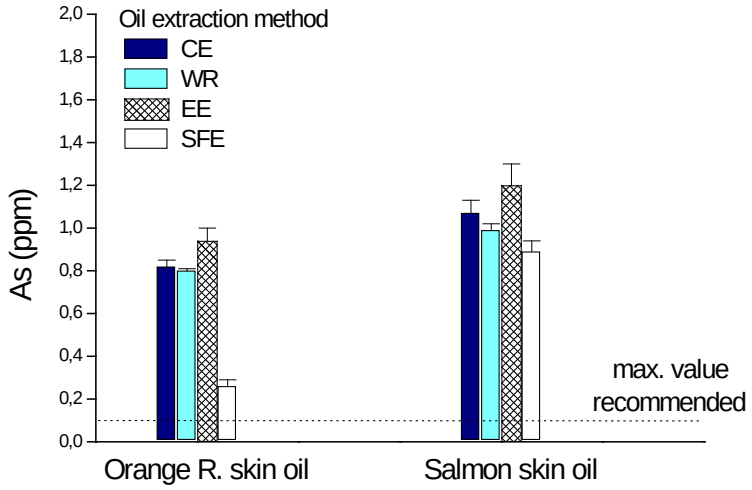


FIGURE 2.30

Total arsenic content found in the oil extracted from orange roughly and salmon offcuts by different methods.

iii) Sensorial properties

Sensorial properties, especially odour and flavour, are related to volatile compounds such as aldehydes, amines or acids. Some of them are produced during storage or spoilage of the marine by-products both by bacterial action over protein, aminoacids and carbohydrates and by enzymatic action or in the lipid oxidation (*see* Table 2.11), whereas other volatile compounds such aldehydes are indicators of the auto-oxidation process that occurs during the oil extraction.

TABLE 2.11

Volatile compounds produced by different fish degradation processes.

Process	Substrate	Compounds produced
Bacterial degradation	Trimethylamine oxide (TMAO)	Trimethylamine (TMA)
	Cysteine	H_2S
	Methionine	CH_3SH, $(CH_3)_2S$
	Carbohydrates and lactate	Acetate, CO_2, H_2O
	Inosine	Hypoxanthine
	Glycine, serine, leucine	Esters, ketones, aldehydes
	Urea	NH_3
Enzymatic action	Trimethylamine oxide (TMAO)	Dimethylamine (DMA)
Autooxidation process	Lipids	Aldehydes
		Ketones
		Alcohols
		Short-chain organic acids
		Alkanes

The influence of the oil extraction method on its volatile compounds content was studied in the case of orange roughy skin oil. Table 2.12 shows a list of the volatile compounds identified in the oils extracted by different methods.

TABLE 2.12

Volatile compounds found in orange roughy offcuts oil extracted by different methods.

Compound		Odour characteristics	Extraction method			
			CEN	WR	EE	SFE
Alkanes	Decane	---	✓	✓	✓	✓
	2-methyl-Decane	---	×	×	×	✓
	3-methyl-Decane	---	×	×	×	✓
	Undecane	---	✓	✓	✓	✓
	Dodecane	---	✓	✓	✓	✓
	Tridecane	---	✓	✓	✓	✓
	Pentadecane	---	✓	✓	✓	✓
	Cyclohexadeccane	---	×	×	×	✓
	2,6,10,14-tetramethyl-Pentadecane	---	✓	✓	✓	✓
Aldehydes	Heptanal	Waxy green	×	✓	×	×
	Hexanal	Green	✓	✓	✓	×
	Nonanal	Fatty floral	×	×	✓	×
Acids	Acetic acid	Vinegar-like	×	×	×	✓
Amines	Dimethylamine	Fishy	×	×	×	✓

It can be observed that hexanal and nonanal are detected in the oils extracted by non-SFE methods, whereas volatile compounds such dimethylamine or acetic acid appeared in the oil extracted by SFE. Both hexanal and nonanal are degradation products generated when the lipid oxidation in oil takes

place, whereas acetic acid and volatile amines such dimethylamine may be generated by the action of endogenous enzymes, such as *S. Putrefaciens*, during fish frozen storage (*Gram, et al.*, 1990). Therefore, because the SFE method permits reducing significantly the lipid oxidation during the extraction process, the formation of hexanal and nonanal is avoided. However, volatile compounds found in the raw material, such dimethylamine or acetic acid, are easily extracted by SC-CO_2 due to their high vapour pressure and, since the process takes place in a recirculation system, they may partially remain in the oil. These volatile compounds could be easily removed from the oil by circulating carbon dioxide at low pressure through the oil.

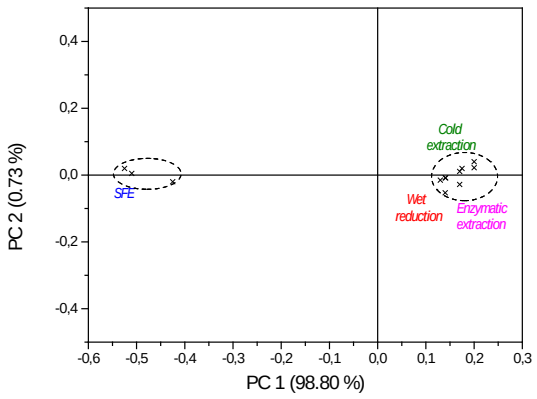


FIGURE 2.31

Principal Component Analysis (PCA) of the data obtained with the electronic nose system for the oil extracted from orange roughly offcuts by different methods.

A clear difference among the orange roughy oil extracted by SFE and the oils obtained by the non-SFE methods used in this work was observed in the Principal Component Analysis (PCA) of the data obtained with the electronic nose system (*see* Figure 2.31). Such differences were also observed in the odour sensorial perception of the orange roughy oils obtained by the different methods.

Figure 2.32 shows the average score given by ten panellists to the different orange roughy oils according to six different odour characteristics. It may be observed that fishy is the attribute detected with more intensity in all the samples, especially in the oil extracted by SFE. This fishy odour is related to the presence of amine compounds that are produced in the raw material during its storage, and could be minimized by using fresh by-products or by deodorizing the oil after extraction as has been mentioned above.

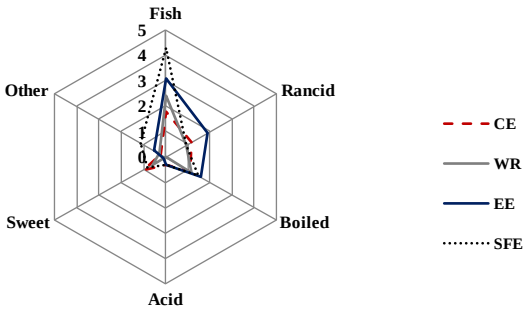


FIGURE 2.32

Sensorial analysis of the oil extracted from orange roughly offcuts by different methods.

2.3.3.4. Stability of the fish oil extracted by SC-CO_2

Fish oil, containing a high level of PUFA, is especially prone to deterioration at a varying velocity, strongly depending on storage conditions. In the present study, two fractions of hake oil obtained by SFE (25 MPa, 313 K) were kept at 4 ºC and darkness and at 20 ºC and natural light, respectively, and several stability parameters were evaluated related to oil stability against hydrolysis and oxidation stability of PUFA.

i) Oil stability against hydrolysis

The stability of hake skin oil against hydrolysis was evaluated by means of the FFA content, since FFA are mainly generated from hydrolytic reactions in the oil.

It was observed that FFA content in oil stored at 4º C did not differ significantly along the first 45 days but, after that, an important increment occurs, especially in the oil stored at higher temperature Figure 2.33.a.

ii) Oxidation stability of PUFA

As it was described in Chapter 1, fish oil with a high amount of PUFA is prone to oxidation, which involves the formation of toxic products and / or volatile compounds related to non-desirable off-flavors.

PUFA oxidation takes places by a complex mechanism of free radical chain reactions catalyzed by different factors such as temperature, light, oxygen or trace metals. Its experimental evaluation was carried out by measuring

different parameters related to the formation of both primary and secondary oxidation products and by determining the sensorial changes in the fish oil odour and colour features.

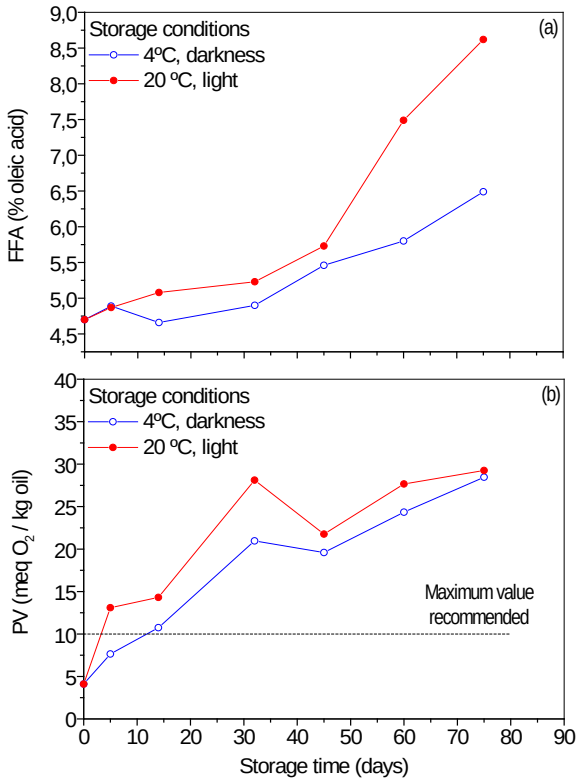


FIGURE 2.33

Evolution of FFA content (a) and PV (b) in hake skin oil stored at 4 ºC in darkness and at 20 ºC in lightness respectively. Hake skin oil extracted with SC-CO_2 at 25 MPa and 313 K.

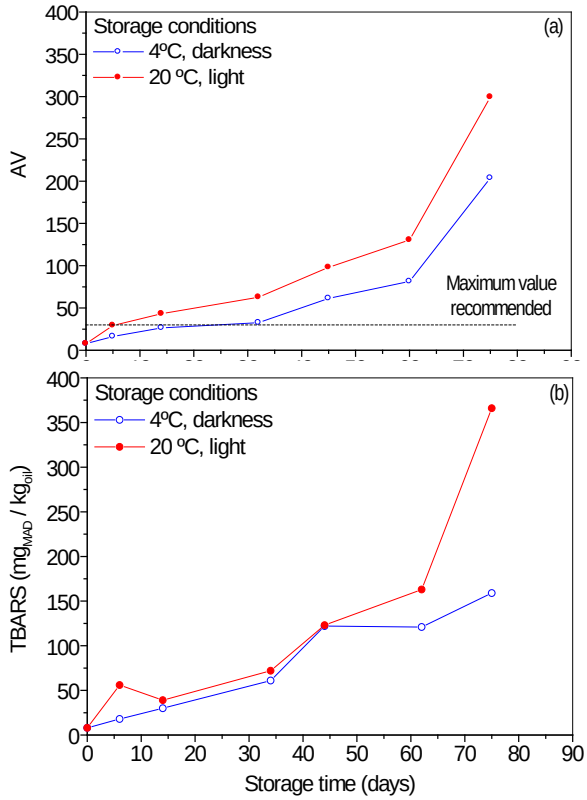


FIGURE 2.34

Evolution of AV (a) and TBARS (b) in hake skin oil stored at 4 ºC in darkness and at 20 ºC in lightness respectively. Hake skin oil extracted with SC-CO_2 at 25 MPa and 313 K.

- *Amount of oil oxidation products*

The amount of oil oxidation products was evaluated by the peroxide value, which is related to the amount of hydroperoxides generated in the primary

stage of PUFA oxidation (*see* Figure 2.33.b); and the anisidine (AV) and thiobarbituric reactive substances (TBARS) values, both of them related to the amount of carbonyl compounds that are generated from hydroperoxides in a subsequent oxidation stage (*see* Figure 2.34).

It is observed that, during the first 35 days of oil storage, PV increases significantly whereas AV and TBARS grows up more slowly, which may be related to the higher production of the primary oxidation products. However, after this time, no significant increment is appreciated in PV but in AV, which increases dramatically mainly in the oil stored at room temperature (20 ºC) and light. Furthermore, it was observed that, after a short period of time (less than 10 days) both PV and AV were above the minimum value established for edible purposes (*BP*, 2008) even when the fish oil was kept preserved of high temperatures and light exposure.

Thus, it must be noticed that, in spite of minimising the oxidative effect of oxygen during oil extraction, only the use of SC-CO_2 is not enough for preventing oil oxidation and, therefore, pro-oxidative conditions must be also minimised as much as possible both during the storage of the raw material and after oil extraction.

- *Sensorial changes*

Sensorial changes in fish oil odour were evaluated by an Electronic Nose (EN) system, which is a sensor-based technology that considers the total headspace volatiles and creates a unique smell print.

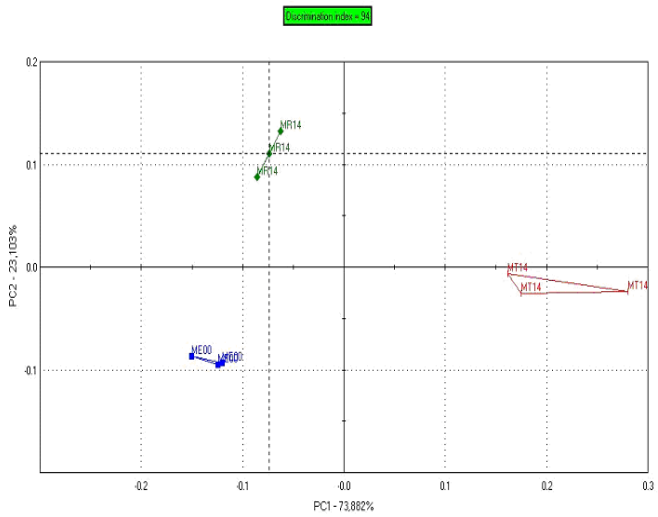


FIGURE 2.35

Plot of the first two Principal Components of the electronic nose sensor responses. ME00 (hake oil after extraction); MR14 (hake oil stored at 4 ºC for 14 days); MT14 (hake oil stored at 20 ºC for 14 days).

Figure 2.35 shows the distribution of the oil samples according the two first Principal Components (PC) of EN sensor response data. PC1 accounted for the 74 % of the total variability and PC2 for 23 %, so any difference in the smell print of the oils are depicted in the graph. The discrimination index of the PC analysis is high, denoting a clear separation of three groups of samples, which corresponds to the initial oil and the two oils stored under different conditions. The storage time at high temperature determine the biggest changes in sensor response, since oil stored at 20 ºC widely differ from oil after extraction in the PC1. Oil stored at 4 ºC present a middle

position along PC1 between those oils, but it also differs in PC2; therefore, storage conditions not only produce modifications in the intensity of volatile compounds responsible for oil smell but also in the type of volatile developed.

TABLE 2.13

Development of colour parameters (lightness (L*) redness (a*) and yellowness (b*)) during oil storage.

Storage time (days)	Temperature (ºC)	CIElab Parameters		
		L*	a*	b*
0		27.01 a	1.07 c	39.88 a
14 (darkness)	4	40.39 b	-0.98 a	46.50 b
14 (light)	20	26.32 a	-0.83 b	40.09 a

Values in each column with common letters are not significantly different ($p < 0.05$)

Color changes were evaluated by means of the three parameters of the CIElab system: lightness (L*), redness (a* value) and yellowness (b* value) (*see* Table 2.13). Lightness does not seem to be affected by storage, along storage under most severe conditions (20 ºC with natural light) L* value did not change. Lightness increased considerably during storage at 4 ºC, but it could be related to precipitation of several particles at the bottom of the vessel enhanced by low temperature. The most significant changes are

observed in a* values, which are related with the tonality of colour, changing from redness (a*) to greenness (-a*) along storage. The characteristic yellow colour of hake oil are denoted by a high value of b* that suffer a slight increase after two weeks of storage related to lipid oxidation.

2.3.4. Economical considerations

During the last decades, different supercritical fluid technologies have been established as interesting for safely processing natural products in the food and pharmaceutical industries. Nowadays, several processes such as coffee decaffeination, hops extraction, essential oils extraction, cork cleaning, pesticides removal from rice, etc., are carried out at commercial scale in different parts of Europe, US and Asia (*Brunner*, 2010, *Perrut*, 2000). Some studies have shown that, in spite of requiring a high investment cost, supercritical fluid extraction of essential oils requires lower processing costs and downstream processing making this process competitive regarding steam distillation (*Pereira, et al.*, 2007). Regarding the processing of fats and oils, SFE may also compete with traditional processes in the case of specialty oils such as nut oils (almond, peanut...), seed oils (apricot, borage, grape, sesame...), cereal oils (wheat germs, rice bran...) or fruit oils (cloudberry, tomato...), which contain bioactive lipid compounds interesting in the food and pharmaceutical industries (*Temelli*, 2009). In the case of marine oil extraction, although SFE leads to high quality oil, the drying step, required before extraction, increases noticeable the production cost and

minimizes competitiveness against alternative extraction processes. Therefore, in this case SFE may be viable not as an isolated process but as part of a whole process involving several steps (fractionation, enzymatic reaction, encapsulation) in order to obtain small volumes of high value omega-3 concentrates used as ingredients in functional foods or as active principles in pharmacology.

2.3.5. Conclusions

The valorisation of marine by-products by recovering their oil has a great interest in the fish industry, especially when the oil is rich in triglycerides and has a high content of omega-3 polyunsaturated fatty acids. The extraction process used to obtain omega-3 rich oils has also shown to be important to obtain the best oil quality regarding lipid oxidation, pollutants contain or sensorial properties.

The comparison of the different oils extracted by SFE previous freeze-drying and other simpler method such cold extraction, wet reduction or enzymatic extraction shows that SFE combined with freeze drying permits improving noticeable the oil quality since it prevents oil lipid oxidation and reduces significantly metal content. The main drawback is the co-extraction of volatile compounds present in the raw material, such amines, which can be reduced increasing the raw material freshness or coupling a subsequently deodorization step. On the other hand, SFE combined with freeze drying is able to extract oil from by-products with a low fat content such as hake

offcuts or jumbo squid liver, and avoids the formation of water wastes rich in proteins or fat that have an important interest both from an economical and environmental point of view. Therefore, in spite of being a more sophisticated technology and implying higher inversion costs, SFE involves some advantages over other simpler and cheaper extraction processes such as cold extraction, wet reduction or enzymatic extraction.

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Chapter 3

Fish Oil Refining & Omega-3 Concentration by Supercritical Fluids

This Chapter partially published as oral communication (*Rubio-Rodríguez, et al.*, 2010) in the 12th European Meeting on Supercritical Fluids in Graz (Austria).

SUMMARY

Comparing with conventional processes, supercritical fluid extraction with SC-CO_2 has shown good results in production of fish oil with a lower amount of impurities, especially heavy metals. However, in some cases other components such as free fatty acids still remain in the oil at higher levels than it would be desired according to quality standards. Furthermore, the amount of omega-3 in natural fish oil is quite lower than the values demanded by the food and pharmaceutical industries; hence a subsequent concentration step is required.

This Chapter is focused on the study of the possibilities of the supercritical fluid technology for fish oil refining and omega-3 concentration. Firstly, a supercritical fluid extraction-fractionation process is proposed as a way to enhance fish oil quality and obtain a lipid fraction rich in triacylglycerides. The last part is the result of the preliminary study carried out in the ACSR in Prague (Czech Republic) about omega-3 concentration through enzymatic hydrolysis-fractionation of fish oil in a SC-CO_2 media.

RESUMEN

En comparación con procesos convencionales, la extracción con CO_2 supercrítico ha dado buenos resultados en la producción de aceite de pescado con un menor contenido en impurezas, especialmente metales pesados. No obstante, en muchos casos otros componentes como ácidos grasos libres siguen estando presentes en el aceite a niveles superiores a los establecidos por los estándares de calidad. Además, la cantidad de omega-3 en el aceite de pescado natural es bastante más baja que los valores demandados por la industria farmacéutica y alimentaria, de ahí la necesidad de etapas posteriores de concentración de omega-3.

Este Capítulo está basado en el estudio de las posibilidades que ofrecen las tecnologías de fluidos supercríticos tanto en el refinado de aceite de pescado como en la obtención de concentrados de omega-3 En primer lugar, se propone un proceso combinado de extracción-fraccionamiento con CO_2 supercrítico como vía para mejorar la calidad del aceite de pescado y obtener una fracción lipídica rica en triacilglicéridos. En la segunda parte, se resumen los resultados de un estudio preliminar llevado a cabo en el ACSR de Praga (República Checa) sobre la obtención de concentrados de omega-3 a través de un proceso de hidrólisis enzimática-fraccionamiento empleando el CO_2 supercrítico como medio de reacción.

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NOMENCLATURE

[E]	Enzyme concentration
[ES]	Enzyme-substrate complex
[P]	Product concentration
[S]	Substrate concentration
2-AG	2-Acylglyceride
a	Specific area in the reactor (m^2 / m^3)
A_c	Cross section of the packed bed reactor (m^2)
AG	Acylglyceride
AV	Anisidine value
$C_{DAG,f}$	Concentration of DAG in bulk fluid at reactor outlet (mol / m^3)
$C_{FFA,o}$	Concentration of FFA in bulk fluid at reactor inlet determined as Eq (3.34)
C_G^*	Glycerol concentration in bulk fluid at reactor outlet (mol / m^3) estimated from the glycerol mass balance (3.42)
C_G	Theoretical glycerol concentration determined according to the solubility of glycerol in SC-CO_2
$C_{MAG,f}$	Concentration of MAG in bulk fluid at reactor outlet (mol / m^3)
$C_{TAG,e}$	Concentration of TAG inside the enzyme particle (mol / m^3)
$C_{TAG,e,o}$	Concentration of TAG inside enzyme particle at reactor inlet (mol / m^3)
$C_{TAG,o}$	Concentration of TAG in bulk fluid at reactor inlet (mol / m^3), determined as Eq. (3.35)
$C_{TAG,o}^*$	Substrate concentration in bulk fluid at reactor inlet (mol / m^3) estimated from the FFA mass balance (3.39)
$C_{TAG,s}$	Concentration of TAG on enzyme particle surface (mol / m^3)
$C_{TAG,s,o}$	Concentration of TAG on enzyme particle surface at reactor inlet (mol / m^3)
C_{TAG}	Concentration of TAG in bulk fluid (mol / m^3)
C_{WP}	Weisz-Prater criterion, defined as Eq. (3.25)

D_{TAG}	Diffusivity of TAG in SC-CO_2 (m^2 / s), determined according to Wilke-Chang equation (3.17)
DAG	Diacylglyceride
$D_{ef,TAG}$	Effective diffusivity of TAG inside enzyme particle (m^2 / s), defined as Eq. (3.28)
DHA	Docosahexaenoic acid (omega-3)
d_p	Average particle diameter of immobilized enzyme (cm)
DPA	Docosapentaenoic acid (omega-3)
EPA	Eicosapentaenoic acid (omega-3)
FA	Fatty acid
FFA	Free Fatty Acids
f_p	Pressure factor for mass transfer coefficient defined as Eq. (3.21)
f_δ	Retardation factor related to the molar conversion, δ and estimated for a three-steps with a fist-order reaction from previous hydrolysis studies (*Sovová, et al.*, 2008).
G	Glycerol
H	Hake skin
H_f	Hindrance factor defined as (3.29)
i.d.	Internal diameter of pressure vessel (mm)
JS	Jumbo squid liver
k_{-1}	Rate of dissociation of the enzyme - substrate complex back to enzyme and substrate (s^{-1})
k_1	Rate of formation of the enzyme - substrate complex (s^{-1})
k_2	Rate of dissociation of the enzyme-substrate complex to enzyme and product (s^{-1})
k_f	External mass transfer coefficient (m / s)
k_i	Distribution coefficient of compound i between separator 1 and 2
K_m	Michaelis-Menten constant (substrate concentration when $V = V_{max} / 2$) (mol / m^3)
K_u	Mass transfer constant ($m^{2.17}$ / $s^{0.17}$ kg), defined by Eq. (3.20)
LCFA	Long Chain Fatty Acid (carbon number, C > 18)

MAG	Monoacylglyceride
M_{CO2}	Amount of carbon dioxide that passes through the reactor during sampling (kg)
M_e	Mass of immobilized enzyme in the reactor (kg)
M_S	Mass of hydrolysate sample (kg)
$M_{w,\ TAG}$	Molecular weight of TAG (taking tripalmitin as reference)
$M_{w,FFA}$	Molecular weight of FFA (taking palmitic acid as reference)
$M_{w,W}$	Molecular weight of water (g / mol)
n	Ratio between the amount of active sites and the amount of free enzyme
$N_{FFA,f}$	Total amount of free fatty acids in the hydrolyzed oil (mol)
$N_{FFA,o}$	Total amount of free fatty acids in fish oil (mol)
$N_{TAG,f}$	Amount of TAG in the hydrolysated fish oil (mol)
$N_{TAG,o}$	Initial amount of TAG in fish oil (mol)
$N_{TAG,r}$	Amount of TAG that are hydrolysed (mol)
PA	Palmitic acid
PBR	Packed Bed Reactor
PV	Peroxide Value (meq O_2 / kg $_{oil}$)
Q	Fluid mass flow rate (g CO_2 / min)
R	Particle pore radius (mm)
r_A	Substrate molecular radius (mm)
Re	Reynolds number ($Re = u \cdot d_p\ \rho / \mu$)
S	Salmon skin
S_1	First separator
S_2	Second separator
Sc	Schmidt number ($Sc = \mu / \rho \cdot D$
SC-CO_2	Supercritical carbon dioxide
SCFA	Short Chain Fatty Acid (carbon number, $C \leq 18$)
SFE	Supercritical Fluid Extraction
S_G	Solubility of glycerol in SC-CO_2 (mg / g)

Sh	Sherwood number ($Sh = k_f \cdot d_p / D$)
S_{FO}	Solubility of fish oil in SC-CO_2 (mg / g)
S_W	Solubility of water in SC-CO_2 (mg / g)
T	Temperature (K)
TAG	Triacylglycerides
T_c	Critical temperature (K)
TOTOX	Total oxidation index (2 PV + AV)
t_r	Residence time (s)
t_s	Sampling time (s)
u	Superficial velocity in reactor (m_{fluid} / s)
V'_{TAG}	Specific reaction rate observed on the enzyme surface (mol / kg s)
V_{TAG}	Specific reaction rate according to Michaelis-Menten mechanism, assuming that all the active points are on enzyme surface (mol / kg s)
V_{max}	Maximum specific reaction rate according to Michaelis-Menten mechanism (mol / kg s)
V_r	Reactor volume (m^3)
v_{TAG}	Reaction rate (mol / m^3 s)
v_{max}	Maximum reaction rate according to Michaelis-Menten mechanism (mol / m^3 s)
w_{in}	Amount of fish oil at the reactor inlet (kg)
$w_{i,S1}$	Amount of compound i in separator 1 (kg)
$w_{i,S2}$	Amount of compound i in separator 2 (kg)
w_{out}	Amount of hydrolyzed oil at reactor outlet (kg)
w_P	Amount of hydrolyzed sample recovered in the trap (kg)
$W_{TAG,z}$	Molar flux of a TAG in the reactor (mol / m^2 s)
x_{FFA}	Mass fraction of FFA in fish oil (mol)
x_{TAG}	Mass fraction of triacylglycerides in fish oil
z	Length in axial direction of the Packed Bed Reactor, PBR (cm)

Greek symbols

α	Wilke-Chang parameter
β	Wilke-Chang parameter
$\beta_{DHA,PA}$	Separation efficiency or selectivity between DHA and palmitic acid defined as Eq. (3.38)
$\beta_{i/FFA}$	Separation efficiency or selectivity among free fatty acids and other neutral lipids defined as Eq. (3.37)
ΔV	Volume element in the reactor ($\Delta V=A_c\Delta_z$
γ	Ratio between substrate molecular ratio, r_A, and particle pore radius, R
δ_{FFA}	Molar conversion of triacylglycerides to free fatty acids, defined as Eq. (3.34)
$\delta_{w,FFA}$	Mass conversion of triacylglycerides to free fatty acids, defined as Eq. (3.38)
ε	Particle porosity
η_I	Internal mass transfer efficiency, defined as Eq. (3.23)
μ	Fluid viscosity (Pa s)
μ_o	Standard SC-CO_2 viscosity defined for 15 MPa and 313 K (Pa s)
ν_A	Global reaction rate (mol / m^3 s)
ρ	Fluid density (kg / m^3)
ρ'_e	Density of active sites in the reactor
ρ_c	Standard SC-CO_2 density defined for 15 MPa and 313 K (kg / m^3)
ρ_e	Bulk density of immobilized enzyme (kg / m^3)
ρ_o	Standard SC-CO_2 density (defined for 15 MPa and 313 K)
τ	Tuortosity
ϕ	Thiele number (defined as Eq. (3.23))

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3.1. Fish Oil Deacidification by SFE-fractionation

Abstract

As it was discussed in Chapter 2, SC-CO_2 under a pressure and temperature conditions of 25 MPa and 313 K respectively can be a good solvent in fish oil extraction, not only for using moderate temperatures, which aim to reduce the lipid oxidation during the extraction process, but also because it allows to extract selectively lipid compounds with low polarity, reducing the level of impurities such as heavy metals in the final product. Moreover, as discussed in *Section 1.2.2.1*, the use of SC-CO_2 as extraction solvent is also advantageous since its solvation power can be easily tunable by changing pressure and temperature conditions. This feature makes SC-CO_2 attractive for oil fractionation, since, according to their different solubility, high molecular compounds, such as triacylglycerides (TAG) may be separated from low molecular compounds such as free fatty acids (FFA) or volatile compounds, responsible for the fish oil acidity. In this section a short study about deacidification of fish oil by a SFE-fractionation process from different fish by-products (hake skin, salmon skin and jumbo squid liver oil) is presented. SFE was carried out at the optimal conditions proposed in *Section 2.2*, and the oil extracted was fractionated by a subsequent despresurization of CO_2 in two separators. Experimental results show that a lipid fraction rich in TAG is mostly recovered in the first separator with a lower acidity (FFA content), since the majority of acid compounds, mainly free fatty acids, reaches the second separator still solubilised in CO_2.

➢ **Section Content**

3.1.1. Introduction

High quality fish oil aimed to food and pharmaceutical industries requires a low level of impurities according to the quality standards. Previous studies have shown that supercritical fluid extraction with CO_2 permits to obtain a less oxidised fish oil and with a lower amount of toxic compounds, such as heavy metals, than the conventional extraction methods (*see* Section 2.3). However, the amount of other impurities such as free fatty acids was still quite high, exceeding in some cases the upper limits required for edible purposes.

Fish oil is a complex mixture of different components, mainly lipids and other volatile compounds or pigments which solubility behaviour in SC-CO_2 is difficult to predict due to the effect of molecular interactions that may increase or decrease the solubility of each component in the mixture comparing with the solubility in the pure state. However, taking into account the relation among solute properties, such as molecular weight, polarity or vapour pressure, and solubility in SC-CO_2, it can be established that, within a same lipid family, solubility increases from triacylglycerides to esters as it is represented in Figure 3.1 for oleic acid series (*Güçlü-Üstündağ, et al.*, 2000). Furthermore, other compounds such as water (*Chrastil*, 1982)or glycerol (*Sovová, et al.*, 1997) show a lower solubility in SC-CO_2 due to their high polarity Figure 3.1.

Hence, this trend in the solubility of fish oil components and the easy tunability of SC-CO_2 density, and therefore its solvation power, by changing temperature and/or pressure, makes supercritical fluid technologies interesting for removing fish oil impurities alternatively to conventional physical and chemical fish oil refining (*Catchpole, et al.*, 2000, *Jakobsson, et al.*, 1991, *Jakobsson, et al.*, 1994, *Kawashima, et al.*, 2006, *Yuqian, et al.*, 2001).

In this section, SFE followed by fractionation in two separators was proposed as an easy way to refine fish oil in order to reduce the amount of impurities, especially free fatty acids, and obtain an oil fraction rich in triacylglycerides.

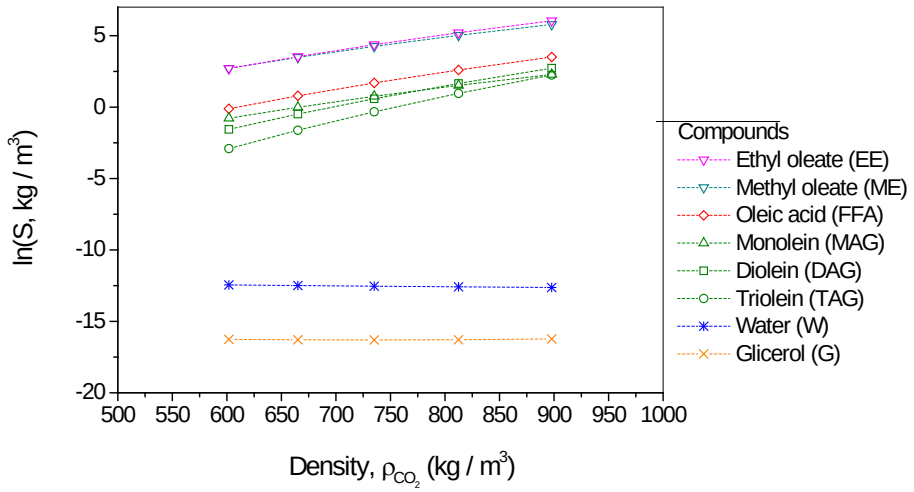

FIGURE 3.1

Solubility of pure lipid compounds in SC-CO_2.

3.1.2. Experimental procedure

3.1.2.1. Raw material and pretreatment

The fractionation was only applied in the case of hake skin, salmon skin and jumbo squid liver oils since they were those which led the best oils regarding triacylglycerides and omega-3 fatty acids content (*see Section 2.3*). Raw material pretreatment was carried out as previously described (*Section 2.2*).

3.1.2.2. Oil extraction – fractionation methods

Oil extraction-fractionation was carried out in a semi-pilot plant under the optimal extraction conditions (25 MPa, 313 K, 175 kg CO_2 / h · kg protein) proposed in a previous study (*see section 2.2*) and the oil extracted was recovered in two separators installed in series. In all the experiments, the first separator was maintained at a pressure range of 8 – 9 MPa and at a temperature range of 308 - 309 K (35 – 36 ºC), in order to aim a SC-CO_2 density between the extractor (around 880 kg / m^3) and the separator 2 (below supercritical density, ρ_c = 468 kg / m^3), as it is show in Figure 3.2.

In all cases, the oil extracted was stored in a closed vessel in darkness at -18 ºC in order to minimize spoiling before characterization.

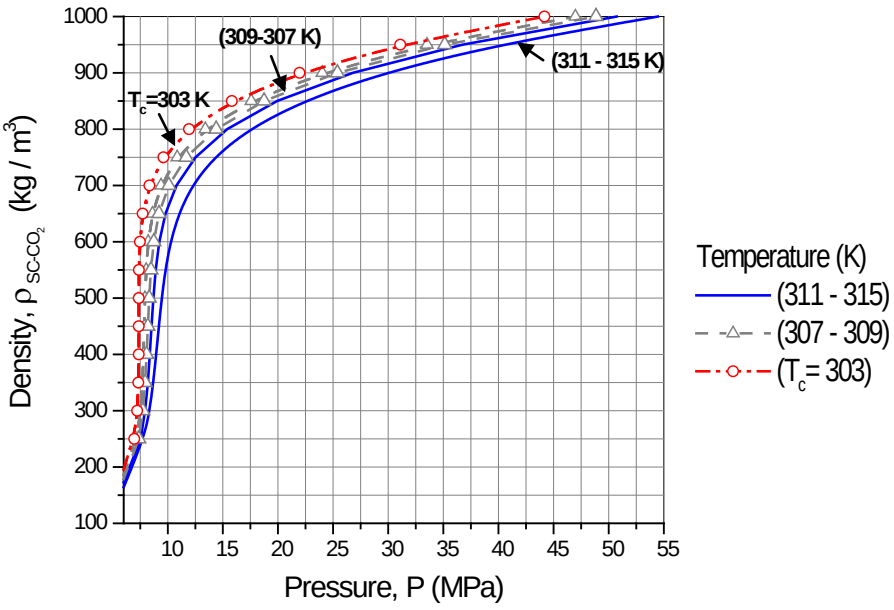

FIGURE 3.2

Density of CO_2 estimated at different pressure and temperature according to the Bender EOS. (*see* Appendix III.1)

3.1.2.3. Oil Characterization. Analytical methods

The quality of the oils obtained by the different extraction methods was evaluated focusing in lipid composition (neutral lipids and fatty acids profile) and oxidation parameters, i.e.: free fatty acids content, peroxide value and anisidine value. The experimental procedure for these methods is described in Appendix I.2.

3.1.3. Results and discussion

3.1.3.1. Amount of oil fraction recovered in each separator and lipid composition

Table 3.1 shows the amount of each oil fraction recovered in both separators in each experiment as well as the mass percentage. It is observed that, in all the cases, a higher amount of lipid compounds are recovered in separator 1, although this amount varies from 63 % in hake skin oil fractionation to 86 % and 83 % in salmon skin oil and jumbo squid liver oil fractionation respectively. These differences may be attributed not only to the instability of the experimental conditions, which causes a high density variation SC-CO_2 especially in separator 1, but also to the different fish oil composition.

TABLE 3.1

Lipid fraction recovered in each separator in fractionation of different fish oils.

	g oil /100 g dry material			%	
	S_1	S_2	total	S_1	S_2
Hake skin	11 ± 1	7 ± 2	18 ± 1	63	37
Salmon skin	44 ± 2	7 ± 1	51 ± 1	86	14
Jumbo squid liver	14 ± 1	3 ± 1	17 ± 1	83	17

The mass percentage distribution of TAG and FFA between the two separators is also presented in Figure 3.3.

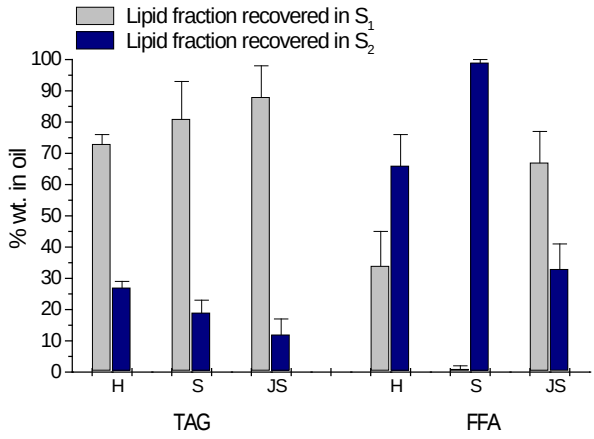

FIGURE 3.3

Mass percentage distribution of triacylglycerides, TAG (left) and free fatty acids, FFA (right) between both separators in fish oil fractionation.

It is observed that, in all the cases, the majority of TAG precipitates when pressure depletes in separator 1, which is explained considering its high molecular weight and low vapour pressure. However, the distribution of fatty acids varies significantly among different fish oils, which may be attributed to the different fatty acid profile. Thus, in the case of hake skin oil and salmon skin oil, in which palmitic and oleic are the main fatty acids, the majority of FFA reach separator 2, whereas in the case of jumbo squid oil, in which palmitic acid, PA, and EPA are the most common found, a large

among of FFA remain in separator 1. These experimental results indicate again that fractionation is highly affected by fish oil composition.

Finally, it is observed that, in all the cases, the ratio among FFA and TAG increases noticeably in the fraction recovered in S_2, and decreases in the fraction recovered in S_1 (Figure 3.4), although in the case of jumbo squid liver oil, this fraction still remains fairly.

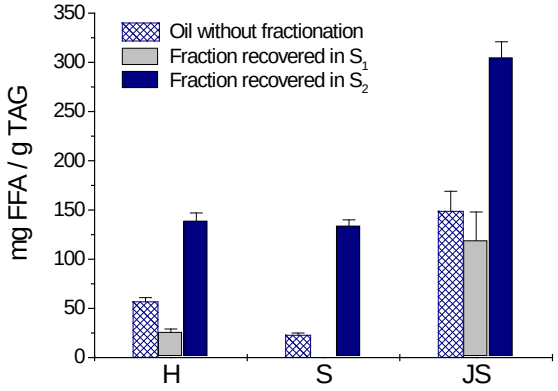

FIGURE 3.4

Ratio among FFA and TAG in fish oils after and before fractionation.

3.1.3.2. Fatty acids profile

A comparison among the fatty acid profile in natural fish oil and in both lipid fractions is presented in Figure 3.5. It is observed that, in general, the concentration of fatty acids is higher in the fraction recovered in separator 1, which is related to the fact that most triacylglycerides remain in this fraction.

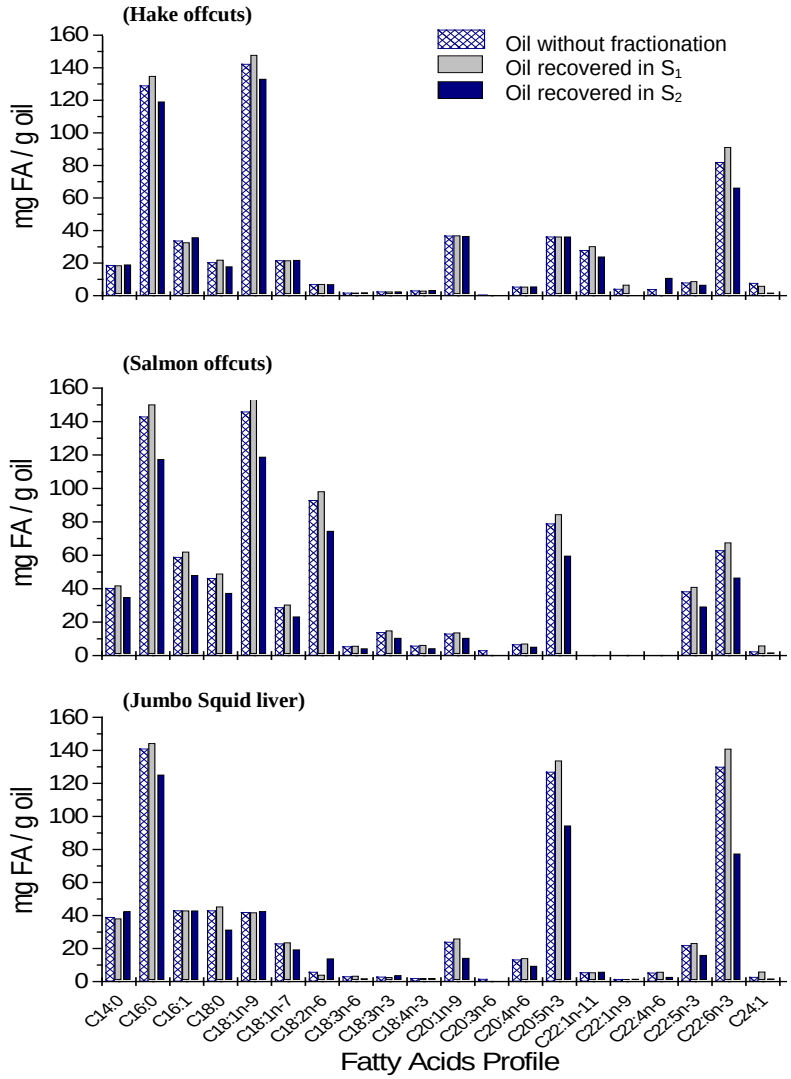

FIGURE 3.5

Comparison among fatty acid profile lipid fractions obtained after fractionation of hake oil, salmon oil and jumbo squid liver oil.

Furthermore, a slightly increment in the ratio among long chain (LCFA) and short chain fatty acids (SCFA) is observed in the fraction recovered in separator 1, which is more important in the case of jumbo squid oil fractionation (Figure 3.6.a), although the ratio among saturated (SFA) an unsaturated (MUFA and PUFA) fatty acids does not show a significant variation within the two lipid fractions (Figure 3.6.b).

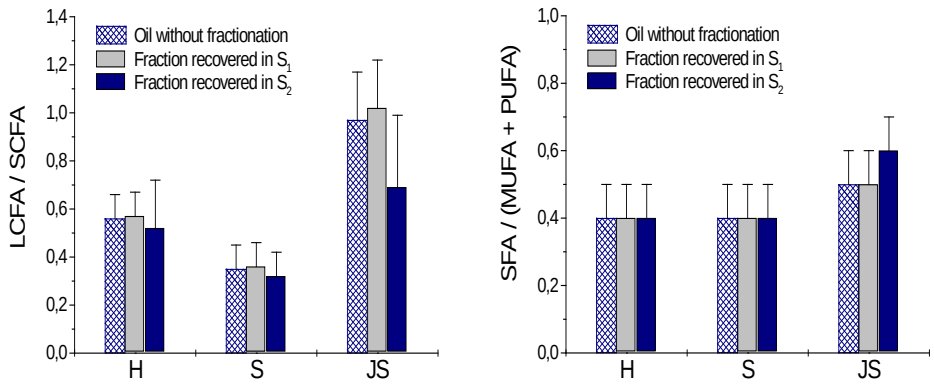

FIGURE 3.6

(a) Ratio among long chain fatty acids (LCFA) and short chain fatty acids (SCFA) in fish oil with and without fractionation. (b) Ratio among saturated fatty acids (SFA) and unsaturated fatty acids (MUFA + PUFA) in fish oil with and without fractionation. LCFA are considered fatty acids with a carbon number, $C > 18$, whereas SCFA are considered those with a carbon number, $C \leq 18$. H: Hake skin oil. S: Salmon skin oil. JS: Jumbo squid liver oil.

3.1.3.3. Oxidation parameters

In order to study the effect of fractionation on fish oil oxidation, different parameters such as free fatty acid content of total oxidation index (TOTOX) where evaluated in the fractions obtained from hake and salmon skin oil.

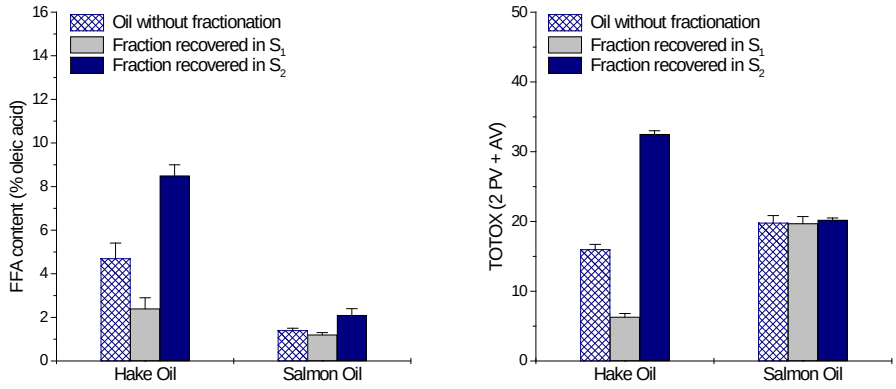

FIGURE 3.7

Comparison among FFA content (left) and total oxidation value, TOTOX (right) determined in oil fractions recovered in separator 1, S_1, and in separator 2, S_2; and oil without fractionation obtained from hake and salmon skin respectively.

As it can be seen in Figure 3.7, hake oil fractionation in two separators offers an interesting advantage since it allowed to obtain a fraction in the first separator with a lower amount of free fatty acids and total oxidation

index (TOTOX) than in the fish oil without fractionating. However, in the case of salmon oil, no significant improvement was observed, especially regarding the TOTOX value, which may be attributed to the similar concentration of non neutral lipid compounds found in both fractions.

3.1.4. Conclusions

Supercritical fluid extraction- fractionation in two separators has been proposed as an easy way to enhance fish oil quality right after extraction, giving good results for reducing FFA in salmon skin oil, hake skin oil and jumbo squid liver oil.

Further experiments about supercritical fluid extraction - fractionation in three separators are expected to improve the separation efficiency and enhance fish oil quality. Additionally, studies on the solubility of pure lipid compounds and lipid mixtures will be carried out in order to determine the equilibrium behaviour and get a better comprehension of the fish oil fractionation process.

3.2. Production of DHA Omega-3 Concentrates as Acylglycerides from Hake Skin Oil by an Enzymatic Reaction – Fractionation Process using SC-CO_2 as Reaction Media

Abstract

As it was treated in *Chapter 1*, the production of omega-3 concentrates such as DHA, presents a great interest both in the pharmaceutical and food industries, especially when they are as acylglycerides (AG), since in this form they are easier absorbed by humans and more stable against oxidation than the relative ethyl esters. Enzymatic reactions with sn-1, 3 specific lipases are an interesting strategy to obtain omega-3 as AG from fish oil. On the other hand, due to its unusual properties (low viscosity, high density and negligible superficial tension) the use of SC-CO_2 as enzymatic reaction media may offer interesting advantages over the same enzymatic reaction carried out in conventional liquid solvent. In this section, a continuous enzymatic hydrolysis-fractionation process in SC-CO_2 is proposed as a method to concentrate DHA as 2-AG from hake oil. Firstly, hydrolysis experiments carried out with a sn-1,3 specific immobilised lipase from *Mucor miehi* at 313 K, different pressures (18 – 25 MPa) and different CO_2 flow rates ($1.5 - 3.3 \cdot 10^{-5}$ kg / s) are presented. In the last part, the results of an enzymatic reaction-fractionation process are reported, which were carried out by keeping the reaction conditions at 313 K, 25 MPa and 0.5 g CO_2/min, and recovering subsequently the lipid mixture at the reactor outlet in two separator vessels by changing pressure (8.3 – 25 MPa) and temperature (308 – 333 K) in separator 1. It was found that the best separation factors among acylglycerides and FFA and DHA and palmitic acid respectively, were obtained working in separator 1 at a density close to its critical point. In this case, DHA was slightly concentrated as acylglyceride in separator 1.

➢ **Section Content**

3.2.1. Introduction

The production of polyunsaturated fatty acids such omega-3, especially DHA concentrates, has acquired a great importance in the last years, both in the food and pharmaceutical industries due to their beneficial in human health. Nowadays, these omega-3 concentrates are commercialised as esters, which are commonly obtained from fish oil trough urea complexation or molecular distillation procedures. However, the production of omega-3 concentrates as 2-acylglycerides (2-AG) has become more interesting since this lipid form is more stable against oxidation (*Boyd, et al.*, 1992, *Wijesundera, et al.*, 2008) and easier to metabolise by humans than the corresponding esters (*Lawson, et al.*, 1988).

Taking into account that, in most fish oils, DHA is bounded preferentially to the sn-2 position of triacylglycerides, hydrolysis or ethanolysis reactions catalysed by sn-1,3 specific lipases offer a promising way to obtain DHA concentrates as 2-AG. On the other hand, the use of SC-CO_2 as enzymatic media has an enormous attractive not only for its properties (no toxicity or low economical cost) and for improving mass transfer, but also because it allows to couple enzymatic reactions with other processes such fractionation which are very interesting for the production of microencapsulated omega-3 concentrates directly from raw material, minimising the oil manipulation and preventing its oxidation.

Enzymatic ethanolysis followed by a fractionation step has been proposed recently as a method to obtain DHA concentrates from hoki oil as

monoacylglycerides (*Weber, et al.*, 2008). However, the high accumulation of some lipids in the reactor made this process difficult to carry out in continuous since, after some time, it is necessary to stop the reaction and clean the reactor to avoid a high hold-up of the less soluble.

In this section, a continuous enzymatic reaction – fractionation process is proposed as a way to concentrate DHA as 2-AG from hake oil, which has a high interest from a practical point of view. The results of this preliminary study have been obtained in collaboration with the research group of Prof. Sovová in the Institute of Chemical Processes Fundamentals AS CR, of Prague (Czech Republic).

3.2.2. Fish oil enzymatic hydrolysis in a Packed Bed Reactor (PBR): Theoretical considerations

Hake oil extracted by SC-CO_2 is a lipid mixture composed basically by triacylglycerides (TAG) with a small amount of free fatty acids (FFA) and other minor neutral lipids such as cholesterol (*see* Section 2.3)

During oil hydrolysis free fatty acids are released from triacylglycerides (TAG) in three steps, producing also diacylglycerides (DAG), monoacylglycerides (MAG) and glycerol (G). Therefore, after hydrolysis the reaction product would be a mixture of FFA, MAG, DAG, G, and unreacted TAG.

$$TAG + H_2O \rightarrow DAG + FFA \quad \textbf{(3.1)}$$

$$DAG + H_2O \rightarrow MAG + FFA \quad \textbf{(3.2)}$$

$$MAG + H_2O \rightarrow G + FFA \quad \textbf{(3.3)}$$

Total hydrolysis:

$$TAG + 3\,H_2O \rightarrow G + 3\,FFA \quad \textbf{(3.4)}$$

Considering the total hydrolysis of TAG in FFA and glycerol in a packed bed reactor (PBR) working at steady state with plug flow (Figure 3.8) the mass balance for a TAG, on the volume element $A_c\Delta_z$ can be expressed as Eq.(3.6) in which $W_{TAG,z}$ is the molar flux of a TAG in the reactor, described as (3.7) according to Fick's first law. Combining Eq. (3.6) and (3.7), dividing by $A_c\Delta_z$ and taking the limit as $\Delta_z \rightarrow 0$, an expression that relates the concentration of TAG to the reactor length is obtained (3.8), which, assuming that axial dispersion in the reactor is much lower than forced axial convection (3.9), can be simplified to the differential equation (3.10) with the boundary condition $C_{TAG} = C_{TAG,o}$, at z = 0.

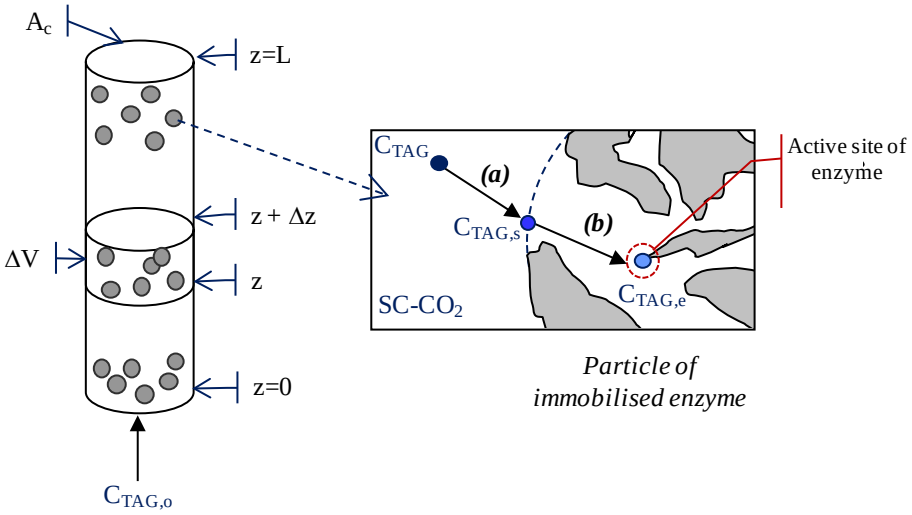

FIGURE 3.8

Scheme of the reaction catalyst by a porous immobilised enzyme in a pack bed reactor (PBR). C_{TAG} = concentration of TAG in bulk, $C_{TAG,s}$ = concentration of TAG on enzyme particle surface, $C_{TAG,e}$ = concentration of TAG inside the enzyme particle. (a) External mass transfer; (b) Internal mass transfer.

Mass balance in a PBR:

$$[\text{rate}]_{in} - [\text{rate}]_{out} = 0 \qquad \textbf{(3.5)}$$

$$A_c W_{TAG,z}\big|_z - A_c W_{TAG,z}\big|_{z+\Delta z} - v_{TAG}\rho_e A_c \Delta_z = 0 \qquad \textbf{(3.6)}$$

$$W_{TAG,z} = -D_{TAG}\frac{dC_{TAG}}{dz} + uC_{TAG} \qquad \textbf{(3.7)}$$

$$D_{TAG}\frac{d^2C_{TAG}}{dz^2} - u\frac{dC_{TAG}}{dz} - v_{TAG}\rho_e = 0 \quad \textbf{(3.8)}$$

$$D_{TAG}\frac{d^2C_{TAG}}{dz^2} << u\frac{dC_{TAG}}{dz} \quad \textbf{(3.9)}$$

$$-u\frac{dC_{TAG}}{dz} - v_{TAG}\rho_e = 0 \Rightarrow \frac{dC_{TAG}}{dz} = -\frac{v_{TAG}\rho_e}{u} \quad \textbf{(3.10)}$$

Thus, the concentration of TAG along the reactor depends directly on the global reaction rate, v_{TAG}, which is be affected not only by the enzymatic kinetics, but also by the external mass transport from the bulk fluid to the outer catalyst surface and the internal diffusion from the outer catalyser surface to the enzyme active point located inside the solid particle. All of these factors are describe below.

i) Enzymatic hydrolysis kinetics

Assuming that the hydrolysis reaction occurs under excess of water, and the substrate A is the limiting reactant, the enzymatic kinetics can be described by the typical one substrate Michaelis-Menten behaviour (Figure 3.9), where [S] and [P] represent the substrate and product concentration respectively.

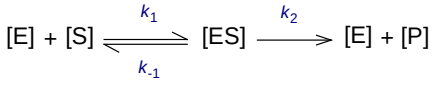

FIGURE 3.9

Scheme for the typical Michaelis-Menten enzymatic mechanism.

Following this mechanism, the specific reaction rate, V_{TAG} is defined as function of the Michaelis-Menten constants V_{max} and K_m according to Eq. (3.11).

$$V_{TAG} = \frac{V_{max}}{K_m + C_{TAG,e}} C_{TAG,e} \quad \textbf{(3.11)}$$

$$V_{max} = \frac{k_2}{[E]} = \frac{v_{max}}{\rho'_e} \quad \textbf{(3.12)}$$

$$K_m = \frac{k_{-1} + k_2}{k_1} \quad \textbf{(3.13)}$$

The constant V_{max} is the maximum specific reaction rate and is related to the amount of active points in the enzyme. This is considered an apparent first order rate constant, and, therefore, depends on the reaction temperature according to the Arrhenius equation. The enzyme concentration, [E], can be estimated as the density of active sites in the reactor, ρ'_e, which is related to the enzyme density, ρ_e, through $\rho_e = n\rho'_e$. In the case of lipase, the ratio of the amount of active sites to the amount of free enzyme can be assumed to be n = 1, so the densities ρ'_e and ρ_e are equal.

The parameter K_m is the Michaelis constant, related to the enzyme-substrate affinity and depends also on the reaction temperature. In the case of oil hydrolysis with immobilised lipase from *Mucor miehei* in SC-CO_2 at 313 K

(40 ºC), the value of K_m reported in the literature is 0.07 mol / m^3 (*Sovová, et al.*, 2008).

ii) External mass transfer effect

Since the enzymatic reaction takes place in a packed bed reactor, at steady state the reaction rate on the enzyme surface, V_{TAG}', is equal to the transport of the substrate A from the bulk fluid to the external surface of the catalyst particle(3.14). This external mass transfer depends directly on the external mass transfer coefficient, $k_f \cdot a$, which can be defined as the correlation (3.15) proposed by Tan et al., (*Tan, et al.*, 1988).

Substituting dimensionless numbers Sh, Re and Sc, Eq. (3.15) gives Eq. (3.16), in which substrate diffusivity in SC-CO_2, D_{TAG}, can be estimated according to Wilke-Chang equation (3.17) where parameters α and β depend on the substrate and, for triolein, has been estimated as $\alpha = 1.986 \cdot 10^{-14}$ K^{-1} and $\beta = -0.685$ (*Funakuzuri, et al.*, 2004).

Thus, the mass transfer coefficient can be simplified as Eq. (3.19) where K_u is a mass transfer constant that depends on the substrate, the enzyme properties and the reaction temperature (3.20) whereas f_p is the pressure factor which relates the SC-CO_2 properties at experimental condition to the standard SC-CO_2 properties selected as 15 MPa and 313 K (3.21).

Combining Eq. (3.14) and (3.19), the rate of external mass transfer can be related to the superficial velocity, u, according to (3.22). Thus, the variation

of experimental reaction rate with the superficial velocity according to (3.22) indicates that external mass transfer resistance is not negligible.

$$V'_{TAG} = k_f a_e (C_{TAG} - C_{TAG,s}) \quad \textbf{(3.14)}$$

$$Sh = 0.83\,Re^{0.83}\,Sc^{1/3} \quad \textbf{(3.15)}$$

$$k_f a = 0.38 u^{0.83} d_p^{-0.17} D_{TAG}^{2/3} \left(\frac{\rho}{\mu}\right)^{0.5} \frac{6(1-\varepsilon)}{d_p \rho_e} \quad \textbf{(3.16)}$$

$$\frac{D_{TAG}}{T} = \alpha \mu^{\beta} \quad \textbf{(3.17)}$$

$$k_f a = \text{const}\ \alpha^{2/3} d_p^{-1.17} T^{2/3} \rho_o^{0.5} \mu_o^{-0.956} \frac{(1-\varepsilon)}{\rho_e} u^{0.83} f_p \quad \textbf{(3.18)}$$

$$k_f a = K_u u^{0.83} f_p \quad \textbf{(3.19)}$$

$$K_u = \text{const}\ \alpha^{2/3} d_p^{-1.17} T^{2/3} \frac{(1-\varepsilon)}{\rho_e} \quad \textbf{(3.20)}$$

$$f_p = \left(\frac{\rho}{\rho_o}\right)^{0.5} \left(\frac{\mu}{\mu_o}\right)^{-0.956} \quad \textbf{(3.21)}$$

$$V'_{TAG} = K_u u^{0.83} f_p (C_{TAG,o} - C_{TAG,s,o}) \quad \textbf{(3.22)}$$

iii) Internal mass transfer effect

Immobilized lipases, such as lipase from *Mucor miehi*, are usually immobilized on a porous material in order to increase the density of active sites in the catalyst (AL-Muftaha, et al., 2005). Therefore, for the enzymatic reaction to take place, the substrate must diffuse trough the porous matrix and reach an active point. This substrate profile within the solid matrix of catalyst involves that the reaction rate observed on the enzyme surface, $V_{TAG'}$, may differs to the reaction rate if the entire interior surface were exposed to external surface, V_{TAG}, according to the internal mass transfer efficiency, ηI (3.23).

$$\eta_I = \frac{V'_{TAG}}{V_{TAG}} = \frac{\text{Observed reaction rate}}{\text{Theoretical reaction rate assuming that all the active points are on enzyme surface}} \quad \textbf{(3.23)}$$

Internal diffusion presents a greater complexity than conventional diffusion in a fluid since porous are only in a fraction of the transversal section of the solid catalyst, and present a complex geometry and different shape and size, which sometimes is in the same magnitude order than the molecules of substrate. Therefore, the profile of the substrate concentration inside the porous particle is strongly depended on the particle size, the effective diffusion of the substrate trough the particle and the kinetic parameters related to the immobilized enzyme. These three factors can be taken into account in a dimensionless parameter called Thiele number, ϕ, which, for a Michaelis-Menten enzymatic mechanism, is defined as (3.24).

$$\phi = \frac{R}{3}\left(\frac{v_{max}}{K_m D_{ef,TAG}}\right)^{1/2} \quad \textbf{(3.24)}$$

$$\eta_I \ \phi^2 = C_{WP} \quad \textbf{(3.25)}$$

$$\frac{\text{Observed reaction rate}}{\text{Reaction rate evaluated at } C_S} \cdot \frac{\text{Reaction rate evaluated at } C_s}{\text{Diffusion rate}} = \frac{\text{Observed reaction rate}}{\text{Diffusion rate}} \quad \textbf{(3.26)}$$

$$C_{WP} = \frac{\nu' \rho_e}{\frac{D_{ef,TAG} C_s}{R^2}} \quad \textbf{(3.27)}$$

$$D_{ef,TAG} = D_{TAG} \frac{\varepsilon}{\tau} H_f \quad \textbf{(3.28)}$$

$$H_f = (1-\gamma)^2 (1 - 2.1044\gamma + 2.089\gamma^3 - 0.948\gamma^5) \quad \textbf{(3.29)}$$

$$\gamma = \frac{\text{substrate molecular radius}}{\text{particle pore radius}} = \frac{r_{TAG}}{R} \quad \textbf{(3.30)}$$

Experimentally, the influence of the internal diffusion on the reaction rate is usually evaluated through the Weisz-Prater criterion, C_{WP}, which relates the observed reaction rate and the substrate diffusion rate inside the catalyst particle (3.25). Diffusion rate inside the particle can be estimated

considering the effective diffusivity, $D_{ef,TAG}$ which is related to the diffusion coefficient in the bulk, D_{TAG}, through the particle porosity, ε, tuortosity, τ, and the hindrance factor, H_f, which depends on the ratio of substrate molecular radius and particle pore radius, γ [Eq. (3.28) - (3.30)].

In the case of enzymes immobilised on a macroporous resin, such as Lipozyme, and substrates with a low molecular volume, such as triacylglycerides, the diffusion rate inside the particle is assumed to be higher than the observed reaction rate, V_{TAG}', which means that $C_{WP} << 1$ and η_I, is close to 1. Actually, previous studies on enzymatic esterification with Lipozyme have reported a value of C_{WP} equal to 0.084, indicating that the internal mass transfer is supposed to be negligible in the global process (*Laudani, et al.,* 2007). Therefore, the concentration of substrate inside the catalyst particle, C_{Ae}, may be approximated to the concentration on the particle surface, C_{As}.

3.2.3. Experimental section

3.2.3.1. Material

i) Substrates

Hydrolysis substrates were hake oil and water.

Hake oil was obtained from hake by-products by SFE with SC-CO_2 at the optimal conditions (25 MPa, 313 K, 175 kg CO_2 / h kg protein) proposed in

a previous study (*see* Section 2.2). It is characterised by a high amount of triacylglycerides and omega-3 fatty acids, especially DHA, as it has been reported before (*see* Section 2.3).

ii) Enzyme

The enzyme used in this study was a Lipozyme RM IM (62 U/g), lipase from *Mucor miehei* immobilised on macroporous anionic exchange resin (Novo Nordisk BioChem Inc.).

TABLE 3.2

Main features of Lipozyme RM IM

Average particle diamter, d_p (cm)	Moisture (% wt.)	Bulk density (kg m^{-3})	Porosity, ε
0.04 ± 0.02	2.5 ± 0.5	400 ± 50	0.53 ± 0.02

The main reasons for choosing this enzyme were:

- It is a sn-1,3 specific lipase (triacylglycerol hydrolase EC3.1.1.3), which means that it allows to hydrolyse preferentially the sn-1,3 position of the triacylglyceride used as substrate.
- It is immobilised in a solid support, so it can be easily isolated from

the reaction mixture and continuous enzymatic reactions may be carried out in a packed bed reactor.

- It can work in a temperature range from 30 to 70 ºC, and shows a good thermal stability, keeping its catalytic activity for three months if it is stored at 25 ºC or for one year if stored at 5 ºC.
- It presents a good stability in carbon dioxide and keeps its catalytic activity after working in SC-CO_2 media for a long time.

Further information about this enzyme is reported in Table 3.2.

iii) Reaction media

Carbon dioxide (99.9%) (Linde Technoplyn, Prague, CR) was used as reaction media, which the advantages of CO_2 against conventional solvents have been reported in Section 3.2.1.

3.2.3.2. Equipment

Enzymatic hydrolysis - fractionation experiments were carried out in the Institute of Chemical Processes Fundamentals AS CR of Prague (Czech Republic). A schematic diagram of the home made apparatus used in this work is shown in Figure 3.10.

The main parts of the equipment are described below:

- ***Pump***: A syringe pump (ISCO Inc., Lincoln, NE) was used for pumping liquid CO_2.

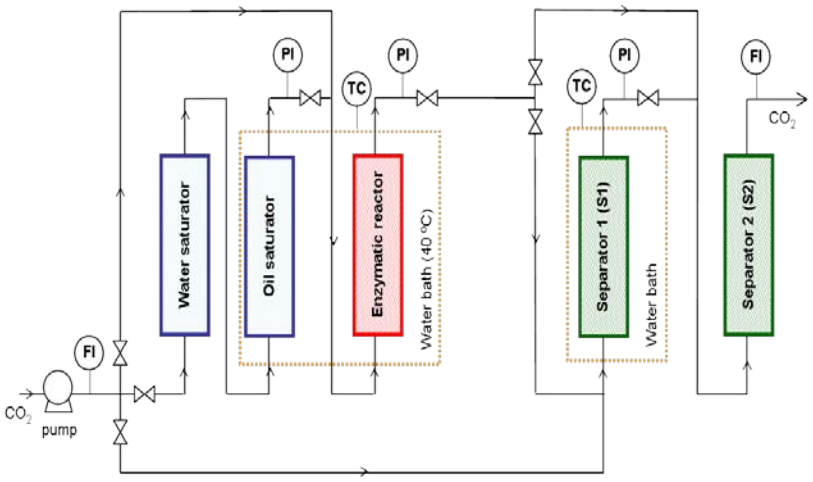

FIGURE 3.10

Scheme of experimental apparatus used in fish oil enzymatic hydrolysis-fractionation.

- ***Pressure vessels***: Pressure vessels were made by stainless steel columns (8 mm i.d.) and with sintered metal filters at both ends (*see* Figure 3.11). The void fraction for all the columns was close to 0.5 and the maximum pressure specifications were 30 MPa.

The function of the pressure vessels was:

- *Substrate saturators*: the first two columns (240 × 8 mm i d) served as substrate saturators. They were filled with layers of glass beads (2 mm i.d.) and glass wool and wetted with 1-2 mL of distilled water in the first column and fish oil in the second column. In all the experiments, the oil saturation column was immersed in the first water bath whose temperature was the

reaction temperature maintained within ± 0.1 ºC, whereas the water saturator column was placed at room temperature in order to minimised the solubility of water in SC-CO_2 and, therefore, the amount of water that reaches the reactor and may cause a loss in enzyme activity.

- *Packed bed reactor*: the third column (80 × 8 mm i.d.), immersed also in the first water bath, was the reactor. It was filled with immobilised enzyme particles mixed with glass beads and distributed in the whole reactor.

- *Separator*: the last column (240 × 8 mm i.d.) was used as first separator (S_1) when the fractionation of the reaction products was desired. It is immersed in a second water bath in order to allow a working temperature different from the reactor. This column was also filled with glass beads and glass wool to achieve a better distribution of the non-soluble lipid fraction and avoid an accumulation at the bottom.

FIGURE 3.11

Stainless steel columns used as pressure vessel in the enzymatic reaction-fractionation apparatus.

- ***Second separator or trap***: a glass vial with Teflon cap and pre-inserted

silicon septum was use as a second separator or trap for lipid compounds after enzymatic reaction. It was keeping at room temperature and pressure conditions, and CO_2 inlet and outlet was carried out by a metallic needle as it is shown in Figure 3.12.

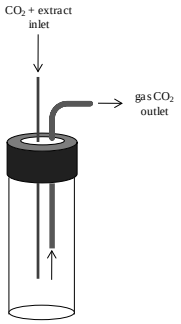

FIGURE 3.12

Scheme of the glass vial used as second separator or trap in the enzymatic reaction-fractionation apparatus.

- ***Heating system***: it was carried out by two different water baths, the first one for heating the oil saturator and the reactor, and the second one for heating the first separator (S_1) in the enzymatic hydrolysis-fractionation experiments.

- ***Valves***: the equipment contained two different types of valves:

- *Chromatographic valves*: these valves allow to send the SC-CO_2 towards different pipes according to the required purpose: cleaning of the enzyme,

hydrolysis reaction, hydrolysis reaction-fractionation or removal of the lipid fraction collected in the first separator.

- *Needle valves*: these valves allow to control the CO_2 flow rate and maintained the desired pressure in the different parts of the equipment.

• ***Piping***: all the apparatus was made using stainless steel pipes (1/18'' internal diameter) with double-ferrule connections for operating pressures under 30 MPa.

• ***Control system***: several devices were installed in order to measurement the amount of carbon dioxide, pressure and temperature. The volume of gaseous carbon dioxide was measured by a gas meter connected in the trap. In each experiment, the total gas volume was converted into mass through the carbon dioxide density at room pressure and temperature. Gas flow rate was established by the movement of the gas meter dial. Digital pressure gauges and laboratory thermometers were used for measuring pressure and temperature respectively.

3.2.3.3. Experimental procedure

The main steps of the experimental procedure were:

1) *Cleaning of the enzyme:* Previous to the reaction, the enzyme was cleaning by passing fresh SC-CO_2 directly through the reactor during 5 minutes.

2) *Hydrolysis reaction*: During reaction, SC-CO_2 carried the dissolved substrates, water and oil, through the reactor, filled with enzyme, where the oil hydrolysis takes place.

   Two sets of experiments were carried out in order to study both, the kinetics of the enzymatic hydrolysis and the fractionation of the reaction products.

   In the first set of experiments, all the reaction products were recovered directly in the second separator, which was maintained under ambient conditions, and the variables studied were the reaction pressure and flow rate.

   In the second set, the enzymatic reaction was carried out at fixed conditions and the reaction products were fractionated in the two consecutive vessels, being the pressure and temperature in separator 1 the parameters studied in this case.

3) *Removal of lipid fraction from separator 1*: At the end of the reaction-fractionation experiments, the lipid fraction recovered in separator 1 is removed by passing fresh SC-CO_2 under the same conditions set in the reactor.

3.2.3.4. Analytical methods

The course of the enzymatic reaction and of the fractionation was followed by determining the total fatty acid amount N_{FFA} (mol), the neutral lipid composition and the fatty acid profile along time.

- The total amount of N_{FFA} (mol) was determined by the colorimetric method according to (*Kwon, et al.*, 1986) using blue cupric acetate–pyridine reagent. The absorbance was measured at 714 nm.
- Neutral lipid composition was determined by liquid chromatography in a HPLC-ELSD (Agilent 1200) using palmitil palmitate (99 %), tripalmitin (> 99 %), dipalmitin (99 %), monopalmitin (99 %) and palmitic acid (99 %) as calibration standards.
- Fatty acids profile was determined by GC-FID in samples previously methylated according to the AOAC method (*AOAC*, 1995.).

Further details about analytical methods are reported in Appendix I.2.

3.2.3.5. Data evaluation

i) Enzymatic hydrolysis

In the enzymatic hydrolysis, the data measured were the sampling time, t_s, the amount of hydrolyzed sample, w_p, and the amount of CO_2 that passes through the reactor, $M_{CO2.}$ From these data, and taking into account the physical properties of SC-CO_2 (Table 3.3), superficial velocity in the

reactor, u, was calculated according to Eq. (3.31). All these data are reported in Table 3.4.

$$u = \frac{Q_{CO_2}}{\rho A_C} \qquad \textbf{(3.31)}$$

Data evaluation was carried out taking into account both, specific reaction rate and molar conversion, which values are reported also in Table 3.4.

The specific reaction rate was calculated as Eq. (3.32) considering the molar amount of free fatty acids determined in the hydrolyzed sample, $N_{FFA,f}$ and the amount of free fatty acids in the fish oil, $N_{FFA,o}$**,** which is estimated as Eq. (3.33) taking into account that the mass fraction of FFA in hake oil was $x_{FFA,o} = 0.04$ and assuming that the average molecular weight of the FFA is the molecular weight of palmitic acid ($M_{w,FFA} = 256.43$ g / mol) for it being the main fatty acid in this type of fish oil.

$$V = \frac{N_{FFA}}{M_e t_s} = \frac{N_{FFA,f} - N_{FFA,o}}{M_e t_s} \qquad \textbf{(3.32)}$$

$$C_{FFA,o} = \frac{M_s \cdot x_{FFA,o}}{M_{w,FFA}} \frac{\rho_{CO_2}}{M_{CO_2}} \qquad \textbf{(3.33)}$$

TABLE 3.3

Physical properties of SC-CO_2 and solubility data of substrates at the different experimental conditions studied (further details in Appendix III)

Series	**A**	**B**	**C**	**D**	**E**
Runs	**1 - 3**	**4 - 6**	**7 - 9**	**10 -12**	**13 -15**
Experimental conditions					
P (MPa)	18	20	25	25	25
T (K)	313	313	313	313	313
Q x 10^5 (kg CO_2 / s)	1.5	1.5	1.5	1	3.3
Properties of CO_2					
ρ (kg / m^3)	820.3	840.6	880.2	880.2	880.2
μ x 106 (Pa s)	74.2	78.4	87.4	87.4	87.4
Solubility data in SC-CO_2					
S_W^* (mg / g)	1.48	1.50	1.52	1.52	1.52
S_W (mg / g)	2.18	2.22	2.27	2.27	2.27
S_{FO} (mg / g)	2.7	3.4	5.4	5.4	5.4
S_G (mg / g)	0.06	0.06	0.07	0.07	0.07

* At the temperature in the water saturator (assumed 20 ºC).

TABLE 3.4

Reaction rates and conversions of enzymatic hake oil hydrolysis at different experimental conditions

Series	Run	P (MPa)	10^5Q (kg $_{CO2}$/s)	t_s (s)	10^3·u (m/s)	$C_{TAG,o}$ (mol/m^3)	$\frac{W_P}{S_{FO}}$	10^3 V (mol/kg s)	δ	f_δ
A	1	18	1.5	2340	35.6	1.37	0.71	0.12	0.78	0.80
	2	18	1.5	6840	36.4	1.61	0.83	0.15	0.84	0.74
	3	18	1.7	10800	40.9	1.38	0.71	0.18	0.98	0.38
B	4	20	1.5	2400	34.7	2.24	0.90	0.17	0.71	0.86
	5	20	1.5	4380	34.7	2.39	0.96	0.19	0.77	0.80
	6	20	1.7	7980	40.2	2.03	0.81	0.22	0.82	0.76
C	7	25	1.5	2400	33.2	3.41	0.87	0.32	0.88	0.68
	8	25	1.4	3180	30.7	3.19	0.82	0.29	0.99	0.38
	9	25	1.8	5220	40.5	3.79	0.97	0.32	0.64	0.88
D	10	25	0.9	4050	19.9	3.16	0.81	0.13	0.64	0.88
	11	25	0.9	5400	19.9	2.81	0.72	0.12	0.71	0.86
	12	25	1.0	11400	21.7	3.04	0.78	0.16	0.77	0.80
E	13	25	3.1	1320	69.4	2.33	0.60	0.47	0.92	0.62
	14	25	3.3	1920	74.7	3.45	0.88	0.49	0.64	0.88
	15	25	3.1	3000	70.6	3.25	0.83	0.52	0.70	0.86

The molar conversion, $\delta_{FFA,}$ defined as the fraction of TAG that is completely hydrolyzed, was calculated according as Eq. (3.34) considering the stoichiometry for the total hydrolysis of TAG into FFA (3.4). The substrate concentration in SC-CO_2 at the reactor inlet, C_o, was estimated as Eq. (3.35) taking into account that the mass fraction of TAG in hake oil was $x_{TAG,o} = 0.7$ and assuming that the average molecular weight of the TAG is the molecular weight of tripalmitin ($M_{w,TAG}$ = 807.35 g/mol) for it being the main triacylglyceride in this type of fish oil. The molar conversion, δ_{FFA},was related to the mass conversion, $\delta_{w,FFA}$, according to Eq.(3.38).

$$\delta_{FFA} = \frac{N_{TAG,r}}{N_{TAG,o}} = \frac{(N_{FFA,f} - N_{FFA,o})}{3} \frac{\rho}{C_{TAG,o} M_{CO_2}} \qquad \textbf{(3.34)}$$

$$C_{TAG,o} = \frac{M_s \cdot x_{TAG,o}}{10^{-3} \cdot M_{w,TAG}} \frac{\rho}{M_{CO_2}} \qquad \textbf{(3.35)}$$

$$\delta_{w,FFA} = \frac{10^{-3} \cdot P_{FFA} \cdot (N_{FFA,f} - N_{FFA,o})}{M_s} = 3 \cdot \left(\frac{M_{w,FFA}}{M_{w,TAG}} \right) \cdot \delta_{FFA} \qquad \textbf{(3.36)}$$

ii) Enzymatic hydrolysis – fractionation

The separation step was evaluated through the relative separation efficiencies or selectivities, $\beta_{i/FFA}$, among free fatty acids and other neutral lipids, i, and between DHA and PA ($\beta_{DHA,PA}$). These factors were calculated

according to taking into account the mass fraction of each component, w_i, in S_1 and S_2 respectively [Eqs. (3.37) and (3.38)].

$$\beta_{i,FFA} = \frac{k_i}{k_{FFA}} = \frac{w_{i,S2}/w_{i,S1}}{w_{FFA,S2}/w_{FFA,S1}} = \frac{\left(w_i/w_{FFA}\right)_{S2}}{\left(w_i/w_{FFA}\right)_{S1}} \quad \textbf{(3.37)}$$

$$\beta_{DHA,PA} = \frac{k_{DHA}}{k_{PA}} = \frac{w_{DHA,S1}/w_{DHA,S2}}{w_{PA,S1}/w_{PA,S2}} = \frac{\left(w_{DHA}/w_{PA}\right)_{S1}}{\left(w_{DHA}/w_{PA}\right)_{S2}} \quad \textbf{(3.38)}$$

3.2.4. Results and discussion

3.2.4.1. Enzymatic hydrolysis of fish oil in SC-CO_2: data analysis and modelling

Firstly, different experiments were carried out without fractionation in order to study the influence of experimental parameters such as pressure and flow rate on the reaction rate and conversion. The range of pressure studied was 18-25 MPa, wherein the Lipozyme from *Mucor miehei* shows a good stability in SC-CO_2 as has been reported in the literature (*Sovová, et al.*, 2003). The flow rate range studied was $1.5 – 3.5 \cdot 10^{-5}$ kg CO_2 / s. In all the experiments, the reaction temperature was fixed at 313 K in order to prevent thermal oxidation of omega-3 fatty acids. The amount of immobilized enzyme used was 0.5 g.

The physical properties of SC-CO_2 (density and viscosity) and solubility data of water, fish oil and glycerol under the experimental conditions explored are reported in (Table 3.3)

3.2.4.2. Amount of hydrolyzed oil recovered

After enzymatic hydrolysis, the reaction mixture is composed by the reaction products generated (FFA, DAG, MAG and G), an excess of substrates, TAG and water, and other unreacted compounds present in fish oil, such as cholesterol or volatile compounds. The recovery of these compounds in the trap after CO_2 depressurization depends on their solubility in CO_2 under supercritical conditions in the reactor and on their solubility in gas CO_2 under the ambient conditions in the trap.

In general, lipid compounds show a good solubility in SC-CO_2 and negligible solubility in gas carbon dioxide, so they are mostly collected in the trap as a liquid phase. Taking into account the free fatty acid mass balance, (3.39), the estimated initial concentration of TAG in the reaction media, $C_{TAG,o}^{*}$, shows a good correlation with the initial concentration determined experimentally, $C_{TAG,o}$ (Table 3.5)

$$C_{TAG.o}^{*} = C_{TAG} + [(2\,(C_{DAGf}) + (C_{MAGf}) + (C_{FFAf} - C_{FFAo})] / 3 \qquad \textbf{(3.39)}$$

$$C_{G}^{*} = [(C_{FFAf} - C_{FFAo}) - (C_{DAGf}) - 2\,(C_{MAGf})] / 3 \qquad \textbf{(3.40)}$$

On the contrary, in all the experiments the final concentration of glycerol in the reaction media, C_G^*, estimated from the glycerol mass balance (3.40) was higher than the theoretical concentration determined according to the solubility of C_G in SC-CO_2, (Table 3.5), which may indicate that most of the glycerol produced during the hydrolysis process remains in the reactor.

TABLE 3.5

Composition of the product in the trap (tri-, di, monoacylglycerides and free fatty acids) and equivalent initial oil and produced glycerol (mol / m^3)

Run	C_{TAG}	C_{FFA}	C_{DAG}	$C_{MAG,f}$	$C_{TAG,0}$	$C_{TAG,0}^*$	C_G	C_G^*
1	2.3 x 10^{-8}	4.5	0.01	0.30	1.4	1.6	1.3	0.53
2	2.9 x 10^{-8}	5.5	0.03	0.44	2.2	2.0	1.5	0.55
3	1.8 x 10^{-8}	8.0	0.25	0.73	3.4	3.1	2.1	0.67
4	5.1 x 10^{-8}	7.7	0.26	0.78	3.0	3.0	2.0	0.67
5	3.6 x 10^{-8}	7.5	0.26	0.77	3.5	2.9	1.9	0.67

Finally, it must be taken into account that the excess of water in the reactor reaches the trap still solubilised in SC-CO_2, although it is mostly removed in the vapour phase since its solubility in CO_2 at ambient conditions is higher than in SC-CO_2, (*Wiebe, et al.*, 1941). This same behaviour is expected to happen to some volatile compounds in fish that present a high vapour pressure.

Thus, if hydrolysis of TAG to DAG or MAG is produced, no significant loses in the reactor are expected and the ratio between the amount of

products at reactor outlet and at reactor inlet (w_{out} / w_{in}) should vary from 1 when no reaction takes places (zero conversion) to $1 + (3\ M_{w,\ W}) / M_{w,TAG} = 1 + 54 / M_{TAG} > 1$ in the case of total hydrolysis (conversion = 1). On the contrary, if TAG are hydrolyzed to FFA and glycerol, w_{out} / w_{in}, should vary in the range from 1 to $1 + (3\ M_{w,W} - M_{w,G}) / M_{w,TAG} = 1\text{-}38 / M_{w,TAG} < 1$.

In this work, the ratio w_{out} / w_{in} can be approximated to w_P / S_{FO}, where w_P is the amount of oil hydrolysed recovered in the trap and S_{FO} is the solubility of fish oil in SC-CO_2. Thus, assuming that fish oil solubility is reached at the saturator outlet and the water entrainer effect on glycerol solubility is neglected, a $w_P / S_{FO} < 1$ (Table 3.4) may indicate that fish oil is hydrolysed into FFA and glycerol. Nonetheless, it must be taken into account that, in some cases, w_P / S_{FO} may be lower than the real w_{out} / w_{in} as long as oil saturation is not reached, and therefore $w_{in} < S_{FO}$, although this effect may be counteracted if solubility of glycerol in SC-CO_2 is increased due to the water effect. Furthermore, higher mass loses may be also justified taking into account the presence of a certain amount of impurities such as cholesterol, pigments or volatile compounds in fish oil, which may be accumulated in the reactor, adsorbed on the catalyst and / or removed from the trap during depressurization.

i) Effect of water

The amount of water is essential in the hydrolysis reaction since it acts as a reactive. However, high amounts of water near the enzyme may cause an

important decrease of its catalytic activity and, therefore, in the reaction yield. This effect may be due to the inhibition of the enzyme, caused by the carbonic acid generated, and to the water film formed in the enzyme support that acts as a repulsive barrier between the substrate and the catalytic site. Thus, working at optimal water content is essential for achieving the highest enzyme activity.

Previous studies have found that, in the case of Lipozyme from *Mucor miehei*, the optimal amount of water is between 8 and 12 % (g / g supported enzyme), which is related to a water activity in the solvent within a range of 0.4-0.7 and to an approximate 40-70 % of CO_2 saturation with water (*Condoret, et al.*, 1997). In these experiments, the concentration of water at the reactor, related to the solubility of water in SC-CO_2 (*see* Table 3.3), was about 64 % of CO_2 saturation with water, which is within the optimal moisture range claimed for Lipozyme in the literature. Therefore, no losses in catalytic activity due to water inhibition are expected.

ii) Study of molar conversion from TAG to FFA

Table 3.4 reports the molar conversions estimated under different conditions according to Eq. (3.37). From these data, it is observed that molar conversion is not affected by pressure nor by flow rate, which may indicate that enzyme activity remains constant in all cases. Furthermore, comparing with previous hydrolysis studies (*Sovová, et al.*, 2008) carried out with the same enzyme, the molar conversions obtained in this work are noticeably

higher, which may be related to the lower oil / enzyme substrate used in the previous works.

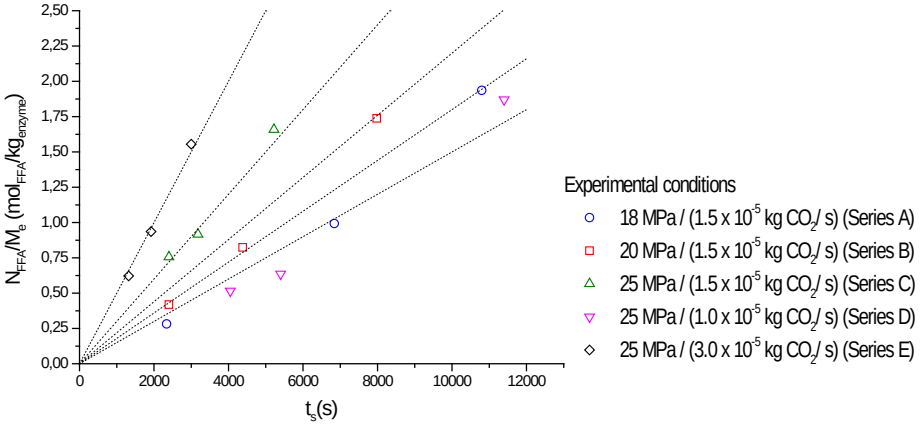

FIGURE 3.13

Amount of free fatty acids produced per kg of mass enzyme along the time in the packed bed enzymatic reactor at different reaction conditions.

iii) Study of specific reaction rate

Molar conversions are fairly high, and therefore the amount of TAG, DAG and MAG in the reaction products is very low, since they have reacted almost completely. Thus, an accurate evaluation of the individual reaction rate constants for the hydrolysis of TAG, DAG, and MAG was not possible. Instead, a study of the global specific reaction rate was carried out considering the amount of FFA recovered in the hydrolyzed samples per

kg of enzyme (N_{FFA} / M_e) after a certain time. The values obtained in different runs are reported in Table 3.4.

Figure 3.13 shows that, under the same experimental conditions, the ratio (N_{FFA} / M_e) increases proportionally with the sampling time, t_s, which proves that the steady state has been reached in the continuous packed bed reactor. On the other hand, from Figure 3.13 can be also concluded that the reaction rate increases when both pressure and flow rate increases.

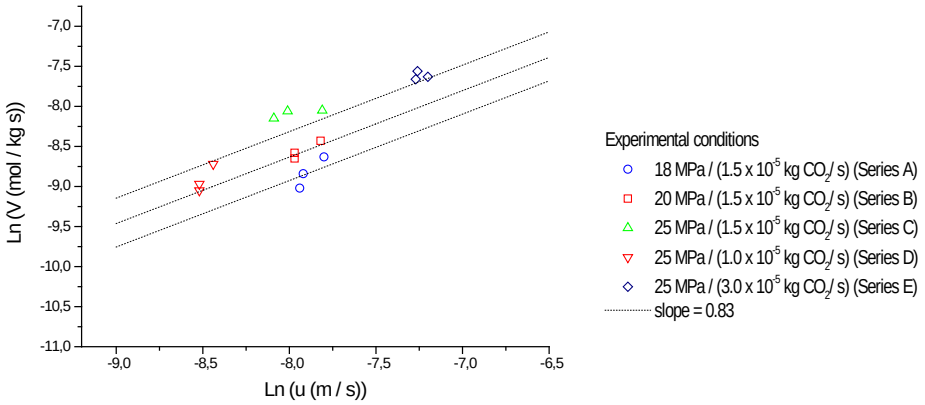

FIGURE 3.14

Dependence among specific reaction rate, V, and superficial velocity in the reactor, u.

The effect of pressure may be explained considering the amount of reactants (water and oil) at the reactor inlet, which is higher the higher the pressure since their solubility in CO_2 increases with pressure, whereas the effect of

flow rate may indicate that the effect of external mass transfer is not negligible as it was observed in previous studies (*Sovová, et al.*, 2008).

Furthermore, the graphical line obtained plotting (ln u) *vs*. (ln V) at a constant pressure of 25 MPa (runs 7 – 15) (Figure 3.14) shows a good correlation with the equation proposed for external mass transfer (Eq. (3.22)).

iv) Data analysis and modeling

In order to obtain a better comprehension on the hydrolysis process, a enzymatic model on Sovová's model has been proposed based,

The main assumptions of this model are:

- The reaction takes place in a packed bed reactor with plug flow working at steady state, in which the axial dispersion is negligible. Measured average reaction rates are converted to reaction rates at the reactor inlet trough a retardation factor, f_δ, described as Eq (3.45). The values of this factor (Table 3.4), related to the molar conversion, δ, were estimated from previous studies taking into account that the TAG hydrolysis takes place in three-steps with a fist-order reaction kinetics (*Sovová, et al.*, 2008).
- The reaction kinetics catalysed by lipase follows a Michaelis-Menten mechanism.
- The external mass transfer resistance plays an important role in the

global reaction process, so substrate concentration is depleted from the bulk fluid to the enzyme surface.

- The internal mass transfer resistance in a macroporous resin is supposed to be negligible as has been reported previously in the literature (*Laudani, et al.*, 2007, *Miller, et al.*, 1991). Therefore, the concentration of substrate inside the particle of immobilised enzyme is assumed to be the same as the concentration on the enzyme surface.

Thus, taking into account the theoretical considerations previously described (*see Section 3.2.2*), the final mathematical model can be described by the following equations [Eqs. (3.41) - (3.45)].

$$V = f_{\delta} \frac{V_{max}}{K_m + C_{TAG,e,o}} C_{TAG,e,o} = f_{\delta} K_u u^{0.83} f_p (C_{TAG,o} - C_{TAG,s,o}) \quad \textbf{(3.41)}$$

$$C_{TAG,e,o} \approx C_{TAG,s,o} \quad \textbf{(3.42)}$$

$$C_{TAG,e,o} = \frac{C_{TAG,o}}{2} \left(-b + \sqrt{b^2 + \frac{4K_m}{C_{TAG,o}}} \right) \quad \textbf{(3.43)}$$

$$b = \frac{K_m}{C_{TAG,o}} - 1 + \frac{V_{max}}{K_u u^{0.83} f_p C_{TAG,o}} \quad \textbf{(3.44)}$$

$$f_{\delta} = \frac{v(\delta)}{v(\delta = 0)} = \frac{\delta C_{TAG,o}}{v(\delta = 0) t_r} \quad \textbf{(3.45)}$$

The variables of the model are the reaction rate, V, the substrate concentration in the bulk at the reactor inlet, $C_{TAG,o}$, the superficial velocity in the reactor, u, and the pressure factor for mass transfer coefficient, f_p, and the retardation factor, f_δ; all of them reported in Table 3.4. The fitting parameters V_{max} and K_u, were in the same order of magnitude that those obtained in previous studies about enzymatic hydrolysis with the same Lipozyme (*Sovová, et al.*, 2008). The parameter K_m was assumed to be 0.07 mol /m^3.

TABLE 3.6

Fitting parameters found in the hake oil enzymatic hydrolysis according to the model described by Sovová et al. (*Sovová, et al.*, 2008).

Raw material	K_m (mol / m^3)	V_{max} x 10^3 (mol / kg s)	K_u ($m^{2.17}s^{-0.17}kg^{-1}$)
Hake oil	0.070	0.75	0.12
Blackcurrant oil (*Sovová, et al.*, 2008)	0.070	1.7 – 4.9	0.23 – 0.72

The reaction curve calculated for the estimated model parameters is represented in Figure 3.15, together with the experimental data. The percentage of absolute relative deviation, % AARD, between the calculated and experimental reaction rates, estimated according to Eq. (3.46), was equal to 26 %.

$$\%AARD = \frac{100}{N}\sum_{i=1}^{N}\frac{\left|y_{exp} - y_{cal}\right|}{y_{exp}} \qquad \textbf{(3.46)}$$

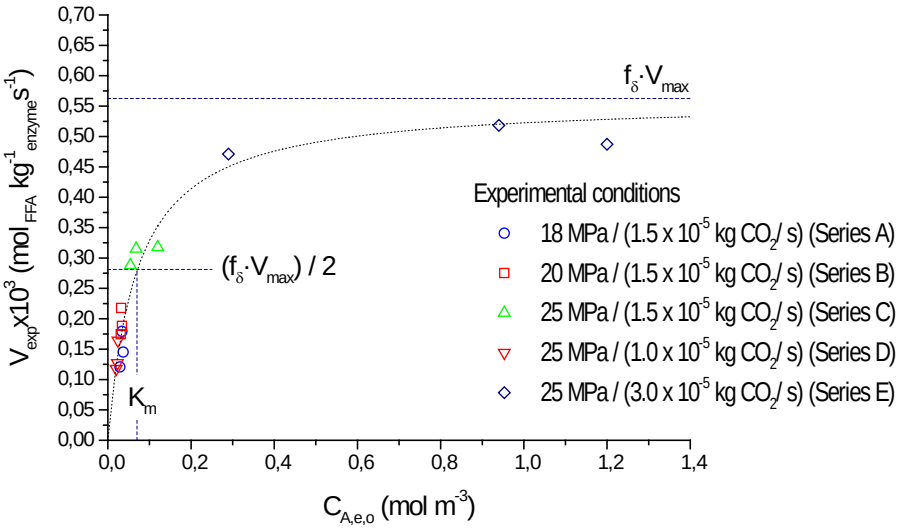

FIGURE 3.15

Modelled reaction rate at different substrate concentration near the enzyme according to the estimated model parameters (Table 3.6).

Finally, it is concluded that, according to these results, at high pressure and flow rate, the estimated substrate concentration near the enzyme, $C_{TAG,e,o}$ is higher than the value of K_m, so the specific reaction rate reaches the maximum value. This high value is related to the higher amount of oil solubilised in SC-CO_2 at the reactor inlet and the lower external mass transfer resistance in the reaction inlet. On the contrary, at low pressures and/or low flow rate, the estimated $C_{TAG,e,o}$ values are lower than K_m and the specific reaction rate is far from the maximum value. Thus, the most suitable reaction conditions were established at 25 MPa and

$1.5 \cdot 10^{-5}$ kg CO_2 / s, since in this case the substrate concentration estimated near the enzyme was close to the K_m value, and therefore the specific reaction rate was around $V_{max}/2$.

3.2.4.3. Enzymatic hydrolysis – fractionation: efficiency of the separation process in omega-3 enrichment

After the preliminary study about enzymatic hydrolysis, another set of experiments was developed focusing on the fractionation step. In all cases, the reaction conditions were fixed at 25 MPa, 313 K and $1.5 \cdot 10^{-5}$ kg CO_2 / s.

The experimental variables, pressure, P and temperature, T, in separator 1, as well as the SC-CO_2 density, ρ, are summarized in Table 3.7.

TABLE 3.7

Experimental conditions in separator 1 studied in the enzymatic hydrolysis – fractionation of hake oil

Run	P (MPa)	T (K)	ρ (kg / m^3)
1	8.3 ± 0.3	313 ± 1	334 ± 59
2	10.2 ± 0.4	313 ± 1	643 ± 27
3	15.5 ± 0.8	313 ± 1	788 ± 14
4	8.3 ± 0.4	308 ± 1	523 ± 104
5	10.2 ± 0.4	308 ± 1	720 ± 12
6	10.0 ± 0.1	333 ± 1	287 ± 4
7	25.0 ± 0.1	333 ± 1	787.2 ± 0.1

i) Influence of process parameters on lipid fraction recovery

Figure 3.16 shows that, at constant temperature, the lipid fraction (% wt.) recovered in separator 1 decreases as pressure in this separator is increased. This was expected since the reaction products are more soluble in CO_2 the higher is the pressure, and they do not precipitate in the separator 1 but reach separator 2 still solubilised in CO_2. At constant temperature, a pressure increase results in an increase of the SC-CO_2 density and, therefore, of its solvation power. Consequently, the reaction products remain solved after separator 1 and are collected in separator 2.

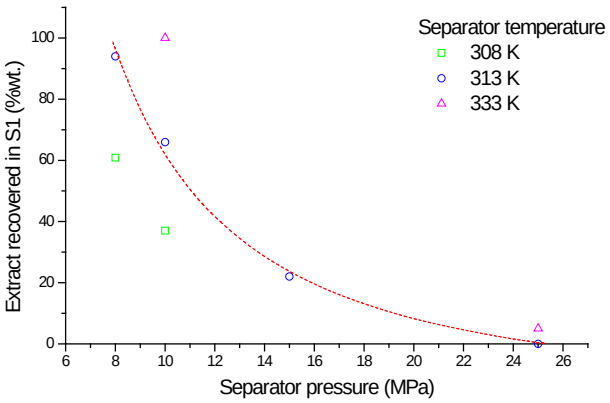

FIGURE 3.16

Mass fraction of the reaction products recovered in separator 1 at different experimental conditions.

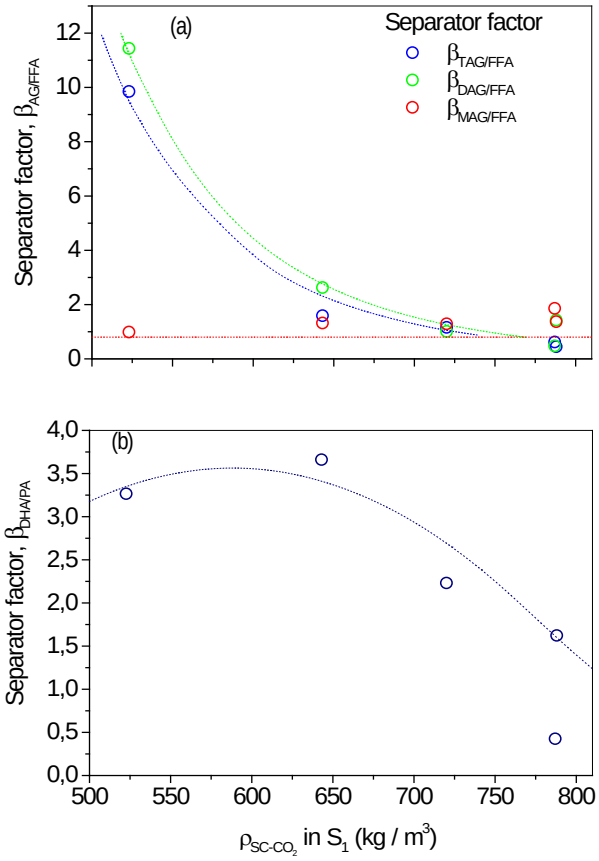

FIGURE 3.17

Influence of SC-CO_2 density in separator 1 on the separator factors among AGs and FFA (a) and DHA and PA (b) respectively.

ii) Separation efficiencies

Figure 3.17.a shows that the best selectivities were found among TAG and FFA, $\beta_{TAG,FFA}$, and DAG and FFA, $\beta_{DGA,FFA}$, respectively when the SC-CO_2

density in separator 1 was close to the CO_2 critical density (468 kg / m^3). This behaviour agrees with the experimental results reported in the literature for other separation processes using SC-CO_2 such batch-fractionation of hake oil ethyl esters (*Jachmanián, et al.*, 2007) or countercurrent extraction of tocopherols (*Brunner*, 1994). No significant differences were found in selectivity β obtained among MAG and FFA at different SC-CO_2 density in separator 1, being close to 1 in all the cases.

Furthermore, Figure 3.17.b shows that, when the density in separator 1 was close to the density in CO_2 critical point, a slight increasing in the selectivity between PA and DHA, $\beta_{PA,DHA}$, is observed.

iii) Fatty acid profile of lipid fractions

Fatty acid profile was determined in the two lipid fractions collected after enzymatic hydrolysis - fractionation process when the density in separator 1 was keeping close to the critical density of CO_2 (Run 4).

The comparison among these two fatty acid profiles and the fatty acid profile of hake skin oil is shown in Figure 3.18. It is observed that the fraction recovered in separator 1 is slightly enriched not only in DHA ($C_{22:6n-3}$) but also in DPA ($C_{22:5n-3}$), another important omega-3. Both fatty acids are increased around 35 and 29 % respectively regarding the whole hake oil. On the contrary, both palmitic acid ($C_{16:1}$) and oleic acid ($C_{18:1n-9}$) decreased in the fraction collected in separator 1 around 22 % and 13 % respect to the hake oil.

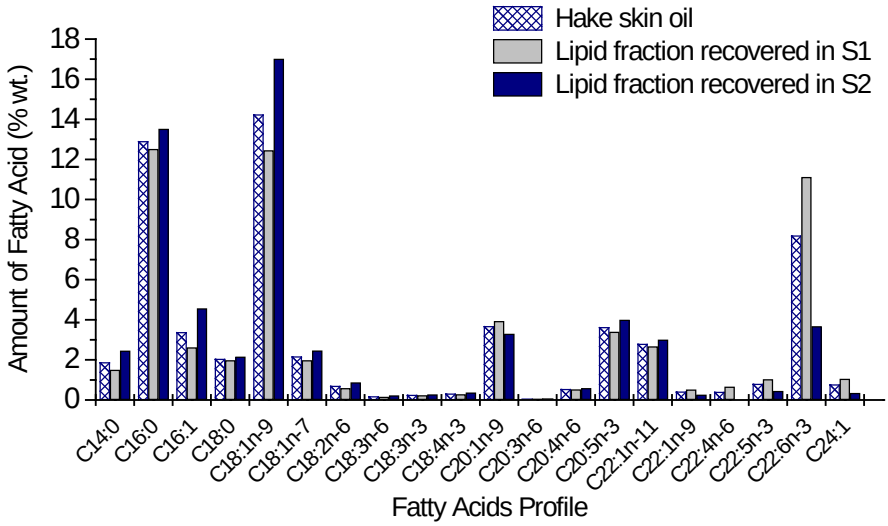

FIGURE 3.18

Comparison among the fatty acid profile in hake oil and in two fractions obtained after the enzymatic hydrolysis – fractionation process. (Enzymatic hydrolysis conditions: at 25 MPa, 313 K and $1.5 \cdot 10^{-5}$ kg CO_2 / s. Experimental conditions in separator 1: 8.3 MPa, 308 K).

3.2.5. Conclusions

A continuous enzymatic hydrolysis - fractionation process in SC-CO_2 is proposed as a way to produce DHA concentrates as 2-AG from hake oil previously extracted by SC-CO_2 (25 MPa and 313 K) from hake

by-products. A preliminary study about the kinetic of the enzymatic hydrolysis was carried out by using an immobilized sn-1,3 specific from *Mucor miehei* at 313 K, different pressures (18 - 25 MPa) and flow rates (1.5 - 3.3 · 10^{-5} kg CO_2 / s). It was observed that the reaction rate increased noticeably with pressure, which indicates that the process is controlled by the amount of reactants at the reactor inlet; and with CO_2 flow rate, which may be due to the increase of the amount of reactants at the reactor inlet but also may indicate that the external mass transfer is not negligible.

Fixing the reaction conditions (313 K, 25 MPa, 1. 5 · 10^{-5} kg CO_2 / s), a subsequently fractionation step was studied in two consecutive separators by changing the pressure (8.3 – 25 MPa) and temperature (308 – 333 K) in separator 1. It was observed that the best separator factor was obtained between TAG and FFA and DGA and FFA when the density in separator 1 was close to the density in the CO_2 critical point. Moreover, under these conditions the best separation factor between DHA and PA was found, which may indicate that DHA is slightly enriched in separator 1 as tri- or diacylglycerides. However, it must be taken into account that, due to its simplicity, this procedure is not profitable to produce DHA concentrates on commercial scale.

Further studies about enzymatic hydrolysis are required in order to aim a lower conversion of TAG at an optimal reaction rate and obtain a higher amount of DAG and MAG. In addition, alternative processes such enzymatic reaction in dense gas, which may improve substantially the

reaction rate, would be worth exploring. Furthermore, the study of enzymatic ethanolysis as alternative to enzymatic hydrolysis has also a great interest in order to improve the fractionation step since, the triacylglyceride solubility in SC-CO_2 is usually further from the ester solubility than from the free fatty acid solubility (*Güçlü-Üstündağ, et al.*, 2000).

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Use of Supercritical Fluids in Fish Oil Stabilization & Formulation

Chapter 4

SUMMARY

Due to the use of moderate temperatures (313 K) and a non-oxidising atmosphere (CO_2), supercritical fluid extraction (SFE) allows to obtain a less oxidised fish oil than conventional physical and chemical extraction methods (*see* Section 2.3.3.3). In addition, storage conditions at low temperatures (4 ºC) and darkness allows to slow down slightly both fish oil hydrolysis and polyunsaturated fatty acids (PUFA) oxidation (*see* Section 2.3.3.4), although, even at these conditions, fish oil obtained by SFE tends to oxidise quite fast, reaching undesirable oxidation levels after a short period of time (less than 10 days).

As it was mentioned in section 1.2.1.4, the high tendency of fish oil to oxidation is an important drawback that limits its application field in both food and pharmaceutical industries and makes necessary the design of an effective stabilisation strategy. On the other hand, the use of fish oil as ingredient in omega-3 enriched food products or nutraceuticals usually requires a previous formulation in a dry solid form able to improve the dispersion capability of the oil within the food matrix and reduce as much as possible the sensorial impact derived from the incorporation of smelly fish oil compounds in the final product.

Fish oil encapsulation in a solid matrix (carbohydrate or protein) and in presence of an antioxidant compound (chitosan) has been recently proposed as an attractive strategy to achieve both fish oil stabilization and formulation as a dry powder. Nowadays, this type of encapsulation is being carried out by conventional processes such as spray-drying. As a contribution to the conventional fish oil encapsulation methods, in this chapter, the novel PGSS-drying process, based on the use of supercritical fluid carbon dioxide, is proposed as a way to obtain fish microcapsules in a carbohydrate matrix.

RESUMEN

Gracias al uso de temperaturas moderadas (313 K) y de una atmósfera no oxidante (CO_2), la extracción con fluidos supercríticos (EFSC) permite obtener aceite de pescado con un menor índice de oxidación que los procesos físico-químicos convencionales de extracción (*ver* Sección 2.3.3.4). Además, la conservación de dicho aceite a bajas temperaturas (4 ºC) y oscuridad permiten ralentizar tanto el proceso de hidrólisis del aceite de pescado como la oxidación de los ácidos grasos poliinsaturados (AGPI) (ver Sección 2.3.3.4), aunque, incluso en estas condiciones, el aceite de pescado obtenido mediante EFSC tiende a oxidarse bastante rápido, alcanzando niveles de oxidación no deseados después de un breve periodo de tiempo (menor de 10 días).

Como se mencionó en la sección 1.2.1.4, la alta tendencia del aceite de pescado a la oxidación es un grave problema que limita su campo de aplicación tanto en la industria alimentaria como en la farmacéutica, haciendo necesario el diseño de estrategias de estabilización efectivas. Por otro lado, el uso de aceites de pescado como ingredientes en alimentos enriquecidos en omega-3 o nutracéuticos requiere, en muchas ocasiones, de una etapa previa de formulado en polvo capaz de mejorar la capacidad de dispersión del aceite en la matriz alimenticia y reducir lo máximo posible el impacto sensorial debido a la incorporación de aromas característicos del pescado en el producto final.

La encapsulación de aceite de pescado en una matriz sólida (carbohidrato o proteína) en presencia de antioxidantes (quitosan) ha sido propuesta recientemente como una estrategia atractiva para conseguir tanto la estabilización del aceite de pescado como su formulado en polvo. Hoy en día, dicho proceso se lleva a cabo mediante técnicas convencionales como el spray-drying. En este capítulo, como contribución a los métodos convencionales de encapsulación de aceite de pescado, se propone el proceso novedoso de PGSS-drying basado en el uso de la tecnología de fluidos supercríticos como vía para obtener microcápsulas de aceite de pescado en una matriz de quitosan / maltodextrina.

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NOMENCLATURE

A_{3340}	Infrared absorbance at 3340 cm^{-1}
Aij	Interaction energy (kJ / mol)
C	CO_2
$d_{0.5}$	Volume median diameter (diameter where 50% of the particle size distribution is above and 50% is below)
DE	Dextrose equivalent
EE	Encapsulation efficiency (defined as Eq. (4.1))
FT-IR	Fourier Transform Infrared
GPR	Gas - emulsion ratio (kg CO_2 / kg emulsion)
HCB	Hydrophilic - CO_2-philic balance
MD	Maltodextrine
o/w	Oil-in-water
P	Pressure (MPa)
PEG	Polyethylene Glycol
PGSS	Particle from Gas Saturated Solutions
$P_{v,H2O}$	Vapour pressure of water
R	Gas constant (R = 8.3145 J / mol K)
RESS	Rapid Expansion of Supercritical Solutions
SAS	Supercritical Antisolvent Process
SC-CO_2	Supercritical carbon dioxide
SEM	Scanning Electron Microscopy
S_H	Surfactant head-group
S_T	Surfactant tail
S_w	Water solubility in SC-CO_2 (g / kg CO_2)
T	Temperature (K)
W	Water
x_{H2O}	Molar fraction of water in vapour phase

y_{H2O}	Molar fraction of water in liquid phase
$\Delta H_{v,H2O}$	Entalphy of vaporization for water

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4.1. Fish Oil Microencapsulation by PGSS-drying

Abstract

As it is discussed in section 2.2.4.6, fish oil extracted with supercritical carbon dioxide (SC CO_2) requires stabilization and formulation in order to be a suitable ingredient in omega-3 enriched products or drugs, which may be achieved by microencapsulating fish oil in a solid matrix, usually by spray-drying technology. As innovation, this chapter deals with the use of supercritical fluid technology, specifically PGSS-drying, in fish oil microencapsulation using a mixture of maltodextrine / chitosan as coating material and two different emulgents or emulsifiers: polysorbate (Tween 80) and lecithin. The effect of different factors related to the initial composition of the fish oil-in-water emulsion or to the processes parameters in the three main stages of the encapsulation process, i.e, mixing between emulsion and carbon dioxide, atomisation through the nozzle and drying in the spray tower are considered, taking into account the physical properties (density, particle size, morphology) of the final products and the efficiency of encapsulated oil. The results of this preliminary study show that PGSS-drying may be worth to be explored as a way in fish oil microencapsulation since it allows to obtain a wide range of particle features easily by changing experimental parameters.

➢ **Section Content**

4.1.1. Introduction

One of the most recent advances in the use of supercritical fluids are particle formation processes. Nowadays, a wide amount of methods to obtain solid particles from pure compounds or composites have been developed, which can be based on the use of a SC-CO_2 as an anti-solvent (SAS process) or in the expansion of a solution in which supercritical CO_2 acts as a solvent (RESS process) or as a solute (PGSS process) (*Weidner*, 2009).

Among all of these techniques, PGSS and PGSS-drying using CO_2 have acquired a great attractive on production of microcapsules which, due to special thermo- and fluid dynamic behaviour of supercritical fluids such as CO_2 (high Joule-Thomson coefficient, low viscosity), may present unique properties difficult to obtain by conventional methods. Moreover, these processes have given interesting results in formulation of different products such as seasoning for fish, praline with flavours, soy sauce, honey or tea, being highly attractive in the food industry (*Weidner*, 2009).

PGSS has been also explored as a way to stabilise sensitive products like soybean oil using PEG 6000 (polyethylene glycol with an average molecular weight of 6000), concluding that, in spite of resulting in a higher specific contact surface between oil and oxygen, oxidation in encapsulated oil is only slightly faster than in bulk oil, which may be related to the protective effect of the shell material. (*Gontaru,* 2009). Other studies have been also developed on encapsulation of lavandin essential oil both in PEG 6000 by PGSS or in octenyl succinic anhydride-modified starch (OSA starch) by PGSS-drying, in order to improve their control release as biocide in agriculture (*Varona, et al.*, 2010).

The formulation of fish oil as solid particles has an enormous interest in both the food and pharmaceutical industries since it allows to broad the application field of marine lipids as active ingredients, improve sensorial properties of omega-3 enriched products and enhance the control release in order to optimise the beneficial effects on human health. A wide range of

substances have been proposed in the literature as coating materials in fish oil encapsulation. Among them, multilayer coating composed by a mixture of a carbohydrate (corn syrup, glucose or maltodextrines) and chitosan has been proposed as an effective barrier for preventing the oxidation of fish oil encapsulated by conventional processes such as spray-drying.

Since the use of supercritical fluids may be proposed as valuable alternative to spray-drying, in this section, the results of a preliminary study about the viability of the PGSS-drying process in the production of fish oil microcapsules with a multilayer coating (carbohydrate / chitosan) are presented (*Klaypradit, et al.*, 2008, *Klinkesorn, et al.*, 2005a, *Klinkesorn, et al.*, 2005b, *Klinkesorn, et al.*, 2005c, *Shen, et al.*, 2010).

This work has been developed in collaboration with the research group of Prof. Petermann at the Ruhr University of Bochum (Germany), and tries to give an overview of the influence of the main process parameters of PGSS-drying in particle physical properties.

4.1.2. Fundamentals of oil encapsulation processes

Oil encapsulation for edible purposes requires the selection of a hydrophilic biopolymer which must be solid under ambient conditions, stable enough to isolate and protect the oil from environmental stress, prevent the oxidation of sensitive compounds and easy to be absorbed by human body.

A wide range of compounds have been proposed as coating materials for oil encapsulation such as proteins, carbohydrates or polyethylene glycol (PEG).

Depending on their physical and chemical properties, oil microencapsulation procedures can be reached by two different ways: dispersing the oil in the molten shell material and solidifying the mixture by decreasing the temperature; or dispersing the oil in an aqueous solution of the shell material and promoting the precipitation of the coating from the resulting oil-in-water emulsion. Both procedures are described below.

4.1.2.1. Encapsulation from oil dispersed in molten shell material.

This procedure is suitable when the desired shell material is a biopolymer that, as PEG 6000, presents a low melting point (50 - 60 ºC) and does not change its properties when solidified after melting.

Commonly, this encapsulation method is carried out in a screw-extruder which conveys the oil dispersed in the molten coating through a die forming and, subsequently, to a cooling device where the coating solidifies. Recently, conventional extruders have been replaced by centrifugal extruders which contain a rotating head with concentric nozzles and allow to obtain smaller and spherical particles from a mixture of oil dispersed in a molten compound (*Goud, et al.*, 2005). Based on melt extrusion, several methods have been proposed in the literature, many of them under patent, to encapsulate fish oil in low molecular weight carbohydrates such as maltose (*Drusch, et al.*, 2009).

Recently, PGSS process (Particle from Gas Saturated Solution) has been also applied in oil encapsulation from molten wall material taking advantage

of the supercritical fluid technology. This encapsulation method involves pumping separately the oil to be encapsulated, the molten shell material, PEG 6000, and the CO_2 into a static mixer which is maintained under pressure and temperature conditions above the CO_2 critical point. Then, the resulting achieved mixture (liquid compound / shell material / CO_2) is sprayed through a nozzle at atmospheric pressure, which causes a rapid expansion of the CO_2. As a consequence, the liquid / shell material mixture is desegregated in small droplets and cooled according to the Joule Thomson effect, which promotes the solidification of shell material. This solidification rate and its relation with the coalescence time of the dispersed liquid droplet are determinant on the effectiveness of the PGSS process to produce closed or porous microcapsules (*Weidner, et al.*, 2004).

Hence, as it was previously described (*Petermann, et al.*, 2007), the formation of closed microcapsules could be possible as long as the solidification of the shell material occurs faster than the phase separation on the outer surface and on the inner surface the phase separation occurs faster than the solidification of the shell material. In this case, if total liquid coalescence takes place, the core material may form a continuous phase surrounded by the solid coating, as it is represented in Figure 4.1 (a), or, most likely, may remain dispersed as small drops within the continuous solid shell material, as shown in Figure 4.1 (b). On the contrary, if on the outer surface the phase separation takes place faster than the solidification of the shell material and on the inner surface the solidification of the shell material occurs faster than the phase separation, open composites or porous

microcapsules will be formed, in which the liquid component remains bounded in the solid matrix by adsorption phenomena and capillary forces Figure 4.1 (c).

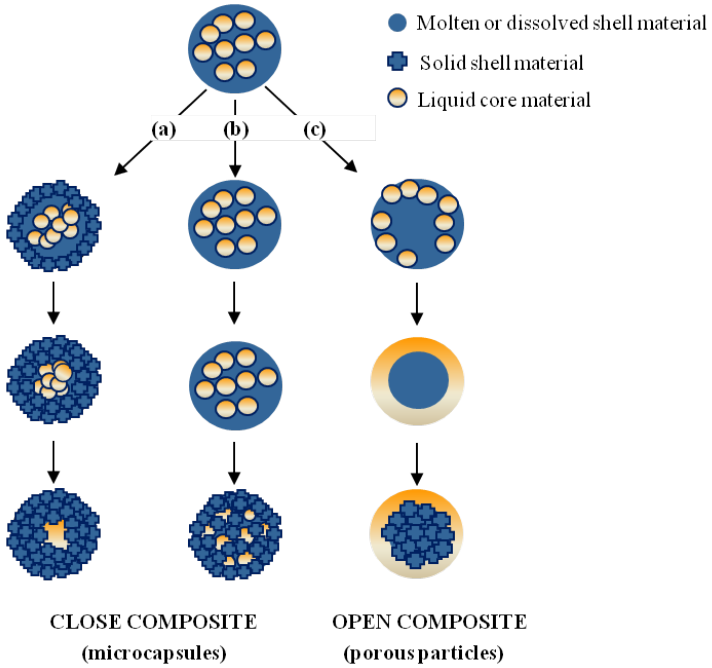

FIGURE 4.1

Scheme of the mechanism of solidification and particle formation from a liquid dispersed in a molten or dissolved solid. Routes (a) and (b) represent the formation of a microcapsule when the coating builds up faster than the phase separation. Route (c) represents the formation of open composites when the phase separation occurs faster than the solidification of the shell material. (Adapted from (*Petermann, et al.*, 2007)).

4.1.2.2. Encapsulation from oil-in-water emulsions.

This method is suitable when the desired coating is a compound that, as high molecular weight carbohydrates or proteins, presents a high melting point and / or its properties may change irreversibly when it melts. In this case, oil encapsulation can be carried out by mixing the oil and an aqueous solution of carbohydrate to form and stable oil-in-water emulsion which is subsequently atomised in small droplets and dried to build up the wall material by water evaporation. Thus, the success of these encapsulation methods is conditioned by both the formation of a stable oil-in-water emulsion and the effectiveness of the particle formation procedure.

i) Formation of an oil-in-water emulsion

In general, emulsion is defined as a mixture of two immiscible liquids which, in the case of oil-in-water emulsions, are oil and water. Although these mixtures are thermodynamically unstable, their kinetic stability can get longer by adding emulsifiers or surfactants, which take advantage of their amphiphilic character, that is, they present a simultaneous affinity for the water phase, through their hydrophilic group or head, and for the oil phase by their hydrophobic group or tail.

Since carbohydrates such as maltodextrine (MD) are not amphiphilic molecules, they have a poor capability for achieving a kinetically stable oil-in-water emulsion, and therefore the addition of other biopolymers able to act as an emulsifier is necessary.

In the last years, emulsifier mixtures, composed by a cationic biopolymer and an anionic or no-ionic biopolymer, have been used to form multilayer oil-in-water emulsions, which allows to enhance the oil stabilization against oxidation and, therefore, are highly interesting in fish oil encapsulation processes.

The formation of stable oil-in-water multilayer emulsions is affected by a wide amount of factors, such as the sign and magnitude of the drop charge, the molecular weight, charge density and flexibility of the biopolymer, the ionic strength, pH and temperature of the media, the stirring method or the order established for adding the different components in the mixture (*Guzey, et al.*, 2006).

On the other hand, the capability of a multilayer oil-in-water emulsions to protect the oil against oxidation depends also on several factors, especially the size and charge of the lipid droplet, since they play an important role in the interactions between metals and lipid hydroperoxides and, therefore, in the formation of the free radicals that promote lipid oxidation (*Ogawa, et al.*, 2003). Size and charge of lipid droplets are strongly affected by the surfactant layer between the oil and water phases, which is conditioned by the interaction between the different biopolymers, either through adsorption mechanism, such as between a cationic and a no-ionic biopolymer (Tween 80 / chitosan) or through electrostatic attraction between a cationic and an anionic biopolymer (lecithin / chitosan). Furthermore, oil stability against oxidation can be also improved by the antioxidant effect carried out

by some biopolymers, especially chitosan (*Xia, et al.*, 2011), as has been demonstrated in recent studies.

The present study is focused on the use of multilayer oil-in-water emulsions with fish oil, maltodextrine (DE 15-20) and a mixture of Tween 80 / chitosan or lecithin / chitosan respectively. In all the cases, chitosan has been chosen as secondary emulgent since it presents a cationic and bioadhesive character, which makes it suitable to form a stable multilayer with both anionic biopolymers (*Schulz, et al.*, 1998) and no-ionic biopolymers (*Klinkesorn, et al.*, 2009), allows to create positively charged barrier among oil and water, which allows to prevent fish oil oxidation by the action of metal cations (*delBlanco, et al.*, 1999); and offers a high antioxidant effect (*Xia, et al.*, 2011).

ii) Mechanism of particle formation

The mechanism of particle formation from an oil-in-water emulsion is also essential for achieving a high efficiency in the final oil encapsulation process.

In conventional spray-drying processes, atomisation is produced by pumping the emulsion through the nozzle and then drying the droplets with hot air or nitrogen in a drying chamber. Thus, the design of both nozzle and drying chamber is crucial to efficiently achieve a dry solid loaded in oil. Nowadays, a wide range of designs for nozzles (2-fluid, 3-fluid, ultrasonic...) and drying chambers (counter current gas flow, co-current gas

flow...) can be found in order to improve the mechanism of emulsion dispersion and the contact between the drying agent and the sprayed emulsion and therefore, the properties of the final product (*Cal, et al.*, 2010).

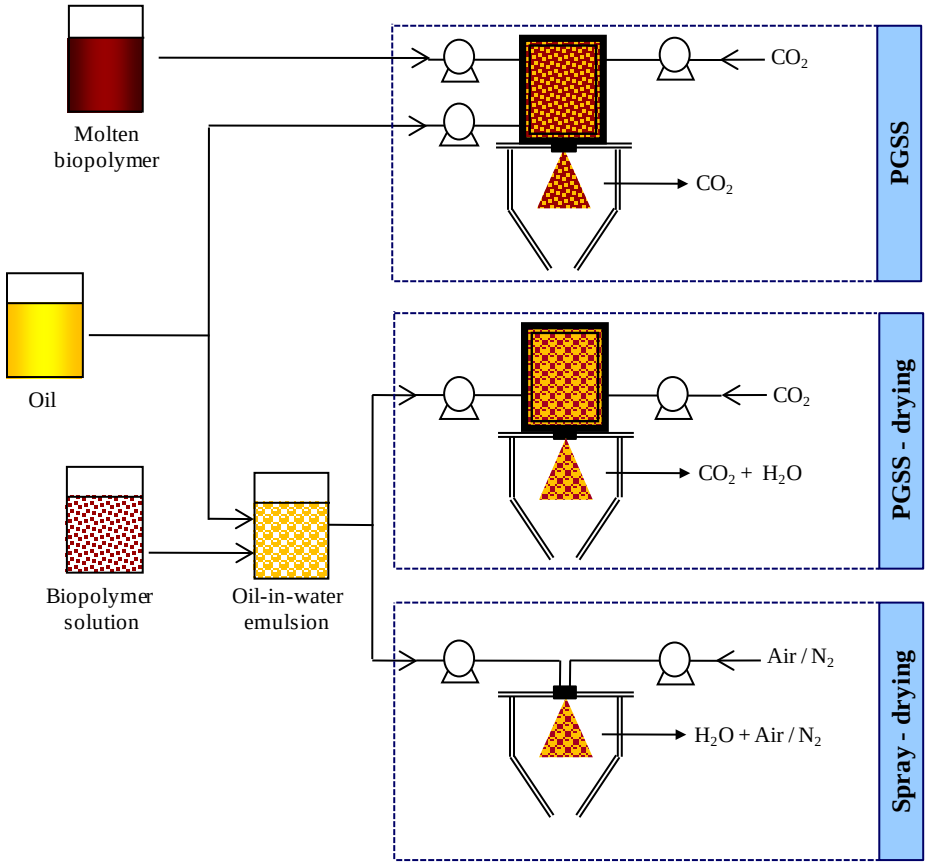

FIGURE 4.2

Schematic comparison among oil encapsulation by PGSS, PGSS-drying and spray-drying processes.

Recently, oil encapsulation by PGSS-drying has been proposed as a hybrid between the PGSS and the spray-drying processes previously described since it involves spraying and drying an oil-in-water emulsion previously admixed with SC-CO_2 in a static mixer (*see* Figure 4.2). The fundamentals of this process consist on the emulsion atomization and subsequent water removal taking the advantage of the unusual properties of SC-CO_2. Thus, encapsulation efficiency and particle properties depend on emulsion stability previous to spraying, atomization mechanism in the nozzle and particle formation in the spray tower.

Emulsion stability, previous to spraying depends on the phase equilibria between the emulsion and the SC-CO_2 mixture and the mass transfer resistance along the mixer. Phase equilibria are affected by the mixture composition, and therefore by the CO_2 / emulsion ratio (GPR), and by the SC-CO_2 properties such as solvation power, which are in turn to pre-expansion pressure and temperature. Mass transfer resistance along the mixer has been described as a complex phenomenon which depends on a wide amount of factors (density and viscosity in the gas and liquid phases, diffusion coefficient in the gas and liquid phases, interfacial tension between different phases...) and may be modulated by changing the emulsion and SC-CO_2 properties, minimizing its effect when the contact between emulsion and CO_2 is improved, for instance, increasing the turbulence in the mixer chamber through an static mixer (*Martín, et al.*, 2010b).

The atomization mechanism in PGSS-drying has been approached to a combination of both effervescent and flash-boiling atomization, since the expansion of the mixture resulting from the mixer chamber presents a similar behaviour to the expansion of a superheated solution saturated with gas. Thus, it is affected by a wide amount of factors related to the phase behaviour of emulsion / CO_2 mixture, the pressure depletion between the mixer and the expansion chamber, the nozzle geometr, etc. (*Martín, et al.*, 2010b).

Finally, particle formation in the spray tower may be approached, as in a RESS process, to a combination of three phenomena: nucleation and condensation, which are mostly important at the nozzle outlet; and coagulation, which is mainly responsible for particle growth inside the expansion chamber (*Helfgen, et al.*, 2003). Moreover, since water vaporisation in PGSS-drying depends on the liquid-vapour equilibria of the water + CO_2 mixture, the efficiency of the drying step, and therefore the solid particle formation, is strongly affected by the pressure, temperature and gas/liquid ratio in the expansion chamber (*Martín, et al.*, 2010b).

In this preliminary study, it has been established a general trend between the experimental process parameters and powder properties (average size and size distribution, moisture, oil encapsulation efficiency, particle morphology...) which helps to obtain a global vision on the viability of the PGSS-drying process to produce fish oil microcapsules. Nonetheless, from this starting point, a more detailed study focused on different aspects such as

the phase behaviour of SC-CO_2 / emulsion mixture, the mass transfer phenomena in the mixer chamber or the atomisation and particle formation mechanism will be required for a better description of the process mechanism and its subsequent modelling and scaling-up.

4.1.3. Experimental section

4.1.3.1. Materials

PGSS-drying was carried out using a multilayer oil-in-water emulsion containing the fish oil (core material), a primary emulgent, a secondary emulgent and maltodextrine (shell material).

- *Core material*: refined anchovy oil provided by Denomega Nutritional Oils AS, (Norway), was used as core material in encapsulation experiments. The chemical composition of this fish oil is reported in Table 4.1.

- *Coating material*: the selected coating was maltodextrine (DE 15-20) provided by Cargill Deutschland GmbH, (Krefeld, Germany). Chemically, this compound is a high molecular weight polysaccharide composed by D-glucose units bounded with α(1→4) glycosidic bonds in chains of variable length (n) depending on the dextrosa equivalente (DE) value (s*ee* Figure 4.3). Its physical appearance at ambient conditions is as a white powder and a high solubility in water.

- *Primary emulgent*: two components were studied as primary emulgents, polysorbate 80, which is a no-ionic surfactant (*see* Figure 4.4 (a)) able to

produce neutral charged oil drops; an lecithin, which is an amphipathic phospholipid (*see* Figure 4.4 (b)) that can act as an anionic surfactant producing negative charged oil drops. Since commercial polysorbate 80 (Tween 80) is a liquid emulgent, it is mixed directly with oil; whereas refined lecithin was used as an aqueous solution of powdered lecithin (3 % wt. in water).

- *Secondary emulgent*: the secondary surfactant used was an acid solution of chitosan (2 % wt. in an aqueous solution of acetic acid 1 M). Chemically, this cationic surfactant is a linear amino-polysaccharide composed by β-(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine (Figure 4.5), which properties have been reviewed in the literature (*Rathkea, et al.*, 1994).

TABLE 4.1

Chemical composition of fish oil used in PGSS-drying experiments

Parameter	Value	Parameter	Value
Acid value (mg KOH)	< 1.0	Dioxins (PCDD/PCDF) (pg TEQ)	< 1.5
Peroxide value (meq /kg)	> 5.0	Dioxines-like PCBs (pg TEQ)	< 3.0
Anisidine value	< 20.0	PCBs (209 congners) (ppm)	< 0.02
Cold test (0 ºC)	> 3	Arsen (As) (ppm)	< 0.05
Unsaponifiable matter	< 1.5	Cadmium (Cd) (ppm)	< 0.05
Colour Gardener	< 6	Mercury (Hg) (ppm)	< 0.05
EPA (%)	> 18	Lead (Pb) (ppm)	< 0.05
DHA (%)	> 12	DDT (ppm)	< 0.10
Total omega-3 (%)	> 35		

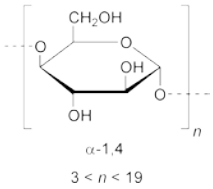

FIGURE 4.3

Chemical structure of maltodextrine monomer.

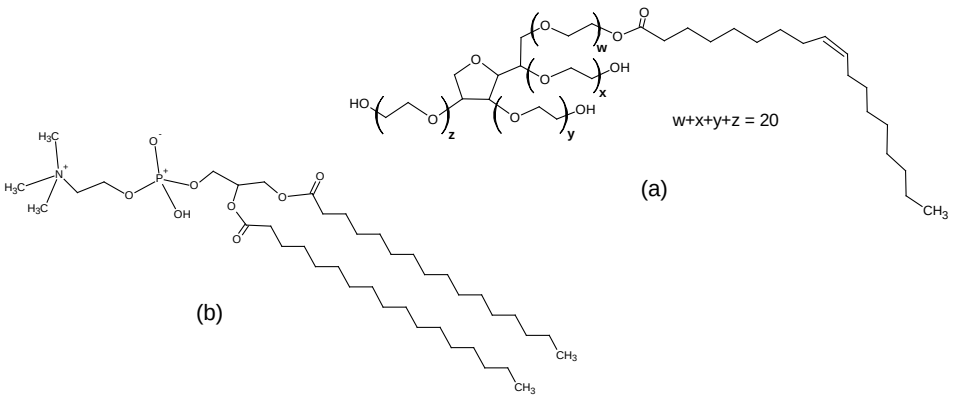

FIGURE 4.4

Chemical structure of (a) polysorbate 80 (Tween 80); (b) lecithin.

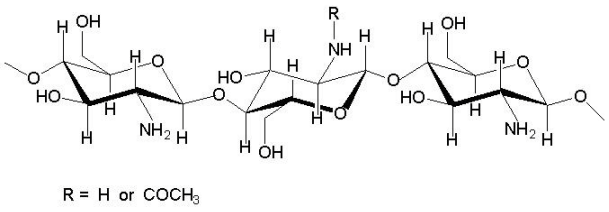

FIGURE 4.5

Chemical structure of chitosan monomer.

4.1.3.2. Preparation of oil-in-water emulsions

The procedure for preparing oil-in-water emulsions was based on the layer-by-layer deposition method previously described in the literature (*Ogawa, et al.*, 2003). This method involves three steps: the formation of a primary emulsion by mixing the oil with the primary emulgent (Tween 80 or aqueous lecithin solution) the dilution of the primary emulsion with the acid chitosan solution to form the secondary emulsion and the mixture of the secondary emulsion with the aqueous maltodextrine solution to form the final emulsion (*see* Figure 4.6).

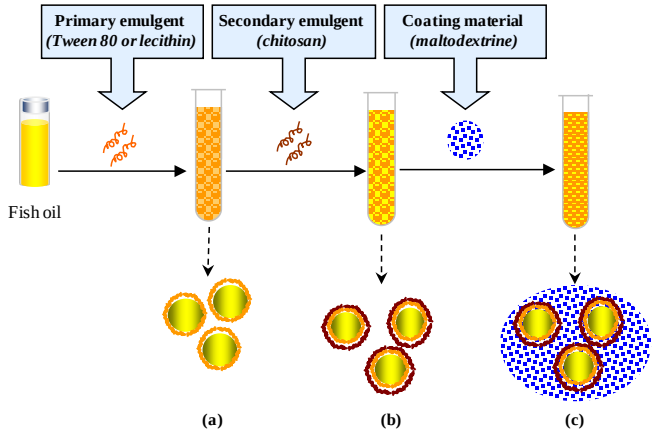

FIGURE 4.6

Mechanism of double-layer formation in oil-in-water emulsions. (a) Oil droplets coated by a monolayer of primary emulgent; (b) Oil droplets coated by a double layer formed from the interactions between primary and secondary emulgent; (c) Multilayer oil-in-water emulsion in a maltodextrine aqueous solution.

In each step, ingredients were blended mechanically during 5 min at 19000 rpm in an ultra-turrax homogenizer (*IKA Labortechnik*, Staufen, Germany). Four different emulsions were studied in this work, which are presented in Table 4.2. In all of them, the amount of chitosan was less than 1 % wt., since at this level no emulsion destabilization by creaming has been evidenced (*Klinkesorn, et al.*, 2009). Emulsion stability was proved at ambient condition, not being observed any phenomena of creaming for more than one hour.

TABLE 4.2

Composition of oil-in-water emulsions prepared for fish oil microencapsulation.

Emulsion	Fish oil (%wt)	Primary emulgent		Chitosan (%wt)	Maltodextrine (%wt)	Water (%wt)	Oil / solid ratio	Oil in water (% wt.)
		Tween 80 (%wt)	Lecithin (%wt)					
A	5.07	0.40	___	0.06	26.16	68.31	0.19	7.4
B	8.53	0.35	___	0.14	33.22	57.76	0.26	14.8
C	2.79	___	1.47	0.03	15.00	80.71	0.17	3.5
D	3.39	___	1.50	0.03	14.53	80.55	0.21	4.2

4.1.3.3. PGSS-drying equipment

Oil encapsulation by PGSS-drying was carried in the laboratory of Thermo-und Fluid dynamic located at the Ruhr University of Bochum (Germany).

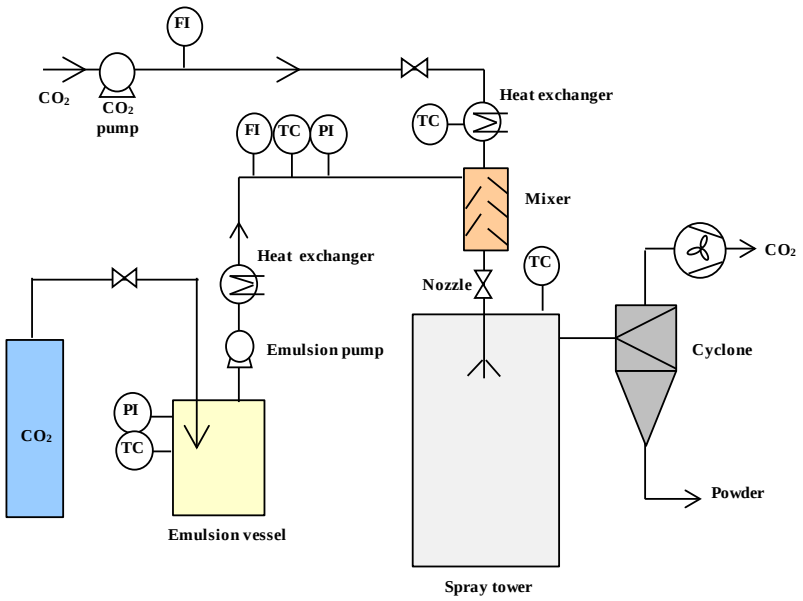

FIGURE 4.7

P&I of the PGSS-drying pilot plant used in this study.

The equipment used was a pilot plant designed for working at a maximum operating pressure and temperature of 20 MPa and 523 K (250 ºC) respectively. The flowsheet of the equipment is presented in Figure 4.7, which main parts are described below.

- *CO_2 line:* Carbon dioxide is pumped from the tank, in which it is stored as liquid state, to the mixer, which is set at a pressure above the critical point of CO_2, by a diaphragm pump. The heating of CO_2 up to the mixer temperature (443 K) is achieved by passing it through a heat exchanger. Measurement of the CO_2 mass flow is carried out by a Coriolis flow meter.

- *Emulsion line*: Fish oil emulsion is stored in a vessel, which is subsequently thermostated at 70 ºC and pressurized with gas CO_2 at 1 bar in order to help the suction of the liquid into the pump. A piston pump was installed to convey the emulsion to the mixer, whereas a platform balance connected to the control system allows to determine and control emulsion mass flow rate.

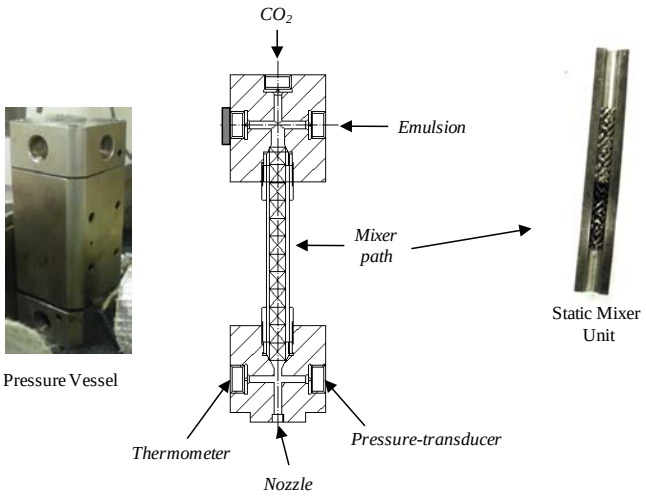

FIGURE 4.8

Design of the mixing chamber and static mixer.

- *Mixing chamber*: Fish oil emulsion and SC-CO_2 are mixed in an autoclave (Figure 4.8) designed for working at high pressures (up to 35 MPa). The mixer path has a length of 20 cm and 8 mm of internal diameter. In order to improve the mixing among emulsion and CO_2, the mixer path was filled by a static mixer (Fluitec Georg AG, Neftenbach, Switzerland), which is also shown in Figure 4.8.

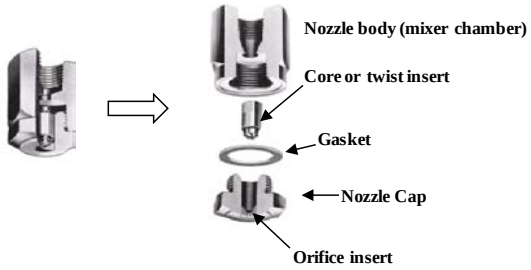

FIGURE 4.9

General scheme of a nozzle design and its main components.

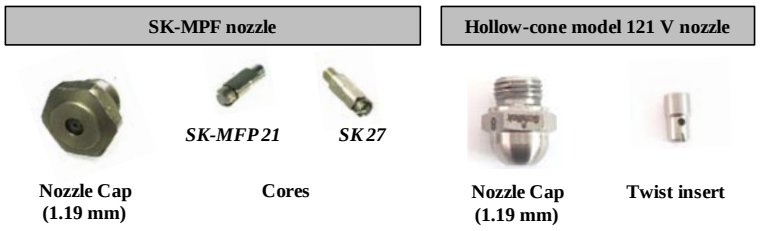

FIGURE 4.10

Main components of the SK-MPF nozzle and the hollow core nozzle (model 121 V) used in this work.

- *Spraying system*: After the mixer chamber, the biphasic emulsion/SC-CO_2 mixture is sprayed and expanded through a nozzle, promoting the atomization of the emulsion in small drops. Different types of hollow cone nozzles were explored: a SK-MPF nozzle, provided by Spraying Systems Deutschland GmbH, (Hamburg, Germany) and a 121V nozzle provided by Düsen-Schlick GmbH (Coburg, Germany). SK-MPF nozzles were composed by three parts: cap, orifice insert, core, gasket and body (*see* Figure 4.9). The orifice insert size was 1.19 mm in all the cases, whereas two different cores were used: SK-MFP 21 (which contains two slots with a nominal width of 0.89 mm) and SK 27 (which contain 4 slots with a nominal width of 0.64 mm). The 121 V nozzle was made of three parts: body (orifice size = 1.2 mm), twist insert (which presents two slots with a diameter < 0.5 mm) and a short screw (*see* Figure 4.10).

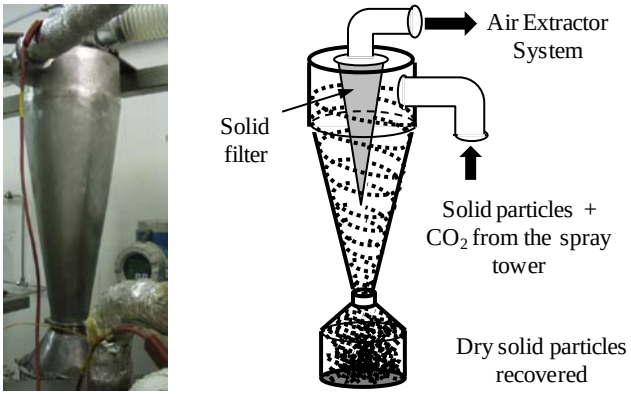

FIGURE 4.11

Design of the cyclone installed in the PGSS-drying pilot plant.

- *Particle recovering*: The sprayed emulsion is recovered in the spray tower, which is an isolated vessel maintained at 1 bar and heated at the desired temperature for drying the particles with CO_2. A gas extractor system connected to the spray tower top allows to extract the gas phase (water / CO_2) and the dry solid particles, which are subsequently separated by a cyclone. In order to avoid small particles loosing, the gas removed is passed through a filter located at the cyclone outlet (Figure 4.11).

4.1.3.4. Experimental procedure of oil encapsulation

In a fish oil encapsulation experiment, approximately 1.5 kg of fish oil emulsion were placed in the emulsion vessel that was later pressurized with CO_2 up to 1 bar and thermostated up to 340 K. Then, CO_2 was heated and pumped in order to pressurize the mixer chamber up to the pre-expansion pressure and fill the spray tower with hot gas CO_2 up the desired drying temperature. After that, the emulsion was pumped to the mixing chamber and sprayed through the nozzle to the spray tower. In all cases, CO_2 flow rate was kept constant, and the gas-emulsion ratio (GPR) was adjusted by changing either the piston volume or the pumping speed of the emulsion pump.

A total of twenty-one experiments were carried out; seven of them with the emulsion A, eleven with emulsion B, one with emulsion C and two with emulsion D. In all the experiments, the pre-expansion temperature was maintained around 443 K. This temperature was selected as high enough to allow, according to the phase equilibria presented in Figure 4.12, a CO_2

phase rich in water but with a negligible oil content, which is interesting for a selective water removal minimizing the oil lost; besides to a liquid phase, (oil-in-water emulsion), with an amount of dissolved CO_2 higher enough to achieve a good dispersion of the oil droplets and an effective atomization in small particles.

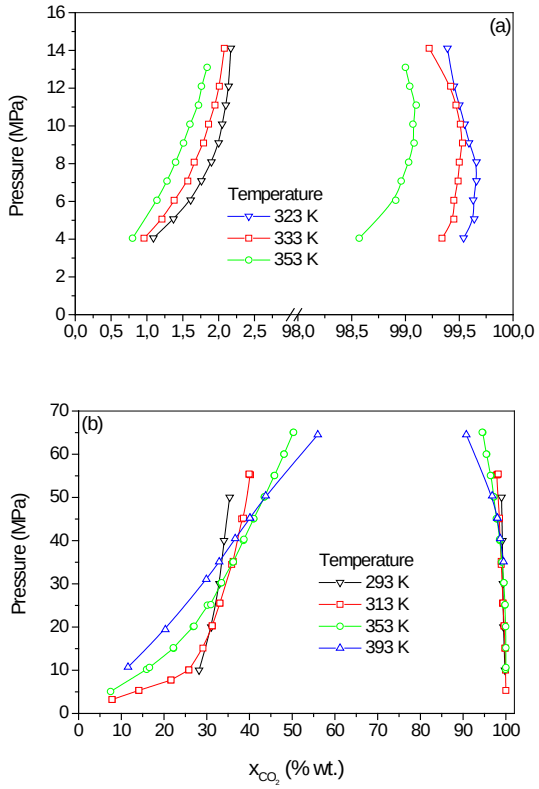

FIGURE 4.12

(a) Binary phase equilibria of water / CO_2. (b) Fish oil / CO_2. Experimental data taken from (*Bamberger, et al.*, 2000) and (*Borch-Jensen, et al.*, 1997) respectively.

Due to its short residence time in the mixing chamber and to the protective effect of the oil-water-emulsion, oil degradation by the use of this high temperature is expected to have a minimal impact on the final oxidation state of the encapsulated fish oil.

The rest of the experimental conditions are reported in Table 4.3.

4.1.3.5. Dry powder characterization

The resulting dry powder obtained was characterized by means of its physical properties, such as density, average particle size (d0.5), moisture content or internal microstructure, which was explored by scanning electron microscopy (SEM).

The water on particle surface was determined qualitatively by FT-IR analysis focused on the absorption at 3340 cm^{-1} (A_{3340}) since this is the wavelength associated to the O-H stretch in the water molecule.

Additionally, encapsulation efficiency (% EE) was determined taking into account both the amount of total oil and the amount of non-encapsulated oil according to Eq (4.1).

$$\%EE = \frac{[Oil]_{TOTAL} - [Oil]_{NON-ENCAPSULATED}}{[Oil]_{TOTAL}} \cdot 100 \qquad \textbf{(4.1)}$$

The amount of non-encapsulated oil was considered the amount of oil released after washing the dry powder with hexane. Since the dry particles recovered in the cyclone presented a low amount of non-encapsulated oil, the amount of oil removed with hexane was determined by a spectrophotometric method instead of gravimetrically. Moreover, due to the small particle size and its high dispersion capability in organic solvents, total oil content in dry powder was determined by the Schmid-Bondymdki-Ratzlaff procedure instead of by conventional soxhlet extraction.

All the methods developed are detailed in Appendix I.3.

4.1.4. Results and discussion

In this section the effect of the main process parameters that controls each step in the global mechanism of the PGSS-drying process is discussed, i.e. the phase behaviour of the mixture after spraying, the atomization step and the water vaporization and their influence on the physical properties of the dry powder recovered (compiled in Table 4.4), and on the internal microstructure shown in Figure 4.13.

4.1.4.1. Influence of emulsion stability in the mixer chamber

According to the mechanism described in section 4.1.2, the morphology of the microcapsules formed in a PGSS process depends on the rate of formation of the coating and on the rate of phase separation in the emulsion.

TABLE 4.3

Experimental conditions of the encapsulation experiments carried out in this work.

Run Nº.	Emulsion	Oil / solids mass ratio	Nozzle core	S.m.	Pre-exp P (MPa)	GPR (kg CO_2 / kg emulsion)	Tspray-tower (K)
1	A	0.19	SK-MFP 21	no	11.5	30	88
2	A	0.19	SK-MFP 21	no	11.6	6	89
3	A	0.19	SK-MFP 21	no	11.7	21	70
4	A	0.19	SK-MFP 21	no	12.6	35	100
5	A	0.19	SK-MFP 21	no	12.7	21	70
6	A	0.19	SK-MFP 21	no	13.0	88	102
7	A	0.19	Hollow cone	no	12.3	31	98
8	B	0.26	SK-MFP 21	no	11.5	30	88
9	B	0.26	SK-MFP 21	no	11.6	50	98
10	B	0.26	SK-MFP 21	yes	12.7	15	79
11	B	0.26	SK-MFP 21	yes	16.7	23	90
12	B	0.26	SK-MFP 21	yes	25.7	18	64
13	B	0.26	SK 27	no	11.6	25	88
14	B	0.26	SK 27	no	11.6	25	105
15	B	0.26	SK 27	no	12.0	23	89
16	B	0.26	SK 27	no	12.4	13	119
17	B	0.26	SK 27	no	13.4	51	103
18	B	0.26	SK 27	no	15.5	48	105
19	C	0.17	SK-MFP 21	no	11.7	29	93
20	D	0.21	SK-MFP 21	no	11.0	27	80
21	D	0.21	SK-MFP 21	no	11.8	42	93

S.m.: Static mixer

TABLE 4.4

Main features of dry solid particles recovered from each experiment.

Run Nº.	Density (kg/m^3)	d0.5 (µm)	Water content		Oil content		% EE
			Moisture (% wt.)	A_{3340}	Total (mg / g	No encapsulated (mg / g solid)	
1	94 ± 6	918 ± 174	6.6 ± 0.4	0.05	11 ± 3	20 ± 6	< 10
2	276 ± 29	154 ± 63	6.7 ± 0.2	0.03	40 ± 4	4 ± 2	90
3	124 ± 10	n.d.	7.8 ± 0.1	0.03	n.d.	n.d.	---
4	134 ± 11	80 ± 57	4.0 ± 0.9	0.01	38 ± 3	10 ± 2	74
5	260 ± 15	99 ± 18	4.7 ± 0.1	< 0.01	14 ± 2	13 ± 1	---
6	168 ± 15	23 ± 3	3.5 ± 0.3	0.01	5 ± 2	6 ± 1	---
7	150 ± 12	32 ± 4	3.6 ± 0.2	0.02	19 ± 5	7 ± 3	63
8	134 ± 11	995 ± 5	3.6 ± 0.4	0.03	9 ± 3	8 ± 1	---
9	189 ± 11	28 ± 4	4.4 ± 0.4	0.02	10 ± 2	6 ± 1	40
10	182 ± 12	31 ± 8	6.6 ± 0.2	0.04	42 ± 4	34 ± 4	19
11	150 ± 2	380 ± 40	4.7 ± 0.4	0.02	4 ± 1	3 ± 1	---
12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
13	253 ± 22	314 ± 27	5.3 ± 0.1	0.01	10 ± 1	12 ± 2	< 10
14	162 ± 13	24 ± 4	4.6 ± 0.2	0.02	4 ± 1	5 ± 2	---
15	193 ± 21	54 ± 14	4.3 ± 0.3	0.03	35 ± 5	14 ± 1	60
16	170 ± 21	44 ± 27	2.8 ± 0.1	0.03	32 ± 3	16 ± 1	50
17	189 ± 11	505 ± 276	3.6 ± 0.1	0.03	7 ± 3	11 ± 1	---
18	134 ± 9	768 ± 380	4.5 ± 0.1	0.01	11 ± 6	12 ± 1	---
19	n.d.	n.d.	5.3 ± 0.2	0.02	9 ± 1	8 ± 2	< 1
20	148 ± 5	1085 ± 31	5.6 ± 0.4	0.01	6 ± 3	9 ± 2	< 1
21	76 ± 3	n.d.	5.0 ± 0.1	0.07	n.d.	n.d.	---

n.d.: no data avalaible

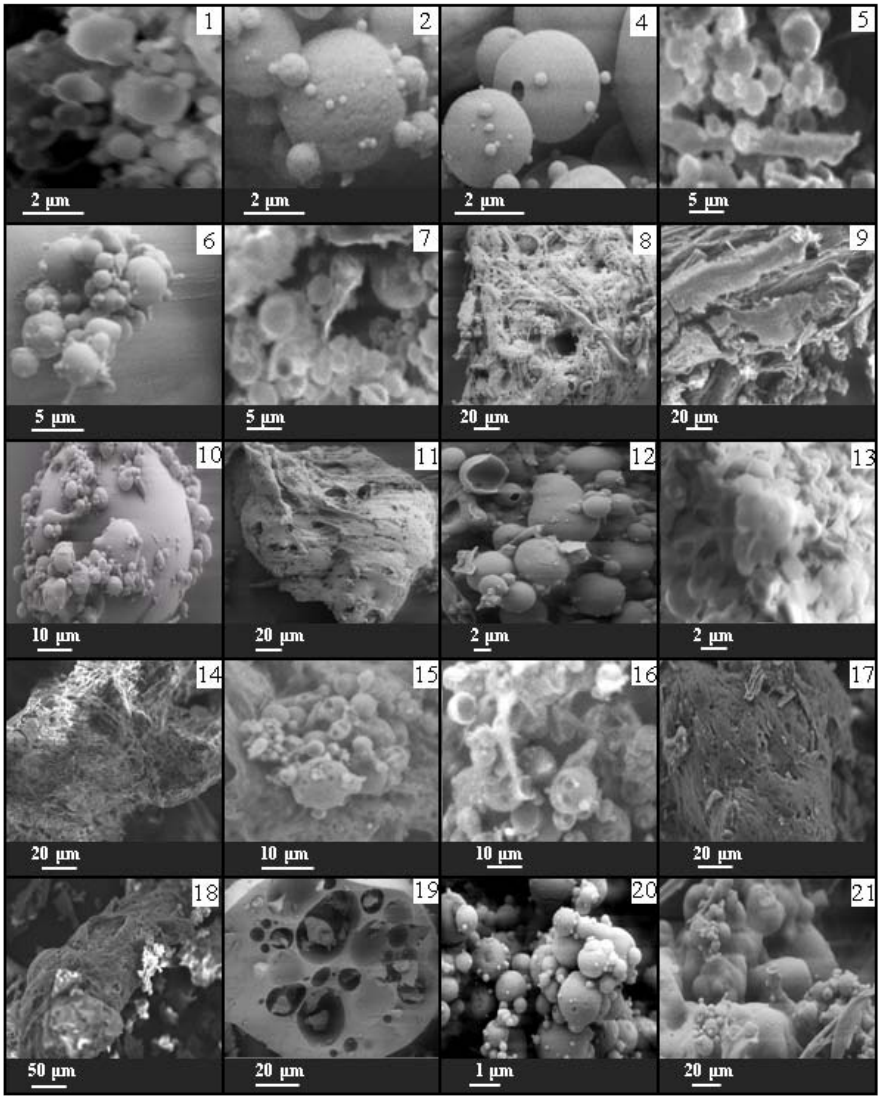

FIGURE 4.13

Internal microstructures observed in dry powder by scanning electron microscopy (SEM). Numbers on right label of each picture correspond to PGSS-drying run.

In PGSS-drying, since the feed is an oil-in-water emulsion, the rate of building up the shell material depends on its precipitation rate from the aqueous solution, and therefore on the efficiency of the water removal; whereas the rate of phase separation depends on the stability of the oil-in-water emulsion and the coalescence rate of the oil droplets.

The stability of the emulsion, previous to spraying, depends on the phase behaviour in the static mixer, which is not only affected by the emulsion composition but also by the conditions in the mixer chamber, i.e. pre-expansion pressure, pre-expansion temperature and gas-emulsion ratio (GPR), since these factors condition the phase behaviour of the CO_2/emulsion mixture.

Previous studies have shown that the stability of an oil-water emulsion under SC-CO_2 is noticeably lower when pressure increases, which is attributed to an increment in the oil-water interfacial tension caused by an increment in the concentration of CO_2 in the emulsion (*Mattea, et al.*, 2010, *Varona, et al.*, 2010). The effect of pressure, as the effect of gas-emulsion ratio (GPR) and temperature, on the emulsion stability may be also approached assuming that, in the CO_2 / emulsion mixture, water is removed from the emulsion to the gas phase, promoting a similar effect that an emulsion destabilization by water evaporation. Thus, higher amounts of CO_2 dissolved in the emulsion promoted a higher amount of water removed and the subsequent formation of a more concentrated emulsion. This concentrated emulsion is expected to be less stable that the initial since a

high concentration of oil droplets may promote a partial destabilization by clumping or flocculation (*Guzey, et al.*, 2006) or even the emulsion breakage due to the compression of the oil drops and the subsequent coalescence in a separated oil layer (*Aranberri, et al.*, 2004). Furthermore, if the concentration of solids such maltodextrine or chitosan is up their saturation value, a solid precipitation in the liquid phase will occur, which involve the clogging inside the mixer chamber or in the nozzle.

Taking into account that at the mixer outlet a biphasic gas / liquid mixture is formed, and that the solubility of oil and shell material in the gas phase is negligible, the ratio among a compound i and water in the remaining liquid phase after water removal can be related to the composition of the initial emulsion and the GPR by Eq.(4.3). Moreover, considering that mass transfer resistance in the mixer is neglected and at the mixer outlet the phase equilibrium has been reached, the mass fraction of water in gas phase can be approximated to the solubility of water in SC-CO_2, S_W, which can be easily estimated as a function of CO_2 density, ρ (kg / m^3) and temperature,T (K) according to Chrastil Eq. (4.5).

$$\left(\frac{x_i}{x_W}\right)_L = \frac{m_e \cdot x_{i,e}}{m_e \cdot x_{W,e} - m_G \cdot x_{W,G}} \qquad \textbf{(4.2)}$$

$$\left(\frac{x_i}{x_W}\right)_L = \frac{1}{\left(\frac{x_w}{x_i}\right)_e - GPR\frac{x_{W,G}}{x_{i,e}}} \quad \textbf{(4.3)}$$

$$x_{W,G} \approx S_W \quad \textbf{(4.4)}$$

$$\ln S_W = 1.549 \cdot \ln \rho_{CO_2} - \left(\frac{2826.4}{T}\right) - 0.807 \quad \textbf{(4.5)}$$

Thus, as it is represented in Figure 4.14(a), 4.15 (a) & 4.16 (a), the increment of oil/water, maltodextrine/water and chitosan/water ratios in the liquid phase at the mixer outlet is affected by GPR, being slightly above a limit GPR value, and by the composition of the initial emulsion, being more noticeable when the oil/water ratio is higher. As a consequence, it can be concluded that working near of a maximum GPR value involves a higher risk of suffering two undesired drawbacks in a PGSS-drying process: emulsion destabilization and clogging. Furthermore, since the solubility of water in CO_2 is higher the higher CO_2 density, and therefore the higher the pressure, the risk of oil phase separation and clogging increases when pre-expansion pressure increases, as can be observed in Figure 4.14 – 4.16.

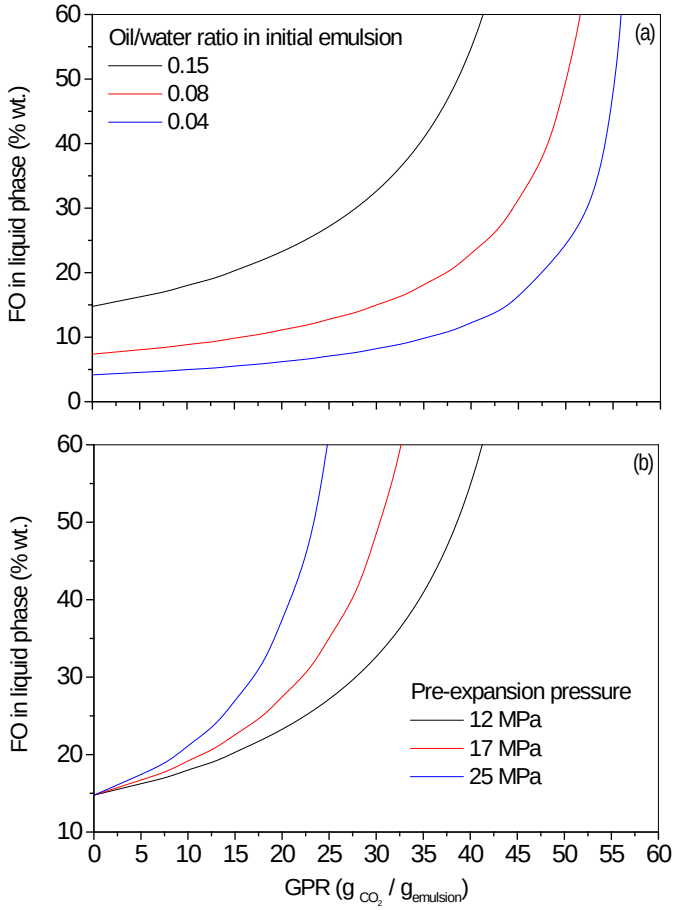

FIGURE 4.14

Theoretical variation of fish oil (FO) content in the liquid phase with the GPR value estimated for (a) different oil / water ratios in the initial emulsion, (b) different pre-expansion pressures. Figure (a) presents the experiments carried out at a constant pressure of 12 MPa whereas Figure (b) presents those at a constant oil/water ratio of 0.15. In both cases, temperature was 343 K.

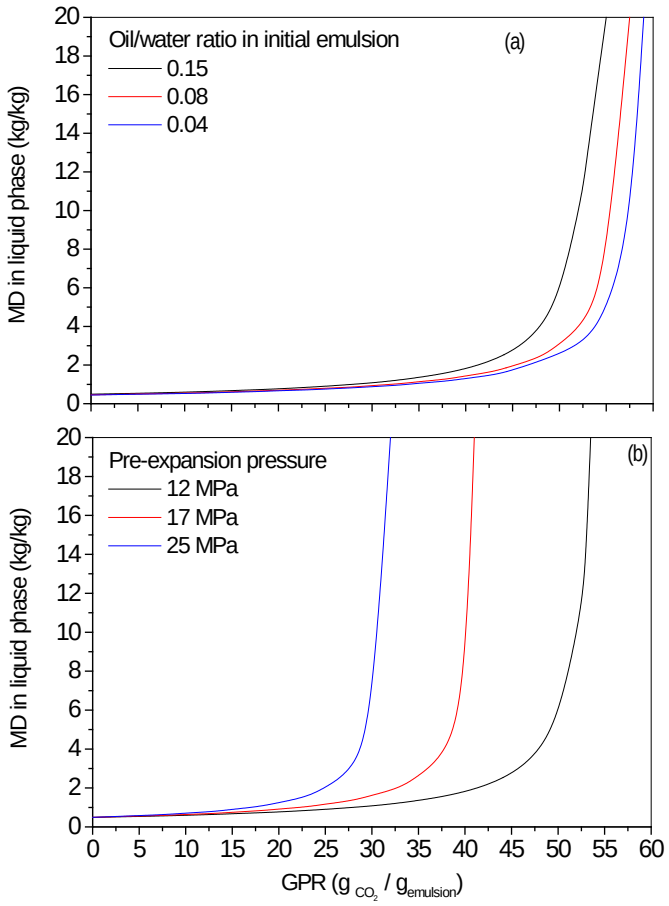

FIGURE 4.15

Theoretical variation of maltodextrin (MD) content in the liquid phase with the GPR value estimated for (a) different oil / water ratios in initial emulsion and (b) different pre-expansion pressures. Figure (a) has been carried out at a constant pressure of 12 MPa whereas Figure (b) has been carried out at a constant oil/water ratio of 0.15. In both cases, temperature was considering 343 K.

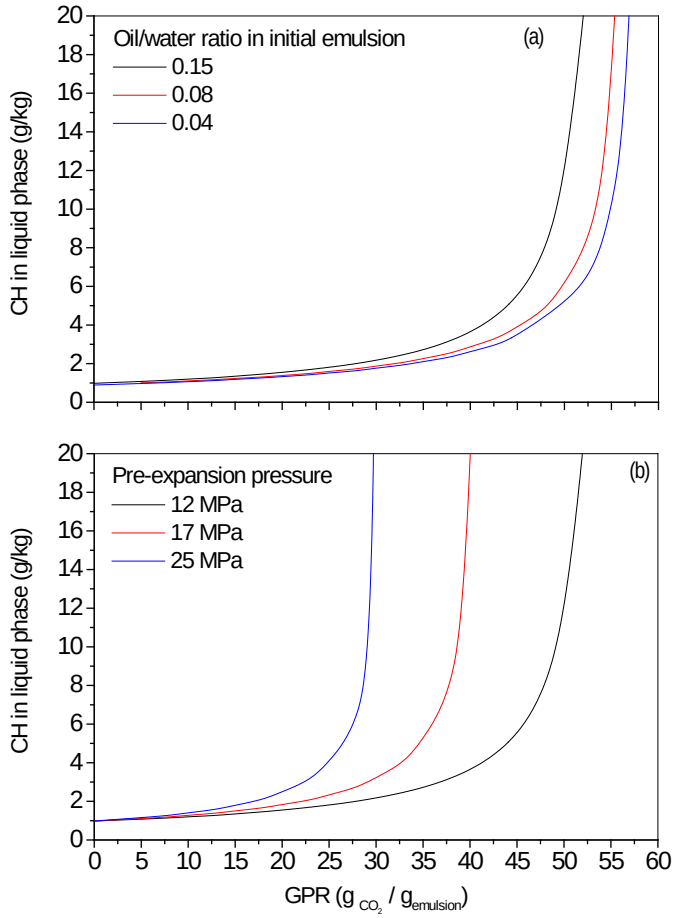

FIGURE 4.16

Theoretical variation of chitosan (CH) content in the liquid phase with the GPR value estimated for (a) different oil / water ratios in initial emulsion and (b) different pre-expansion pressures. Figure (a) has been carried out at a constant pressure of 12 MPa whereas Figure (b) has been carried out at a constant oil/water ratio of 0.15. In both cases, temperature was considering 343 K.

The ratio between the emulsion destabilization and emulsion composition may explain why at the same pressure, in the powder obtained from emulsion A, with an oil/water ratio of 0.19, a spherical microstructure is observed even at high GPR value whereas in the powder obtained from emulsion B, with an oil/water ratio of 0.26, the spherical structure is observed only when GPR is low (around 15), although, in this case, the encapsulation efficiency was lower than the one obtained from emulsion A at the same experimental conditions. On the other hand, the effect of GPR on emulsion stability may explain why in the powder obtained in runs 1-5, 7, 13, 15 & 16, characterised by a spherical microstructure, the amount of non-encapsulated oil, mostly remaining on the particle surface, increases when GPR increases, as it is observed Figure 4.17; The influence of pressure may justify that in Run 10, carried out at 25.7 MPa, a total oil phase separation occurs at the mixer outlet and, consequently, almost all the oil sprayed remains in the spray tower as a sticky solid, whereas the dry powder recovered is composed mostly by maltodextrine.

The use of a static mixer in the mixer chamber is also another variable to take into account in PGSS-drying experiments. Previous studies have reported that working with a static mixture increases the turbulence in the mixer chamber and minimises the effect of mass transfer resistance, making more effective the contact between the CO_2 and the aqueous solution and therefore, increasing the water removal to the gas phase up to reaching the value established by the thermodynamics equilibrium (*Martín, et al.*, 2010b).

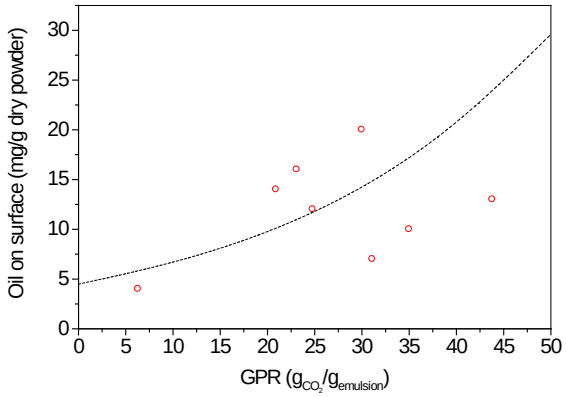

FIGURE 4.17

Influence of GPR on the amount of oil on surface determined in dry powder with predominant spherical microstructure (Runs 1-5, 7, 13, 15 & 16)

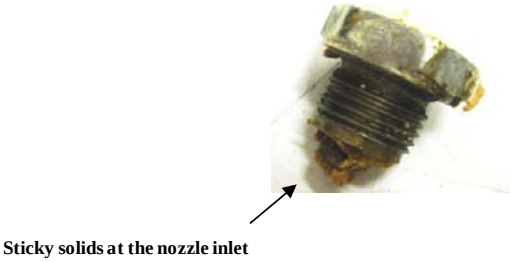

FIGURE 4.18

Clogging effect observed inside the nozzle in Runs 10-12.

This enhancement of mass transfer implies that, in our case, working with static mixer promotes the clogging of the mixer chamber and therefore in

the nozzle, as it was observed after a short time in runs 10-12 (Figure 4.18), increasing the risk of emulsion destabilization, which explains that, even at low pressure and GPR, the powder recovered presents a high amount of non-encapsulated oil (Run 10).

4.1.4.2. Effect of nozzle geometry

In PGSS processes, as in RESS processes, the nozzle has a great importance since it controls the expansion of emulsion-SC-CO_2 mixture from the mixing chamber to the spray tower and, therefore, influences on solid properties such as the particle size.

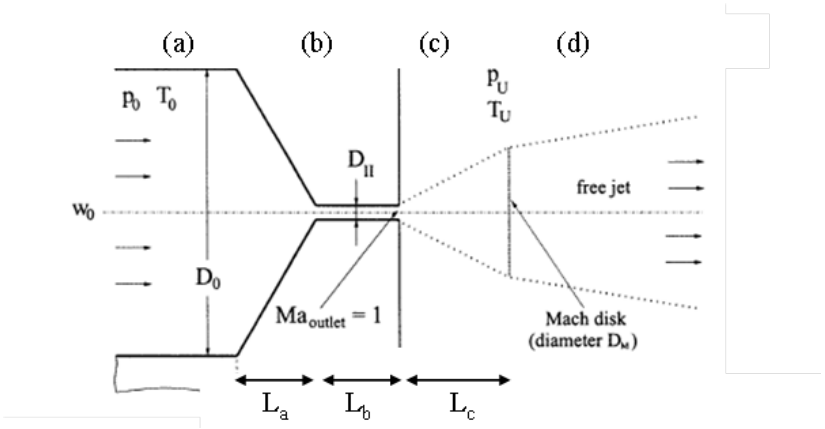

FIGURE 4.19

Scheme of different parts an expansion device. (a) Inlet region; (b) capillary nozzle; (c) supersonic free jet and (d) subsonic free jet. (Adapted from (*Helfgen, et al.*, 2003))

Previous studies have reported that the expansion path of a nozzle can be divided in four different zones: inlet, capillary section, supersonic free jet and subsonic free jet, as it is represented in Figure 4.19, being the capillary section one the factors that plays a greater influence on the particle size of the final solid. It also has been demonstrated that bigger capillary sections involve longer residence times in the nozzle, which is related to the formation of solid particles with a bigger average particle size (*Helfgen, et al.*, 2003)

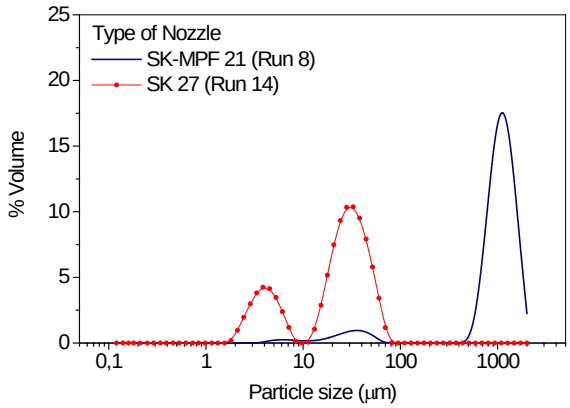

FIGURE 4.20

Particle size distribution found in dry solid particles obtained with a wide nozzle (SK-MPF 21, Run 8) and with a narrow nozzle (SK 27, Run 14).

This phenomenon may explain the different average particle size and particle size distribution observed between run 8, carried out with the

SK-MPF 21 nozzle, and run 14, carried out with the SK 27 nozzle. In both cases, the experimental conditions were similar as well as the solid features (*see* Table 4.3 and Table 4.4), but the use of a nozzle with a triangular path and a wider slot (SK MPF 21) in run 8 may be the main reason why the average particle size in the solid obtained in this case is almost ten times bigger than the average particle size found in the solid obtained in run 14 (*see* Figure 4.20). A similar conclusion may be established comparing the average particle size of the solid obtained in runs 4, carried out with nozzle SK MPF 21, and 7, carried out with the hollow cone nozzle, although, due to the higher agglomeration, deduced from the bimodal particle size distribution, this effect is not so clear (*see* Figure 4.21).

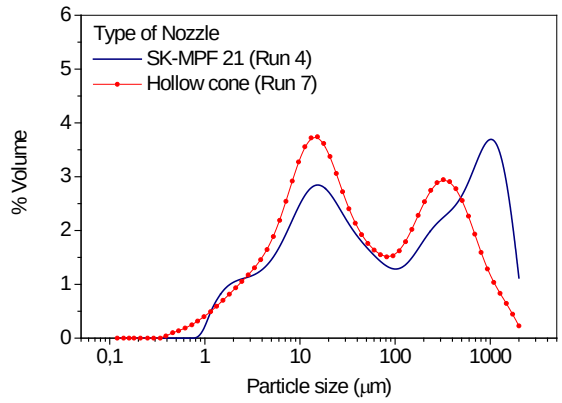

FIGURE 4.21

Particle size distribution found in dry solid particles obtained with a wide nozzle (SK-MPF 21, Run 4) and with a narrow nozzle (hollow cone nozzle, Run 7).

4.1.4.3. Influence of expansion temperature

In PGSS-drying processes, water evaporation is an important step since it promotes the precipitation of the coating material and therefore the solid particle formation. Furthermore, an effective water evaporation is essential to achieve a dry powder and prevent solid agglomeration.

Water evaporation takes places in the spray tower and depends on expansion pressure, expansion temperature and gas-emulsion ratio (GPR). Assuming that at the low pressure in the spray tower (1 atm) the water - CO_2 mixture has an ideal solution behavior, a theoretical temperature-composition phase equilibrium diagram (Figure 4.22) can be estimated according to Raoult's law (4.6), where y_{H2O} and x_{H2O} represent the molar fraction of water in liquid phase and vapor phase respectively, P is the total pressure of the liquid phase (1 atm at ambient conditions) and $P_{v,H2O}$ is the vapor pressure of water which depends on temperature conditions. Taking into account that the vapor pressure of water at T_1 = 373 K is $(P_{v,H2O})_1$ = 1 atm, the vapor pressure $(P_{v,H2O})_2$ at a temperature T_2 can be estimated according to the integrated Clausius-Clayperon equation (4.7), where $\Delta H_{v,H2O}$ is the enthalpy of vaporization for water at 373 K and 1 atm ($\Delta H_{v,H2O}$ = 47.467 kJ / mol), and R is gas constant (R = 8.3145 J / mol K).

$$y_{H_2O} P = x_{H_2O} P_{v,H_2O} \qquad \textbf{(4.6)}$$

$$\frac{(P_{v,H_2O})_1}{(P_{v,H_2O})_2} = \exp\left[\frac{\Delta H_{v,H_2O}}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)\right] \qquad \textbf{(4.7)}$$

Figure 4.22 allows to deduce that particle drying may be possible by working with low gas-emulsion rate (GPR) and increasing temperature in spray-tower or working at low temperature and increasing GPR, providing that the temperature and CO_2 mass percentage is in the single vapour phase region above the dew point curve in which the first drop of water appears.

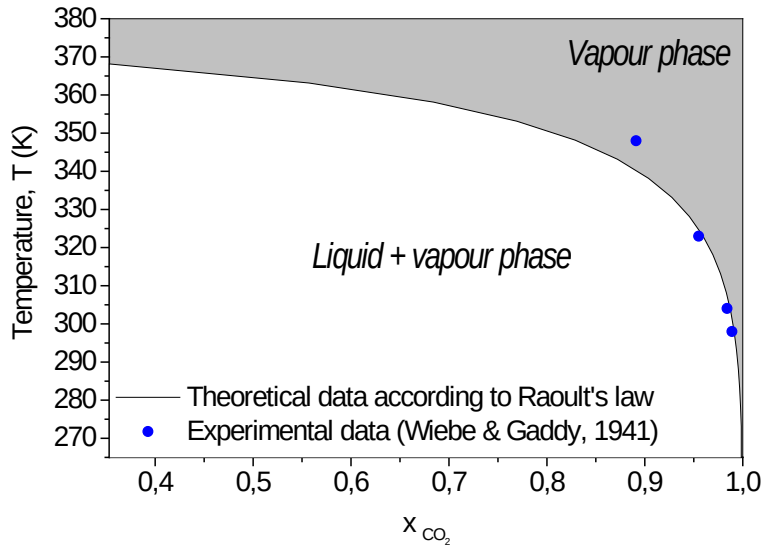

FIGURE 4.22

Phase diagram for CO_2 / water mixtures.

In order to decrease expansion temperature as much as possible and prevent fish oil oxidation, PGSS-drying experiments were developed at a GPR high enough to get a water - CO_2 mixture with more than 95 wt % of CO_2. Thus, according to Figure 4.22 an expansion temperature of 323 K would be enough for complete water evaporation.

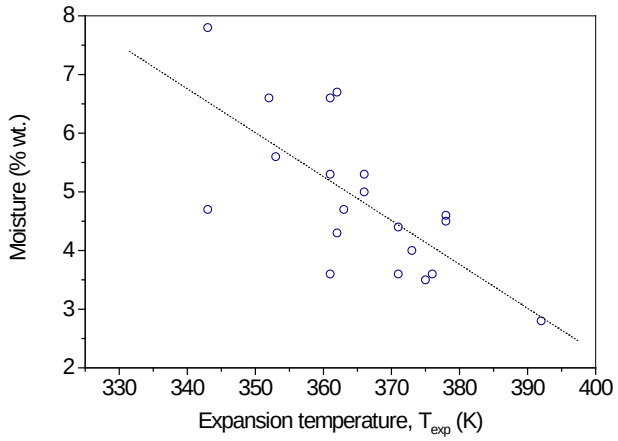

FIGURE 4.23

Moisture content found in the dry powder recovered at different expansion temperatures.

Experimentally, it is observed that residual moisture in powder decreases when expansion temperature increases (*see* Figure 4.23), being around 363 K the temperature required for achieving a residual moisture of around 5 % wt. and more than 390 K the temperature require for reaching a

moisture content less than 3 % wt. The difference between this temperature and the theoretical temperature predicted by the vapor / liquid equilibria is higher than this same difference observed in micronization process by PGSS-drying (*Martín, et al.*, 2010a, *Meterc, et al.*, 2008), which may be attributed to the molecular interaction between water and the other components of the emulsion.

Furthermore, it must be taking into account that theoretical phase equilibria data predicted by Raoult´s law shows a good fit to the experimental data reported by Wiebe & Gaddy (*Wiebe, et al.*, 1941)only at low temperatures and low water content, which are the conditions close to the ideal gas solution; whereas at high temperatures, experimental water solubility in gas CO_2 is slightly lower than the water solubility predicted by the Raoult´s law, which may also explain why the amount of residual water in particles was higher than the expected value.

4.1.4.4. Influence of the hydrophilic character of the primary emulgent

The mixture of emulgents in the oil-in-water multilayer emulsion is another variable that affects the efficiency of the oil encapsulation process since it plays an important role on emulsion properties and stability. The use of chitosan/lecithin mixtures in the formation of secondary emulsions with oil and maltodextrine has an especial interest on the enhancement of encapsulated fish oil stability since, additionally to chitosan, lecithin has also shown a protection effect against oil oxidation (*Judde, et al.*, 2003).

In this work, fish oil encapsulation was carried out by using two different emulsions stabilised with lecithin/chitosan: emulsion C and D.

In Figure 4.13, runs (20) & (21), it is shown the internal microstructure of the particles obtained from emulsion D, which oil:lecithin:chitosan proportion was 1:0.4:0.01. It can be observed that, as it was discussed in Section 4.1.4.1, higher pressures and GPR values promote the formation of a fibrous microstructure (run 21) due to the lower stability of the emulsion; whereas lower pressures and GPR values allows to obtain spherical microstructures (run 20). Nonetheless, even in this case the encapsulation efficiency was quite low, which can be attributed to the highly porous surface responsible forthe high agglomeration observed in Figure 4.13 (20).

On the other hand, Figure 4.13 (19) shows the internal microstructure of the powder obtained in run 19, carried out with lecithin/oil ratio slightly higher (emulsion C). In this case, an amorphous microstructure with large holes is observed in dry powder.

This unexpected microstructure may be explained taking into account that the use of lecithin decreases the stability of the emulsion under SC-CO_2 media due probably to its anionic character, which may affect to the phase behaviour and curvature of the surfactant layer.

Previous studies, reviewed recently by Johnston et al., (*Johnston, et al.*, 2009), have shown that the surfactant curvature under SC-CO_2 media depends on the interaction energy (Aij) between the surfactant tail (S_T),

surfactant head-group (S_H), water (W) and CO_2 (C) according to the hydrophilic-CO_2-philic balance (HCB) of the surfactant (Eq. (4.8)).

$$\frac{1}{HCB} = \frac{A_{S_TC} - A_{S_TS_T} - A_{CC}}{A_{S_HW} - A_{S_HS_H} - A_{WW}} \qquad \textbf{(4.8)}$$

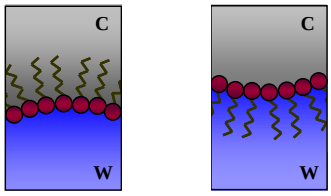

FIGURE 4.24

Scheme of the variation of the curvature of the emulsifier layer depending on the HCB value, and the formation of an water-in-CO_2 emulsion, W/C, (left) or a CO2-in-water emulsion, C/W (right) (*Johnston, et al.*, 2009).

Thus, as it is represented in Figure 4.23, if surfactant has a CO_2-philic character, such as Tween 80, the balance 1 / HCB > 1, which means that the interface is concave with respect to water phase and the resulting mixture is a water-in-CO_2 emulsion (W/C). On the contrary, if the surfactant has a hydrophilic character, such as lecithin, the balance 1 / HCB < 1, which

involves that the interface is concave with respect to CO_2 and the resulting mixture is a CO_2-in-water emulsion (C/W).

This change in the curvature of the surfactant layer, and the subsequent emulsion destabilization, when an non-ionic emulgent such as Tween 80 is changed by an ionic one, such as lecithin, keeping constant the rest of the process variables, may explain why the powder obtained in run 20 is made of an agglomeration of small porous spheres, which lead to a poor encapsulation efficiency, or why the powder obtained in run 21, carried out at higher GPR, is characterised by a fibrous microstructure composed mostly by maltodextrine. Furthermore, an excess of the ionic emulgent in the emulsion may promote the emulsion inversion and the formation of a CO_2-in-water at the mixer outlet, which subsequent expansion leads to the formation of big holes in the dry solid, as it is observed in the powder obtained in run 19.

4.1.5. Conclusions

Fish oil encapsulation in a maltodextrine coating has been carried out by PGSS-drying, using different multilayer oil-in-water emulsions stabilized with a mixture of non-ionic/cationic emulgents (Tween 80 / chitosan) and anionic/cationic emulgents (lecithin / chitosan).

Fish oil encapsulation by PGSS-drying is a complex process, which efficiency is strongly depended on the emulsion stability along the mixer chamber, the atomization mechanism and the vapour/liquid equilibria of the

CO_2 / water mixture in the drying chamber. Emulsion stability along the mixer is related to the phase behaviour of the CO_2 / emulsion mixture, and therefore to pre-expansion pressure, pre-expansion temperature and the gas-emulsion ratio (GPR). At a constant temperature, it has been observed that low pre-expansion pressures and low GPR values allows to obtain spherical microstructures and improve encapsulation efficiency, especially when the oil / water ratio in the initial emulsion is low and a non-ionic biopolymer is used as primary emulgent, which may be related to a higher emulsion stability at the mixer outlet. On the contrary, amorphous and porous microcapsules, with very low oil encapsulation efficiency, were mostly observed in the dry solid obtained from an emulsion with a higher oil/water ratio, especially at higher pre-pressures and GPR values, which may be attributed to a phenomena of oil drop flocculation or even oil coalescence in the liquid phase as a consequence of a higher amount of water removed to the gas phase. The amount of water removed from the emulsion to the gas phase can be also increased improving the contact between CO_2 and emulsion trough a static mixing, promoting oil phase separation and solid precipitation in the mixer chamber. Emulsion atomization is controlled by the pressure depletion between the mixer and the expansion chamber and by the nozzle design. At the same experimental conditions, it has been observed that a nozzle with a quadratic path and a narrow slot diameter allows to obtain smaller particle size. Solid drying is affected by the liquid/vapour equilibria of a water / CO_2 mixture, which depends on the expansion pressure, the expansion temperature and the GPR

value. In this case, keeping constant the expansion pressure (1 atm) and working with a high CO_2 / water ratio, a high temperature of 363 K was required for achieving a moisture content less than 3 % wt. This high temperature, compared to the temperature required in a conventional micronization process by PGSS-drying, may be attributed to the molecular interaction between water and the other components of the emulsion. Finally, the use of an emulsion stabilized by lecithin/chitosan was also studied, showing worse results in both particle morphology and oil encapsulation efficiency. These results may be attributed to the poorer stability of these emulsions under SC-CO_2 due to the anionic character of the lecithin and therefore to higher affinity for the water phase.

As a final conclusion, PGSS-drying may be a valuable process to produce fish oil microcapsules of fish oil in a coating of maltodextrine / chitosan from fish oil-in-water emulsion. Nonetheless, further studies about properties of oil-in-water multilayer emulsions and their stability under SC-CO_2 will be required in order to get a better comprehension of the PGSS-drying encapsulation process, and to optimise encapsulation efficiency and yield. Moreover, a subsequent study about the stability of encapsulated oil will be required in order to test if the coating material formed in the PGSS-drying process is suitable enough to protect fish oil against oxidation and increase its shelf life.

Finally, a comparison among encapsulation efficiency and particle features (moisture, average particle size and distribution, morphology) obtained by

both PGSS-drying and conventional microencapsulation processes, mainly spray-drying, must be taking into account in order to establish the profitability and competitiveness of the PGSS-drying process and its implementation on large scale.

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Final Remarks
&
Future Challenges

GENERAL CONCLUSIONS

This work has been focused on the use of supercritical fluid technologies in order to obtain lipid extracts with a high content of omega-3 fatty acids and formulated as dry powder from fish by-products.

From the different experimental studies carried out in this project, the following general conclusions can be established:

- Fish off-cuts from salmon or hake may be a valuable raw material for obtaining quality fish oil with a high amount of omega-3 fatty acids and a low content of impurities such as free fatty acids (FFA), cholesterol or wax esters.
- Supercritical fluid extraction with CO_2 may be an attractive process to obtain fish oil from fish by-products, being the optimal extraction conditions, in a semi-pilot plant, 25 MPa, 313 K and around 175 kg CO_2 /h kg raw material. The main drawback of the process is the previous freeze-drying step required to remove the water from the raw material. However, against conventional physical and chemical processes, it has the advantage of minimising fish oil oxidation, as a consequence of the use of moderate temperatures and a non-oxidising atmosphere; and reducing the amount of heavy metals, especially arsenic, due to the selectivity of SC-CO_2 for extracting non-polar compounds.
- The coupled extraction-fractionation process with SC-CO_2 allows to reduce the amount of fish oil impurities, mainly volatile compounds and FFA, being an alternative method to the conventional physical and chemical refining procedures.
- The coupled extraction-enzymatic reaction-fractionationation process in SC-CO_2 media allows to increase slightly the amount of omega-3 fatty acids in fish oil, mainly DHA, keeping the natural form of AG. This method may be a valuable alternative to conventional processes that concentrate omega-3 as ethyl esters (EE).

- PGSS-drying technology, based on the use of SC-CO_2, allows to obtain fish oil microencapsulated in a solid matrix, such as maltodextrine / chitosan, similar than those used in conventional microencapsulation processes. It has been observed that the efficiency of fish oil microencapsulation by PGSS-drying is strongly depended on a wide range of factors such as emulsion stability along the mixing chamber, the atomization mechanism and the vapour / liquid equilibria of the CO_2 / water mixture in the drying chamber. Thus, the optimization of these process parameters is the key for making the PGSS-drying a competitive method for producing microencapsulated fish oil against the current spray-drying.

Future challenges proposed are:

- Scaling-up the supercritical fish oil extraction process in order to increase the oil production volume.
- Improving the refining procedure by working with a new separator in series, in order to achieve a better lipid fractionation after fish oil extraction.
- Enhancing the reaction rate of the enzymatic hydrolysis by increasing the amount of oil in the reaction media, which may be achieve carrying out the enzymatic reaction in dense gas instead of in supercritical fluid media (*see* Figure 4.12).
- Performing an enzymatic ethanolysis as alternative to enzymatic hydrolysis in order to improve the efficiency of the omega-3 concentration process since, according to its solubility in SC-CO_2, 2-AG are expected to be better isolated from EE than from FFA (*see* Figure 3.1).
- Studing the phase behavior of fish oil – in - water emulsion under SC-CO_2 in order to get a better comprehension of the emulsion stability in a PGSS-drying process and be able to optimize the microencapsulation method.

CONCLUSIONES GENERALES

El presente trabajo se ha basado en el uso de la tecnología de fluidos supercríticos para la obtención de extractos lipídicos con un alto contenido en ácidos omega-3 y formulados en polvo a partir de subproductos de la pesca.

Como conclusiones generales del trabajo, se puede decir que:

- Las pieles de pescados como salmón o merluza pueden ser una materia prima rentable para la obtención de aceite de pescado de calidad con un alto contenido en omega-3 y un bajo nivel de impurezas como ácidos grasos libres (AGL), colesterol o ceras.
- La extracción con dióxido de carbono supercrítico, CO_2-SC, puede ser un proceso atractivo para extraer aceite de pescado a partir de subproductos, siendo las condiciones óptimas de extracción, para una planta semi-piloto, 25 MPa, 313 K y 175 kg CO_2 /h kg materia prima. El principal inconveniente del proceso es la etapa previa de liofilización necesaria para reducir el contenido en agua de la materia prima. Sin embargo, frente a los métodos de extracción físico-químicos convencionales, tiene la ventaja de minimizar la oxidación del aceite de pescado, gracias al uso de temperaturas moderadas y de una atmósfera no oxidante; y de reducir el contenido en metales pesados, especialmente arsénico, debido a la selectividad del CO_2-SC en la extracción de compuestos no polares.
- El proceso combinado de extracción – fraccionamiento con CO_2-SC permite reducir el contenido de impurezas en el aceite de pescado, especialmente AGL y compuestos volátiles, siendo una vía alternativa a procesos de refinado físico-químicos convencionales.
- El proceso en continuo de reacción enzimática -fraccionamiento en medio CO_2-SC permite obtener a partir de aceite de pescado una fracción lipídica enriquecida en ácidos omega-3, principalmente DHA, en forma de 2-acilglicéridos (2-AG), siendo

una alternativa novedosa a los métodos convencionales basados en la obtención de concentrados de omega-3 en forma de ésteres etílicos (EE).

- La tecnología de PGSS-drying permite microencapsular aceite en matrices sólidas similares a las utilizadas en los métodos convencionales de microencapsulación. La eficacia de este proceso de microencapsulación depende de una gran variedad de factores (estabilidad de la emulsión a lo largo de la cámara de mezcla, mecanismo de atomización, equilibrio líquido / vapor de la mezcla CO_2 / agua en la torre de secado…). Por lo tanto, la optimización de estos parámetros de proceso es la clave para conseguir que la obtención de aceite de pescado microencapsulado por PGSS-drying sea una vía competitiva frente al método actual de secado por pulverización (spray-drying)

Como retos futuros se propone:

- El escalado del proceso de extracción de aceite de pescado con CO_2-SC con el objetivo de aumentar el volumen de producción de aceite.
- La mejora del proceso de refinado trabajando con un nuevo separador en serie, lo cual permitiría un fraccionamiento más efectivo después de la extracción del aceite.
- Aumentar la velocidad de la reacción enzimática aumentando la cantidad de aceite en el medio, lo cual se podría conseguir trabajando con CO_2 disuelto en el aceite (medio expandido) en lugar de como disolvente del aceite (medio supercrítico) (*ver* Figura 4.12).
- Llevar a cabo la etanólisis enzimática como alternativa a la hidrólisis para mejorar la eficacia del proceso de concentrado de omega-3, ya que, según la solubilidad en CO_2-SC, los 2-AG se separarían más fácilmente de los EE que de los AGL (*ver* Figura 3.1).

- Estudiar el comportamiento de la emulsión aceite de pescado-agua en medio CO_2-SC como vía para poder optimizar la eficacia de la microencapsulación del aceite de pescado por PGSS-drying.

Analytical Methods for Characterization of Fish by-products, Fish Oils & Microcapsules

Appendix I

TABLE OF CONTENTS

NOMENCLATURE

AcOH	Acetic Acid
AOAC	The Association of Official Analytical Chemists
AV	Anisidine Value
b.p.	Boling poin
BP	British Pharmacopeia
BSE	Back-scattered electrons
CHOL	Cholesterol
$d_{0.5}$	Volume median diameter (diameter where 50% of the particle size distribution is above and 50% is below)
DAG	Diacylglycerides
FAME	Fatty Acid Methyl Ester
FFA	Free fatty acid content (% oleic acid)
FT-IR	Fourier Transform - Infrared
GC-FID	Gas Chromatography - Flame Ionization Detector
GC-MS	Gas Chromatography - Mass Spectrophotometry detector
HPLC-ELSD	High Pressure Liquid Chromatography - Evaporative Light Scattering Detector
ICP-MS	Inductively coupled plasma mass spectroscopy
IS	Internal Standard
ISO	International Organization for Standardization
IUPAC	The International Union of Pure and Applied Chemistry
MAD	Malonaldehyde
MAG	Monoacylglycerides
MTBE	methyl tert-butyl ether
MUFA	Monounsaturated Fatty Acids
PCA	Principal Components Analysis
PDMS-AC	Polydimethyl- siloxane monoacetate

PUFA	Polyunsaturated Fatty Acids
PV	Peroxide Value (meq O_2 / kg oil)
SEM	Scanning electron microscopy
SFA	Saturated Fatty Acids
SPDE	Solid Phase Dynamic Extraction
TAG	Triacylglycerides
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Tricloroacetic Acid
TEP	Tetraetoxipropane
UV-Vis	Ultraviolet-visible
WE	Wax ester

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I.1. Characterization of Fish By-products

➢ **Section Content**

I.1.1. Determination of Moisture Content

Moisture content, defined as the loss in mass of the sample on heating at 105 °C, was determined by the AOAC Official Methods 934.01 (*AOCS*, 1990).

I.1.1.1. Material and equipment

Moisture content was measured by drying sample in a oven. Samples were place in covered aluminium dishes (64 mm diameter, 44 mm depth). Weight was measured in an analytical balance (± 0.0001 g).

I.1.1.2. Analytical procedure

An amount of sample (3 to 6 g) was weighed into a pre-weighed covered aluminium dish and dried for 16 - 18 hours at 100 – 105 °C in an oven. After that, it was cooled to room temperature in a desiccator and weighed again. The determination was carried out in triplicate.

I.1.1.3. Calculations

Moisture content, expressed as % wt. of raw material, is determined according to Eq.(I.1) where w_o is the weight of the empty covered aluminium dish; w_1 is the weight of dish + sample before drying and w_2 is the weight of dish + sample after drying.

$$\%\text{moisture} = \frac{m_1}{m_o} = \frac{(w_2 - w_1)}{(w_1 - w_o)} \cdot 100 \qquad \textbf{(I.1)}$$

I.1.2. Determination of fat content by Soxhlet extraction

Fat in fish by products has been carried out by Soxhlet extraction according to the AOAC Official Method 991.36 (*AOAC*, 2000).

This method allows to determine fat and oil from raw materials taking into account the high solubility of neutral lipids in organic solvents with low polarity and boiling point, such as petroleum ether. In order to improve the extraction efficiency, solid samples are previously dried.

The soxhlet extraction procedure is based on a continuous extraction of fat from a solid raw material using a warm organic solvent.

Continuous solvent extraction is carried out by solvent reflux in a Soxhlet extraction device (*see* Figure I. 1.a) in which the solvent is evaporated from a vessel, cooled in a condenser and recovered in the extraction thimble where solid sample is placed. As the warm solvent drips back down it fills the extraction thimble, removes a fraction of fat from the solid sample and,

as soon as it reaches a maximum level, the solvent, loaded in fat, runs back down to the vessel by a siphon side arm, completing an extraction cycle. Soxhlet extraction finishes when, after a series of several cycles, the remaining fat in the solid is negligible.

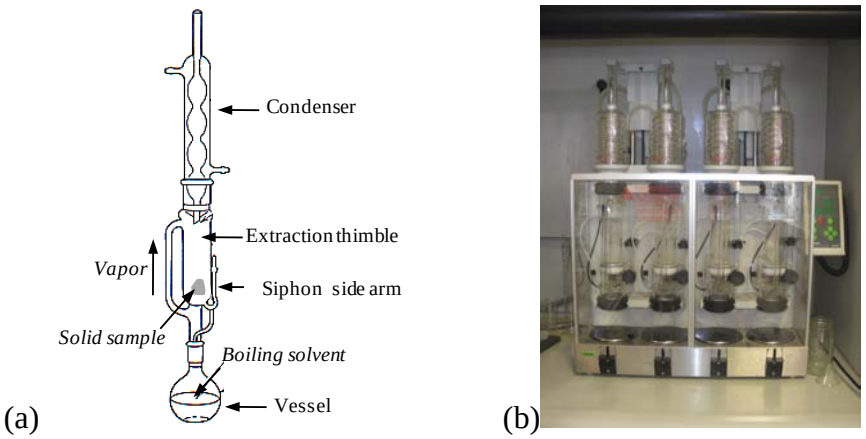


FIGURE I. 1

(a) Conventional soxhlet extraction apparatus. (b) Automatic Soxhlet extractor (Büchi extraction system B-811)

The number of extraction cycles, and therefore the analysis time, may be noticeably reduced by in an automatic soxhlet extraction apparatus, especially if the extraction thimble is continuously warmed.

I.1.2.1. Material and equipment

Soxhlet extractions were performed in an automatic soxhlet extraction equipment (Büchi extraction system B-811) (*see* Figure I. 1. b).

I.1.2.2. Reagents and solutions

Oil extraction was carried out with petroleum ether (b.p. 40 / 60 ºC)

TABLE I. 1

Warm extraction program performed for fat analysis in automatic soxhlet equipment Buchi B-811.

Stage	Time (min)	Heating level	
		Vessel	Extraction thimble
Extraction	120	9	2
Rinsing	10	9	---
Drying	10	2	---

Established to petroleum ether in the manual of the equipment

I.1.2.3. Sample preparation

All the samples were cut and dried in an oven as reported in the method for determination of moisture content.

I.1.2.4. Analytical procedure

After moisture content analysis, the dried sample was wrapped up in the covered aluminium dish and put inside the extraction chamber of the soxhlet extractor. The glass vessel, previously weighed, was filled with 120 mL of petroleum ether and hold in the extraction device. Fat extractions were performed in "warm program" which allows to warm both the solvent vessel and the extraction thimble. The total time for the analysis was up to 140 min distributed in three stages: extraction, rinsing and drying (*see* Table I. 1). After finishing the extraction program, the glass vessels with the fat removed were cooled down to room temperature and weighed.

I.1.2.5. Calculations

Fat content, expressed as % wt. of dry raw material is determined according to Eq. (I.2), where V_1 is the weight of the empty glass vessel; V_2 is the weight of the glass vessel + fat and m is m_o, the total amount of sample, if fat content is expressed as % total wt., or m_1, the weight of the dry sample, if fat is expressed as % wt. of dried material.

$$\%\text{fat} = \frac{(V_2 - V_1)}{m} \cdot 100 \qquad \textbf{(I.2)}$$

I.1.3. Determination of heavy metals content

The amount of heavy metals in oil samples was determined by ICP-MS previous wet digestion in a microwave oven.

I.1.3.1. Material and equipment

Measurements of heavy metals were ordered to the R&D laboratory of Burgos University which is responsible of an ICP-MS equipment (Agilent 7500 i) and of a microwave oven (Ethos Sel, Milestone) provided with ten Teflon vessels (HPR-1000/10 S).

I.1.3.2. Reagents

Wet digestion was carried out with nitric acid, HNO_3 65 % suprapur® (Merck, Germany). Standard solutions of heavy metals (Fe, Zn, Cu, Cd, Hg, Pb & As) were also use for calibration.

I.1.3.3. Experimental procedure

i) Sample digestion

A wet digestion was carried out over the samples in order to destroy the organic matter. About 20 mg of sample were treated with 10 mL of HNO_3 65 % suprapur® (Merck, Germany) in a microwave oven. The temperature program selected involved three heating steps (from room temperature to 80 ºC in 4 min, from 80 ºC to 130 ºC in 7 min and from 130 ºC to 170 ºC in 5 min) followed by a constant heating at 170 ºC for 10 min and a final ventilation step.

ii) Heavy metal measurement

After digestion, the samples were diluted to 25 mL with Milli-Q water, and measured by ICP-MS (Agilent 7500 i).

I.1.3.4. Calculations

The amount of heavy metals in sample was determined from the calibration curve. Results are reported in ppm (mg metal / kg oil).

I.2. Characterization of Fish Oil and Omega-3 Concentrates

➢ **Section Content**

I.2.1. Determination of moisture and total volatile matter

The moisture and total volatile matter content in oil is defined as the loss in mass of the sample on heating at 105 °C.

Measurement of moisture and volatile content in fish oil was carried according to IUPAC standard method (*IUPAC*, 1964). This procedure is similar to the gravimetrical method described in Section I.1.1.

I.2.2. Determination of free fatty acid content

Free fatty acid content is defined as the fraction of fatty acids which is not attached to other molecules as in acylglycerides, wax esters or phospholipids.

This parameter is related to the oil rancidity since free fatty acids came usually from the hydrolysis of acylglycerides due to the moisture, temperature and / or the action of lipases.

Depending on sample availability, the free fatty acid content was determined either by acid-basic titration or by a colorimetric method.

I.2.2.1. Titration Method

In this method, the free fatty acid content or acid value is determined from the amount of potassium hydroxide required to neutralize one gram of fat. This procedure has been the one used to determine FFA content in fish oil characterization.

i) Material

The main material required were Erlenmeyer flaskes (10 mL) and a glass burette (10 mL)

ii) Reagents and solutions

The reagents and solutions required were:

Ethanol-ethyl ether mixture (1:1) used as oil solvent.

Phenolphthalein solution (1 g of phenolphthalein dissolved in 100 mL of ethyl alcohol), used as an acid-basic indicator for a pH range of 8.3 - 10.0.

Standard alcoholic potassium hydroxide solution 0.1 N, used as basic titrator. This solution is prepared dissolving should 6.6 g of KOH (85 % wt.) in 1 l of ethanol until colourless and stored in a brown glass bottle before use.

iii)Experimental procedure

Accurately 1 to 2 g of cooled oil sample was weighed in a 10 mL Erlenmeyer flask and dissolved in 5 mL of the ethanol-ethyl ether (1:1) mixture. Then, 3 drops of phenolphthalein indicator solution were added to the mixture and titration was performed against standard aqueous potassium hydroxide 0.1 N, shaking vigorously up to a persisting pink colour appears.

iv) Calculations

Total free fatty acid content, expressed as % wt. of oleic acid, is calculated according to Eq. (I.3), where V and N are the volume in mL and the normality of the standard potassium hydroxide solution, and w is the weight of the oil sample.

$$\%FFA(oleicacid) = \frac{V \cdot N \cdot 282{,}5}{10 \cdot w} \qquad \textbf{(I.3)}$$

I.2.2.2. Colorimetric method

This method, developed by Kwon & Rhee (*Kwon, et al.*, 1986), is based on the colour change that is produced when fatty acids (FFA) are converted to copper soaps by the reaction with a cupric acetate-pyridine. Since it requires a lower amount of sample than the titration method, it was chosen for measuring the FFA content in the hydrolysated oil and therefore for determining the enzymatic activity of a lipase.

i) Material and equipment

Measures were carried out in a spectrophotometer.

ii) Reagents and solutions

The required reagents were:

Cupric acetat-pyridine reagent, used as both cupper and colour reagent for FFA determination. It was prepared by filtering an aqueous solution of cupric acetate (5 % w/v) and adjusting its pH value to 6.1 by adding pyridine.

Isooctane, used as lipid solvent.

iii)Standard solution and calibration samples

Standard solutions were prepared dissolving different amounts of oleic acid standard (2.0 - 50.0 μmol) in 5 mL of isooctane. Calibration samples were prepared by mixing each standard solution with 1.0 mL of cupric-acetate

reagent in a vortex and, when the separation between aqueous and organic phases was clear, isolating the upper organic layer which contains the FFA.

iv) Oil samples pre-treatment

Oil samples were prepared by dissolving a total amount of 10 - 20 mg of oil or hydrolysate sample in 5 mL of isooctane, mixing this solution with 1.0 mL of cupric-acetate reagent in a vortex and taking the upper organic layer.

v) Experimental procedure and calculations

After making the calibration curve by measuring the absorbance of the different calibration samples at 715 nm, the absorbance upper organic layer resulting from oil sample was measured at the same wave length. The resulting calibration curve presented a linear relation among the absorbance, A715, and the concentration of oleic acid, c (μmol / 5 mL isooctane) as it is in Eq.(I.4). The amount of FFA, expressed as mol of palmitic acid per g of oil sample, is calculated according to Eq. (I.4), where m' is the amount of oleic acid estimated from the calibration curve, in mol, and m is the amount of oil sample, in g.

$$[\mathrm{FFA}]_{\mathrm{sample}} = \frac{m'}{m} \qquad \textbf{(I.4)}$$

I.2.3. Determination of peroxide value (PV)

Peroxide value (PV) is a measure of the hydroperoxides compounds generated in the earlier stages of the oil oxidation process, which can be determined indirectly by measuring the amount of iodine, I_2, liberated when the hydroperoxides, ROOH, contained in oil reacts with iodine ion, I^- (I.5).

$$2\ I^- + H_2O + ROOH \rightarrow ROH + 2\ OH^- + I_2 \quad \textbf{(I.5)}$$

In this work, the I_2 generated in oil samples was measured by titration with sodium thiosulphate, $Na_2S_4O_6$, according to AOCS Official Method Cd 8-53 (*AOCS*, 1990)

I.2.3.1. Materials

The main material required was Erlenmeyer flaskes (250 mL) and a glass burette (25 mL)

I.2.3.2. Reagents and solutions

The reagents and solutions required were: chloroform and glacial acetic acid, used for oil dissolving; fresh saturated aqueous solution of potassium iodide (KI), aqueous solution of sodium thiosulphate (0.01 N), used as titrator; an aqueous solution of almidon (10 g / L), used as indicator redox.

I.2.3.3. Procedure

A certain amount of oil (2 g) was weighed in an Erlenmeyer flask and dissolved in 10 mL of chloroform. Then, 150 mL of glacial acetic acid and 1 mL of saturated KI aqueous solution were added. The mixture was agitated during 1 minute and then stored preserved of light during 5 minutes more. After that, the mixture was diluted by adding 75 mL of distilled water and 3 drops of almidon indicator solution, and immediately titrated with $Na_2S_4O_6$ up solution became violet.

I.2.3.4. Calculations

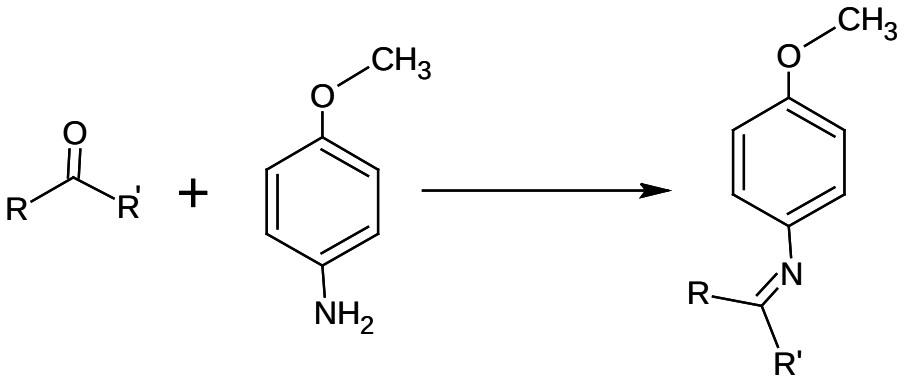
The peroxide value, expressed as miliequivalent of O_2 per kg of sample, was determined according to Eq. (I.6), where V is the volume of $Na_2S_4O_6$ solution in mL, N in the normality of Na2S4O6 solution and m is the weight of oil in g.

$$PV = \frac{V \cdot N}{m} \cdot 1000 \qquad \textbf{(I.6)}$$

I.2.4. Determination of anisidine value

The anisidine value (AV) is a measure of the concentration of non-volatile carbonyl compounds, mainly 2-alkenals, which are secondary products of the oil oxidation. In this work, AV has been measured according to the method developed by the British Pharmacopoeia Commission, (*BP*, 2008), which is based on the reductive amination reaction that takes places between

the carbonyl group of the 2-alkenal and the amine group of p-anisidine (p-methoxyaniline). This reaction leads to the formation of a Schiff base or imine (Figure I. 2), which presents a high absorption at 350 nm.



carbonyl compound *p-anisidine* *Schiff base or imine*

FIGURE I. 2

Chemical reaction between a carbonyl compound and p-anisidine.

I.2.4.1. Material and equipment

Absorbance measurements were carried out in a UV-Vis spectrophotometer.

I.2.4.2. Reagents and solutions

The reagents required were hexane, used as oil solvent; an a solution of p-anidisina (0.25% w/v in glacial acetic acid).

I.2.4.3. Experimental procedure

A sample solution (A) was prepared by dissolving 0.5 g of oil in 25 mL of hexane. The absorbance of this solution was measured at 350 nm using hexane as blank solution. Then, solution B was prepared by mixing 5 mL of solution A with 1 mL of p-anisidine solution (0.25 w / v in glacial acetic acid), shaked and stored protected from light. The absorbance of this solution was also measured at 350 nm using a mixture of 5 mL hexane and 1 mL of p-anisidine solution as blank.

I.2.4.4. Calculations

Ansidine value (AV) is determined according to Eq. (I.7) where A_A and A_B are the absorbances of sample A and sample B respectively, and m is the weight of oil sample.

$$AV = \frac{(1{,}2A_B - A_A)}{m} \cdot 25 \qquad \textbf{(I.7)}$$

I.2.5. Analysis of Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric acid reactive substances (TBARs) are related to those compounds formed during lipid perodixation that reacts with 2-thiobarbituric acid (TBA) and, therefore, are an indicator the oxidative stress of lipids.

The method described is based on the official method 19.90 established by the AOAC (*AOAC*, 2000).

I.2.5.1. Materials and equipment

The main lab material required was Eppendorf plastic tubes (25 mL) for centrifuging and PYREX® glass tubes (25 mL) with leak-tight and Teflon-lined screw caps.

Centrifuging was carried out in a centrifuge (Eppendorf Centrifuge 5804) provided by an Eppendorf Fixed-angle Rotor F-34-6-38 with a radius of 115 mm.

Absorbance of the samples were measured in UV-Vis Spectrophotometer

Vortex and water bath were also required for mixing and heating samples.

I.2.5.2. Reagents and solutions

The main reagents used were cyclohexane for oil dissolving and an aqueous solution of thiobarbituric acid (TBA) / tricloroacetic acid (TCA) (7.5 % w / v TCA / 0.34 % w / v TBA). In order to aim the complete TBA dissolving in distilled water a few amount of sodium hydroxide, NaOH, was added.

I.2.5.3. Experimental procedure

i) Calibration procedure

The calibration method was carried out by using tetraetoxipropane (TEP) as standard compound. A primary solution with a TEP concentration of 0.5 mg / mL, was prepared by dissolving 25 mg of TEP (97 % w / w) in 50 mL of sulphuric acid, H_2SO_4 (1 % w / v). A first calibration solution, STD-6, with a concentration of 40 µg/mL, was prepared diluting 2 mL of solution A in 25 mL of H_2SO_4 (1 % w/v). The rest of calibration solutions were preparing by diluting subsequently 10 mL of the previous solution in 25 mL of H_2SO_4 (1 % w / v). The final TEP concentration in each calibration solution is reported in Table I. 2.

TABLE I. 2

Calibration solutions of tetraetoxipropane (TEP) for TBARs quantification.

Calibration solution	Initial TEP concentration (µg/mL)	TEP concentration after reaction, c_{TEP} (µg/mL)
STD-5	41.44	7.5
STD-4	16.58	3.0
STD-3	6.63	1.2
STD-2	2.65	0.5
STD-1	1.06	0.2
STD-0	0.00	0.0

Calibration procedure was carried out by taking 1 mL of each calibration solution in a plastic Eppendorf tube with 3.5 mL of cyclohexane and 5.5 mL of TCA-TBA mixture, and, after agitating in a vortex, centrifuging at 805 x g (2500 r.p.m.) during 15 minutes. After that, the upper organic layer was removed and the bottom aqueous layer was transferred to a PYREX® glass tube and heated at 100 ºC during 10 minutes, observing the appearance of pink colour. Finally, the absorbance of each solution was measured at 532 nm. This absorbance was related according to the Lambert-Beer's law to the concentration of TEP, cTEP, in the aqueous phase resulting after reaction with TCA-TBA. The optimal absorbance range (0.3-0.8) was obtained experimentally for a concentration range between 0.5 and 1.2 μg TEP / mL (*see* Figure I. 3).

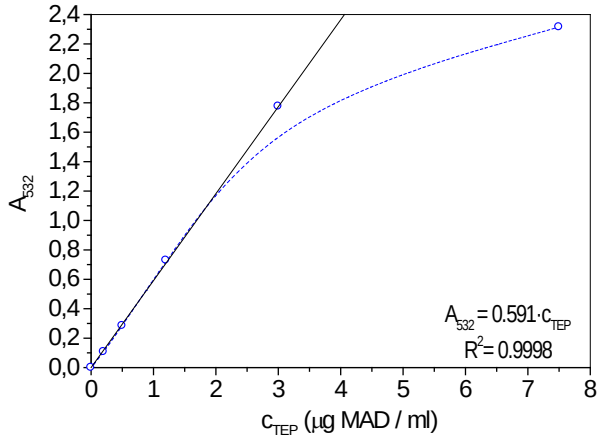


FIGURE I. 3

Calibration curve for TBARs determination. A_{532} = 0.591; c_{TEP}; R^2= 0.9998

ii) Sample assay

The determination of TBARs in oil samples was carried out by weighing 0.1 g of oil in a plastic Eppendorf tube and following the same procedure described in section I.2.5.3 for preparing the calibration curve. If necessary, the aqueous solution after reaction with TCA-TBA was diluted in order to maintain the sample absorbance within the optimal range for the minimal error.

I.2.5.4. Calculation

The amount of TBARs in oil, expressed as $\mu g_{\text{malonaldehyde}}$ (MAD) / kg, is calculated according to Eq. (I.8)), where c_{TPE} is the concentration of oil sample solution, in μg / mL, determined from calibration curve, V is the volume of TCA-TBA aqueous solution (5.5 mL), f_x is the dilution factor, and m is the weight of oil sample in g.

$$\text{TBARs} = 1000 \cdot \frac{c_{TPE} \cdot V \cdot f_x}{m} \qquad \textbf{(I.8)}$$

I.2.6. Determination of trace metal content

The amount of trace metal (Fe, Cu, Zn, As, Cd, Hg and Pb) in oil is determined by ICP-MS previous wet digestion in a microwave oven, as it is described in Section I.1.3.

I.2.7. Determination of fatty acid profile by GC-FID

The fatty acid profile of fish oil was determined qualitatively and quantitatively by a gas chromatographic method following the official method proposed by the AOAC (*AOAC*, 1995). This method required a previous oil methylation step carried out by reaction with boron trifluoride in methanol, BF3-MeOH, and alkali, NaOH-MeOH. The resulted methyl esters were separated in a gas chromatograph (GC) and detected in a flame ionization detector (FID). Identification of fatty acids was carried out by comparison of retention times of standard compounds, whereas quantification was developed using tricosanoate (C23:0) as the internal standard.

FIGURE I. 4

GC-FID equipment.

I.2.7.1. Material and equipment

Gas chromatography was performed in a gas chromatograph (6890 N Network GC System, Hewlett Packard) equipped with an auto-sampler (7683B series) and a flame ionization (FID) detector (see Figure I. 4). The column used was a fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.).

Oil methylation was carried out in PYREX® glass tubes with leak-tight and Teflon-lined screw caps and a water bath was used for heating.

I.2.7.2. Reagents

Oil methylation was performed by using boron trifluoride (BF_3) 12 % in methanol and an alcoholic solution of sodium hydroxide 0.5 M, prepared dissolving 2.0 g NaOH in 100 mL methanol. Isooctane was used as solvent of methylesters and a saturated solution of sodium chloride (36 g NaCl / 100 mL of distilled water) was used to improve aqueous phase-organic phase separation.

I.2.7.3. Standards

An isooctane solution of C23:0 (1 g / mL) was used as internal standard (IS) for quantitative analysis, which was prepared by weighting 25 mg (± 0,1 mg) of C23:0 methyl ester in a 25 mL volumetric flask and diluting with isooctane.

I.2.7.4. Experimental procedure

i) Sample pre-treatment

Accuracy, 1ml of internal standard solution was added into a glass tube and evaporated in gentle stream of N_2. When solvent was removed, 25 mg (± 0.1 mg) of fish oil were weighed into glass tube containing the solid internal standard, and 1.5 mL of NaOH-MeOH solution was added. The glass tube was blanket with nitrogen, cap, mixed and heated 5 minutes in a water bath at 100 °C. Then, it was cooled down and 2 mL BF_3 in methanol were added, mixed and heated in the water bath at 100 °C. After 30 minutes heating, the mixture was cooled, diluted with 1 mL of isooctane, and mixed in a vortex for 30 s. Immediately, the mixture was diluted in 5 mL of saturated NaCl solution and agitated. When the separation between the two phases was clear, the upper organic layer was transferred into a glass vial for autosampler injection in the GC column.

ii) Chromatographic method

CG-FID was performed by using a split injection of (50:1) at 250 ºC and helium, He, (1.8 mL / min) as carrier gas. The temperature program for the column is detailed in Table I. 3.

The FID detector was also heated at 250 ºC. Flame was obtained by a mixture of hydrogen, H_2 (40 mL / min) and air (400 mL / min). Nitrogen,

N_2, (20 mL / min) was used as make-up gas. Control parameters and data analysis was carried out with a GC-Chemstation® Software (Agilent Tech.).

TABLE I. 3

Temperature program used for the oven in the analysis of fatty acids by GC-FID.

	Rate (ºC / min)	Value (ºC)	Hold time (min)
Initial		180	20
Ramp 1	1	200	1
Ramp 2	5	220	20

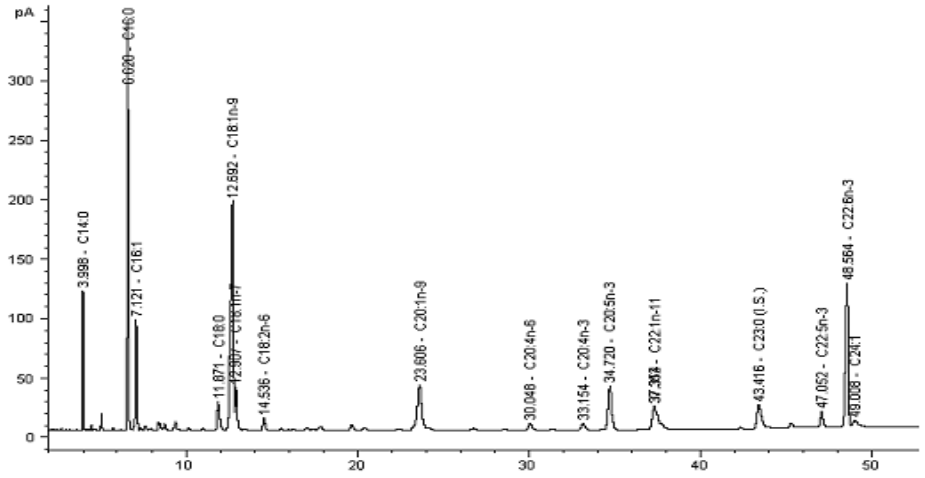


FIGURE I. 5

GC-FID chromatogram of fish oil (hake oil).

iii)Identification of the main chromatographic peaks

A total of 20 fatty acids usually present in common fish oil samples were identified by means of standard fatty acids methyl esters (FAME's) as it is shown in Figure I. 5. The retention times for each fatty acid are presented in Table I. 4.

TABLE I. 4

Retention times of standard lipids compounds indentified by GC-FID.

Type of fatty acid	Chemical formula	Common name	Retention time (min)
SFA	C14:0	Myristic acid	3,9
SFA	C16:0	Palmitic acid	6,6
MUFA	C16:1	Palmitoleic acid	7,1
SFA	C18:0	Stearic acid	11,9
MUFA	C18:1n-9	Oleic acid	12,6
MUFA	C18:1n-7	Vaccenic acid	12,8
PUFA	C18:2n-6	Linoleic acid (LA)	14,4
PUFA	C18:3n-6	γ-linolenic acid (GLA)	16,9
PUFA	C18:3n-3	a-linolenic acid (ALA)	17,7
PUFA	C18:4n-3	Stearidonic acid	19,5
MUFA	C20:1n-9	Gadoleic acid (gondoic?)	23,5
PUFA	C20:3n-6	dihomo-γ-linolenic acid (DGLA)	28,5
PUFA	C20:4n-6	Araquidónico	29,9
PUFA	C20:5n-3	EPA	34,5
MUFA	C22:1n-11	___	37,3
MUFA	C22:1n-9	___	37,7
(IS)	C23:0	Tricosanoic acid	43,4
PUFA	C22:4n-6	Adrénic acid	43,9
PUFA	C22:5n-3	DPA	46,9
PUFA	C22:6n-3	DHA	48,4
MUFA	C24:1	Nervónico	49,0

SFA: Saturated Fatty Acids, MUFA: Monounsaturated Fatty Acids, PUFA: Polyunsaturated Fatty Acids, IS: Internal Standard

iv) Quantification of fatty acids

The amount of each fatty acid in the oil sample is quantified according to the Eq. (I.9), in which A_x is the area counts of each fatty acid, FA; A_{IS} is the area counts of internal standard; CF_x is the theoretical detector correction factor (assumed to be 1); wis is the weight of IS added to sample, mg, w_s is the weight of sample, mg; and 1.08 is the correction factor to express results as mg of fatty acid instead of methyl ester.

$$mg_{FA} / g_{oil} = \frac{A_X \cdot W_{IS} \cdot CF_x}{A_{IS} \cdot W_S \cdot 1.08} \cdot 1000 \qquad \textbf{(I.9)}$$

I.2.8. Determination of neutral lipids profile by HPLC-ELSD

Neutral lipids were determined qualitatively and quantitatively by High Pressure Liquid Chromatography (HPLC) with an Evaporative Light Scattering Detector (ELSD) according to the method proposed by Schaefer (*Schaefer, et al.,* 2003).

I.2.8.1. Material and equipment

Identification of neutral lipid was carried out by comparison with retention times of standard compounds, whereas quantification was carried out by the external standard method.

I.2.8.2. Equipment

Liquid chromatography was developed in a HPLC system (Agilent 1200) formed by a quaternary pump and an autoinjector (see Figure I. 6). The separations were carried out at in a normal-phase column (Lichrospher Diol 5 mm, 4 × 250 mm) and the detection was performed in an evaporative light scattering detector (Agilent 1200 series). Data were assessed by Chemstation® software.

FIGURE I. 6

HPLC-ELSD equipment.

I.2.8.3. Reagents

Neutral lipid separation was carried out by means of a binary gradient among two mobile phases: mobile phase A, composed by isooctane; and

mobile phase B composed by a mixture of methyl tert-butyl ether (MTBE) and acetic acid (AcOH) (99.9 / 0.1).

I.2.8.4. Standards and calibration solutions

A total of 10 neutral lipid standards (squalene, palmitil palmitate, cholesteryl palmitate, tripalmitine, α-tocopherol, palmitic acid, 1,3-dipalmitin, cholesterol, 1,2-dipalmitin and monopalmitin), were used both in peak identification and in lipid calibration.

Calibration solutions were prepared by weighting accurately a certain amount of each neutral lipid and dissolving it in 50 mL hexane.

I.2.8.5. Experimental procedure

i) Sample preparation

Accurately, 0.1 g of oil was weighed in a volumetric flask and dissolved in 50 mL hexane.

ii) Chromatographic method

Neutral lipid separation was carried out at room temperature in the normal phase column and the detection was performed in an ELS-detector at 45 ºC and 3.5 bar using N_2 as nebulizer gas. Separation was developed at room temperature in 35 minutes according to the binary solvent gradient presented in Table I. 5.

TABLE I. 5

Binary solvent gradient used in neutral lipid analysis by HPLC-ELSD.

Accumulated time (min)	Flow rate (mL/min)	% Mobile Phase B
0	1	0
1	1	0
10	1	10
22	1	44
30	1	100
35	1	0

iii)Identification of chromatographic peaks

The chromatographic method developed aimed to separate ten different neutral lipids (see Figure I. 7). Identification of each neutral lipid series and other related compounds was carried out by comparison of the retention time of the standard compounds presented in Table I. 6.

TABLE I. 6

Retention times of standard lipids compounds detected by HPLC-ELSD.

Compound	Retention time (min)		Compound	Retention time (min)
Squalene	2.8		Palmitic acid	16.8
Palmitil palmitate	3.7		1,3-dipalmitin	19.2
Cholesteryl palmitate	4.2		Cholesterol	20.1
Tripalmitine	8.9		1,2-dipalmitine	20.5
α-tocopherol	13.6		Monopalmitine	29.6

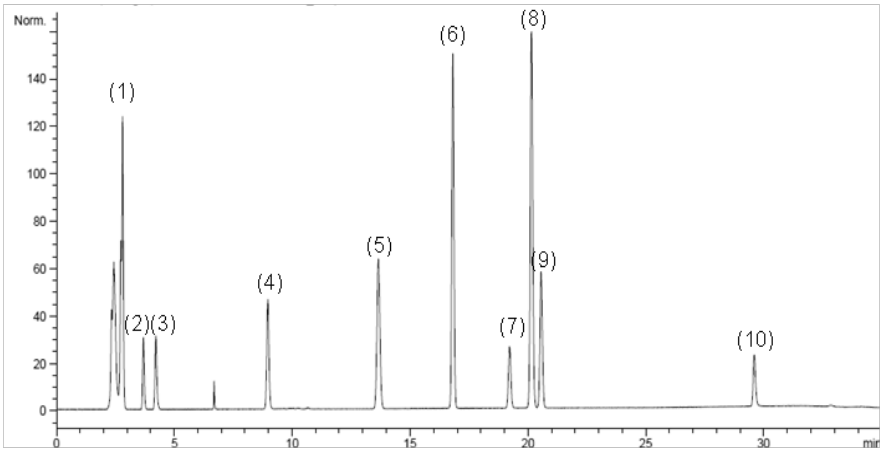


FIGURE I. 7

HPLC-ELSD chromatogram of pure neutral lipids. (1) squalene, (2) palmitil palmitate, (3) cholesteryl palmitate, (4) tripalmitine, (5) α-tocopherol, (6) palmitic acid, (7) 1,3-dipalmitin, (8) cholesterol, (9) 1,2-dipalmitin, (10) monopalmitin.

iv) Quantification

Quantitative analysis of six neutral lipids (wax ester, free fatty acids, tri-, di- and monoacylglycerides and cholesterol) was performed by external calibration of relative standard compounds, that is, palmitil palmitate, palmitic acid, tri-, di- and monopalmitine and cholesterol respectively. As it is presented in Figure I. 8-I. 10, calibration curves showed a good correlation according to the exponential relationship described for an evaporative light scattering detector as it has been described in the literature (*Megoulas, et al.*, 2005).

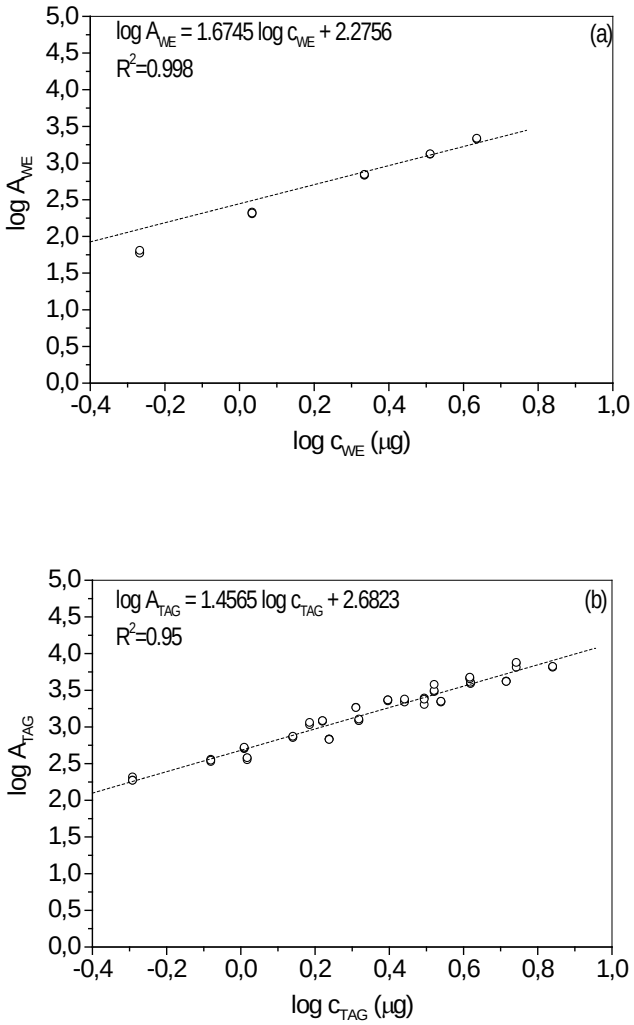


FIGURE I. 8

(a) Calibration curves of wax esters (WE) and (b) triacylglycerols (TAG) taking palmitil palmitate and tripalmitina respectively as standard compound.

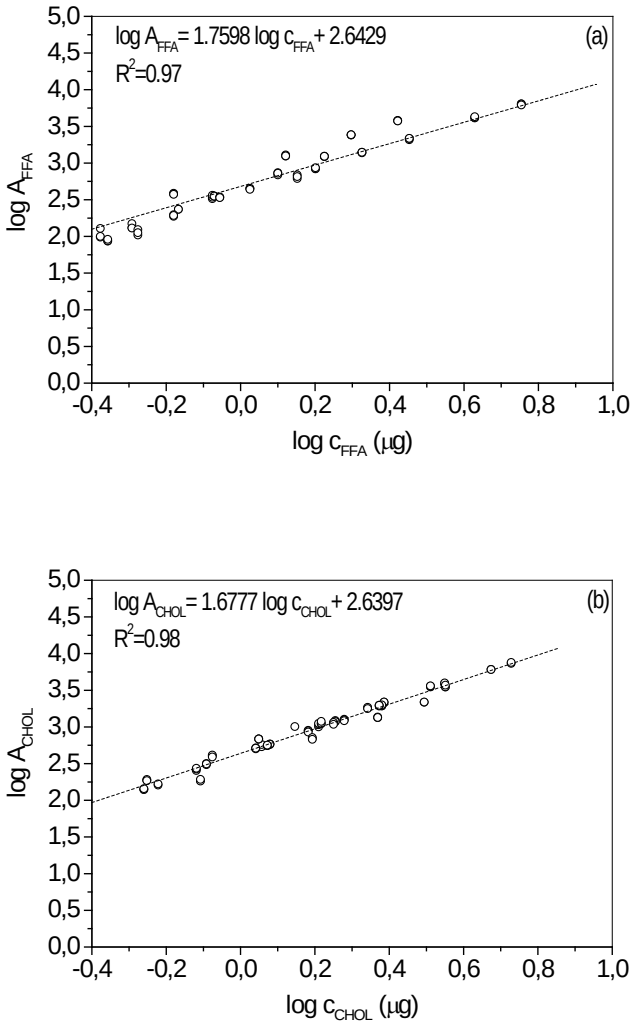


FIGURE I. 9

(a) Calibration curves of free fatty acids (FFA) taking palmitic acid as standard compound; and (b) cholesterol (CHOL).

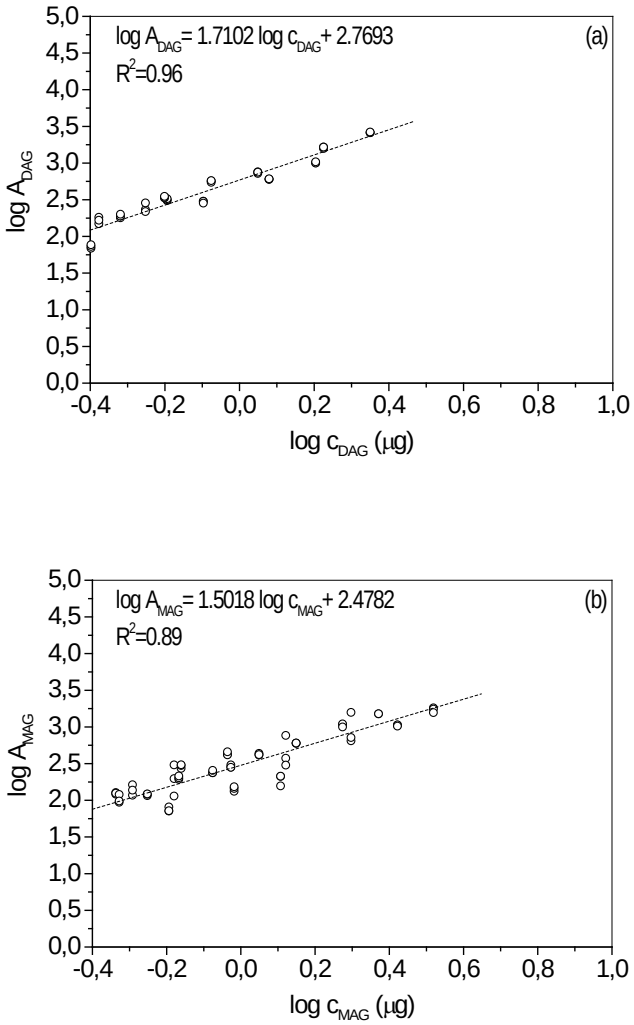


FIGURE I. 10

(a) Calibration curves of 1,2-diacylglycerides (DGA) and (b) monoacylglycerides (MAG) taking dipalmitin and monopalmitin as standard compound respectively.

The amount of each neutral lipid in oil sample, expressed as % wt. of the related lipid standard, was determined by extrapolation of the relative calibration curve.

I.2.9. Volatile compounds profile

Volatile compounds were determined qualitatively by Solid Phase Dynamic Extraction (SPDE) sampling and gas chromatography coupled to a mass spectrophotometry detector (GC-MS).

I.2.9.1. Material and equipment

Oil sampling was carried out in a Solid Phase Dynamic Extractor (Chromtech, Idstein, Germany). Separation of volatile compounds was performed is a gas chromatograph, GC (Agilent Technologist 6890N Series) with a HP5 capillary column (50 m length x 0.32 mm I.D fused silica capillary column coated with 1.05 μm film thickness (Quadrex Corporation. New Haven. USA). Detection was developed in a mass spectrophotometric detector, MS, (Agilent Technologist 5973i) (Figure I. 11).

Glass vials (10 mL) with a 19mm butyl/PTFE septum were used for oil stored previous to the analysis and the samples of volatile compounds were taken by SPDE needle coated with polydimethyl- siloxane monoacetate (PDMS-AC).

FIGURE I. 11

SPDE-GC-MS equipment used for volatile compounds analysis.

I.2.9.2. Experimental procedure

A certain amount of oil sample (0.5 g) was placed in a 10 mL glass vial which was then sealed with the septum. After 1 min of equilibrating time at 70 ºC, volatile compounds were extracted from the head space by the SPDE needle and subsequently injected in the GC, where they were thermally desorbed at 250 ºC. Separation of volatile compounds was performed increasing the temperature of the column from 40 to 240 ºC at a rate of 3 ºC/min, and isolated volatile compounds were detected by the MS-detector. Molecular ionisation was achieved at 70 eV under SCAN-mode (1 scans / s), and the mass range studied was from 30 to 250 m / z. Identification of each peak was performed by means of standard

compounds and a Hewlett-Packard Chemstation equipped with the Wiley 275 library.

I.2.10. Determination of overall smell print

The overall smell print analysis is a sensorial method which allows to classify different kinds of oils according to their odour and flavour features detected by electronic sensors.

FIGURE I. 12

Electronic nose equipment.

I.2.10.1. Equipment

The smell print was obtained in a electronic nose αFOX 4000 (AlfaMOS, Toulouse, France) with a sensor array of 18 metal oxide sensors

(Figure I. 12). Glass vials (10 mL) with a 19 mm butyl/PTFE septum were used for oil storing previous to the analysis.

I.2.10.2. Experimental procedure

Oil samples were placed in a glass vial which was then sealed with a septum. The vials with samples were incubated under agitation (cycles 5 s on and 2 s off and 500 rpm) in an oven at 50 ºC for generating the equilibrated headspace. After that, headspace is injected at 60 ºC using synthetic air (150 mL/min) as the carrier gas.

I.2.10.3. Data analysis

The response of the 18 sensors was analysed mathematically by means of Principal Components Analysis (PCA), taking into account the two first components.

I.2.11. Determination of colour

The colour of the extracted oil was measured in quadruple by means of reflectance spectra in the L*a*b* scale, as recommended the CIE (*CIE*, 1976).

I.2.11.1. Equipment

Colour was measured a Spectrophotometer (Konica Minolta).

I.2.11.2. Experimental procedure

For measurements, samples were placed in a white cup and covered with optical glass. CIE L*a*b* colour coordinates (considering standard illuminant D65 and observer 10°) were then calculated.

I.2.11.3. Data analysis

Colour changes were evaluation by means of the lightness (L*) and the coordinates greenness-redness (a*) and blueness-yellowness (b*).

I.2.12. Sensory Evaluation

The sensorial analysis proposed is based on the human perception of a trained panel against the off-flavor of fish oil and lipid extracts, specifically related to the rancidity of fish oil. It was carried out according to the standard rule proposed by ISO (*ISO*, 1987).

I.2.12.1. Experimental procedure

Sensorial characterization was performed by 10 panellists previously trained in descriptive analysis of fishy off-flavors. The assessors evaluated a total of six sensory descriptors (fishy, rancid, cooked, acid, sweet and others), on a 5-point intensity scale with a range from a minimum of 0 to a maximum intensity of 5. The evaluation was performed in the sensorial laboratory of the University of Burgos, where the fish oil samples (0.5 mg) were

presented randomised at room temperature in blind bottles numbered with a code of three digits

I.3. Caracterisation of Microencapsulated Fish Oil

➢ **Section Content**

I.3.1. Determination of moisture and total volatile matter

The moisture and total volatile matter content in oil is defined as the loss in mass of the sample on heating at 105 °C.

Measurement of moisture and volatile content in fish oil was carried according to the AOAC Official Methods 934.01(*AOAC*, 2000), which was described in Section I.1.1.

I.3.2. Analysis of particle surface by FT-IR

Fourier Transform Infrared analysis of particles surface aims to identify molecular compounds and therefore to characterise particle surfaces. This instrumental analysis is based on the capability of molecular bonds to

vibrate at a discrete energy level related to a certain infrared frequency, which allows to associate molecular structures with IR spectra.

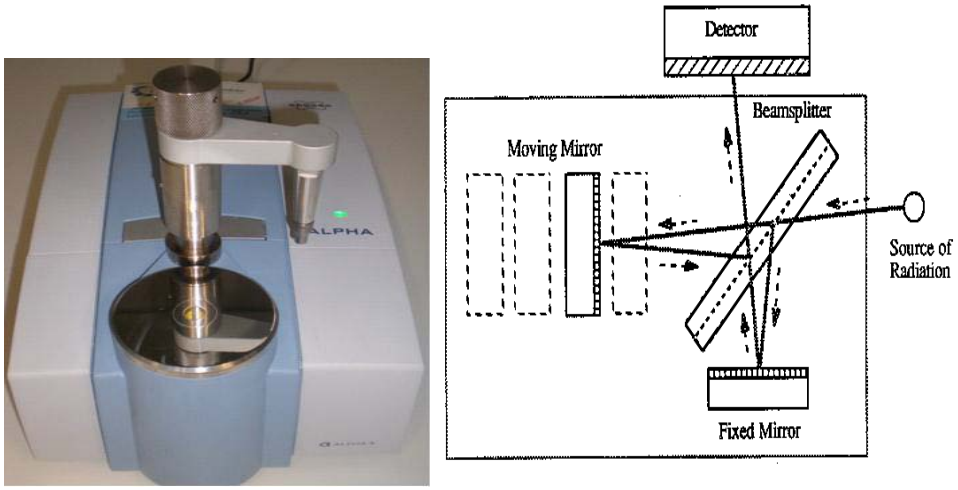


FIGURE I. 13

FT-IR equipment (left) and scheme of an FT-IR analysis (right).

I.3.2.1. Equipment

FT-IR was carried out in an Alpha FT-IR spectrometer provided by a Eco ATR module with a one reflection ZnSe ATR crystal and a slip-clutch pressure applicator with 360 º rotation (*see* Figure I. 13). This module allowed to analyze both solid and liquid compounds within a pH range from 4 to 8 without sample pre-treatment.

I.3.2.2. Experimental procedure

A few amount of sample was placed on the ZnSe ATR crystal previously clean and dry. In the case of solid samples, they were fixed by the slip-clutch pressure applicator. Subsequently, the sample was passed through a beam containing several infrared frequencies within a range from 600 to 4000 cm^{-1}. The light absorbed at each frequency was detected as an interferogram and transformed by the OPUS/Mentor software interface in a FT-IR spectrum according to the Fourier transform algorithm.

I.3.2.3. Data analysis

Characterization of solid particles surface was carried by comparison the FT-IR spectra with the relative spectra obtained from pure fish oil and water (see Figure I. 14). Fish oil on particles surface was detected through the narrow peak at 1746 cm^{-1}, which is characteristic of the ester group, whereas water amount of particle surface was identified by a wide peak around 3350 cm^{-1} related to the –OH group.

FT-IR absorption was conversed from % transmittance to absorbance by Eq. (I.10).

$$\text{Absorbance, } A = -\log(\text{Transmitance}) \quad \textbf{(I.10)}$$

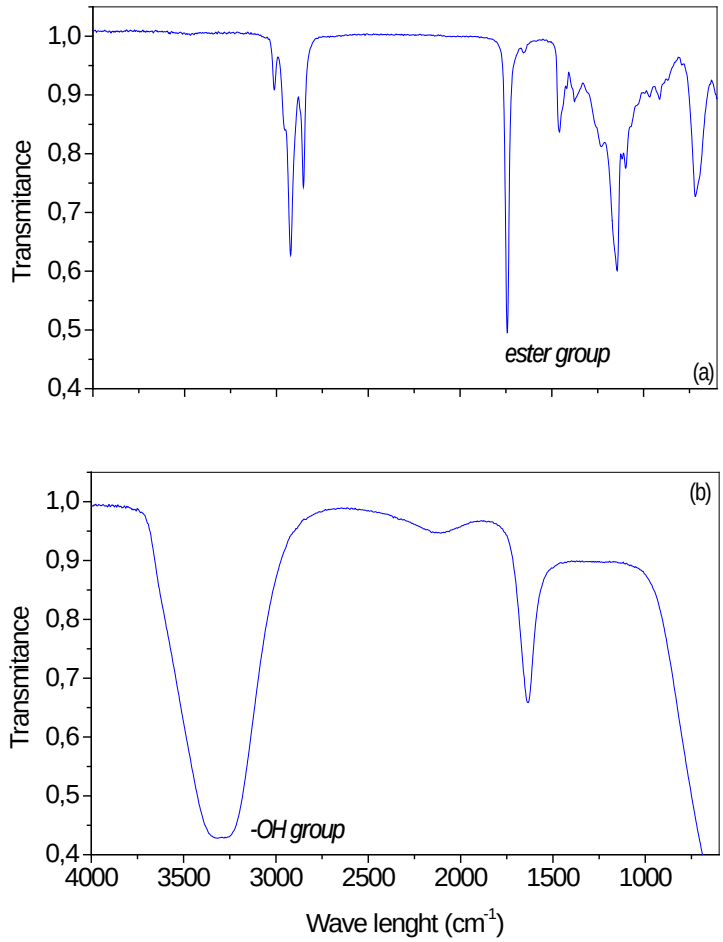


FIGURE I. 14

FT-IR spectra of the fish oil used in encapsulation experiments (a) and pure water (b).

I.3.3. Analysis of bulk density

Bulk density was determined following the hand tapping method described by ISO (*ISO*, 1993).

I.3.3.1. Material

Measurements were carried out by using a dry and clean graduated glass cylinder (25 mL). An analytical balance (± 0.0001 g) was also required.

I.3.3.2. Experimental procedure

A graduated glass cylinder (25 mL), previously weighed, was filled with the powder and tapped by repeatedly striking onto a hard surface. When a constant volume was reached, the full cylinder was weighed again. The method was repeated three times.

I.3.3.3. Calculations

Bulk density, ρ_{bulk}, expressed as kg/m^3, was determined according to Eq (I.11), where m_o is the weight of the empty glass cylinder; m_f is the weight of the cylinder full with solid; and V is the volume of the glass cylinder.

$$\rho_{bulk} = \frac{m_f - m_o}{V} \qquad \textbf{(I.11)}$$

I.3.4. Determination of average particle size

Particle size and particle size distribution of solids were determined via laser diffraction method according to the official method proposed by ISO (*ISO*, 2009).

This technique is based on the fact that particles passing through a laser beam are able to scatter light at a certain angle. The scattered angle increases logarithmically as particle size decreases, whereas the intensity of scattered light decreases with the cross-sectional area of the particle.

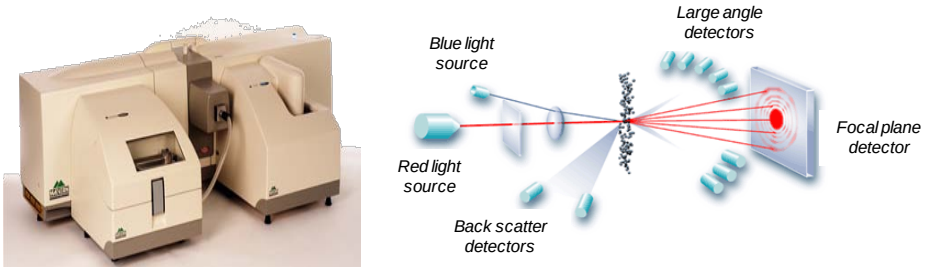


FIGURE I. 15

Particle size analyzer Malvern Mastersizer 2000 with a dry powder feeder Scirocco 2000 (left) and scheme of particle size analyzer by laser diffraction (right) (adapted from www.Malvern.com).

I.3.4.1. Equipment

The measurements of particle size were carried out in a Malvern Mastersizer 2000 composed by a laser beam, which provides a source of coherent and intense light at a fixed wavelength, and a series of detectors,

which measure the intensity of the scattered light over a wide range of angles. The equipment also presents a dry powder feeder Scirocco 2000, which allows to measure the particle size of solid samples using a variable feed-rate vibrating tray and compressed air (Figure I. 15-left).

I.3.4.2. Experimental procedure

A certain amount of solid sample was place in the powder feeder up to fill the feeder sieve. Then, the feeder was closed and the solid was dispersed in air at a pressure of 2 bar. This dispersion was passed through a focused laser beam (wave length = 10 mm), which is scattered by the particles at a certain angle. The intensity of the scattered light, which is inversely proportional, is measured by the detector. The scheme of the process is shown in Figure I. 15-right.

I.3.4.3. Data analysis

Data analysis was carried out by the Mastersize 2000 Software SOPs, which gives particle size related to volume frequency or to cumulative volume. Particle size *versus* volume frequency allows to determine if the particle size distribution is normal, which indicates that there is not particle agglomeration. Particle size *versus* cumulative volume allows to estimate the average particle size expressed as $d_{0.5}$. (Figure I. 16).

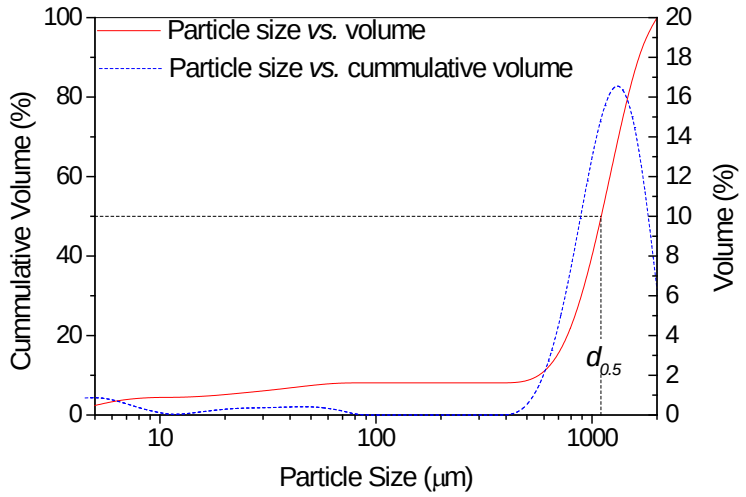


FIGURE I. 16

Particle size vs. volume and cumulative volume. Average particle size, d0.5, represents the particle size value where the 50 % of the distribution is above and the 50 % is below.

I.3.5. Analysis of solid microstructure by Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is commonly used for imaging and characterization of solid microstructures. This instrumental technique involves to produce a high-energy beam of electrons which is condensed and focused through magnetic lens as a fine spot. This spot impacts on the sample surface and interacts to the sample atoms, producing different

signals such as secondary electrons or back-scattered electrons (BSE), which are detected and transformed in electrical signals that give a topographic image (*see* Figure I. 17).

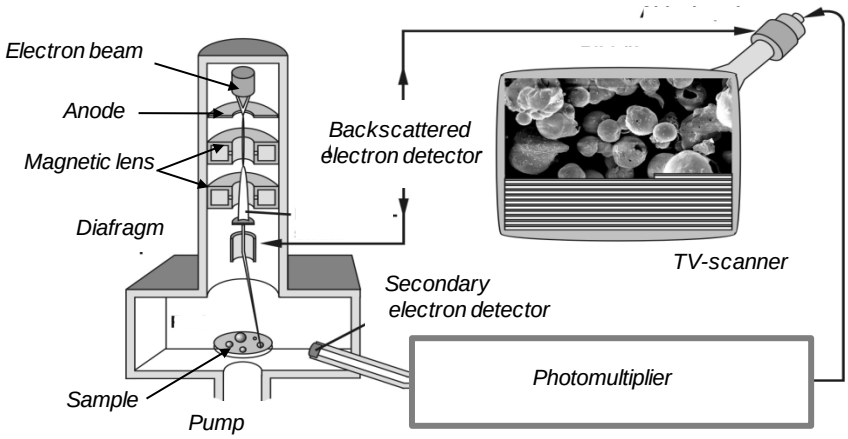


FIGURE I. 17

Scheme of a Scanning Electron Microscope (SEM) equipment (Adapted from (Föcker, 2004))

I.3.5.1. Equipment

The analysis of microstructure was ordered to the Centre of Analysis of the Geology, Mineralogy and Geophysics of the Ruhr University in Bochum (Germany). The equipment used was a LEO (Zeiss) 1530 Field Emission Scanning Electron Microscope (FESEM) with patented GEMINI objective lens (see Figure I. 18). This equipment was able to work with acceleration

voltages from 0.2 to 30 kV and achieving different resolutions (1.0 nm at 20 kV, 2.5 nm at 1 kV or 5 nm at 0.2 kV). It also was provided of two pumps (normal direct dive rotary pump and high-vac turbo pump), an infrared CCD chamber viewing camera with two 19" monitors and a joystick stage controller. At least four SEM pictures were taken from each sample by changing the scale, magnification level, EHT voltage level of the electron beam or working distance from the sample to the beam tip.

FIGURE I. 18

LEO 1530 Gemini SEM (Zentrales Rasterelektronenmikroskop, Ruhr Universität – Bochum, Germany)

I.3.5.2. Sample pre-treatment

A small amount of sample was placed on a mounting stub, which was then screw in a mounting plate and introduced in the SEM equipment. In order to achieve the electrical conductivity required for the electron beam to get a

proper image, particles were previously sputtered with a thin layer of either carbon or gold by using a carbon coater or a gold coater (BioRad SC-500 sputter coater) (see Figure I. 19)

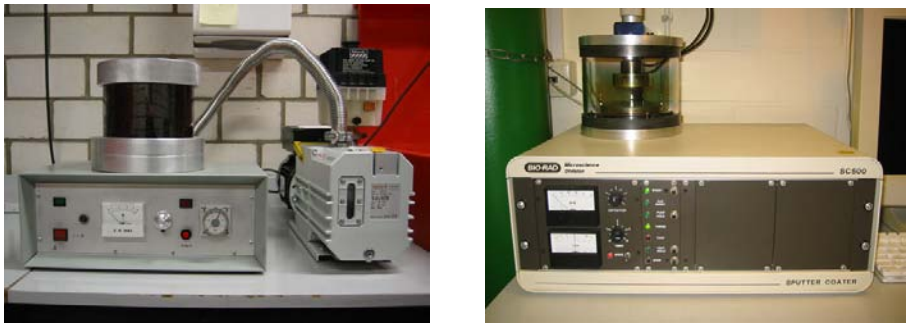


FIGURE I. 19

Carbon coater (left) and gold coater (right). (Zentrales Rasterelektronenmikroskop, Ruhr Universität – Bochum, Germany)

I.3.5.3. Data analysis

Particle morphology was characterised according to Brandin and Kappler (*Brandin*, 2006) by two factors: factor M, related to form, and factor P, related to porosity. The range for factor M was established from 0, in the case of irregular and agglomerate particles, to 1 for a spherical close shape. The range established for factor P varies from 0, in the case of a sponge form, to 1 in the case of smooth surfaces.

I.3.6. Determination of no-encapsulated oil

No-encapsulated oil in oil microcapsules is defined as the fraction of total oil that is removed by washing the solid with an organic solvent such as hexane. Since the oil extracted by the organic solvent is too low to be determined gravimetrically, it was measured by UV-Vis spectrometry taking in to account the absorbance of fish oil at 297 nm, which is due to the presence of unsaturated compounds.

I.3.6.1. Equipment

Solid washing was carried out in a centrifuge (Eppendorf Centrifuge 5804) provided by an Eppendorf Fixed-angle Rotor F-34-6-38 with a radius of 115 mm. Fish oil dissolved in hexane was determined by measuring the sample absorption in a UV-Vis spectrometer at 297 nm.

TABLE I. 7

Standard addition solutions for quantification of fish oil on particle surface.

N°	Fish oil solution (mL)	Sample solution(mL)	Total volumen (mL)	Fish oil concentration added (mg /mL)
A1_0	0	2	10	0.000
A1_1	0,5	2	10	0.211
A1_2	1	2	10	0.421
A1_3	1,5	2	10	0.632
A1_4	2	2	10	0.842

I.3.6.2. Reagents

Hexane was used as fish oil solvent. Calibration solutions were prepared from an organic solution of the fish oil used in encapsulation experiments (0.004 g / mL hexane).

I.3.6.3. Experimental procedure

i) Removal of the no-encapsulated fish oil

Accurately, 1 g of solid sample was weighed in a 50 mL conical Falcon® tube and 25 mL of hexane were added. The mixture was centrifuged during 20 minutes at 1159.2 x g (3000 r.p.m) and, after that, the hexane was filtered in a glass funnel through a filter disc 80 g / m^2 (grade 288, Sartorius Stedim Biotech).

ii) Quantification of the no-encapsulated fish oil

In order to avoid the matrix effect over sample detection, no-encapsulated fish oil removed in hexane was quantified by means of the standard addition method (*Harris*, 2003). A total of five calibration samples (see Table I. 7) were prepared by adding, in a 10 mL volumetric flask, 2 mL of sample solution and different amounts of fish oil, filling up to 10 mL with hexane. The absorbance of each sample was measured at 286 nm and registered.

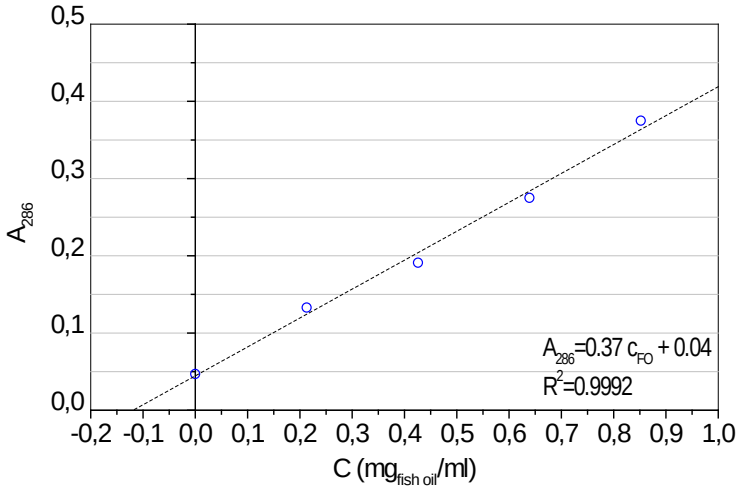


FIGURE I. 20

Calibration curve obtained according to standard addition method for the determination of fish oil on particle surface. Regression equation: A286 = 0.37 c + 0.04, R2= 0.9992.

I.3.6.4. Calculations

For each sample, the absorbance of the calibration solution, A_{286}, was plotted versus the amount of standard oil, c (see Figure I. 20). The absorbance value at zero concentration added, C_a, is related to the unknown concentration in the sample, hence, C_a concentration is estimated as the absolute value of the x-intercept.

I.3.7. Determination of total oil in solid

Total oil is defined as the amount of oil removed with an organic solvent after destroying the coating material. In this case, since the coating material is mainly composed by a polysaccharide (maltodextrine), the method followed to determine the total oil content was an acid digestion, which has been proposed to measure oil in flour.

I.3.7.1. Material

Sample digestions were performed in glass Kjeldahl tubes. PYREX® glass tubes (50 mL) with leak-tight and teflon-lined screw caps were used to mix the sample and the organic solvent in a vortex and glass flasks were used to solvent evaporation.

I.3.7.2. Equipment

A Kjeldahl digester was used to heat the sample with the acid. Solvent evaporation was performed in a rotary evaporator (Rotavapor® R-210 BÜCHI Labortechnik AG).

I.3.7.3. Reagents

Acid digestion was carried out by using chlorhydric acid (HCl, 36 % w / v) to destroy the polysaccharide matrix and ethanol was used to break the oil-in-water emulsion. Oil was extracted by ethyl ether.

I.3.7.4. Procedure

A certain amount of solid sample (1 g) was placed in a Kjeldhal tube and mixed with 2 mL of ethanol. Then, 10 mL of HCl were added and the mixture was heated at 100 ºC during 10 min. After that, the mixture was cooled down to the room temperature, diluted with 10 mL of ethanol and transferred to a Pyrex glass tube. Then, 25 mL of ethyl ether were added, the glass tube was closed and the mixture was shaken in the vortex during 5 min. After phase separation, 10 mL of the upper organic layer were transferred to a glass vessel and put in an oven up to constant weight.

I.3.7.5. Calculations

The total amount of oil in solid sample, expressed as mg / g solid, was determined as Eq (I.12), were m_o is the weight (g) of empty glass vessel, m_f is the weight of glass vessel (g) with the oil removal, m_s is the weight of solid sample (g), V_1 is the total amount of organic solvent added (mL), V_2 is the amount of the organic solvent aliquot taken to the glass vessel (mL).

$$\mathrm{Oil}_{\mathrm{total}} = \frac{V_1}{V_2}\frac{(m_f - m_o)}{m_s}1000 \qquad \textbf{(I.12)}$$

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Fish Species used in this work

Appendix II

II.1 Kingklip

Scientific name	*Genypterus capensis*
Phylum	Chordata
Class	Actinopterygii
Order	Ophidiiformes
Family	Ophidiidae
Enviroment	Bathydermersal
Depth range (m)	50 – 500
Capture zone	Eastern Altantic (Namibian coast)

II.2 Hake

Scientific name	*Merluccius capensis –Merluccius paradoxus*
Phylum	Chordata
Class	Actinopterygii
Order	Gadiformes
Family	Merlucciidae
Enviroment	Bathydermersal
Depth range (m)	50 - 1000
Capture zone	Eastern Altantic (Namibian coast)

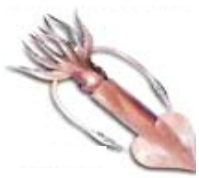
II.3 Salmon

Scientific name	*Salmo salar*
Phylum	Chordata
Class	Actinopterygii
Order	Salmoniformes
Family	Salmonidae
Enviroment	Benthopelagic
Depth range (m)	0 – 210
Capture zone	Chile (aquicultre)

II.4 Orange Roughy

Scientific name	*Hoplostethus atlanticus*
Phylum	Chordata
Class	Actinopterygii
Order	Beryciformes
Family	Hoplostethus
Enviroment	Bathypelagic
Depth range (m)	180 – 1809
Capture zone	Eastern Altantic (Namibian coast)

II.5 Jumbo Squid



Scientific name	*Dosidicus gigas*
Phylum	Mollusca
Class	Cephalopoda
Order	Teuthida
Family	Ommastrephidae
Enviroment	Bathydemersal
Depth range (m)	200-700
Capture zone	Eastern Pacific (Gulf of California)

Appendix III

Physical Properties of Carbon Dioxide & Solubility Data of Pure Compounds in SC-CO_2

III.1 Density of SC-CO_2: the Bender EOS

Density of SC-CO_2, ρ, expressed in g/cm^3, as was estimated for a temperature, T (K) and pressure, P (MPa) conditions, according to the Bender EOS (*Bender*, 1971).

$$P = T \cdot \rho \cdot [R + B \cdot \rho + C \cdot \rho^2 + D \cdot \rho^3 + E \cdot \rho^4 + F \cdot \rho^5 + (G + H \cdot \rho^2) + \rho^2 \exp(-a_{20} \cdot \rho^2)] \quad \textbf{(III.1)}$$

Where:

$$B = a_1 - \frac{a_2}{T} - \frac{a_3}{T^2} - \frac{a_4}{T^3} - \frac{a_5}{T^4} \quad \textbf{(III.2)}$$

$$C = a_6 + \frac{a_7}{T} + \frac{a_8}{T^2} \quad \textbf{(III.3)}$$

$$D = a_9 + \frac{a_{10}}{T} \quad \textbf{(III.4)}$$

$$E = a_{11} + \frac{a_{12}}{T} \quad \textbf{(III.5)}$$

$$F = \frac{a_{13}}{T} \quad \textbf{(III.6)}$$

$$G = \frac{a_{14}}{T^3} + \frac{a_{15}}{T^4} + \frac{a_{16}}{T^5} \quad \textbf{(III.7)}$$

$$H = \frac{a_{17}}{T^3} + \frac{a_{18}}{T^4} + \frac{a_{19}}{T^5} \quad \textbf{(III.8)}$$

The parameters of the EOS for CO_2 are reported in Table III. 1.

TABLE III. 1

Coefficients of the Bender EOS for CO_2 (Source: (*Brunner*, 1994))

Coefficient	Value	Coefficient	Value
R	0.188918	a_{10}	$-0.39436077 \cdot 10^{3}$
a_1	0.22488558	a_{11}	0.12115286
a_2	$0.13717965 \cdot 10^{3}$	a_{12}	$0.10783386 \cdot 10^{3}$
a_3	$0.14430214 \cdot 10^{5}$	a_{13}	$0.43962336 \cdot 10^{2}$
a_4	$0.29630491 \cdot 10^{7}$	a_{14}	$-0.36505545 \cdot 10^{8}$
a_5	$0.20606039 \cdot 10^{9}$	a_{15}	$0.19490511 \cdot 10^{11}$
a_6	$0.45554393 \cdot 10^{-1}$	a_{16}	$-0.29186718 \cdot 10^{13}$
a_7	$0.7704284 \cdot 10^{2}$	a_{17}	$0.24358627 \cdot 10^{8}$
a_8	$0.40602371 \cdot 10^{5}$	a_{18}	$-0.3754653 \cdot 10^{11}$
a_9	0.40029509	a_{19}	$0.11898141 \cdot 10^{14}$
		a_{20}	$0.50000000 \cdot 10^{1}$

III.2 Solubility data

Solubility of pure compounds in supercritical CO_2, S, expressed in g / Lwas estimated according to Chrastill equation (III.9), where ρ is the density of the SC-CO_2, expressed in g / L, T is the temperature, expressed in K, and k, a and b are the correlation parameters (*see* Table III. 2)

$$S = (\rho)^k \exp\left(\frac{a}{T} + b\right) \qquad \textbf{(III.9)}$$

TABLE III. 2

Correlation parameters for solubility of pure compounds in SC-CO_2

Compound	Equation parameters			Reference
	k	a	b	
Water	0.549	-2826.4	-0.807	(Chrastil, 1982)
Glicerol	1.591 - 0.679 ρ + 0.419 ρ^2	-1672	-13.22	(Sovová, et al., 1997)
Oleic acid	7.92	-3982	-38.1	(Güçlü-Üstündağ, et al., 2000)
Monolein	10.68	-7925	-45.8	(Güçlü-Üstündağ, et al., 2000)
Diolein	10.48	-4601	-54.3	(Güçlü-Üstündağ, et al., 2000)
Triolein	10.28	-2057	-61.5	(Güçlü-Üstündağ, et al., 2000)
Oleic acid (EE)	7.78	-1947	-40.9	(Güçlü-Üstündağ, et al., 2000)

EE: Ethyl ester

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Curriculum Vitae

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Education

2006-2010 Ph.D. student in the program "Advances on Food Science and Biotechnology", University of Burgos, Burgos, Spain.

2006-2007 Master in Food Biotechnology and Safety, University of Burgos, Burgos, Spain.

2000-2005 B. Sc. in Chemistry, University of Valladolid, Valladolid, Sapin.

Employment

Since 2010 Research scientist assistant in the Department of Biotechnology and Food Sciece of Burgos University, Burgos, Spain

2006-2010 Spanish research fellowship FPI in the Department of Biotechnology and Food Sciece of Burgos University, Burgos, Spain

2005-2006 Assistant Scholar in the Department of Analytical Chemistry at Faculty of Chemistry of University of Valladolid, Valladolid, Spain.

Short stays in European Universities and / or Institutes of Research

2009 Institute of Chemical Processes Fundamentals de la ASCR, Praga (Czech Republic). 12 weeks.

2010 Institut für Thermo- und Fluiddynamik - Fakultät für Maschinenbau - Ruhr Universität, Bochum (Germany). 24 weeks.

Publications

Journals

Rubio-Rodríguez, N., de-Diego-Rupérez, S., Beltrán, S., Jaime, I., Sanz, M.T. and Rovira, J. (2008) Supercritical fluid extraction of the omega-3 rich oil contained in hake (*Merluccius capensis – Merluccius paradoxus*) byproducts: study of the influence of process parameters on the extraction yield and oil quality. *Journal of Supercritical Fluids*, 47, 215-226.

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Poster presentations

Sara de Diego, Nuria Rubio, Sagrario Beltrán, Isabel Jaime and Jordi Rovira. *Comparación de distintos métodos de extracción lipídica de pieles de pescado.* IV Congreso Nacional de Ciencia y Tecnología de los Alimentos. La Seguridad y la Calidad de los Alimentos. La Laguna (Spain). June, 2007.

Nuria Rubio-Rodríguez, Sara de Diego, Sagrario Beltrán, Isabel Jaime, Jordi Rovira, Maria Teresa Sanz and Jose Luis Cabezas. *Supercritical Carbon Dioxide Extraction of Oil from Hake Skin. 5th International Symposium on High Pressure Processes Technology and Chemical Engineering.* Segovia (Spain). June, 2007.

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