UNIVERSITY OF GRANADA

DOCTORAL PROGRAM IN CHEMISTRY

OBTENTION OF FUNCTIONAL COMPOUNDS FROM FISH OIL AND PROTEIN

DOCTORAL THESIS

ROCÍO MORALES MEDINA

2016

Editor: Universidad de Granada. Tesis Doctorales
Autora: Rocío Morales Medina
ISBN: 978-84-9163-062-3
URI: http://hdl.handle.net/10481/44537

“I am among those who think that Science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, even though such machinery has its own beauty.”

Marie Curie (1867 – 1934)

Acknowledgements

This Ph.D. thesis was carried out at the Bioreactors Research Group Laboratory of the Department of Chemical Engineering at the University of Granada. This research work was financed by the Spanish Ministry of Economy and Competitiveness under the project entitled “Obtention of bioactive peptides and oils enriched in PUFA from fish discards”.

First of all, I am deeply grateful to my supervisors Prof. Emilia M. Guadix and Dr. Antonio Guadix, for giving me the opportunity to work with them and for their guidance, strong support and valuable advices during these four years.

I am deeply owed to Prof. Stephan Drusch for giving me the chance to be a member of his work team during my research stays at the Technical University of Berlin which were financed by the DAAD and by the Spanish Ministry of Economy and Competitiveness. Many thanks to Dr. Anja Oechsle and Dr. Frederic Tamm for their collaboration to the success of this research. Also, I would like to thank Sandra, Addi, Janine, Friederike, Sabrina, Miss Kern and all my colleagues in Berlin. Thanks for the humongous amount of good moments that we shared together.

I am grateful to my colleagues at the Department of Chemical Engineering. Particularly I would like to thank to Prof. Fernando Camacho for his constant guidance and orientation since the very beginning of this research. Also, I would like to thank to Dr. Raúl Pérez for all the time and energy devoted to this project. Also, I am grateful to Dr. Maria del Mar Muñío for her constant support and help. My gratitude also to M.Sc. Guillermo de León, Dr. Ignacio Moya, Dr. Pedro García, Dr. Francisco Ríos and Dr. Javier Espejo for his unvaluable help and support.

I would like to thank my close friends, for understanding all my absences and being a constant presence in my life.

Finally, my deepest thanks go to my family for its unconditional love and support during my whole life.

Table of contents

Acknowledgements 7

Table of contents 9

Resumen 15

1. INTRODUCCIÓN 17
1.1. Descartes de pesca 17
1.2 Aceite de pescado 19
1.2.1. Revalorización de aceite de pescado: concentrados en PUFA y lípidos estructurados 19
1.2.2. Oxidación y estabilización de aceite de pescado 22
1.3 Hidrolizados de proteína de pescado 23
1.3.1 Capacidad antioxidante de los hidrolizados de pescado 25
1.3.2 Actividad anithipertensiva de los hidrolizados de pescado 26
2. OBJETIVOS 26
3. MATERIALES Y MÉTODOS 28
4. RESULTADOS Y DISCUSIÓN 28
4.1. Revalorización del aceite de pescado 28
4.1.1. Caracterización estacional del sustrato 28
4.1.2. Obtención de aceite enriquecido en PUFA 31
4.1.3. Producción de lípidos estructurados (MLM) partiendo de descartes de sardina 36
4.2. REVALORIZACIÓN DE LA PROTEÍNA DE PESCADO 40
4.2.1. Caracterización estacional del sustrato 40
4.2.2. Estudio cinético de la hidrólisis enzimática de proteína de jurel empleando una mezcla de proteasas de origen animal y bacteriano 40
4.2.3. Evaluación de las propiedades tecnológicas y antioxidantes de los hidrolizados de proteína de pescado para su aplicación como estabilizantes en la microencapsulación de aceite de pescado 49
5. BIBLIOGRAFÍA 51

Summary 59

1. INTRODUCTION 61
1.1. Fish discards 61
1.2 Fish oil 63
1.2.1. Fish oil up-grading: PUFA concentrates and structured lipids 63
1.2.2. Fish oil oxidation and stabilization 65
1.3 Fish protein hydrolysates 67
1.3.1 Antioxidant hydrolysates 68
1.3.2 Antihypertensive hydrolysates 69
2. OBJECTIVES 69
3. MATERIALS AND METHODS 72
4. RESULTS AND DISCUSSION 72
4.1. Fish oil up-grading 72
4.1.1. Substrate seasonal characterization 72

4.1.2. Obtention of oil enriched in PUFA 74
4.1.3. Production of MLM structured lipids from sardine discards 79
4.2. Fish protein up-grading 82
4.2.1. Substrate seasonal characterization 82
4.2.2. Kinetic study of the enzymatic hydrolysis of horse mackerel with a mixture of bacterial and animal proteases 82
4.2.3. Evaluation of technological and antioxidant properties of protein hydrolysates as stabilizers for the microencapsulation of fish oil 89
5. REFERENCES 91

I. Lipids from Marine Sources 99

1. FISHERIES WASTE REUTILIZATION: AN ECONOMIC AND ENVIRONMENTAL CHALLENGE TO FACE 101
2. MARINE LIPIDS 102
2.1. Fatty Acids and Triglycerides 102
2.2. Phospholipids 104
3. SOURCES OF MARINE LIPIDS 104
3.1. Tuna 105
3.2. Salmon 106
3.3. Sardine 106
3.4. Mackerel 107
3.5. Cod 108
3.6. Lipid composition of bones 109
4. HEALTH BENEFITS ASSOCIATED TO MARINE LIPIDS 110
4.1. Cardiovascular Benefits 112
4.2. Omega-3 Fatty Acids' Role in Mental Health 112
4.2.1. Early Human Development 112
4.2.2. Depression 113
4.2.3. Maintenance of Cognitive Function: Alzheimer 113
4.3. Prevention of Type 2 Diabetes 113
4.4. Prevention of Allergy and Asthma 113
4.5. Prevention of Cancer 114
5. LIPIDS OXIDATION: A SETBACK TO DEAL WITH 114
6. LIPIDS REVALORIZATION 116
6.1. Production of Omega-3 Concentrates 116
6.1.1. Low temperature crystallization (winterization) 116
6.1.2. Urea complexation 117
6.1.3. Distillation methods 118
6.1.4. Supercritical Extraction 119
6.1.5. Enzymatic Processes 120
7. STRUCTURED LIPIDS 121
7.1. Digestion and absorption of lipids 122
7.2. Chemical Synthesis 124
7.3 Enzymatic Synthesis 124
7.3.1. Acidolysis 125
7.3.2. Interestification 126
7.3.3. Two Steps Process: Alcoholysis and Esterification 127
7.3.4. Two-Steps Process: Hydrolysis and Esterification 129
8. REFERENCES 130

II. Seasonal Variations in the Regiodistribution of Oil Extracted from Small-Spotted Catshark and Bogue 139

1. INTRODUCTION 141
2. MATERIALS AND METHODS 143
2.1. Raw materials 143
2.2. Proximate composition and oil extraction 144

2.3. Fatty acid profile and lipid composition 144
2.4. Oxidative and nutritional indices 145
2.5. Determination of the positional distribution of fatty acids in TAG 145
2.6. Statistical analysis 146
3. RESULTS AND DISCUSSION 146
3.1. Proximate composition 146
3.2. Fatty acids profile and nutritional indices 147
3.3. Regiospecific distribution of fatty acids in TAG 153
4. CONCLUSIONS 156
5. REFERENCES 156

III. Nutritional Indexes, Fatty Acids Profile and Regiodistribution of Oil Extracted from Four Discarded Species of the Alboran Sea: Seasonal Effects 159

1. INTRODUCTION 161
2. MATERIALS AND METHODS 163
2.1 Materials 163
2.2. Proximate composition and oil extraction 163
2.3. Fatty acid profile and lipid composition 164
2.4. Oxidative and nutritional indexes 164
2.5. Determination of the positional distribution of fatty acids in TAG 165
3. RESULTS AND DISCUSSION 165
3.1. Proximate composition 165
3.2. Fatty acids profile and nutritional indexes 166
3.3. Regiospecific distribution of fatty acids in TAG. 167
4. CONCLUSIONS 172
5. REFERENCES 173

IV. Mass Transfer Model of Sardine Oil Polyunsaturated Fatty Acids (PUFA) Concentration by Crystallization at Low Temperature 177

1. INTRODUCTION 179
2. MATERIALS AND METHODS 181
2.1. Materials 181
2.2. Free fatty acids production 181
2.3. Fatty acid profile analysis and lipid class composition 182
2.4. Winterization 182
3. THEORY: MODEL OF THE LIQUID FRACTION MASS EVOLUTION 183
4. RESULTS AND DISCUSSION 185
4.1. Experimental data 185
4.2. Model of the global mass evolution of the liquid fraction 188
4.3. Model of PUFA, EPA and DHA concentration for each fraction 190
5. CONCLUSIONS 193
6. REFERENCES 195

V. Lumped model of the lipase catalyzed hydrolysis of sardine oil to maximize polyunsaturated fatty acids content in acylglycerols 198

1. INTRODUCTION 201
2. MATERIALS AND METHODS 203
2.1. Oil, lipases and chemicals 203

2.2. Enzymatic hydrolysis of triacylglycerols 203
2.3. Study of the acyl migration of monoacylglycerols 204
2.4. Determination of the fatty acids profile and lipid class composition 205
2.4.1. Positional distribution of fatty acids in TAG 206
2.5. Kinetic model of the triacylglycerols hydrolysis 206
2.6. Statistical analysis 208
3. RESULTS AND DISCUSSION 209
3.1. Degree of concentration of PUFA in each acylglycerol and FFA fractions 209
3.2. Hydrolysis kinetic model 212
3.3. Kinetic model of the acylmigracion in monoacilglycerols 218
4. CONCLUSIONS 220
TABLE OF ABBREVIATIONS 221
APPENDIX. CHARACTERISTIC OF THE PARTICLES OF IMMOBILIZED ENZYME 221
5. REFERENCES 222

VI. Development of an Up-grading Process to Produce MLM Structured Lipids from Sardine Discards 225

1. INTRODUCTION 227
2. MATERIALS AND METHODS 229
2.1. Materials 229
2.2. Production Process 229
2.2.1. Oil Content of sardines and fish oil extraction 233
2.2.2. Omega-3 concentration: free fatty acids (FFA) production and winterization 233
2.2.3. Enzymatic esterification 233
2.2.4. Diacylglycerols and triacylglycerols purification 234
2.3. Characterization of the oil 235
2.3.1. Lipid Composition and fatty acids profile 235
2.3.2. Determination of the positional distribution of fatty acids in the triacylglycerols 236
2.3.3 Oxidative state of the oils: peroxide and anisidine values 236
3. RESULTS AND DISCUSSION 236
3.1. Process overview: yield and oxidative stability 236
3.1.1. Individual yields 237
3.1.2. Oxidative evolution of oil through the process 238
3.2. Enzymatic treatment: re-esterification 242
3.3. Fatty acid composition and regiodistribution 244
4. CONCLUSIONS 247
5. REFERENCES 247

VII. Fish Discards as a Source of Health Promoting Biopeptides 251

1. INTRODUCTION: DISCARDING PRACTICES IN THE FISHING INDUSTRY 253
1.1. Definition of discards. Negative impact of discards on the environment 253
1.2. Assessment of discard rates: focus on the Alboran Sea 254
1.3. Fisheries regulations on discards: the EU's Zero discard policy 255
2. PRETREATMENTS TO PROCESS FISH DISCARDS AND BY-PRODUCTS 256
3. UPGRADING OF DISCARDED MATERIALS: FOCUS ON THE PROTEIN FRACTION 259
3.1. Introduction: Recovery of the protein fraction from fish materials 259
3.2. Identification of bioactive peptides 260
3.3. Bioavility of active peptides: simulated digestion assays 264
3.4. ACE Inhibitory activity of FPH from discarded species 267
3.5. Antioxidant activity of FPH from discarded species 269
3.6. Antiocholesterolemic activity of FPH from discarded species 271
4. APPLICATION OF FISH PROTEIN HYDROLYSATES IN THE FOOD INDUSTRY 273
4.1. Technological properties of FPH from discarded species 274

4.1.1. Solubility 274
4.1.2. Emulsifying and foaming capacity 275
4.1.3. Fat binding and water holding capacity 277
4.2. Application of FPH as chemical and physical stabilizers in emulsions and microcapsules 277
5. REFERENCES 278

VIII. Artificial Neuronal Networks Modelling of the Enzymatic Hydrolysis of Horse Mackerel Protein Using Protease Mixtures 287

1. INTRODUCTION 289
2. MATERIALS AND METHODS 291
2.1. Characterization and pretreatment of the substrate 291
2.2. Enzymes and hydrolysis procedure 291
2.3. Experimental design 292
2.4. ANN model of the degree of hydrolysis 293
2.5. Evolutionary algorithm 294
3. RESULTS AND DISCUSSION 298
3.1. Experimental data 298
3.2. Artificial neural network model of the degree of hydrolysis 299
3.3. Optimization of the degree of hydrolysis by evolutionary algorithm 301
4. CONCLUSIONS 304
5. REFERENCES 305

IX. Modeling of the Production of ACE Inhibitory Hydrolysates of Mediterranean Horse Mackerel (*T.mediterraneus*) Using Protease Mixtures 309

1. INTRODUCTION 311
2. MATERIALS AND METHODS 313
2.1. Proximate composition of the raw material 313
2.2. Production of the fish protein hydrolysates (FPH) 313
2.3. Experimental design 314
2.4. Crossed mixture-process models for DH and ACEI 315
2.5. Determination of the ACE inhibitory activity of the FPH 316
2.6. *In vitro* digestion of the FPH 316
2.7. Size exclusion Chromatography (SEC) 317
2.8. Optimization of DH and ACEI by evolutionary algorithm 317
3. RESULTS AND DISCUSSION 317
3.1. Curves of hydrolysis 317
3.2. Modelization of the degree of hydrolysis 320
3.3. Modelization of the ACE inhibitory activity 324
3.4. *In vitro* digestion of the hydrolysates 327
4. CONCLUSIONS 330
5. REFERENCES 330

X. Multiobjective Optimization of the Antioxidant Activities of Horse Mackerel Hydrolysates Produced with Protease Mixtures 335

1. INTRODUCTION 337
2. MATERIALS AND METHODS 339
2.1. Raw material and reagents 339
2.2. Substrate pretreatment 339
2.3. Enzymes and hydrolysis procedure 340

2.4. Experimental design and mathematical modeling 340
2.5. Determination of the degree of hydrolysis 342
2.6. Determination of the antioxidant activity 343
2.6.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay 343
2.6.2. Fe^{3+} reducing power assay 344
2.6.3. Iron (Fe^{2+}) chelating activity assay 344
2.7. Size exclusion chromatography (SEC) 344
2.8. Multiobjective optimization 345
3. RESULTS AND DISCUSSION 346
3.1. Modeling and optimization of the antioxidant properties 346
3.1.1. DPPH scavenging activity and Fe^{3+} reducing power 347
3.1.2. Fe^{2+} Chelating activity 354
3.2. Multi-objective optimization 357
4. CONCLUSIONS 359
5. REFERENCES 360

XI. Functional and Antioxidant Properties of Hydrolysates of Sardine (*S. pilchardus*) and Horse Mackerel (*T. mediterraneus*) for the Microencapsulation of Fish Oil by Spray-drying. 365

1. INTRODUCTION 367
2. MATERIALS AND METHODS 369
2.1. Characterisation of the composition of the fish muscle meat and the fish protein hydrolysate 369
2.2. Enzymatic hydrolysis of fish muscle meat 371
2.3. Determination of the antioxidant activity and the interfacial tension of the fish protein hydrolysates 371
2.4. Preparation of FPH-stabilised emulsions and evaluation of the stability of the liquid emulsions 373
2.5. Emulsion preparation and spray-drying of emulsions for microencapsulation of fish oil 374
2.6. Storage and determination of the hydroperoxide content of microencapsulated fish oil over time 374
2.7. Statistical analysis 375
3. RESULTS AND DISCUSSION 375
3.1. Proximate composition and amino acid composition of fresh muscle meat and lyophilized FPH 375
3.2. Molecular weight distribution of muscle and FPH by SDS-PAGE 376
3.3. *In vitro* antioxidant properties of FPH 378
3.4. Interfacial activity of FPH 380
3.5. Selection of pH and composition of the emulsion for microencapsulation by spray-drying 382
3.6. Characterisation of fresh emulsions, microcapsules and reconstituted emulsions after spray-drying 383
3.7. Lipid oxidation of fish oil in spray-dried emulsions stabilised by FPH 384
4. CONCLUSIONS 387
5. REFERENCES 388

Conclusiones Finales 391

Final Conclusions 397

Resumen

1. INTRODUCCIÓN

1.1. Descartes de pesca

Se define descarte de pesca como la porción del total de material orgánico de origen animal en la captura pesquera, la cual es desaprovechada o vertida al mar por cualquier razón (Kelleher, 2005). Los descartes se componen, en parte, de capturas accesorias, (como por ejemplo, equinodermos, peces con bajo valor comercial o esponjas marinas), de especies que no presentan la talla mínima para su desembarco y/o de especies que exceden el volumen fijado por la cuota de pesca. Prácticamente, tras el proceso de selección, la mayoría de los individuos devueltos al mar están muertos o moribundos (Groenewold et al., 2000).El porcentaje de descartes respecto al total de la captura de una determinada especie depende de varios factores: el tipo de red usada, la regulación pesquera e incluso su demanda en los mercados locales.

En el caso más específico de España, centrándonos en la costa sureste del mar Mediterráneo (Mar de Alborán), la tasa de descarte alcanza valores del 23% para la pesca por arrastre y del 10 % en la pesquería de cerco. Estos valores representan una subutilización de los recursos marinos, en un área en la que las capturas se redujeron a la mitad en la última década (FROM, 2008). Entre las especies descartadas algunas tienen interés comercial, como por ejemplo la sardina (*Sardina pilchardus*), el jurel (*Trachurus mediterraneus*) o la bacaladilla (*Micromesistius poutassou*) (Fig. 1). Estas especies se descartan debido a requerimientos de talla mínima, cuotas de pesca o prácticas de "high grading" (es decir, descarte de pesca comercial de talla legal y con poco valor). Por otro lado, otras especies como el aligote (*Pagellus acarne*), la boga (*Boops boops*) o la pintarroja (*Scyliorhinus canicula*) son descartadas debido a que son especies accesorias con poco valor comercial (Fig. 1) (Carbonell, Martín, Ranieri, & Team, 1998).

La práctica del descarte tiene un impacto negativo en la productividad del sector pesquero y, además, causa una serie de problemas ambientales ya que altera la cadena trófica del ecosistema y contribuye a la diseminación de componentes tóxicos y parásitos localizados en las vísceras de las especies devueltas al mar (Blanco, Sotelo, Chapela, & Pérez-Martín, 2007).

Por todo ello, por parte de la Unión Europea se ha realizado una profunda reforma implantándose la llamada legislación de cero descartes. Esta reforma propone una serie de medidas cuyo objetivo final es eliminar los descartes de las pesquerías pertenecientes a la Unión Europea (EU, 2013). Tan ambicioso objetivo se ha implantado de manera progresiva. En 2014, se prohibieron los descartes de especies pelágicas (sardina, jurel o caballa, entre otros). En 2015, se vetó la práctica del descarte de especies demersales como por ejemplo: bacalao, merluza o lenguado. En 2019, la política de cero descartes estará totalmente implantada. Como consecuencia, un considerable volumen de especies marinas, compuesto principalmente de especies accesorias, sin interés comercial llegan a puerto a diario.

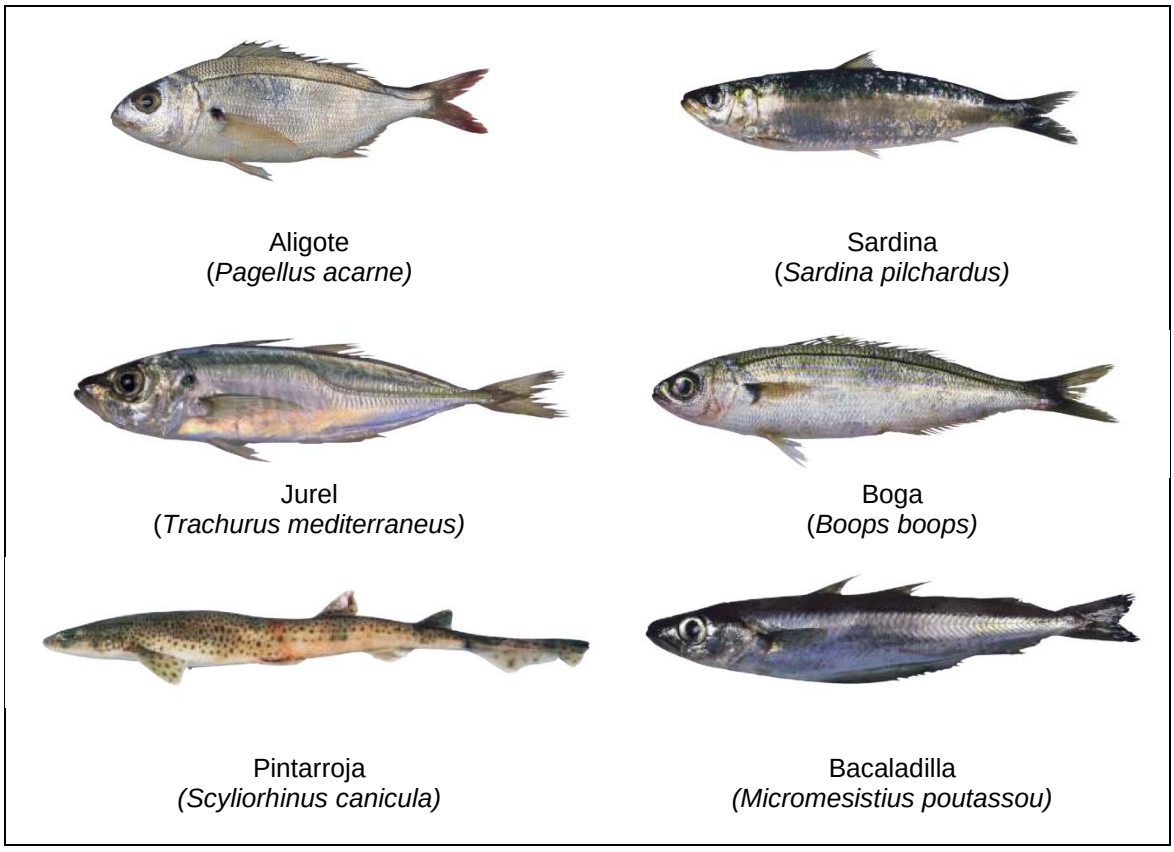


Figura 1. Ejemplos de especies de descarte en el Mar de Alborán.

Paralelamente a esta nueva normativa, se han desarrollado una serie de medidas técnicas con el objeto de minimizar la tasa de descarte: (i) el diseño y uso de artes de pesca más selectivos, (ii) el aumento de control a bordo o (iii) la revalorización de los descartes mediante el incremento de su venta comercial a través de su trasformación en otros compuestos de mayor interés en el mercado (EU, 2011; Seafish, 2016).

En este marco, los descartes pueden considerarse como una materia prima de bajo coste para la obtención de productos con valor añadido. Así, a partir de descartes de pesca se

pueden producir productos de interés para la industria farmacéutica y alimentaria como son los hidrolizados de proteína de pescado y aceites modificados. Por lo tanto, se han de desarrollar y optimizar soluciones técnicas que permitan convertir dichos descartes en compuestos funcionales.

1.2 Aceite de pescado

El aceite de pescado tiene un valor nutricional único debido al alto contenido en ácidos grasos poliinsaturados (PUFA) que presenta. Dentro de esta familia, se destacan los ácidos grasos Omega-3 que ejercen numerosos efectos beneficiosos sobre la salud humana. Más concretamente, sus propiedades se han relacionado, en la literatura científica, con los ácidos eicosapentanoico (EPA, C20:5n-3) y docosahexanoico (DHA, C22:6n-3) (Ruxton, Reed, Simpson, & Millington, 2007). Ambos ácidos grasos refuerzan el sistema cardiovascular (Jump, Depner, & Tripathy, 2012), favorecen el correcto desarrollo visual en los neonatos (Weichselbaum, Coe, Buttriss, & Stanner, 2013) y fortifican el sistema cerebral (Bradbury, 2011).

Industrialmente, el aceite de pescado se extrae por el método de prensado en húmedo que consta de tres etapas: (i) precalentamiento de la materia prima (85 a 95 ºC), (ii) extracción por prensado y (iii) centrifugación (Bimbo, 2007; Rubio-Rodriguez et al., 2010). Como productos de este proceso se obtienen aceite crudo y una torta con alto contenido proteico. El aceite crudo ha de refinarse con el fin de alcanzar los estándares de calidad exigidos para su uso (Johnson, 2008). Este refino, tradicionalmente, se realiza siguiendo las etapas de: desgomado (cuyo objetivo es separar los fosfolípidos), neutralización de ácidos grasos libres, decolorización empleando tierras activas (cuya función es separar pigmentos y productos de oxidación) y deodorización por medio de destilación a vacío (con el fin de retirar los compuestos volátiles) (Bimbo, 2012; Rubio-Rodriguez et al., 2010).

1.2.1. Revalorización de aceite de pescado: concentrados en PUFA y lípidos estructurados

Los aceites concentrados en Omega-3 han de contener, como mínimo, 600 mg de ácidos grasos Omega-3 por gramo de aceite (Rubio-Rodriguez et al., 2010). Son considerados productos de muy alto valor añadido con numerosas aplicaciones en la industria alimentaria y farmacéutica (Dillon, Aponte, Tarozo, & Huang, 2013; Kralovec, Zhang,

Zhang, & Barrow, 2012; Kuratko & Salem, 2013; Lembke, 2013). El proceso de concentración se lleva a cabo una vez el aceite ha sido refinado y descontaminado.

Los concentrados de Omega-3 se producen por métodos físicos y enzimáticos. En el primer caso, la etapa de concentración se basa en las diferentes propiedades físicas (por ejemplo, punto de fusión o ebullición) que los ácidos grasos poseen en función de su grado de insaturación o/y de su longitud de cadena. La eficiencia de estos métodos aumenta muy significativamente al usar como materia prima ésteres o ácidos grasos libres (FFA) en lugar de triglicéridos (TAG). Por ello, como pretratamiento, el aceite de pescado se hidroliza o esterifica antes de realizar la concentración por métodos físicos (Lembke, 2013). Como post-tratamiento, los concentrados han de re-esterificarse en la forma de triglicéridos ya que éstos se metabolizan y digieren mejor que los ésteres o ácidos grasos (Small, 1991). Los procesos físicos más usados en la producción de concentrados de Omega-3 son: la winterización a baja temperatura, la complejación con urea, la extracción con fluidos supercríticos, la cromatografía líquida o de fluidos supercríticos y la destilación molecular (Lembke, 2013; Rubio-Rodriguez et al., 2010; Fereidoon Shahidi & Wanasundara, 1998).

Los procesos enzimáticos se basan en la gran especificidad que las lipasas poseen. Las lipasas se clasifican en dos grandes grupos en función de su regioselectividad: (i) lipasas sn-1(3) específicas, que tienen una gran tendencia a reaccionar con ésteres colocados en las posiciones extremas del esqueleto de la glicerina y (ii) lipasas no específicas o aleatorias, que reaccionan indistintamente con los tres enlaces de la glicerina. Sin embargo, las lipasas con selectividad a la posición central son muy poco comunes (Rogalska, Cudrey, Ferrato, & Verger, 1993; Sugihara, Shimada, & Tominaga, 1991).

En el aceite de pescado, los ácidos grasos poliinsaturados presentan cierta regioselectividad hacia la posición central (sn-2) (Brockerhoff, Hoyle, Hwang, & Litchfield, 1968; Olley, Dunstan, & Kolakowska, 2002). En un principio, el uso de lipasas 1,3-específicas permitiría producir concentrados de PUFA en forma de acilgliceroles, es decir, triglicéridos (TAG), diglicéridos (DAG) o monoglicéridos (MAG). En la concentración de PUFA mediantes procesos enzimáticos, el rendimiento está muy altamente influido por la regiodistribución de los ácidos grasos del aceite empleado como materia prima. Por ello, es difícil describir tendencias, rendimientos o procesos de manera general y se recomienda llevar a cabo una completa caracterización del aceite usado como sustrato. Dicha caracterización debe incluir un análisis regioespecífico de los ácidos grasos que lo

constituyen. Los concentrados de PUFA en forma de acilgliceroles son compuestos de alto valor nutricional con aplicación en la industria alimentaria y farmaceútica. Como triglicéridos se emplean como suplemento nutricional, mientras que en la forma de diglicéridos o monoglicéridos se pueden usar como emulsificantes con valor nutricional. Además, estos concentrados pueden ser el sustrato idóneo para la producción de lípidos estructurados (SL). Se define como SL a una grasa o aceite sintético o modificado que contiene ácidos grasos de cadena larga (L) y de cadena media (M) esterificados en posiciones específicas del esqueleto de la glicerina. El término modificado se refiere a cualquier cambio de composición y/o distribución de los ácidos grasos originales. Estos cambios estructurales dotan al nuevo lípido de unas propiedades nutricionales y/o fisicoquímicas que difieren del original (Kim & Akoh, 2015). Los lípidos estructurados tipo MLM tienen aplicación en el ámbito de la nutrición clínica y están compuestos por un ácido graso poliinsaturado de cadena larga en la posición central y dos de cadena media en las extremas. Al estar colocados, los PUFA, en la posición central se metabolizan y absorben más fácilmente (Xu, 2000).

La eficiencia de la digestión y absorción de los ácidos grasos depende de la estructura del triglicérido. Christensen, Hoy, Becker, & Redgrave, (1995) describieron que el EPA y DHA ingerido en la forma de MLM era más rápidamente absorbido y metabolizado que otros triglicéridos con similar composición pero con regiodistribución aleatoria. La lipasa pancreática, segregada durante la digestión, es 1,3-regioespecífica y, presenta cierta selectividad hacia los ácidos grasos de cadena media (Bottino, Vandenburg, & Reiser, 1967). Por ello, los ácidos de cadena media se liberan al medio como ácidos grasos libres, se absorben rápidamente y se transportan por la vena porta. Por otro lado, los 2-monoglicéridos que se producen tras la hidrólisis se absorben por el sistema linfático (Small, 1991). Los lípidos estructurados tipo MLM se usan como fuente de energía rápida para pacientes que sufren de síndrome de malabsorción (Babayan, 1987), para la formulación de productos de nutrición infantil (Innis, 2011) y el diseño de alimentos hipocalóricos.

Los lípidos estructurados se producen por métodos enzimáticos a través de (i) procesos de una etapa (acidólisis o interesterificación) o de (ii) procesos de dos etapas en los que se combina la hidrólisis o alcohólisis con una posterior etapa de esterificación (Akoh & Kim, 2008).

1.2.2. Oxidación y estabilización de aceite de pescado

La causa fundamental del deterioro de alimentos es la microbiana. Las técnicas actuales de procesado permiten controlarla; por ello, la oxidación se ha convertido en el principal mecanismo químico responsable de la descomposición de los alimentos con la consecuente disminución de vida útil del producto y la pérdida de propiedades organolépticas y nutricionales (Schaich, Shahidi, Zhong, & Eskin, 2013; Shahidi & Zhong, 2008).

La reacción de oxidación es catalizada en presencia de calor, luz, iones metálicos, oxígeno y enzimas lipoxigenasas (Frankel, 2005). Por tanto, puede llevarse a cabo por medio de una gran variedad de mecanismos como la auto-oxidación, la foto-oxidación o a través de procesos térmicos o enzimáticos. De ellos, la auto-oxidación es el principal mecanismo de deterioro de alimentos (Shahidi, 2000). Además, la composición del aceite afecta críticamente a la velocidad de esta oxidación. Así, un aumento de la disponibilidad de hidrógenos bis-alílicos acelera muy notablemente la oxidación (Frankel, 2005). Aceites con alto contenido en PUFA son más susceptibles a su deterioro. A su vez, la regiodistribución de los ácidos grasos en el esqueleto de glicerina ejerce cierta influencia en la velocidad de oxidación, siendo ésta menor cuando el ácido graso se encuentra en la posición central (Wijesundera et al., 2008).

La auto-oxidación lipídica es un mecanismo radicalario que consta de tres etapas: iniciación, propagación y terminación. Es un proceso de auto-propagación y de auto-aceleración que se inicia muy lentamente y cuya velocidad crece de manera exponencial (Shahidi & Zhong, 2010). En primer lugar, los ácidos grasos insaturados pierden un átomo de hidrógeno y se transforman en radicales libres. Seguidamente, en la etapa de propagación, se producen los hidroperóxidos (compuestos primarios de oxidación). Estos compuestos son muy inestables y se descomponen formando los denominados compuestos secundarios de oxidación (aldehídos, cetonas, alcoholes, ácidos orgánicos volátiles y compuestos epóxicos, entre otros) (Shahidi & Zhong, 2010). Los hidroperóxidos son sustancias inodoras e insípidas mientras que los compuestos secundarios de oxidación son los responsables del enranciamiento y de los malos olores, detectándose, algunos de ellos, a niveles muy bajos (Jacobsen & Skall Nielsen, 2007). Por último, los radicales libres interaccionan entre sí formando especies estables no radicalarias mediante reacciones de condensación que tienen lugar a baja temperatura (Frankel, 2005).

Para usos industriales, el aceite ha de estabilizarse para protegerlo de la autoxidación y alargar su vida útil. Existen un amplio número de sustancias antioxidantes con capacidad de inhibir parcialmente las reacciones de oxidación, como: secuestrantes o neutralizadores de radicales, desactivadores de peróxidos (u otro tipo de compuesto que contenga oxígeno con capacidad reactiva), agentes reductores o compuestos quelantes de iones metálicos (Shahidi & Zhong, 2006).

La estabilización se puede llevar a cabo añadiendo directamente antioxidantes al aceite (Serfert, Drusch, & Schwarz, 2009) y también empleando procesos de emulsificación o microencapsulación. En estas técnicas, el aceite se dispersa en una fase acuosa que es estabilizada físicamente empleando emulsificantes. En lo que respecta al uso de antioxidantes en sistemas dispersos, se ha demostrado que algunos de los antioxidantes que son muy eficaces al usarlos en matrices oleosas continuas no tienen poder antioxidante en sistemas dispersos secados por atomización (Serfert et al., 2009). En los sistemas dispersos, la ubicación de los antioxidantes es crítica, siendo éstos más eficientes cuando se localizan en la interfase aceite-agua que es donde la oxidación comienza (Oehlke, Heins, Stöckmann, Sönnichsen, & Schwarz, 2011).

La producción de emulsiones o microcápsulas no sólo sirve para estabilizar el aceite sino que también proporciona una vía para la incorporación del aceite de pescado en alimentos. El aceite microencapsulado se puede añadir a la formulación de productos sólidos como pan o productos en polvo (por ejemplo, formulaciones infantiles) (Jacobsen & Skall Nielsen, 2007). El aceite en forma de emulsión se puede incorporar a alimentos líquidos o semi-líquidos (Let, Jacobsen, Sørensen, & Meyer, 2007).

1.3 Hidrolizados de proteína de pescado

Las proteínas procedentes del pescado se pueden clasificar en tres grupos: (i) proteínas miofibrilares como la actina, miosina, tropomiosina y troponina (proteínas estructurales solubles en disoluciones salinas con elevada fuerza iónica que representan entre un 66 y 77% de la masa total), (ii) proteínas sarcoplasmáticas como la mioglobina o las enzimas (solubles en agua y en disoluciones tampón diluidas, alcanzan hasta un 30 % del total de proteína) y (iii) proteínas del estroma (insolubles en disoluciones salinas concentradas, representan entre un 2 y 3 % del total) (Kristinsson & Rasco, 2000).

En las últimas décadas, un número elevado de investigaciones han estudiado el potencial de los hidrolizados de proteínas de pescado (FPH) para su aplicación en la industria alimentaria (Halim, Yusof, & Sarbon, 2016). Estos hidrolizados se producen por diversas vías: fermentación bacteriana, autolisis, hidrólisis química o enzimática. La hidrólisis enzimática se considera como la técnica más adecuada debido a (i) las condiciones suaves de operación (pH y temperatura), (ii) la anulación de reacciones secundarias debido a la alta selectividad de las enzimas y (iii) el alto valor nutricional del producto final.

La hidrólisis enzimática es catalizada por proteasas capaces de romper el enlace peptídico liberando grupos carboxilo y amino, los cuales participan en equilibrios ácido-base (Ec.1):

$$\mathrm{P1-CO-NH-P2+\ H_2O \Rightarrow P1-COOH+P2-NH_2}$$

$$\mathrm{P1-COOH\ \Leftrightarrow P1-COO^- + H^+} \qquad [1]$$

$$\mathrm{P2-NH_2 + H^+\ \Leftrightarrow P2-NH_3^+}$$

Como consecuencia de la continua rotura, las proteínas quedan reducidas a péptidos de menor peso molecular (Adler Nissen, 1986). Los hidrolizados de proteínas de pescado han demostrado tener excelentes propiedades fisicoquímicas, tecnológicas y bioactivas (Halim et al., 2016; Nalinanon, Benjakul, Kishimura, & Shahidi, 2011). Las proteínas de pescado sin modificar no presentan estas actividades, ya que las secuencias de péptidos responsables de las propiedades tecnológicas o bioactivas son innaccesibles (Kim & Wijesekara, 2010). Las capacidades tecnológicas y bioactivas están muy altamente determinadas por el tratamiento enzimático, que a su vez está influenciado por: (i) la matriz elegida como sustrato (es decir, especie seleccionada, origen proteico: músculo, piel, cabeza…), (ii) el tipo y la especificidad de la proteasa seleccionada y (iii) las condiciones de hidrólisis (pH, temperatura, relación enzima:sustrato, tiempo…).

Las propiedades tecnológicas se definen como “las propiedades fisicoquímicas de las proteínas en matrices alimentarias durante su procesado, almacenamiento y consumo” (Halim et al., 2016). De éstas, las más relevantes para su aplicación industrial son la capacidad emulsificante y espumante, la capacidad ligante de aceite y agua. Los hidrolizados de proteína de pescado se han usado en la formulación de espumas (Nalinanon et al., 2011). Mediante la adición de estos hidrolizados es posible mejorar la textura de ciertos alimentos (Taheri, Anvar, Ahari, & Fogliano, 2013), por ello se utilizan en la industria cárnica (Pires & Batista, 2013).

La hidrólisis de proteína de pescado para producir compuestos con propiedades bioactivas ha ganado importancia en los últimos años, con el objetivo de obtener productos con alto valor añadido (Thorkelsson, Slizyte, Gildberg, & Kristinsson, 2009). Un gran número de estudios han descrito la capacidad antioxidante (Sathivel et al., 2003; Wu, Chen, & Shiau, 2003), antihipertensiva (Jung et al., 2006; Ono, Hosokawa, Miyashita, & Takahashi, 2006), antimicrobiana (Fleury, Defer, & Bourgougnon, 2008) o anticolesterolémica (Naqash & Nazeer, 2011) de los hidrolizados de pescado.

1.3.1 Capacidad antioxidante de los hidrolizados de pescado

Los compuestos antioxidantes son capaces de ralentizar la oxidación lipídica en matrices alimentarias y desempeñan un rol crucial en la prevención de la oxidación de tejidos desde un punto de vista fisiológico.

Los antioxidantes actúan mediante diferentes mecanismos: transferencia electrónica o mediante quelación de metales, entre otros. Los metales catalizan la oxidación lipídica, siendo el poder catalítico de los metales reducidos mucho más intenso que en el caso de los metales oxidados (Shahidi & Zhong, 2008). Existen compuestos sintéticos con alto poder antioxidante, como el butilhidroxitolueno (BHT) o el butilhidroxianisol (BHA). Sin embargo, su uso en matrices alimentarias se ha comenzado a limitar debido a efectos secundarios peligrosos (Ito, Fukushima, & Tsuda, 1985). Por ello, uno de los principales retos científicos actuales es el desarrollo de compuestos antioxidantes naturales que no presenten efectos secundarios.

La actividad antioxidante de un hidrolizado proteico depende del tamaño molecular de los péptidos que lo conforman y de la composición y distribución de los aminoácidos en la secuencia peptídica. Recientemente, numerosos autores han descrito diferentes hidrolizados con intensa actividad antioxidantes producidos de especies como el arenque (*Clupea harengus),* (Sathivel et al., 2003); merluza (*Ohnius belengerii*) (Mendis, Rajapakse, & Kim, 2005) o carbonero (*Pollachius virens*) (Chabeaud, Vandanjon, Bourseau, Jaouen, & Guérard, 2009).

Se han identificado, además, secuencias peptídicas que actúan como eficientes antioxidantes; como por ejemplo, Asn-His-Arg-Tyr-Asp-Arg, Gly-Asn-Arg-Gly-Phe-Ala-Cys-Arg-His-Ala (0.856 kDa y 1.10 kDa, respectivamente) obtenidas de proteína de jurel y de piel de corvina (Sampath Kumar, Nazeer, & Jai Ganesh, 2011).

1.3.2 Actividad anithipertensiva de los hidrolizados de pescado

La hipertensión afecta a un tercio de la población y es un factor de riesgo que puede desencadenar enfermedades cardiovasculares (Kearney et al., 2005). La presión sanguínea se puede reducir inhibiendo a la enzima convertidora de la angiotensina (ECA). Dicha enzima participa en la regulación de la presión sanguínea interviniendo en el sistema renina-angiotensina, transformando el decapéptido inactivo Angiotensina I en un potente vasoconstrictor denominado Angiotensina II. Un gran número de compuestos sintéticos (captopril, enalapril, alacepril o lisinopril) se emplean para inhibir a la ECA. Éstos presentan como desventaja ciertos efectos secundarios como tos, alteración del gusto o erupciones cutáneas (Kim & Wijesekara, 2010). Por ello, hay un gran interés en la producción y desarrollo de compuestos antihipertensivos procedentes de fuentes naturales.

En este contexto, los hidrolizados de proteína de pescado se consideran una posible materia prima alternativa (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). Los péptidos con capacidad antihipertensiva están, generalmente, constituidos por entre 2 y 12 aminoácidos y contienen residuos hidrofóbicos (como Phe, Trp, Tyr o Pro) en el carbono terminal. Diferentes péptidos antihipertensivos producidos a partir de proteína de pescado se han identificado, como la secuencia Ser-Pro-Arg-Cys-Arg obtenida de hidrolizados de pez lagarto (Wu et al., 2012) o Gly-Ile-Pro-Gly-Ala-Pro y Ala-Pro-Gly-Ala-Pro producidos a partir de gelatina de raya común (Lassoued et al., 2015).

2. OBJETIVOS

El principal objetivo de esta tesis doctoral es desarrollar nuevos procesos para la obtención de compuestos funcionales a partir de descartes de pesca. Esta investigación se ha centrado en los dos componentes principales de los descartes: los lípidos y las proteínas.

Con el fin de revalorizar la fracción lipídica, se han seleccionado los siguientes objetivos:

- Revisión bibliográfica de los procesos de revalorización de descartes centrados en los lípidos marinos (Chapter I).
- Caracterización estacional del aceite extraído de seis especies de descarte (*Sardina pilchardus*, *Trachurus mediterraneus*, *Pagellus Acarne*, *Micromesistius poutassou*, *Boops boops*, and *Scyliorhinus canicula*) evaluando su valor nutricional y la

regiodistribución de los ácidos grasos en el esqueleto de la glicerina (Capítulo II y III).

- Obtención de aceite enriquecido en ácidos grasos poliinsaturados:
    - Concentración por medio de winterización a baja temperatura (Capítulo IV).
    - Concentración por medio de procesos enzimáticos en sistemas microacuosos (Capítulo V).
- Producción de lípidos estructurados (MLM) usando como materia prima descarte de sardina. Caracterización del estado oxidativo, rendimiento por etapa y regiodistribución de los ácidos grasos del producto purificado (Capítulo VI).

Se han seleccionado los siguientes objetivos para llevar a cabo la revalorización de los compuestos proteicos:

- Revisión bibliográfica de las propiedades y aplicaciones de los hidrolizados de proteína de pescado (Capítulo VII).
- Caracterización estacional del contenido proteico de seis especies de descarte (*Sardina pilchardus*, *Trachurus mediterraneus*, *Pagellus Acarne*, *Micromesistius poutassou*, *Boops boops*, and *Scyliorhinus canicula*) (Capítulo II y III).
- Estudio cinético de la hidrólisis enzimática de proteína de jurel empleando una mezcla de proteasas de origen bacteriano y animal centrado en:
    - Modelado de la hidrólisis por medio de redes neuronales artificiales (Capítulo VIII).
    - Modelado de la hidrólisis empleando un modelo mixto de proceso y mezcla (Capítulo IX).
    - Optimización de la producción de péptidos antihipertensivos (Capítulo IX).
    - Optimización de la producción de péptidos antioxidantes (Capítulo X).
- Evaluación de las propiedades tecnológicas y antioxidantes de hidrolizados proteicos de sardina y jurel para su empleo como estabilizantes en la microencapsulación de aceite de pescado (Capítulo XI).

3. MATERIALES Y MÉTODOS

Seis especies de peces procedentes del Mar de Alborán se emplearon para el estudio inicial: sardina (*Sardina pilchardus*), jurel (*Trachurus mediterraneus*), aligote (*Pagellus Acarne),* bacaladilla (*Micromesistius poutassou),* boga *(Boops boops)* y pintarroja (*Scyliorhinus canicula*). El resto de materiales necesarios se especifican en cada capítulo.

En la Tabla 1, se muestran los principales métodos que se han empleado en esta tesis doctoral.

4. RESULTADOS Y DISCUSIÓN

4.1. Revalorización del aceite de pescado

4.1.1. Caracterización estacional del sustrato

Para la adecuada elección de la técnica de revalorización ha sido necesario analizar los distintos sustratos poniendo especial atención en la composición global de los ácidos grasos así como en su regiodistribución.

Como se apuntaba en los objetivos, seis especies de descarte del Mar de Alborán se seleccionaron como posibles materias primas para la producción de alto valor nutricional. Para evaluar su calidad, el contenido lipídico, la composición global de ácidos grasos y su regiodistribución, se han analizado, a lo largo del año. Además se han calculado los índices de aterogenicidad, trombogénico y de hipo-hipercolesterolemia. La extracción del aceite se ha llevado a cabo por prensado hidráulico. El aceite crudo extraído en todos los casos estaba compuesto mayoritariamente por TAG (>99%).

Según su contenido lipídico, las especies se han clasificado en (i) grasas (sardina > 8% de lípidos), (ii) semi-grasas (aligote, jurel y boga con un contenido en grasa entre 4 y 8%) y (iii) magras (bacaladilla y pintarroja, < 4%) (Ackman, 2005).

El contenido lipídico varió considerablemente en función de la especie y la estación. La sardina presentó mayor contenido lipídico y mayor variabilidad estacional (12.45 ± 7.11 wt%).

Table 1. Resumen de las pirncipales técnicas de análisis usadas en esta investigación doctoral.

Análisis	Descripción	Capítulo
Composición proximal	Contenido proteico, lipídico, en cenizas y agua (A.O.A.C., 2006)	II, III, VI, VIII-XI
Grado de Hidrólisis	Método de pH-stato	VIII-XI
Perfil de los ácidos grasos del aceite	Esterificación (Rodríguez-Ruiz, Belarbi, Sánchez, & Alonso, 1998) Cromatografía gaseosa directa (Camacho Paez, Robles Medina, Camacho Rubio, González Moreno, & Molina Grima, 2002)	II- VI
Composición lipídica (MAG, DAG,TAG, FFA)	Cromatografía en capa fina	II-VI
Composición lipídica (1-MAG, 2-MAG)	Cromatografía en capa fina	VI
Composición lipídica de compuestos saturados (MAG, DAG, TAG)	Cromatografía líquida de ultra alta presión y espectrometría de masas (Moya-Ramírez, García-Román, & Fernández-Arteaga, 2016)	VI
Contenido en FFA	Valoración química (ISO 660:2009)	VI
Regiodistribución de los ácidos grasos en TAG	Alcohólisis empleando la lipasa Novozyme 435 (Shimada et al., 2003)	II-VI
Índice de peróxidos	Método espectrofotométrico (Drusch et al., 2012)	VI, XI
Índice de anisidina	Método espectrofotométrico (ISO 6885:2006)	VI
Fraccionamiento de los acilgliceroles	Columna de cromatografía líquida (Köse, Tüter, & Aksoy, 2002)	VI
Inhibición de la enzima ECA	Método espectrofotométrico basado en una reacción enzimática (Shalaby, Zakora, & Otte, 2006)	IX
Actividad quelante	Método espectrofotométrico (Decker & Welch, 1990)	X, XI
Capacidad reductora	Método espectrofotométrico (Oyaizu, 1986)	X,XI
Capacidad inhibitoria del radical DPPH	Método espectrofotométrico basado en la interacción del hidrolizado con el radical DPPH (Picot et al., 2010)	X, XI
Digestión *in vitro*	Hidrólisis enzimática (Garrett, Failla, & Sarama, 1999)	IX
Distribución de pesos moleculares de hidrolizados proteicos	Cromatografía de excluisón molecular por tamaño empleando cromatografía líquida rápida de proteína y SDS- PAGE	IX-XI
Composición de los amino ácidos	Cromatografía de fase reversa (Liu, Chang, Yan, Yu, & Liu, 1995)	XI

En cuanto al contenido lipídico del aligote y la pintarroja permanecieron prácticamente constantes a lo largo del año con valores de 5.3±0.5 wt% y 1.9±0.2 wt%, respectivamente. Estas diferencias se deben principalmente a los hábitos alimentarios de cada especie, su comportamiento migratorio, cambios reproductivos y periodo de desove (Brockerhoff et al., 1968). En concreto, en el caso de la sardina, ésta presentó su mínimo contenido lipídico en primavera (2.5 %), justo después del periodo de puesta.

En lo que respecta a la composición global de los ácidos grasos, de manera general, los PUFA fueron la fracción mayoritaria (26.3 to 46.4 wt%), hecho que demuestra el valor nutricional de estos aceites. El contenido de PUFA alcanzó un máximo en invierno, en el caso de todas las especies estudiadas excepto la pintarroja. Esto concuerda con la relación inversamente proporcional descrita entre temperatura y grado de insaturación de los ácidos grasos que constituyen un tejido (Malins & Wekell, 1970). En relación a la composición de los ácidos grasos individuales, como tendencia global, los más abundantes fueron el ácido palmítico (C16:0), EPA (C20:5n-3) y DHA (C22:6n-3).

Los tres índices estudiados demostraron el valor nutricional de estos aceites y que podían denominarse como sanos (Subhadra, Lochmann, Rawles, & Chen, 2006).

De manera general, los ácidos grasos poliinsaturados presentaron cierta regioselectividad hacia la posición central del esqueleto de glicerina, cuya composición media ha sido 52.1±8.4 mol%. Esto implica que una media del 47.2±7.9 mol% del total de PUFA se encontraban esterificados en el enlace sn-2. Dicho valor es superior al estequiométrico (33.33%), lo que demuestra la regioselectividad de la familia de los ácidos grasos poliinsaturados. No todos los PUFA mostraron la misma selectividad. Así, por ejemplo, el contenido medio de EPA en la posición central sólo alcanzó un 17.1±5.1 mol% lo que muestra una selectividad hacia las posiciones sn-1(3). Por el contrario, el ácido graso DHA, mostró una intensa selectividad hacia el enlace sn-2, con más de un 70% de la cantidad global esterificada en esta posición.

De todas las especies estudiadas, los aceites extraídos de la boga y pintarroja presentaron un contenido considerablemente alto de PUFA esterificados en la posición central de la glicerina. Por ello, estos aceites se han propuesto como materia prima para la síntesis de lípidos estructurados por medio de procesos en los que se preserve el perfil central de los ácidos grasos constituyentes del triglicérido. Así, el empleo de alcohólisis (Munio et al.,

2009) o una hidrólisis selectiva en un sistema microacuoso serían posibles procesos de revalorización.

El aceite extraído de la sardina, aligote, jurel o bacaladilla presentó propiedades nutricionales y un alto contenido global en PUFA, aunque distribuido de manera aleatoria en los tres enlaces de la glicerina. Estos aceites son candidatos para producir concentrados de PUFA por métodos físicos, en los cuales la regiodistribución del aceite sustrato no es relevante.

4.1.2. Obtención de aceite enriquecido en PUFA

Basándonos en la regiodistribución de los PUFA en los aceites extraídos se han propuesto dos técnicas diferentes: la cristalización a baja temperatura de ácidos grasos libres y la hidrólisis enzimática de triglicéridos en sistemas microacuosos para la producción de aceites enriquecidos en PUFA.

La winterización o cristalización fraccionada consiste en la eliminación de los compuestos de alto punto de fusión (es decir, los ácidos grasos saturados, SFA) mediante el descenso de la temperatura (Gunstone, Harwood, & Dijkstra, 2012). Para una determinada longitud de cadena, el punto de fusión de un ácido graso libre disminuye al aumentar el número de insaturaciones (Akoh, 2005; Gunstone et al., 2012). A bajas temperaturas, los ácidos grasos saturados cristalizan mientras que los poliinsaturados permanecen en la fase líquida (Wanasundara, & Shahidi, 2005).

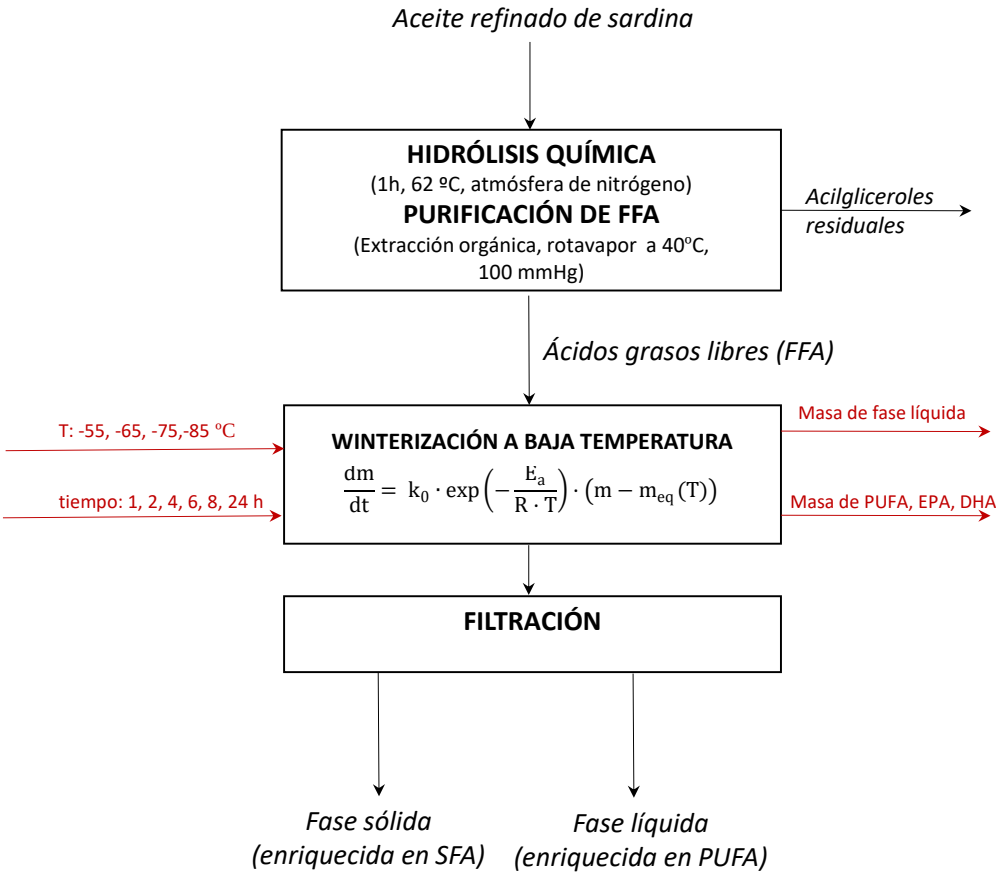
La hidrólisis de triglicéridos catalizada por lipasas consiste en la reacción de triglicéridos y agua para producir diglicéridos, monoglicéridos y ácidos grasos libres. Mediante el uso de una lipasa 1,3- específica y un aceite cuyos ácidos grasos poliinsaturados presenten alta regioselectividad hacia la posición 2, se puede producir una fracción de acilgliceroles enriquecidos en PUFA en la posición central.

En ambos estudios se ha utilizado un aceite refinado de sardina suministrado por Industrias Afines S.L. como aceite modelo.

4.1.2.1. Cristalización (winterización) de ácidos grasos libres a baja temperatura

Como se muestra en la Fig. 2 el proceso de winterización consta de las siguientes etapas: (i) producción de ácidos grasos libres por hidrólisis química y purificación por medio de extracciones líquido-líquido, (ii) winterización y (iii) filtración a vacío.

La mayor concentración de PUFA (>80 wt%, con un factor de concentración de 1.7 con respecto al aceite original) se obtuvo a -85 ºC y tras 24 horas de winterización. Un incremento de la temperatura aumentó la masa recuperada en la fracción líquida y disminuyó muy notablemente la concentración de PUFA. En el sobrenadante, el contenido de SFA disminuyó muy notablemente en todos los casos (de un 31.2 a un 6% en peso). Los ácidos grasos monoinsaturados (MUFA) sólo precipitaron a -85 ºC.



[6]

Figura 2. Diagrama de los experimentos realizados para modelizar el proceso de winterización.

En la fracción sólida, se detectó una cantidad de PUFA que permaneció constante independientemente del tiempo y la temperatura (0.057 ± 0.006 g). Esto puede deberse a diferentes fenómenos como la intersolubilización, agregación u oclusión de PUFA durante la cristalización de los ácidos saturados. De esta manera, se pueden formar cristales mixtos (que contienen SFA y PUFA) a temperaturas mayores de las esperadas para ácidos grasos puros (Wanasundara et al., 2005).

La evolución de la masa de la fracción líquida se ha modelado en función del tiempo y la temperatura siguiendo la teoría de difusión-reacción de transferencia de materia. Esta teoría expone que el crecimiento de la superficie de los cristales tiene lugar en dos etapas:

nucleación y crecimiento (Marangoni & Wright, 2005; Mullin, 2001). La Ec. 2 muestra el modelo adaptado al sistema que se estudia en este trabajo:

$$\frac{dm}{dt} = K_{G\cdot A} \cdot (m - m_{eq}) \quad [2]$$

Donde m representa a la masa (g), t el tiempo (min), $K_{G\cdot A}$ es el coeficiente global (h^{-1}) y m_{eq} la masa que se alcanza en el equilibrio (g). Los datos de equilibrio (m_{eq}) se estimaron ajustando los datos experimentales a una función racional y evaluando esta función a tiempo infinito. El coeficiente global $K_{G\cdot A}$, ha sido calculado analíticamente integrando la Ec.2 para cada temperatura estudiada. La relación de este coeficiente con la temperatura se ha modelado siguiendo una ecuación tipo Arrhenius (Chang, Wu, & Kimura, 2006; Mersmann, 2001). Teniendo en cuenta todo lo anterior, la Ec.2 puede expresarse en función de la temperatura y el tiempo:

$$\frac{dm}{dt} = k_0 \cdot \exp\left(-\frac{E_a}{R \cdot T}\right) \cdot (m - m_{eq}(T)) \quad [3]$$

Donde k_0 es el factor pre-exponencial (h^{-1}), E_a la energía de activación de la cristalización ($kJ \cdot mol^{-1}$), R la constante de los gases ideales ($kJ \cdot mol^{-1} \cdot K^{-1}$) y T la temperatura (K).

La Ec. 4 permite predecir la masa de la fracción líquida en función de la temperatura y el tiempo:

$$\frac{}{dt} = 5.23 \cdot \exp\left(-\frac{}{T}\right) \cdot (m - 0.022 \cdot T + 1.4029) \quad [4]$$

La concentración de PUFA y de los ácidos grasos EPA y DHA tuvo lugar debido al descenso de la masa líquida. De hecho, las masas de estas fracciones permanecieron prácticamente constantes independientemente del tiempo y la temperatura empleados. Por ello, no es posible emplear un modelo de transferencia de materia para describir el incremento de la concentración de estos compuestos en la fase líquida. Se ha propuesto la siguiente ecuación para predecir las fracciones másicas de PUFA, EPA y DHA:

$$X_i = \frac{mass_{i,initial} - mass_{i,sol}}{mass_{L,t}} \quad [5]$$

Donde i se refiere al conjunto de ácidos grasos poliinsaturados, al EPA o al DHA, X es la fracción másica de dichos compuestos en la fase líquida, $mass_{i,initial}$ es la masa inicial del compuesto i, $mass_{i,sol}$ la masa media del compuesto i que ha quedado ocluida en la fracción

sólida y $mass_{L,t}$ la masa total de aceite en la fracción líquida para un tiempo t (calculada por integración de la Ec. 4). El término $mass_{i,sol}$ se ha calculado como la masa media en la fracción sólida de todos los datos obtenidos a diferentes tiempos y temperaturas. Con este modelo, se pueden predecir las fracciones másicas con errores menores del 10%.

4.1.2.2. Hidrólisis enzimática de triacilglicéridos en sistemas microacuosos para maximizar el contenido en PUFA en acilgliceroles

En este caso, el objetivo era producir diacilglicéridos y monoacilglicéridos con alto contenido en PUFA. Para ello, se llevó a cabo la hidrólisis de aceite de sardina catalizada por la lipasa *Rhizomucor miehei*.

La concentración del agua es una de las variables más importantes que se ha de controlar en sistemas micro acuosos dado que afecta a la estructura de la enzima y a las condiciones del sistema ya que facilita la difusión de los sustratos y afecta al equilibrio de la reacción (Hari Krishna & Karanth, 2002). La actividad de agua es la propiedad más conveniente para caracterizar el contenido de agua en sistemas microacuosos (Xia, 2009). Por ello, tres actividades de agua (0.3, 0.6 y 0.8) se evaluaron para determinar y modelar su influencia en la composición final de los productos de la hidrólisis del aceite de sardina.

El aceite de sardina empleado como sustrato contenía principalmente PUFA (43 mol%) y SFA (33.83 mol%). Con respecto a la regiodistribución de los ácidos grasos, la posición sn-2 contenía un 47.84 mol% de ácidos grasos saturados siendo los poliinsaturados los siguientes (30.25 mol%). El DHA fue el ácido graso que presentó mayor regioespecifidad hacia la posición central, con un 70 % del total localizado en la posición sn-2.

A todos los niveles de actividad de agua ensayados, los PUFA se concentraron un factor máximo de 1.2 en la fracción de tri- y diglicéridos. La selectividad de la enzima se mostró de manera mucho más clara en el caso del ácido graso DHA. Este compuesto se concentró en las fracciones de TAG y DAG alcanzando factores de concentración de hasta 1.94.

Estos resultados se justifican teniendo en cuenta la regioselectividad de la enzima, la regiodistribución de los ácidos grasos en la estructura de glicerina del aceite empleado como sustrato y las propias características físicas de los ácidos grasos (como por ejemplo el número de insaturaciones o la longitud de cadena) que pueden causar ciertos impedimentos estéricos.

Para el cálculo del modelo cinético que predice la influencia de la actividad de agua se han propuesto las siguientes reacciones reversibles de segundo orden:

$$\mathrm{TAG} + \mathrm{W} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \mathrm{DAG} + \mathrm{FFA} \quad [6]$$

$$\mathrm{DAG} + \mathrm{W} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \mathrm{MAG} + \mathrm{FFA} \quad [7]$$

En las que TAG, DAG, MAG, FFA y W representan a los triglicéridos, diglicéridos, monoglicéridos, ácidos grasos libres y agua, respectivamente. Este modelo permite calcular el contenido global de TAG, DAG, MAG y FFA así como su concentración en ácidos grasos saturados y monoinsaturados. En el caso de los PUFA, éstos no cumplían con la estequiometría de la reacción (es decir, FFA=2·MAG + DAG) ya que su concentración en los diglicéridos era mayor que en los ácidos grasos libres. Por lo tanto, los PUFA se han modelado haciendo un balance molar a todo el sistema:

$$C_{i,GLOBAL} = C_{i,SFA} + C_{i,MUFA} + C_{i,PUFA} \quad [8]$$

Donde $C_{i,GLOBAL}$, $C_{i,SFA}$ y $C_{iMUFA,}$ se han calculado a través del modelo desarrollado partiendo de las reacciones 6 y 7.

En todos los casos, los datos teóricos y experimentales presentaron bajos valores de desviación estándar (<16%). La constante cinética directa k_1 era del mismo orden de magnitud tanto para el sistema global como para los SFA y MUFA (0.031±0.006 min^{-1}). Por tanto, la liberación del primer ácido graso del triglicéridos debe ser independiente a la naturaleza del ácido graso. La constante cinética directa (k_2), sí varió en función de las fracciones modeladas (0.42±0.04; 0.82±0.2 and 1.05 ± 0.17 min^{-1} para el sistema global, SFA y MUFA, respectivamente). El hecho de que la constante cinética directa fuera menor en el sistema global muestra la resistencia de los PUFA a ser hidrolizados, posiblemente por la regioselectividad hacia la posición sn-2 y por la rigidez que les confiere los dobles enlaces, que puede provocar impedimentos estéricos.

Una de las mayores limitaciones que presenta la modificación selectiva de lípidos es la migración de grupos acilo, es decir "el movimiento espontáneo de un grupo acilo desde un grupo hidroxilo a otro adyacente" (Fureby, Virto, Adlercreutz, & Mattiasson, 1996). Por lo tanto, la acil migración implica el movimiento de grupo acilo de la posición sn-1(3) a la posición sn-2 y viceversa (Xu, 2000).

La cinética de acil-migración de los 2-monoglicéridos a 1,(3)-monoglicéridos se ha estudiado y modelado siguiendo una reacción reversible de primer orden:

$$2-\mathrm{MAG} \underset{k_{-m}}{\overset{k_m}{\leftrightarrow}} 1-\mathrm{MAG} = 2-\mathrm{MAG} \underset{k_{-m}}{\overset{k_m}{\leftrightarrow}} 3-\mathrm{MAG} \quad [9]$$

En esta ecuación k_my k_{-m} son las constantes cinéticas directa e inversa. El mecanismo de acil migración mostró ser independiente de la actividad del agua, así como del soporte sobre el que la enzima estaba inmovilizada. Las fracciones de SFA, MUFA y PUFA presentaron constantes cinéticas y de equilibrio muy similares (k_m = 0.018 ± 0.0018 min^{-1} y K_m = 3.24 ± 0.82). En el equilibrio termodinámico, la proporción molar en 1(3)-MAG y 2-MAG era 86:14 (mol:mol), relación que es muy similar a la anteriormente descrita en la literatura (9:1) (Boswinkel, Derksen, K, & Cuperus, 1996).

La hidrólisis enzimática produjo concentrados de PUFA con un rendimiento mucho menor que los obtenidos vía winterización a baja temperatura. Por tanto, un proceso que emplee ácidos grasos libres concentrados como sustrato para la producción de lípidos estructurados (MLM) se ha propuesto para la revalorización de los descartes.

4.1.3. Producción de lípidos estructurados (MLM) partiendo de descartes de sardina

Se han seleccionado los lípidos estructurados tipo MLM debido a su alta aplicación y demanda en la industria farmacéutica. En este trabajo, se han producido lípidos estructurados con ácido caprílico (C8:0) como ácido graso de cadena media (M) y los ácidos grasos libres concentrados por winterización como ácidos grasos de cadena larga (L).

Para producir dichos lípidos estructurados partiendo de descartes de sardinas se han llevado a cabo los siguientes pasos: (i) extracción de aceite de pescado mediante prensado, (ii) concentración de los ácidos grasos Omega-3 en la forma de ácidos grasos libres vía winterización a baja temperatura, (iii) esterificación enzimática en dos etapas (Ec. 10 y 11) y (iv) purificación de los triglicéridos mediante cromatografía líquida en columna (Fig. 3):

$$\text{Glicerina} + \frac{}{3}\ \text{Ácido caprílico} \xleftrightarrow{\text{inmobilizada}} \mathrm{TAG} + 1{,}3\ \mathrm{DAG} + 1\mathrm{MAG} + \mathrm{FFA} + H_2O \quad [10]$$

$$1{,}3\ \mathrm{DAG} + \frac{1}{3}\ \text{FFA concentrados} \xleftrightarrow{Novozyme\ 435} \mathrm{TAG} + 1{,}3\ \mathrm{DAG} + \mathrm{FFA} + H_2O \quad [11]$$

También se ha estudiado la estabilidad oxidativa del aceite en cada etapa (índice de peróxidos y de anisidina), así como la composición y regiodistribución de los ácidos grasos en el esqueleto de glicerina de los triglicéridos producidos.

4.1.3.1. Descripción global: rendimiento y estabilidad oxidativa

La etapa de extracción por prensado hidráulico, combinada con un precalentamiento a 30ºC de 30 min, presentó un rendimiento del 39.5% (en peso referido al contenido total de aceite de las sardinas). Se pueden obtener mayores rendimientos aumentando la intensidad del pretratamiento, es decir, calentando a mayor temperatura durante tiempos más prolongados. Sin embargo, el aumento de temperatura acelera la oxidación del aceite e implica una pérdida de calidad (García-Moreno, Morales-Medina, et al., 2014). Tras la concentración, se obtuvo una fracción líquida con un rendimiento de 61.6% en peso con respecto a la masa total de ácidos grasos winterizados. Este bajo rendimiento se debe, sobre todo, al fraccionamiento de los ácidos grasos saturados que quedan en la fase sólida. Sólo un 6.5% en peso de los PUFA pasaron a la fase sólida durante la etapa de concentración. Las etapas de esterificación y purificación presentaron rendimientos altos, superiores al 80%.

Los valores máximos de índice de peróxidos (PV) y anisidina (AI) para un aceite crudo son 20 meq/kg de aceite y 60, respectivamente (Bimbo, 1998), mientras que un aceite refinado ha de presentar valores mucho menores (PV < 5 meq/kg aceite y AI < 20), (Ackman, 2005). En el proceso propuesto, el aceite crudo extraído cumplía con la estabilidad oxidativa exigida para los aceites refinados (Fig. 3), debido a las condiciones tan suaves de extracción. La etapa de concentración (winterización a -85ºC junto con la evaporación del disolvente a 100 mmHg) mejoró los valores de PV y AI de los ácidos grasos concentrados, mientras que empeoró, ligeramente, el índice de peróxidos de los ácidos grasos que permanecieron en la fase sólida. Debido a la baja volatilidad que los hidropéroxidos poseen (Jacobsen & Nielsen, 2007), éstos han podido cristalizarse y permanecer en la fase sólida. El aceite sufrió la máxima oxidación en la etapa de esterificación, a pesar de haberse empleado condiciones suaves (37ºC, 24 h, uso de botes topacio, y burbujear nitrógeno para desplazar el oxígeno antes de cerrar el recipiente), alcanzándose valores superiores a los requeridos para un aceite refinado (Fig. 3).

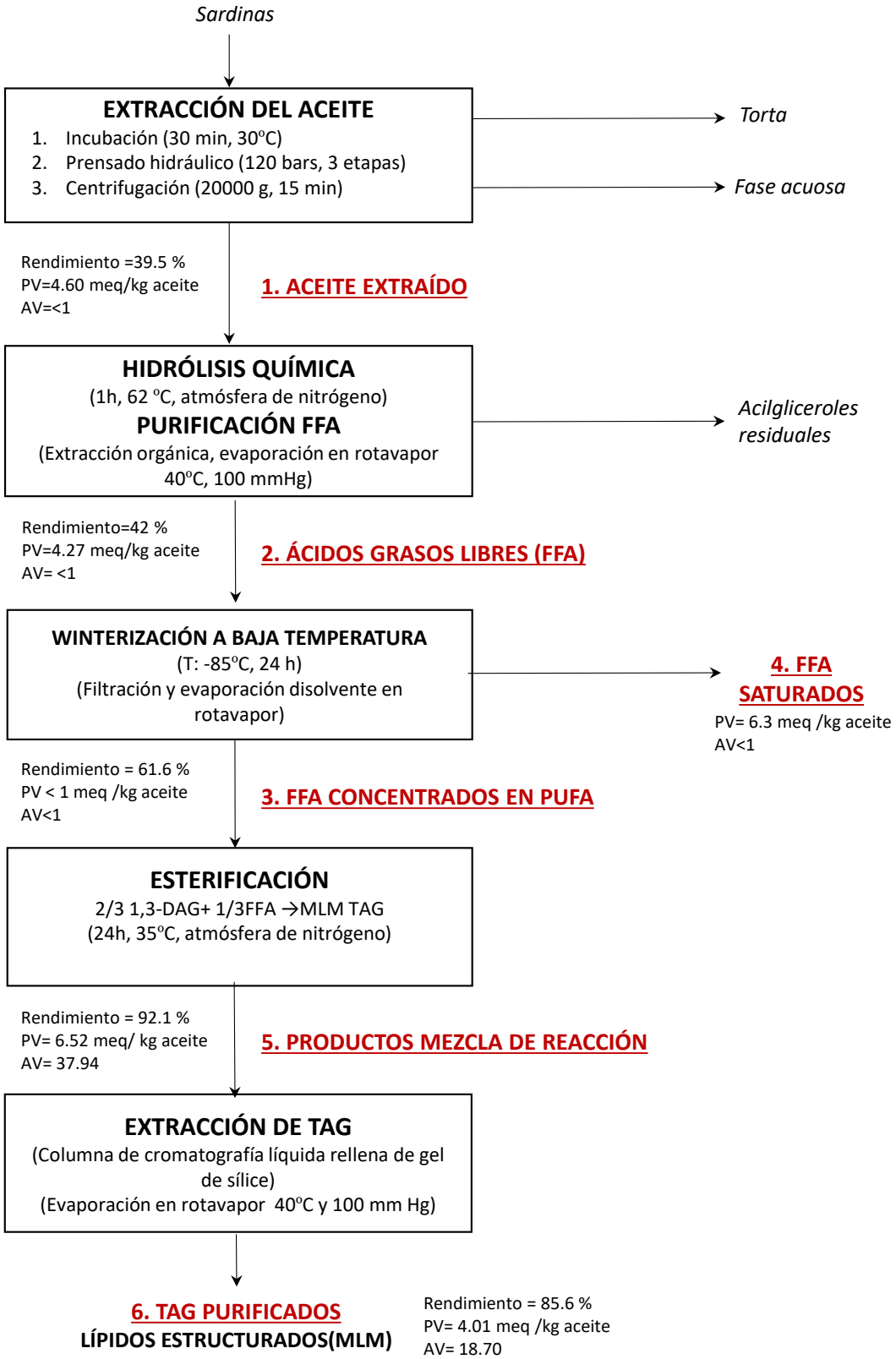


Figura. 3. Diagrama del proceso propuesto par la producción de lípidos estructurados (MLM) partiendo de sardinas. PV y AV se refieren al índice de peróxidos y anisidina respectivamente.

La etapa de separación de los triglicéridos producidos (cromatografía líquida en columna seguida de la evaporación del disolvente a 40ºC y 100 mmHg), mejoró la estabilidad oxidativa hasta valores de aceite refinado. Es posible que el uso de gel de sílice como fase estacionaria, resultara en una parcial absorción de los compuestos primarios de oxidación (hidroperóxidos). También se observó un descenso en el índice de anisidina, es decir, de compuestos volátiles que pudieron evaporarse durante la eliminación del disolvente (40ºC y 100 mmHg). Futuros estudios se han de centrar en la adición de antioxidantes tras la etapa de concentración, con el fin de minimizar el grado de oxidación del aceite producido.

4.1.3.2. Composición y regiodistribución de los ácidos grasos

El aceite extraído estaba compuesto de ácidos grasos poliinsaturados (41.67 mol%), siendo el DHA (16.7 mol%) y EPA (12.47 mol%) los más abundantes de esta fracción.

Tras la winterización a baja temperatura, el contenido en PUFA se incrementó un factor de 1.6 veces. Así, se obtuvo una fase líquida que contenía 610 mg de Omega3 /g de aceite, valor superior al requerido para los concentrados (600 mg/g de aceite).

Con respecto a la esterificación, el producto final estaba compuesto por un 60% mol de triglicéridos (lo que corresponde a un 90% en masa). La composición global de los ácidos grasos constituyentes de los lípidos estructurados era muy próxima a la teórica esperada (66 mol% de ácido caprílico y 33% del concentrado de ácidos grasos libres producidos por winterización). Sin embargo, la composición de las fracciones de ácidos grasos libres y diglicéridos difería de la esperada. Los ácidos grasos libres tenían un alto contenido en ácido caprílico (59 mol%), cuando ésta debería ser mínima. Por otro lado, los diglicéridos contenían cantidades significativas de C16:1n-7, C18:1n-9, EPA y DHA, cuando éstos deberían estar compuestos exclusivamente de ácido caprílico. Además, los triglicéridos contenían alrededor de 25 mol% de ácido caprílico en la posición central. Todos estos datos se pueden justificar bien debido a que los diglicéridos empleados eran una mezcla de 1,2 y 1,3-DAG debido a la acilmigración o debido a una reacción paralela de acidolisis. Se debería profundizar en el estudio del proceso enzimático para elucidar el mecanismo controlante.

Como control negativo, se realizó también una esterificación en una única etapa. En ese caso, los triglicéridos producidos contenían un 73% de ácido caprílico en la posición central. Por lo tanto, a pesar de que el proceso en dos etapas presentaba ciertas

limitaciones, éste supuso una gran mejora en la regioselectividad del proceso en comparación a la esterificación en una etapa.

4.2. REVALORIZACIÓN DE LA PROTEÍNA DE PESCADO

4.2.1. Caracterización estacional del sustrato

Al igual que se hizo para la fracción lipídica, el contenido proteico de las especies de descarte seleccionadas se analizó estacionalmente durante un año. Contrariamente a lo que ocurría con los lípidos, el contenido proteico permaneció prácticamente constante a lo largo de todo el año. La pintarroja era la especie con mayor porcentaje de proteína (con un valor medio anual de 20.6±1.9% en peso) seguido del aligote, boga, bacaladilla, sardina y jurel (cuyos porcentajes de proteína en peso eran 18.5±1.6, 18.1±2.0, 18.1±0.6, 17.5±2.0 and 17.5±0.9 %, respectivamente).

Las actividades antioxidante y antihipertensiva de los hidrolizados proteicos de estas especies ha sido estudiado con anterioridad por este grupo de investigación empleando subtilisina y tripsina (García-Moreno, Batista, et al., 2014; García-Moreno, Espejo-Carpio, Guadix, & Guadix, 2015). Los hidrolizados de jurel fueron los que mayor actividad antihipertensiva presentaron, así como elevada actividad antioxidante (García-Moreno, Batista, et al., 2014; García-Moreno et al., 2015). Se observó, además un efecto sinérgico al emplear mezclas de tripsina y subtilisina. Por lo que, esta especie se eligió como sustrato para la producción de péptidos con propiedades antihipertensiva y antioxidantes empleando distintas mezclas de proteasas.

Los hidrolizados de sardina fueron los que mayor actividad antioxidante mostraron (García-Moreno, Batista, et al., 2014). De ahí que, tanto la sardina como el jurel fueran elegidos como materias primas para la producción de hidrolizados capaces de estabilizar física y oxidativamente emulsiones y/o microcápsulas de aceite de pescado.

4.2.2. Estudio cinético de la hidrólisis enzimática de proteína de jurel empleando una mezcla de proteasas de origen animal y bacteriano

En esta sección, se ha propuesto un diseño mixto de proceso y mezcla compuesto por la siguientes variables de entrada: porcentaje de subtilisina en la mezcla de proteasas (0, 25, 50, 75 and 100%), la concentración de proteína en el reactor (2.5 – 5 y 7.5 g/L) y la temperatura (40, 47.5 y 55ºC). En todos los casos, la relación enzima-sustrato se mantuvo

constante e igual a 1% en peso. Como variables de respuesta se han seleccionado el grado de hidrólisis (DH), la actividad antihipertensiva (capacidad inhibidora de ECA) y la actividad antioxidante (actividad inhibidora de DPPH, capacidad reductora de Fe^{3+} y actividad quelante de Fe^{2+}).

Hay que añadir que en el caso del modelo desarrollado empleando redes neuronales artificiales (ANN) se ha incluido el tiempo como variable de entrada (0 a 240 min).

La hidrólisis de proteína de pescado es una reacción heterogénea en la que tienen lugar de manera simultánea una serie de fenómenos físicos (solubilización del sustrato o desactivación enzimática) y químicos (rotura de los enlaces peptídicos, inhibición por sustrato o por producto). Los modelos mecanísticos basados en la teoría de Michaelis-Menten, tienen un amplio rango de aplicabilidad (Cavaille & Combes, 1995; Qian, Zhang, & Liao, 2011; Valencia, Pinto, & Almonacid, 2014; Zhou, Chen, & Li, 2003). Hay que resaltar que la complejidad del modelo aumenta notablemente en función del número de fenómenos que se incluyan. En el diseño experimental propuesto en este trabajo, el objetivo era modelizar el grado de hidrólisis y las bioactividades en función de las variables de entrada anteriormente enumeradas. Por lo que, además de los fenómenos nombrados con anterioridad, otros factores como la distribución de pesos moleculares y la composición y distribución de aminoácidos en la secuencia peptídica deberían de tenerse en cuenta dando lugar a particulares, poco generalizables.

Por tanto, la utilización de modelos empíricos como la metodología de superficies de respuesta (RSM) o las redes neuronales artificiales (ANN) resulta de gran utilidad (Abakarov, Teixeira, Simpson, Pinto, & Almonacid, 2011; Baş, Dudak, & Boyaci, 2007). Las superficies de respuesta se usan habitualmente para modelizar y optimizar la hidrólisis de proteínas (Valencia, Espinoza, Ceballos, Pinto, & Almonacid, 2015). Su principal ventaja radica en el bajo número de datos experimentales que se requieren para llevar a cabo el cálculo (Keskin Gündoğdu et al., 2014). Como desventaja, presentan el inconveniente de que, usualmente, en estos métodos se modela empleando funciones polinómicas (cuadráticas o cúbicas) y no todos los procesos biológicos pueden ser apropiadamente modelados (Baş et al., 2007).

La modelización empleando ANN es una alternativa con gran capacidad, especialmente en el caso en que el volumen de datos sea grande (Baş et al., 2007; G. Zhang, Eddy Patuwo, & Y. Hu, 1998; Y. Zhang, Xu, & Yuan, 2009). Las redes neuronales artificiales son

modelos empíricos inspirados en el cerebro humano, en los que las neuronas se organizan en capas interconectadas entre sí por funciones matemáticas.

4.2.2.1. Modelo empleando ANN

La estructura de la red neuronal artificial que se empleó estaba compuesta de una capa de entrada, una oculta y una de salida, como se muestra en la Fig. 5. Las cuatro variables de entrada empleadas fueron concentración de proteína en el reactor, temperatura, porcentaje de subtilisina en la mezcla de proteasas y tiempo. El número de neuronas de la capa oculta se ensayó entre 1 y 10, empleándose como función de transferencia la función sigmoide. Finalmente, la única neurona de la capa de salida proporcionaba el valor del grado de hidrólisis.

La ecuación 12 muestra la ecuación global asociada a esta red neuronal artificial:

$$DH = \sum_{k=1}^{10} \omega_k \cdot \Phi\left(\sum_{i=1}^{4} w_{ki} \cdot X_i + b_k\right) + \beta \qquad [12]$$

Para entrenar la red neuronal artificial se dividieron los datos experimentales en tres subgrupos. El primero, que incluía un 70% de los datos, se utilizó para entrenamiento de la red. Para ello, se empleó el algoritmo de Levenberg-Marquardt para minimizar el error cuadrático medio (MSE). El segundo grupo, cuyo objetivo era la validación del modelo, estaba compuesto por un 15% de los datos. El tercer grupo de datos, se empleaba para probar la capacidad predictiva de la red.

Como resultado, el aumento del número de neuronas en la capa oculta disminuyó el MSE del subconjunto de prueba y mejoró el coeficiente de determinación (r^2). En consecuencia, se seleccionó la red neuronal artificial compuesta por 10 neuronas en la capa oculta (r^2 = 0.987).

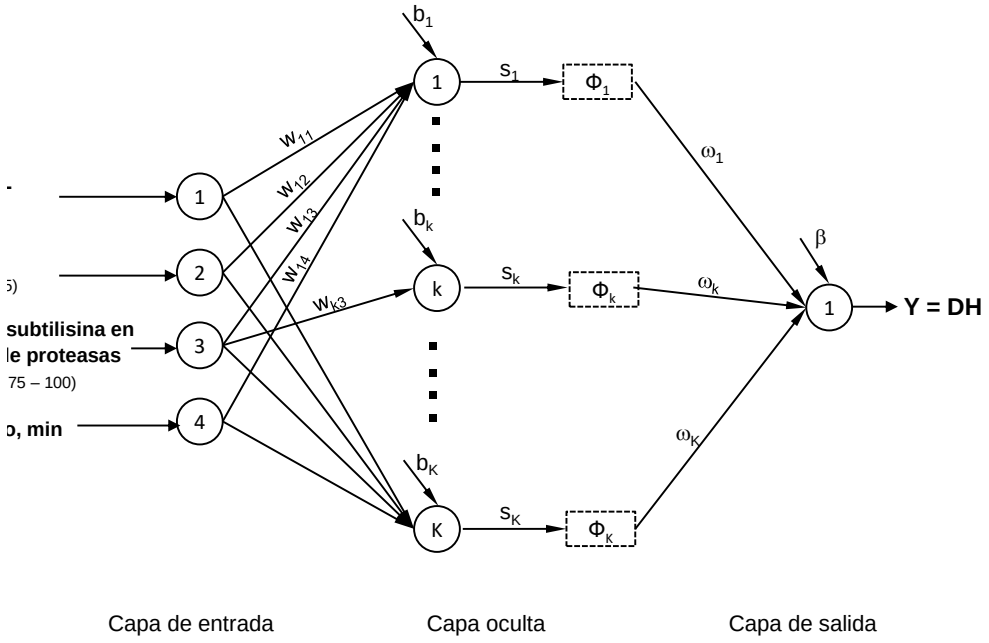


Figura. 5. Diagrama de la estructura de la red neuronal artificial y representación esquemática de los parámetro del modelo.

4.2.2.2. Modelo mixto de proceso y mezcla:

El modelo mixto de variables de proceso y mezcla se ha empleado para modelizar el grado final de hidrólisis (tras cuatro horas de reacción) y las actividades antihipertensivas y antioxidantes de los hidrolizados producidos según el diseño de experimentos descrito con anterioridad. Como se muestra en la Fig. 6, las variables de proceso se modelaron siguiendo un diseño factorial mientras que la composición del complejo enzimático siguió un modelo de mezcla. Mediante la combinación de ambos sub-modelos, se obtuvo el modelo mixto de proceso y mezcla mostrado en la Ec. 13.

$$Y(X_1, X_2, S, T) = (\alpha_0 + \alpha_1 \cdot S + \alpha_2 \cdot T + \alpha_{12} \cdot S \cdot T + \alpha_{11} \cdot S^2 + \alpha_{22} \cdot T^2) \cdot (\beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_{12} \cdot X_1 X_2) \quad [13]$$

Donde Y se refiere a cualquiera de las variables de respuesta (DH, actividades antioxidantes y antihipertensiva), X_1 y X_2 es el porcentaje de subtilisina y tripsina en la mezcla de enzimas, S es la concentración de sustrato en el reactor y T la temperatura.

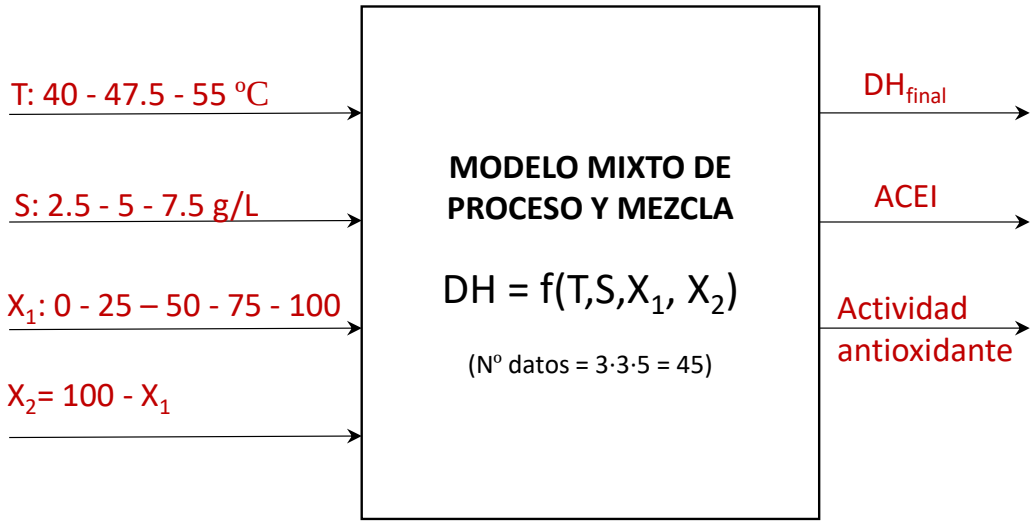


Figura 6. Diagrama del diseño de experimentos propuestos para modelar las variables de salida: DH, activitdad antihpertensiva y la actividad antioxidante (Capacidad inhibitoria de DPPH, capacidad reductora y actividad quelante) en función de la temperatura, concentración de sustrato y contenido de subtilisina y tripsina en la mezcla de proteasas.

Tras simplificar el modelo anterior y expresarlo exclusivamente en función de los coeficientes significativos, el DH final se puede expresar según la Ec. 14 (r^2 =0.9913).

$$+ 4.66 \cdot 10^{-3} \cdot X_2 \cdot S^2 + 7.02 \cdot 10^{-5} \cdot X_1 \cdot X_2 \cdot T - 1.20 \cdot 10^{-6} \cdot X_1 \cdot X_2 \cdot T^2 \quad [14]$$

El máximo DH predicho (17.1%) empleando el modelo calculado mediante ANN, se obtenía para una concentración de proteína de 2.54 g/L, 40ºC, tras 4 horas de hidrólisis y empleando una mezcla de proteasas que contenía 38.3 % de subtilisina y 61.7 % de tripsina. Al optimizar el modelo mixto de proceso y mezcla, el valor máximo de grado de hidrólisis predicho era ligeramente inferior (15.94%) obteniéndose a 2.5 /L, 40ºC y con un 41.2% de subtilisina en la mezcla enzimática. En ambos modelos, los óptimos predichos fueron muy similares y prueban que mediante la adición de tripsina a la mezcla de enzimas se pueden alcanzar grados de hidrólisis elevados a temperaturas inferiores. El hecho de reducir la temperatura de operación presenta ventajas tanto económicas como nutricionales, ya que la proteína se degrada menos.

Los datos experimentales mostraron que las bajas concentraciones (2.5 g/L) favorecían al grado de hidrólisis; hecho que podría relacionarse con una posible inhibición por sustrato o con una mayor solubilidad del sustrato. Un aumento de la temperatura de reacción disminuyó el grado de hidrólisis, comportamiento que es el resultado de la doble influencia que la temperatura ejerce en la solubilidad y en la estabilidad de las enzimas. Así, la solubilidad aumenta a temperaturas mayores lo que provoca que haya más enlaces

peptídicos susceptibles a la hidrólisis y, consecuentemente, que el grado de hidrólisis sea mayor. Paralelamente, a temperaturas elevadas se produce la desactivación térmica de las enzimas, hecho que debe ser el responsable de la disminución del grado de hidrólisis a temperaturas mayores. En el caso de la tripsina, ésta presenta su máxima actividad a 37 ºC (Najafian & Babji, 2012) por lo que es esperable que a 55 ºC ésta sufra desactivación.

No se observó una tendencia común en la influencia que la temperatura ejercía sobre la actividad de la mezcla de protesas aunque en algunos casos se apreció un efecto sinérgico al emplear mezcla de proteasas. Esta sinergia puede estar relacionada con la selectividad de las enzimas, la tripsina hidroliza enlaces vecinos a residuos de lisina-arginina (Olsen, Ong, & Mann, 2004) mientras que la subtilisina presenta un rango mucho más amplio, mostrando selectividad hacia los enlaces adyacentes a residuos aromáticos o ácidos (Adamson & Reynolds, 1996). Por ello, la combinación de ambas enzimas puede resultar en al aumento de enlaces peptídicos disponibles para la hidrólisis.

4.2.2.3. Optimización de la producción de péptidos antihipertensivos

Como se ha hecho anteriormente para el grado de hidrólisis final, el porcentaje de inhibición de la enzima ECA (ACEI) se modeló siguiendo un modelo mixto de proceso y mezcla, dando lugar a la siguiente ecuación (r^2=0.9972):

$$\begin{aligned} \mathrm{ACEI} = {} & 0.3708 \cdot \mathrm{X_1} - 2.61 \cdot 10^{-4} \cdot \mathrm{X_1} \cdot \mathrm{S} \cdot \mathrm{T} + 4.28 \cdot 10^{-5} \cdot \mathrm{X_1} \cdot \mathrm{T}^2 + 0.0139 \cdot \mathrm{X_2} \cdot \mathrm{T} \\ & - 1.77 \cdot 10^{-3} \cdot \mathrm{X_2} \cdot \mathrm{S} \cdot \mathrm{T} + 5.38 \cdot 10^{-3} \cdot \mathrm{X_2} \cdot \mathrm{S}_2^2 + 0.0266 \cdot \mathrm{X_1} \cdot \mathrm{X_2} \\ & - 5.49 \cdot 10^{-3} \cdot \mathrm{X_1} \cdot \mathrm{X_2} \cdot \mathrm{S} - 4.19 \cdot 10^{-4} \cdot \mathrm{X_1} \cdot \mathrm{X_2} \cdot \mathrm{T} + 3.71 \cdot 10^{-5} \cdot \mathrm{X_1} \cdot \mathrm{X_2} \cdot \mathrm{S} \cdot \mathrm{T} \\ & + 3.84 \cdot 10^{-4} \cdot \mathrm{X_1} \cdot \mathrm{X_2} \cdot \mathrm{T}^2 \end{aligned} \quad [15]$$

Donde X_1y X_2 representan al porcentaje de subtilisina y tripsina en la mezcla enzimática, T es la temperatura (ºC) y S la concentración de proteína (g/L).

El valor experimental más elevado de ACEI (56.12%) se predijo para una concentración de 2.5 g/L, 55ºC y empleando solamente tripsina como enzima. Estas condiciones fueron iguales a las predichas por el modelo siendo el valor máximo teórico 55.3%. Bajo estas condiciones (tripsina a 55ºC) el grado de avance de la rotura de enlaces peptídicos era muy limitado, alcanzándose valores de grado de hidrólisis alrededor de 9%. El modelo predijo hidrolizados con actividad antihipertensiva superior al 50% al emplear tripsina pura a temperaturas superiores a 48ºC.

A bajas temperaturas y concentraciones, la actividad antihipertensiva aumentaba con el contenido de subtilisina en la mezcla de proteasas (por ejemplo, se obtuvo 46% de ACEI a

40ºC y empleando 21.6% de subtilisina). Estas condiciones favorecían la hidrólisis del sustrato, llegando a valores finales de DH superiores a 15.8%.

La actividad antihipertensiva de un determinado péptido depende de una serie de factores como su hidrofobicidad, composición y distribución de los aminoácidos y la composición de los residuos. La mayoría de los péptidos con actividad antihipertensiva que se han identificado, en la literatura, son de bajo peso molecular: di o tripéptidos (Li, Zhou, Huang, Sun, & Zeng, 2012; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). La ACEI de un hidrolizado se ve incrementada por la presencia de péptidos que contienen residuos hidrofóbicos (como Pro, Phe o Tyr) en la secuencia tripéptidica del carbono terminal, ya que así se favorece la interacción con los centros activos de la enzima convertidora de la angiotensina (Li, Le, Shi, & Shrestha, 2004).

Con el fin de determinar la influencia que la digestión gastroinstentinal ejerce en la actividad antihipertensiva de los hidrolizados se seleccionaron aquellos que presentaron máxima actividad para cada una de las temperaturas ensayadas. Estos hidrolizados fueron digeridos *in vitro* para comparar el valor de IC_{50} antes y después de la digestión. Los valores de IC_{50} estuvieron comprendidos entre 253 y 330 µg/mL y no se observaron diferencias significativas entre los hidrolizados crudos y digeridos. Este resultado prueba que la digestión gastrointestinal no afecta a la capacidad antihipertensiva de los hidrolizados producidos, dato de gran interés ya que la digestión es uno de los procesos que disminuyen la actividad de los péptidos bioactivos.

4.2.2.4. Optimización de la producción de péptidos antioxidantes

La oxidación puede tener lugar por diferentes mecanismos, por lo tanto, la medida de la actividad antioxidante debe realizarse empleando diferentes análisis que engloben dichos mecanismos (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002).

En esta tesis doctoral se han empleado tres métodos para determinar la capacidad antioxidante: capacidad inhibitoria del radical DPPH, capacidad reductora de Fe^{3+}y actividad quelante de Fe^{2+}. El ensayo de inhibición de DPPH es un test basado en la capacidad de captación de radicales, generalmente se emplea para determinar la habilidad de una determinada sustancia para estabilizar radicales libres, a través de la trasferencia de electrónica.

El ensayo que determina la capacidad reductora pertenece a los análisis basados en el potencial redox y evalúa la capacidad que tiene un compuesto para donar electrones a los

radicales libres. Por otro lado, los metales catalizan la oxidación lipídica ya que descomponen los hidroperóxidos y producen radicales alquilo y especies con oxígeno reactivo (Shahidi & Zhong, 2010). De ahí que los compuestos quelantes de metales actúen como antioxidantes previniendo el efecto catalítico de los metales.

En este capítulo las tres propiedades antioxidantes que se han enumerado se modelaron dando lugar a las ecuaciones 16 a 18 (r^2 >0.97):

$$\text{DPPH, \%} = 3.56 \cdot X_1 - 3.2 \cdot 10^{-1} \cdot X_1 \cdot S + 2.7 \cdot 10^{-2} \cdot X_1 \cdot S^2 - 9.0 \cdot 10^{-2} \cdot X_1 \cdot T + 8.9 \cdot 10^{-4} \cdot X_1 \cdot T^2 + 2.0 \cdot X_2 - 4.0 \cdot 10^{-1} \cdot X_2 \cdot S + 2.5 \cdot 10^{-2} \cdot X_2 \cdot S^2 - 1.4 \cdot 10^{-2} \cdot X_2 \cdot T + 2.3 \cdot 10^{-3} \cdot X_2 \cdot S \cdot T - 2.8 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot S \quad [18]$$

$$\text{Capacidad reductora de } Fe^{3+} = 1.4 \cdot 10^{-2} \cdot X_1 - 2.2 \cdot 10^{-3} \cdot X_1 \cdot S + 1.9 \cdot 10^{-4} \cdot X_1 \cdot S^2 + 3.0 \cdot 10^{-4} \cdot X_2 \cdot T - 3.1 \cdot 10^{-6} \cdot X_2 \cdot T^2 + 1.2 \cdot 10^{-3} \cdot X_1X_2 - 2.7 \cdot 10^{-4} \cdot X_1X_2 \cdot S + 8.8 \cdot 10^{-6}X_1X_2 \cdot S^2 - 1.9 \cdot 10^{-5} \cdot X_1X_2 \cdot T + 3.5 \cdot 10^{-6} \cdot X_1 \cdot X_2 \cdot S \cdot T \quad [19]$$

$$\text{Actividad quelante de } Fe^{2+} = 3.7 \cdot X_1 + 1.4 \cdot 10^{-1} \cdot X_1 \cdot S - 1.4 \cdot 10^{-2} \cdot X_1S^2 - 1.7 \cdot 10^{-1} \cdot X_1 \cdot T + 1.9 \cdot 10^{-3} \cdot X_1 \cdot T^2 + 1.02 \cdot X_2 - 3.0 \cdot 10^{-1}X_2 \cdot S + 1.4 \cdot 10^{-2} \cdot X_2 \cdot S^2 - 1.1 \cdot 10^{-2} \cdot X_2 \cdot T + 3.4 \cdot 10^{-3} \cdot X_2 \cdot S \cdot T + 7.2 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot S^2 + 3.1 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot T - 1.4 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot S \cdot T \quad [20]$$

A pesar de que la capacidad inhibitoria de DPPH y la capacidad reductora actúan por mecanismos diferentes, ambos están relacionados con la transferencia electrónica y, de forma general, se comportaron de manera paralela ante las distintas variables de entrada. Efectivamente, ambas propiedades presentaron su máximo absoluto y teórico a la mínima concentración (2.5g/L) y a 40ºC. El efecto de la mezcla de enzimas fue diferente para cada propiedad. Mientras que la capacidad inhibitoria de DPPH máxima (80.1% medido a una concentración de 3 mg de hidrolizado/mL) se obtuvo al emplear tripsina pura, la capacidad reductora se maximizó (1.25 medido a una concentración de 10 mg de hidrolizado/mL) al emplear una mezcla enzimática compuesta de un 58% de subtilisina y un 42% de tripsina. Como tendencia general, se puede concluir que aquellos hidrolizados producidos a bajas temperaturas presentaron mejores propiedades antioxidantes, exceptuando la capacidad reductora de los hidrolizados producidos con una concentración de sustrato de 7.5 g/L.

Una alta capacidad inhibitoria de DPPH se relaciona con la presencia de aminoácidos hidrofóbicos y a la presencia de Val, Leu, Ile, Ala, Phe or Lys en el nitrógeno terminal (Suetsuna et al., 2000). En el caso del músculo de jurel, estos residuos representan un 30 %

(en peso) de la composición total de los aminoácidos (Morales-Medina, Tamm, Guadix, Guadix, & Drusch, 2015). La hidrólisis de proteína catalizada por subtilisina libera residuos de Met, Phe, Tyr y Trp incrementando la actividad inhibitoria de DPPH. Además, a baja temperatura (<45ºC), la tripsina amplía su selectividad (además de reaccionar con Lis y Arg, también reacciona con Tyr, Trp y Phe) lo que da lugar a hidrolizados con una mayor actividad inhibitoria de DPPH.

De las tres propiedades estudiadas, la actividad quelante era la más intensa y, como en las propiedades anteriores, ésta depende de la composición y distribución de los aminoácidos en el hidrolizado. Así, residuos terminales como Glu, Asp, Lys or Arg pueden actuar como quelantes (Liu et al., 2010). En el caso del jurel, estos aminoácidos representan un 54 % (en peso) del total de sus aminoácidos constituyentes (Morales-Medina et al., 2015), hecho que justifica el elevado valor. El máximo (47.44 %, medida a 0.6 mg de hidrolizado/mL) se obtuvo a 55 ºC empelando una concentración de sustrato de 5 g/L y añadiendo exclusivamente subtilisina. Con referencia a la mínima concentración evaluada (2.5 g/L) el óptimo local también se obtuvo a 55 ºC y empleando subtilisina pura. Por el contrario, a la concentración estudiada más elevada, el máximo local se obtuvo a 40 ºC y 55% de subtilisina.

Se realizó además una optimización biobjetivo con el fin de que las siguientes condiciones se cumplieran:

$$
\begin{aligned}
&\textit{Maximiza DPPH, \% } (S,T,X_1, X_2)\\
&\qquad \textit{Capacidad reductora, } (S, T, X_1, X_2)\\
&\textit{Con las siguientes restricciones:}\\
&\qquad 2.5 \leq S \leq 7.5\\
&\qquad 40 \leq T \leq 55\\
&\qquad 0 \leq X_1 \leq 100\\
&\qquad X_2 = 100 - X_1\\
&\qquad \textit{Actividad quelante } (S, T, X_1, X_2) = \mathcal{E}
\end{aligned}
\qquad [19]
$$

Para un valor fijo de actividad quelante, tanto la capacidad inhibitoria de DPPH como la capacidad reductora se maximizaron de manera individual. Las condiciones que dieron lugar a los valores máximos de actividad quelante coincidían con los menores de capacidad inhibitoria de DPPH y reductora, y viceversa. Este comportamiento opuesto puede estar

principalmente relacionado con los aminoácidos que determinan cada una de estas actividades.

4.2.3. Evaluación de las propiedades tecnológicas y antioxidantes de los hidrolizados de proteína de pescado para su aplicación como estabilizantes en la microencapsulación de aceite de pescado

La microencapsulación de aceite de pescado mediante el secado por atomización en presencia de antioxidantes alarga la vida útil del aceite (Serfert et al., 2009). Dado que la oxidación se inicia en la interfase, hay un gran interés en el uso y desarrollo de compuestos que posean, conjuntamente, actividad emulsificante y antioxidantes como es el caso de algunas proteínas y de los hidrolizados proteicos. En este contexto, los hidrolizados de proteína de pescado (FPH), se han propuesto como estabilizantes potenciales para el proceso de microencapsulación de aceite de pescado gracias a las propiedades tecnológicas y antioxidantes que presentan.

Para el estudio de este último capítulo se han seleccionado como sustratos la proteína de músculo extraída de la sardina y del jurel. Esta proteína, para cada especie, se hidrolizó hasta alcanzar grados de hidrólisis de 5 y 10% (FPH(5) y FPH(10)) empleando dos proteasas diferentes: subtilisina y tripsina. Los FPH se emplearon como agentes emulsificantes y antioxidantes en la producción de emulsiones (con una contenido de aceite del 5% y de proteína del 2% en peso) y subsecuentemente, se estabilizaron por secado por atomización dando lugar a microcápsulas con un contenido lipídico de 14.33 % en peso. También, se analizaron las propiedades antioxidantes de cada FPH, en concreto: capacidad inhibitoria del radical DPPH, capacidad reductora de Fe^{3+} y actividad quelante de Fe^{2+} a varias concentraciones (desde 1 a 30 mg/mL). Las tres actividades antioxidantes dependieron linealmente de la concentración empleada. En el caso de la capacidad inhibitoria de DPPH, la actividad aumentó linealmente en el rango de 1 a 10 mg/mL (r^2> 0.99), mientras que mostró un comportamiento asintótica a concentraciones superiores. En cuanto a las otras dos propiedades, éstas mantuvieron la linealidad con la concentración en todo el rango estudiado (r^2>0.95).

Previamente a la microencapsulación hubo que determinar las condiciones a las que los hidrolizados estabilizaban de manera satisfactoria a la emulsión. Para ello, se varió el pH (2, 3 y 8) y el contenido proteico de la emulsión (0.5, 1 y 2%). Los hidrolizados con DH 10 no produjeron emulsiones estables en ninguna de las condiciones anteriores, mientras que

los producidos a DH 5, con un contenido proteico de 2% (en peso) y a pH 2 fueron los más estables y, por lo cual, se seleccionaron para el proceso de estabilización.

El impacto que el secado por atomización tuvo en la estabilidad física de la emulsión se analizó midiendo la distribución de tamaños de gota antes y después del secado por atomización. Al no observarse diferencias significativas entre ambas distribuciones, se concluyó que el proceso de secado era un paso inocuo con respecto a la integridad física de la emulsión. En todos los casos, el proceso de microencapsulación presentó valores muy elevados de eficiencia (98 %), lo que demostraba que la integridad física de la interfase no había sido dañada durante el secado.

Tras 12 semanas de almacenamiento a temperatura ambiente y 33% de humedad, el contenido de hidroperóxidos varió entre 114±18 ta 136±17mmol/kg de aceite para los hidrolizados de jurel producidos con subtilisina y con tripsina, respectivamente. La pendiente de curva (contenido de peróxidos por día) se puede emplear como una aproximación de la velocidad de oxidación. Ésta varió desde 1.42 mmol/(kg de aceite·día) al emplearse hidrolizados de sardina producidos con subtilisina como agentes estabilizantes hasta 1.48 mmol/(kg de aceite día) al estabilizar las microcápsulas con los hidrolizados de jurel producidos con subtilisina. El rango de velocidades cambió en muy poca medida en función del tipo de hidrolizado usado.

A pesar de haber demostrado que es físicamente posible estabilizar aceite de pescado usando como estabilizantes hidrolizados de pescado, no se observó ningún tipo de relación entre la velocidad de oxidación y las propiedades antioxidantes. Así, los hidrolizados de jurel y sardina producidos con subtilisina mostraron una velocidad de oxidación muy similar. Ambos hidrolizados tenían valores muy similares de capacidad de captación de radicales libres, mientras que los primeros tenían mejor capacidad reductora. La ausencia de una correlación entre las propiedades antioxidantes y la velocidad de oxidación puede deberse a que las diferencias de capacidades antioxidantes eran muy bajas. Además, las medidas de actividad antioxidante *in vitro* suelen realizarse en sistemas que no contienen lípidos. Por ello, se obvia el hecho de que la proteína se orientará en la interfase cambiando su configuración espacial y, posiblemente, sus propiedades (Decker et al., 2005). Se han de realizar más estudios, comparando actividades antioxidantes *in vivo* e *in vitro*, con el fin de determinar si las medidas antioxidantes realizadas *in vitro* son una herramienta útil para la predicción de la oxidación en aceites microencapsulados.

5. BIBLIOGRAFÍA

A.O.A.C. (2006). Official Methods of Analysis of the AOAC, Association of Official Analytical Chemists,. Washington DC.

Abakarov, A., Teixeira, A., Simpson, R., Pinto, M., & Almonacid, S. (2011). Modeling of squid protein hydrolysis: artificial neural network approach. *Journal of Food Process Engineering*, 34(6), 2026–2046. http://doi.org/10.1111/j.1745-4530.2009.00567.x

Ackman, R. G. (2005). Fish oils. In F. Shahidi (Ed.), Bailey's Industrial Oil and Fat Products (6th ed., pp. 279–317). Hoboken, NJ, USA: John Wiley & Sons.

Adamson, N. J., & Reynolds, E. C. (1996). Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. *Enzyme and Microbial Technology*, 19(3), 202–207. http://doi.org/10.1016/0141-0229(95)00232-4

Adler Nissen. (1986). Enzymic hydrolysis of food proteins. London: Elsevier Applied Science Publishers LTD.

Akoh, C. C. (2005). Handbook of Functional Lipids. CRC Press.

Akoh, C. C., & Kim, B. H. (2008). Structured lipids (pp. 841–872). Conference Proceedings, CRC Press.

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. (2002). Methods for testing antioxidant activity. *The Analyst*, 127(1), 183–198. http://doi.org/10.1039/b009171p

Babayan, V. K. (1987). Medium chain triglycerides and structured lipids. Lipids, 22(6), 417–420. http://doi.org/10.1007/BF02537271

Baş, D., Dudak, F. C., & Boyaci, I. H. (2007). Modeling and optimization IV: Investigation of reaction kinetics and kinetic constants using a program in which artificial neural network (ANN) was integrated. *Journal of Food Engineering*, 79(4), 1152–1158. http://doi.org/10.1016/j.jfoodeng.2006.04.004

Bimbo, A. P. (1998). Guidelines for Characterising Food Grade Fish Oil. INFORM - International News on Fats, Oils and Related Materials, 9(5), 473–483. Retrieved from http://www.iffo.net/system/files/Guidelines Food grade fish oil 1998-3.pdf

Bimbo, A. P. (2007). Processing of marine oils. In H. Breivik (Ed.), Long-Chain Omega-3 Specialty Oils (pp. 77–107). Bridgwater, England: The Oily Press.

Blanco, M., Sotelo, C. G., Chapela, M. J., & Pérez-Martín, R. I. (2007). Towards sustainable and efficient use of fishery resources: present and future trends. *Trends in Food Science & Technology Technology*, 18(1), 29–36. Journal Article. http://doi.org/10.1016/j.tifs.2006.07.015

Boswinkel, G., Derksen, J. T. P., K, van't R., & Cuperus, F. E. (1996). Kinetics of acyl migration in monoglycerides and dependence on acyl chainlength. *J. Am. Oil Chem. Soc*., 73(6), 707–711.

Bottino, N. R., Vandenburg, G. A., & Reiser, R. (1967). Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. *Lipids*, 2(6), 489–493. Journal Article. http://doi.org/10.1007/BF02533177

Bradbury, J. (2011). Docosahexaenoic Acid (DHA): An Ancient Nutrient for the Modern Human Brain. *Nutrients*, 3(5), 529–554. http://doi.org/10.3390/nu3050529

Brockerhoff, H., Hoyle, R. J., Hwang, P. C., & Litchfield, C. (1968). Positional distribution of fatty acids in depot triglycerides of aquatic animals. Lipids, 3(1), 24–9. http://doi.org/10.1007/BF02530964

Camacho Paez, B., Robles Medina, A., Camacho Rubio, F., González Moreno, P., & Molina Grima, E. (2002). Production of structured triglycerides rich in n-3 polyunsaturated fatty acids

by the acidolysis of cod liver oil and caprylic acid in a packed-bed reactor: Equilibrium and kinetics. *Chemical Engineering Science*, 57(8), 1237–1249. Journal Article.

Carbonell, A., Martín, P., Ranieri, S. de, & Team, W. (1998). Discards of the Western Mediterranean trawl fleets.

Cavaille, D., & Combes, D. (1995). Characterization of β-galactosidase from Kluyveromyces lactis. *Biotechnology and Applied Biochemistry*, 22(1), 55–64.

Chabeaud, A., Vandanjon, L., Bourseau, P., Jaouen, P., & Guérard, F. (2009). Fractionation by ultrafiltration of a saithe protein hydrolysate (Pollachius virens): Effect of material and molecular weight cut-off on the membrane performances. *Journal of Food Engineering*, 91(3), 408–414.

Chang, S.-H., Wu, S.-K., & Kimura, H. (2006). Crystallization Kinetics of Ti50Ni25Cu25 Melt-Spun Amorphous Ribbons. *Materials Transactions*, 47(10), 2489–2492. http://doi.org/10.2320/matertrans.47.2489

Chen, H., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative Properties of Histidine-Containing Peptides Designed from Peptide Fragments Found in the Digests of a Soybean Protein. *Journal of Agricultural and Food Chemistry*, 46(1), 49–53. http://doi.org/Doi 10.1021/Jf970649w

Christensen, M., Hoy, C., Becker, C., & Redgrave, T. (1995). Intestinal absorption and lymphatic transport of eicosapentaenoic (EPA), docosahexaenoic (DHA), and decanoic acids: dependence on intramolecular triacylglycerol structure. *Am J Clin Nutr*, 61(1), 56–61.

Cornell, J. A. (2002). Experiments with mixtures : designs, models, and the analysis of mixture data. New York: John Wiley & Sons, Inc.

Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring antioxidant effectiveness in food. *Journal of Agricultural and Food Chemistry*, 53(10), 4303–10. http://doi.org/10.1021/jf058012x

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, 38(3), 674–677. http://doi.org/10.1021/jf00093a019

Dillon, J. T., Aponte, J. C., Tarozo, R., & Huang, Y. (2013). Purification of omega-3 polyunsaturated fatty acids from fish oil using silver-thiolate chromatographic material and high performance liquid chromatography. *Journal of Chromatography A*, 1312, 18–25. http://doi.org/10.1016/j.chroma.2013.08.064

Drusch, S., Serfert, Y., Berger, A., Shaikh, M. Q. Q., Rätzke, K., Zaporojtchenko, V., & Schwarz, K. (2012). New insights into the microencapsulation properties of sodium caseinate and hydrolyzed casein. *Food Hydrocolloids*, 27(2), 332–338. http://doi.org/10.1016/j.foodhyd.2011.10.001

EU. (2011). Proposal for a regulation of the European Parliament and of the Council on the Common Fisheries Policy. COM, 425.

EU. (2013). Regulation (EU) no 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Official Journal of the European Union, 22–28.

Fleury, Y., Defer, D., & Bourgougnon, N. (2008). Antimicrobial peptides and antiviral activities from marine fished or farmed animals. In J. P. Bergé (Ed.), Added Value to Fisheries Waste (1st ed., pp. 163–196). Kerala: Transworld Research Network.

Frankel, E. (2005). Lipid oxidation (The Oily Press Lipid Library).

FROM-Fondo de Regulación y Organización del Mercado de los Productos de la Pesca y los, & cultivos Marinos. (2008). Asistencia técnica para la realización de una medida innovadora para

la recuperación, gestión y valorización de los descartes generados por la flota española que faena en los caladeros atlántico y mediterráneo.

Fureby, A. M., Virto, C., Adlercreutz, P., & Mattiasson, B. (1996). Acyl Group Migrations in 2-Monoolein. *Biocatalysis and Biotransformation*, 14(2), 89–111. http://doi.org/10.3109/10242429609106879

García-Moreno, P. J., Batista, I., Pires, C., Bandarra, N. M., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2014). Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species. *Food Research International*, 65, Part C, 469–476. http://doi.org/10.1016/j.foodres.2014.03.061

García-Moreno, P. J., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2015). Production and identification of angiotensin I-converting enzyme (ACE) inhibitory peptides from Mediterranean fish discards. *Journal of Functional Foods*, 18, 95–105. http://doi.org/10.1016/j.jff.2015.06.062

García-Moreno, P. J., Morales-Medina, R., Pérez-Gálvez, R., Bandarra, N. M., Guadix, A., & Guadix, E. M. (2014). Optimisation of oil extraction from sardine (*Sardina pilchardus*) by hydraulic pressing. International *Journal of Food Science & Technology*, 49(10), 2167–2175. http://doi.org/10.1111/ijfs.12527

Garrett, D. A., Failla, M. L., & Sarama, R. J. (1999). Development of an *in Vitro* Digestion Method To Assess Carotenoid Bioavailability from Meals. *Journal of Agricultural and Food Chemistry*, 47(10), 4301–4309. http://doi.org/10.1021/jf9903298

Groenewold, S., Damalas, D., Maravelias, C. D., Osio, G. C., Maynou, F., Sbrana, M., Casey, J. (2000). Effects on benthic scavengers of discards and damaged benthos produced by the beam-trawl fishery in the southern North Sea. *ICES Journal of Marine Science*, 57(5), 1395–1406. http://doi.org/10.1006/jmsc.2000.0914

Gunstone, F. D., Harwood, J. L., & Dijkstra, A. J. (2012). The Lipid Handbook with CD-ROM, Third Edition. CRC Press.

Halim, N. R. A., Yusof, H. M., & Sarbon, N. M. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology*, 51, 24–33. http://doi.org/10.1016/j.tifs.2016.02.007

Hari Krishna, S., & Karanth, N. G. (2002). Lipases and Lipase-Catalyzed Esterification Reactions in Nonaqueous Media. Catalysis Reviews, 44(4), 499–591. http://doi.org/10.1081/CR-120015481

Innis, S. M. (2011). Dietary triacylglycerol structure and its role in infant nutrition. *Advances in Nutrition*, 2(3), 275–83. http://doi.org/10.3945/an.111.000448

Ito, N., Fukushima, S., & Tsuda, H. (1985). Carcinogenicity and Modification of the Carcinogenic Response by BHA, BHT, and Other Antioxidants. CRC *Critical Reviews in Toxicology*, 15(2), 109–150. http://doi.org/10.3109/10408448509029322

Jacobsen, C., Rustad, T., Nielsen, N. S., Falch, E., Jansson, & S. Storr, I. (2009). Processing of marine lipids and factors affecting their quality when used for functional foods. In J. B. Luten (Ed.), Marine functional food (pp. 89–114). Wageningen: Wagenigen Academic Publishers.

Jacobsen, C., & Skall Nielsen, N. (2007). Optimization of oxidative stability of omega-3 enriched food. In H. Breivik (Ed.), Long-Chain Omega-3 Specialty Oils (21st ed., pp. 197–219). Bridgwater, England: The Oily press Lipid Library.

Johnson, L. A. (2008). Recovery, refining, converting, and stabilizing edible fats and oils (pp. 205–243). Conference Proceedings, CRC Press.

Jump, D. B., Depner, C. M., & Tripathy, S. (2012). Omega-3 fatty acid supplementation and cardiovascular disease Thematic Review Series: New Lipid and Lipoprotein Targets for the

Treatment of Cardiometabolic Diseases. *Journal of Lipid Research*, 53(12), 2525–2545. http://doi.org/10.1194/jlr.R027904

Jung, W.-K., Mendis, E., Je, J.-Y., Park, P.-J., Son, B. W., Kim, H. C. Kim, S.-K. (2006). Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (Limanda aspera) frame protein and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry*, 94(1), 26–32. http://doi.org/10.1016/j.foodchem.2004.09.048

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K., & He, J. (2005). Global burden of hypertension: analysis of worldwide data. *The Lancet*, 365(9455), 217–223. http://doi.org/10.1016/S0140-6736(05)17741-1

Kelleher, K. (2005). Discards in the world's marine fisheries. An update. Rome: FAO Fisheries and Aquaculture Department, Rome.

Keskin Gündoğdu, T., Deniz, I. İ., Calışkan, G., Sahin, E. S., Azbar, N., Çalışkan, G.,Azbar, N. (2014). Experimental design methods for bioengineering applications. *Critical Reviews in Biotechnology*, 8551(31), 1–21. http://doi.org/10.3109/07388551.2014.973014

Kim, B. H., & Akoh, C. C. (2015). Recent Research Trends on the Enzymatic Synthesis of Structured Lipids. *Journal of Food Science*, 80(8), C1713–C1724. http://doi.org/10.1111/1750-3841.12953

Kim, S.-K., & Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: A review. *Journal of Functional Foods*, 2(1), 1–9. http://doi.org/10.1016/j.jff.2010.01.003

Köse, Ö., Tüter, M., & Aksoy, H. A. (2002). Immobilized *Candida antarctica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresource Technology*, 83(2), 125–129. http://doi.org/10.1016/S0960-8524(01)00203-6

Kralovec, J. A., Zhang, S., Zhang, W., & Barrow, C. J. (2012). A review of the progress in enzymatic concentration and microencapsulation of omega-3 rich oil from fish and microbial sources. *Food Chemistry*, 131(2), 639–644. http://doi.org/10.1016/j.foodchem.2011.08.085

Kristinsson, H. G., & Rasco, B. A. (2000). Biochemical and functional properties of Atlantic salmon (Salmo salar) muscle proteins hydrolyzed with various alkaline proteases. *Journal of Agricultural and Food Chemistry*, 48(3), 657–666.

Kuratko, C. N., & Salem, N. (2013). Docosahexaenoic acid from algal oil. *European Journal of Lipid Science and Technology*, 115(9), 965–976. http://doi.org/10.1002/ejlt.201300060

Lassoued, I., Mora, L., Nasri, R., Jridi, M., Toldrá, F., Aristoy, M.-C.,Nasri, M. (2015). Characterization and comparative assessment of antioxidant and ACE inhibitory activities of thornback ray gelatin hydrolysates. *Journal of Functional Foods*, 13, 225–238. http://doi.org/10.1016/j.jff.2014.12.042

Lembke, P. (2013). Production Techniques for Omega-3 Concentrates. In F. De Meester, R. R. Watson, & S. Zibadi (Eds.), Omega-6/3 Fatty Acids (pp. 353–364). Humana Press.

Let, M. B., Jacobsen, C., Sørensen, A.-D. M., & Meyer, A. S. (2007). Homogenization conditions affect the oxidative stability of fish oil enriched milk emulsions: lipid oxidation. *Journal of Agricultural and Food Chemistry*, 55(5), 1773–80. http://doi.org/10.1021/jf062391s

Li, G.-H., Le, G.-W., Shi, Y.-H., & Shrestha, S. (2004). Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research*, 24(7), 469–486. http://doi.org/10.1016/j.nutres.2003.10.014

Li, Y., Zhou, J., Huang, K., Sun, Y., & Zeng, X. (2012). Purification of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide with an antihypertensive effect from loach (Misgurnus anguillicaudatus). *Journal of Agricultural and Food Chemistry*, 60(5), 1320–5. http://doi.org/10.1021/jf204118n

Liu, H., Chang, B., Yan, H., Yu, F., & Liu, X. (1995). Determination of amino acids in food and feed by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and reversed-phase liquid chromatographic separation. *Journal of AOAC International*, 78, 736–744.

Liu, Q., Kong, B., Xiong, Y. L., & Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry*, 118(2), 403–410. http://doi.org/10.1016/j.foodchem.2009.05.013

Malins, D. C., & Wekell, J. C. (1970). The lipid biochemistry of marine organisms. *Progress in the Chemistry of Fats and Other Lipids*, 10, 339–363. http://doi.org/10.1016/0079-6832(70)90011-X

Marangoni, A. G., & Wright, A. J. (2005). Physical Properties of Fats and Oils. In Handbook of Functional Lipids (pp. 135–162). CRC Press.

Martínez-Maqueda, D., Miralles, B., Recio, I., & Hernández-Ledesma, B. (2012). Antihypertensive peptides from food proteins: a review. *Food & Function*, 3(4), 350. http://doi.org/10.1039/c2fo10192k

Mendis, E., Rajapakse, N., & Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of Agricultural and Food Chemistry*, 53(3), 581–587.

Mersmann, A. (2001). Crystallization Technology Handbook. CRC Press.

Morales-Medina, R., Tamm, F., Guadix, A. M., Guadix, E. M., & Drusch, S. (2015). Functional and antioxidant properties of hydrolysates of sardine (S. pilchardus) and horse mackerel (T. mediterraneus) for the microencapsulation of fish oil by spray-drying. *Food Chemistry*, 194, 1208–1216. http://doi.org/10.1016/j.foodchem.2015.08.122

Moya-Ramírez, I., García-Román, M., & Fernández-Arteaga, A. (2016). Waste Frying Oil Hydrolysis in a Reverse Micellar System. ACS *Sustainable Chemistry and Engineering*, 4(3), 1025–1031.

Mullin, J. W. (2001). Crystallization. In J. W. Mullin (Ed.), Crystallization (4th Editio, pp. 216–288). Oxford: Butterworth Heinemann. http://doi.org/10.1016/B978-075064833-2/50008-5

Munio, M. del M., Robles, A., Esteban, L., Gonzalez, P. A., & Molina, E. (2009). Synthesis of structured lipids by two enzymatic steps: Ethanolysis of fish oils and esterification of 2-monoacylglycerols. *Process Biochem.*, 44, 723–730. Journal Article. http://doi.org/10.1016/j.procbio.2009.03.002

Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. *Peptides*. ttp://doi.org/10.1016/j.peptides.2011.11.013

Nalinanon, S., Benjakul, S., Kishimura, H., & Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry*, 124(4), 1354–1362. http://doi.org/10.1016/j.foodchem.2010.07.089

Naqash, S. Y., & Nazeer, R. A. (2011). Evaluation of bioactive properties of peptide isolated from Exocoetus volitans backbone. International *Journal of Food Science & Technology*, 46(1), 37–43. http://doi.org/10.1111/j.1365-2621.2010.02443.x

Oehlke, K., Heins, A., Stöckmann, H., Sönnichsen, F., & Schwarz, K. (2011). New insights into the antioxidant activity of Trolox in o/w emulsions. *Food Chemistry*, 124(3), 781–787. http://doi.org/10.1016/j.foodchem.2010.06.095

Olley, J., Dunstan, G. A., & Kolakowska, A. (2002). Fish Lipids. In Chemical and Functional Properties of Food Lipids. CRC Press.

Olsen, J. V, Ong, S. E., & Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular & Cellular Proteomics : MCP*, 3(6), 608–14. http://doi.org/10.1074/mcp.T400003-MCP200

Ono, S., Hosokawa, M., Miyashita, K., & Takahashi, K. (2006). Inhibition properties of dipeptides from salmon muscle hydrolysate on angiotensin I-converting enzyme. *International Journal of Food Science and Technology*, 41(4), 383–386.

Oyaizu, M. (1986). Studies on products of browning reactions: Antioxidative activities of browning products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, 44, 307–315. http://doi.org/http://dx.doi.org/10.5264/eiyogakuzashi.44.307

Picot, L., Ravallec, R., Fouchereau-Péron, M., Vandanjon, L., Jaouen, P., Chaplain-Derouiniot, M. Bourseau, P. (2010). Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. *Journal of the Science of Food and Agriculture*, 90(11), 1819–26. http://doi.org/10.1002/jsfa.4020

Pires, C., & Batista, I. (2013). Functional Properties of Fish Protein Hydrolysates. In R. Pérez-Gálvez & J. P. Bergé (Eds.), Utilization of fish waste (pp. 59–75). Boca Raton, Florida: CRC Press - Taylor & Francis Group.

Qian, J., Zhang, H., & Liao, Q. (2011). The properties and kinetics of enzymatic reaction in the process of the enzymatic extraction of fish oil. *Journal of Food Science and Technology*, 48(3), 280–284. http://doi.org/10.1007/s13197-010-0128-8

Rodríguez-Ruiz, J., Belarbi, E.-H. H., Sánchez, J. L. G., & Alonso, D. L. (1998). Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotechnology Techniques*, 12(9), 689–691. http://doi.org/10.1023/A:1008812904017

Rogalska, E., Cudrey, C., Ferrato, F., & Verger, R. (1993). Stereoselective hydrolysis of triglycerides by animal and microbial lipases. Chirality, 5(1), 24–30.

Rubio-Rodriguez, N., Beltran, S., Jaime, I., De, D. S. M., Sanz, M. T., & Carballido, J. R. (2010). Production of omega-3 polyunsaturated fatty acid concentrates: A review. *Innovative Food Sci. Emerging Technol.*, 11, 1–12. Journal Article. http://doi.org/10.1016/j.ifset.2009.10.006

Ruxton, C. H. S., Reed, S. C., Simpson, J. A., & Millington, K. J. (2007). The health benefits of omega-3 polyunsaturated fatty acids: A review of the evidence. *Journal of Human Nutrition and Dietetics*, 20(3), 275–285. Journal Article. http://doi.org/10.1111/j.1365-277X.2007.00770.x

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: *Meat and fish. Nutrients*, 3(9), 765–791.

Sampath Kumar, N. S., Nazeer, R. A., & Jai Ganesh, R. (2011). Functional properties of protein hydrolysates from different body parts of horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*). Mediterranean *Journal of Nutrition and Metabolism*, 5(2), 105–110. http://doi.org/10.1007/s12349-011-0078-3

Sathivel, S., Bechtel, P. J., Babbitt, J., Smiley, S., Crapo, C., Reppond, K. D., & Prinyawiwatkul, W. (2003). Biochemical and functional properties of herring (*Clupea harengus*) byproduct hydrolysates. Journal of Food Science, 68(7), 2196–2200.

Schaich, K. M., Shahidi, F., Zhong, Y., & Eskin, N. A. M. (2013). Lipid Oxidation. Biochemistry of Foods (Third Edit). Elsevier. http://doi.org/10.1016/B978-0-08-091809-9.00011-X

Seafish. (2016). The Landing Obligation made simple.

Serfert, Y., Drusch, S., & Schwarz, K. (2009). Chemical stabilisation of oils rich in long-chain polyunsaturated fatty acids during homogenisation, microencapsulation and storage. *Food Chemistry*, 113(4), 1106–1112. http://doi.org/10.1016/j.foodchem.2008.08.079

Shahidi, F. (2000). Antioxidants in food and food antioxidants. *Die Nahrung*.

Shahidi, F., & Wanasundara, U. N. (1998). Omega-3 fatty acid concentrates: Nutritional aspects and production technologies. Trends in Food Science and Technology, 9(6), 230–240. Journal Article. http://doi.org/10.1016/s0924-2244(98)00044-2

Shahidi, F., & Zhong, Y. (2006). Antioxidants from marine by-products. In F. Shahidi (Ed.), Maximising the value of marine by-products (pp. 397–412). Cambridge: Woodhead Publishing. http://doi.org/10.1533/9781845692087.2.397

Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC International*, 91(4), 914–931.

Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews*, 39(11), 4067–79. http://doi.org/10.1039/b922183m

Shalaby, S. M., Zakora, M., & Otte, J. (2006). Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. *The Journal of Dairy Research*, 73(2), 178–86. http://doi.org/10.1017/S0022029905001639

Shimada, Y., Ogawa, J., Watanabe, Y., Nagao, T., Kawashima, A., Kobayashi, T., & Shimizu, S. (2003). Regiospecific analysis by ethanolysis of oil with immobilized *Candida antarctica* lipase. *Lipids*, 38(12), 1281–1286. http://doi.org/10.1007/s11745-003-1190-x

Small, D. M. (1991). The effects of glyceride structure on absorption and metabolism. *Annual Review of Nutrition*.

Subhadra, B., Lochmann, R., Rawles, S., & Chen, R. (2006). Effect of dietary lipid source on the growth, tissue composition and hematological parameters of largemouth bass (*Micropterus salmoides*). *Aquaculture*, 255(1–4), 210–222. http://doi.org/10.1016/j.aquaculture.2005.11.043

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry*, 11(3), 128–131. http://doi.org/10.1016/S0955-2863(99)00083-2

Sugihara, A., Shimada, Y., & Tominaga, Y. (1991). A novel Geotrichum candidum lipase with some preference for the 2-position on a triglyceride molecule. *Applied Microbiology and Biotechnology*, 35(6), 738–740. http://doi.org/10.1007/BF00169887

Taheri, A., Anvar, S. A. A., Ahari, H., & Fogliano, V. (2013). Comparison the functional properties of protein Hydrolysates from poultry byproducts and rainbow trout (*Onchorhynchus mykiss*) viscera, 12(1), 154–169.

Thorkelsson, G., Slizyte, R., Gildberg, A., & Kristinsson, H. G. (2009). Fish proteins and peptide products: processing methods, quality and functional properties. In J. B. Luten (Ed.), Marine functional Food (1st vol 2, pp. 115–133). Wageningen: Wagenigen Academic Publishers.

Valencia, P., Espinoza, K., Ceballos, A., Pinto, M., & Almonacid, S. (2015). Novel modeling methodology for the characterization of enzymatic hydrolysis of proteins. *Process Biochemistry*, 50(4), 589–597. http://doi.org/10.1016/j.procbio.2014.12.028

Valencia, P., Pinto, M., & Almonacid, S. (2014). Identification of the key mechanisms involved in the hydrolysis of fish protein by Alcalase. *Process Biochemistry*, 49(2), 258–264. http://doi.org/10.1016/j.procbio.2013.11.012

Wanasundara, U. N., Wanasundara, P. K. J. P. D., & Shahidi, F. (2005). Novel Separation Techniques for Isolation and Purification of Fatty Acids and Oil By-Products. In Bailey's Industrial Oil and Fat Products. John Wiley & Sons, Inc.

Weichselbaum, E., Coe, S., Buttriss, J., & Stanner, S. (2013). Fish in the diet: A review. *Nutrition Bulletin*, 38(2), 128–177. http://doi.org/10.1111/nbu.12021

Wijesundera, C., Ceccato, C., Watkins, P., Fagan, P., Fraser, B., Thienthong, N., & Perlmutter, P. (2008). Docosahexaenoic acid is more stable to oxidation when located at the sn-2 position of

triacylglycerol compared to sn-1(3). *JAOCS, Journal of the American Oil Chemists' Society*, 85(6), 543–548.

Wu, H.-C., Chen, H.-M., & Shiau, C.-Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, 36(9–10), 949–957. http://doi.org/10.1016/S0963-9969(03)00104-2

Wu, S., Sun, J., Tong, Z., Lan, X., Zhao, Z., & Liao, D. (2012). Optimization of Hydrolysis Conditions for the Production of Angiotensin-I Converting Enzyme-Inhibitory Peptides and Isolation of a Novel Peptide from Lizard Fish (*Saurida elongata*) Muscle Protein Hydrolysate. Marine Drugs, 10(12), 1066–1080. http://doi.org/10.3390/md10051066

Xia, X., Wang, C., Yang, B., Wang, Y.-H., Wang, X., 2009. Water activity dependence of lipases in non-aqueous biocatalysis. *Appl. Biochem. Biotechnol*. 159, 759–67. doi:10.1007/s12010-009-8618-8

Xu, X. (2000). Enzymatic production of structured lipids: Process reactions and acyl migration. *INFORM - International News on Fats, Oils and Related Materials*, 11(10), 1121–1130.

Zhang, G., Eddy Patuwo, B., & Y. Hu, M. (1998). Forecasting with artificial neural networks: *International Journal of Forecasting*. http://doi.org/10.1016/S0169-2070(97)00044-7

Zhang, Y., Xu, J.-L., & Yuan, Z.-H. (2009). Modeling and Prediction in the Enzymatic Hydrolysis of Cellulose Using Artificial Neural Networks. In Fifth International Conference on Natural Computation, 2009. ICNC '09 (Vol. 2, pp. 158–162). http://doi.org/10.1109/ICNC.2009.334

Zhou, Q. Z., Chen, X. D., & Li, X. (2003). Kinetics of lactose hydrolysis by beta-galactosidase of Kluyveromyces lactis immobilized on cotton fabric. *Biotechnology and Bioengineering*, 81(2), 127–33. http://doi.org/10.1002/bit.10414

Summary

1. INTRODUCTION

1.1. Fish discards

Fish discards are defined as "that proportion of the total organic material of animal origin in the catch, which is thrown away, or dumped at sea for whatever reason (Kelleher, 2005)". Discards are composed by non-target species (*e.g.* echinoderms, fish species of low commercial value, marine sponges), juvenile individuals below minimum landing size or target species over fishing quota. Also, most of the individual returned to the sea are died or dying (Groenewold et al., 2000). The discard rate (*i.e.* ratio of discarded fish compared to the total catch) of a given species depends on several factors as fishing gear, local markets or fishing regulations.

The last FAO report (Kelleher, 2005) estimated a yearly tonnage of discards around 7.3 million tons, representing 8% of worldwide catches. In Spain, more specifically in the case of southwest Mediterranean coast (Alboran Sea), discard rates arise up to 23% for trawling and 10% for purse seine fisheries. This represents an underutilization of fishing stocks in an area where fish catches have been reduced to a half during the past decade (FROM, 2008). Among the discarded species, some of them are target species such as sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*) or blue withing (*Micromesistius poutassou*) (Fig. 1). These species are discarded due to minimum landing size requirements, fishing quota or high grading practices. Other discards comprise non targeted species of low commercial value, such as axillary seabream (*Pagellus acarne*), bogue (*Boops boops*) or small-spotted catshark (*Scyliorhinus canicula*) (Fig. 1) (Carbonell, Martín, Ranieri, & Team, 1998).

Discarding not only has a negative impact on future fishing productivity, but also presents a number of environmental problems since it alters marine trophic chains and contribute to the dissemination of toxic compounds and parasites present in fish viscera (Blanco, Sotelo, Chapela, & Pérez-Martín, 2007).

In this context, the European Commission has undertaken a depth reform in the policy of the common fisheries, adopting the so-called zero-discard policy (*i.e.* a set of measures aimed to totally eliminate the discards in EU fisheries (EU, 2013). This reform proposes the incorporation of the zero-discard policy in a progressive adaptation.

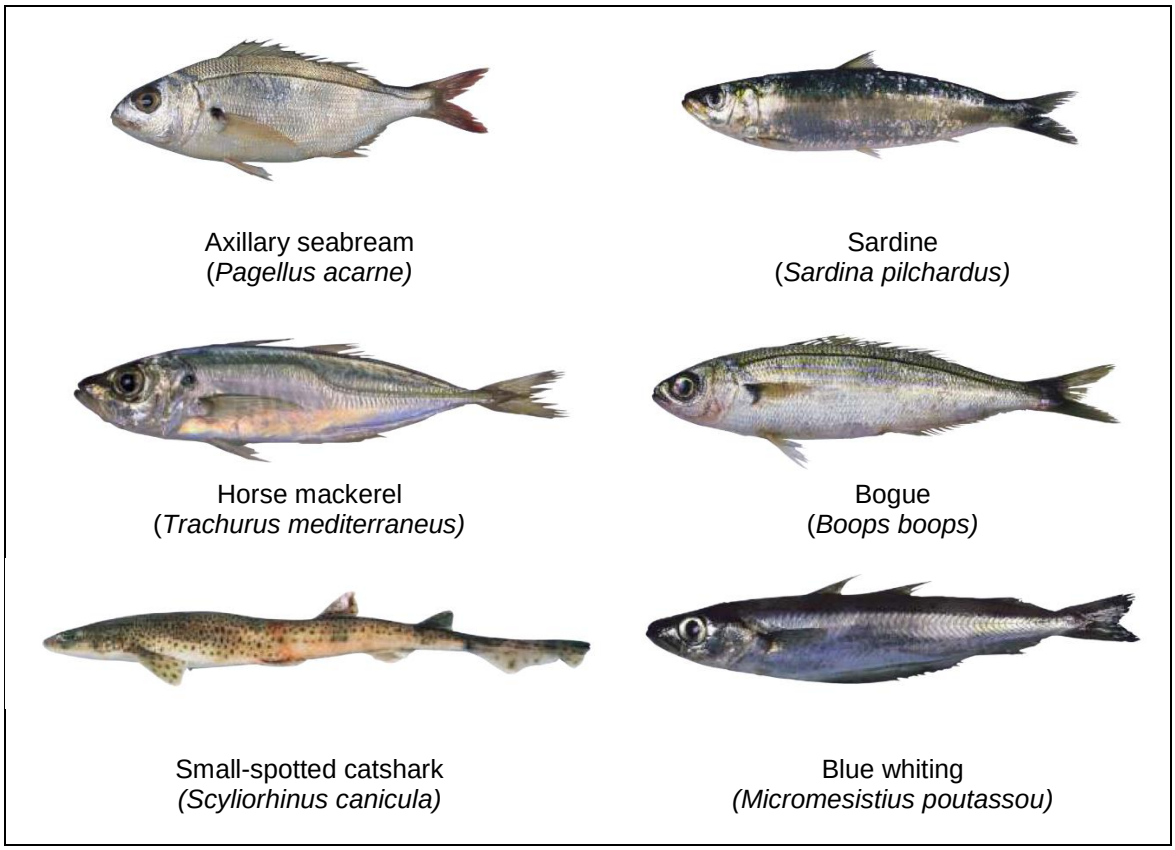


Figure 1. Example of common discarded species in the Alboran Sea.

Effectively, discards of pelagic species (*i.e.* sardine, horse mackerel or mackerel) were banned in 2014 while discards of demersal species such as cod, hake and sole were forbidden in 2015. Finally, discarding of the rest of species wil be banned by 2019. Hence, a considerable volume of fish (mainly composed of non-target species), with no commercial value, will be landed.

Consequently, a set of technical measures have been proposed together with the discard bans such as the improvement of the selective of fishing gears, the increase of the coverage of observers on board and the up-grading of discards by promoting their commercial value (EU, 2011; Seafish, 2016).

In this context, discards can be regarded as raw materials of low cost that can be employed as a substrate for the obtention of added value compounds. Effectively, products as fish protein hydrolysates and modified fish oils, with high value for the food and pharmaceutical industry, can be produced from discards. Therefore, technical solutions able to convert the fish discards into added-value compounds must be developed and optimized.

1.2 Fish oil

Fish oil presents a unique composition due to its high content of polyunsaturated fatty acids (PUFA). Among all PUFA, those belonging to the Omega-3 fraction are the most relevant due to the beneficial role that they exert on human health. Omega-3 PUFA, more specifically eicosapentanoic (EPA, C20:5n-3) and docosahexanoic acid (DHA, C22:6n-3), have been widely reported in the scientific literature as compounds responsible for numerous beneficial effects on human health (Ruxton, Reed, Simpson, & Millington, 2007). Both, EPA and DHA play a positive role on the cardiovascular system (Jump, Depner, & Tripathy, 2012). Additionally, DHA presents a positive influence on the visual development of neonates (Weichselbaum, Coe, Buttriss, & Stanner, 2013) and on the brain system functions (Bradbury, 2011).

Industrially, fish oil is extracted by employing the wet reduction method. This method is composed of three steps (i) cooking of raw materials (85 to 95ºC), (ii) pressing stage and (iii) centrifugation (Bimbo, 2007; Rubio-Rodriguez et al., 2010). The crude extracted fish oil is then refined to accomplish with the quality standards (Johnson, 2008). As a secondary product, a cake rich in protein is also obtained. Oil refining has traditionally been conducted as follows: degumming (removal of phospholipids), neutralization of free fatty acids, bleaching with activated clays (removal of oxidation products and pigments) and deodorization by vacuum distillation (elimination of the volatile compounds) (Bimbo, 2012; Rubio-Rodriguez et al., 2010).

1.2.1. Fish oil up-grading: PUFA concentrates and structured lipids

There is an increasing demand of products with high concentration of EPA and DHA. An Omega-3 concentrated oil must content at least 600 mg of Omega-3 per gram of oil (Rubio-Rodriguez et al., 2010). These concentrates find very valuable applications as supplements in the food industry and also present pharmaceutical applications (Dillon, Aponte, Tarozo, & Huang, 2013; Kralovec, Zhang, Zhang, & Barrow, 2012; Kuratko & Salem, 2013; Lembke, 2013). Concentration process is conducted after crude oil refining and decontamination.

Omega-3 concentrates are produced by physical and enzymatic approaches. The former are based on the difference of physical properties (*i.e.* melting point) that fatty acids present depending on the degree of unsaturation or on the chain length. To enhance the efficiency of these processes, prior to the concentration step, triacylglycerols (TAG) are converted

into methyl esters or free fatty acids (FFA) (Lembke, 2013). Additionally, as post treatment, the concentrates must be re-esterified into TAG since, that way, polyunsaturated fatty acids (PUFA) are better metabolized and digested (Small, 1991). The most relevant physical techniques employed for the concentration of Omega-3 are crystallization at low temperature, urea complexation, supercritical fluid extraction, liquid and supercritical fluid chromatography and molecular distillation (Lembke, 2013; Rubio-Rodriguez et al., 2010; Shahidi & Wanasundara, 1998).

Enzymatic processes take advantage on the specificity of lipases. Lipases are usually classified into two main groups depending on their regioselectivity: (i) sn-1(3) specifics, which mainly react with esters at the external positions of the glycerol and (ii) non-regiospecific of random lipases that can react equally with the three bonds of the glycerol. However, lipases with selectivity towards the central bond are quite uncommon (Rogalska, Cudrey, Ferrato, & Verger, 1993; Sugihara, Shimada, & Tominaga, 1991).

In the case of regiodistribution of fatty acids of fish oils, it has been described that long-chain PUFA are preferentially esterified at the sn-2 position (Brockerhoff, Hoyle, Hwang, & Litchfield, 1968; Olley, Dunstan, & Kolakowska, 2002). Hence, the use of specific 1,(3) lipases might lead to the concentration of PUFA in the acylglycerols form (*i.e*. triacylglycerols, diacylglycerols or monoacylglycerols). However, the yield of concentration will be highly affected by the regiodistribution of the fatty acids of the fish oil selected as substrate, consequently, it is difficult to generally describe the yield and selectivity of these processes. Consequently, a proper characterization fatty acids regiodistribution of the oil is required for the selection of enzymatic method.

Concentrates in the form of acylglycerols are nutritional compounds of great interest for the food and pharmaceutical industry. As TAG, they are employed as nutritional supplements. Additionally, as mono- or diacylglycerols (MAG, DAG) they can be used as healthy food emulsifiers. Furthermore, these concentrates might be employed as a source for producing structured lipids, *i.e.* "modified or synthetic oils and fats containing long-chain fatty acids (L) and medium-chain fatty acids (M), in which each group is located specifically at the sn-2 or sn-1(3) positions of the glycerol backbone" (Xu, 2000). The term "modification" refers to any change in the composition and/or positional distribution of the fatty acids (Kim & Akoh, 2015). These structural changes endow the structural lipids with specific nutritional, physicochemical or textural attributes, which substantially differ from those of the original substrate (Kim & Akoh, 2015). For clinical nutritional purposes,

MLM structured lipids are of special interest since the long-chain fatty acids are more efficiently metabolized when located at the sn-2 position of the acylglycerol (Xu, 2000).

Effectively, the structure of triacylglycerols affects the efficiency of the digestion and absorption of fats. Christensen, Hoy, Becker, & Redgrave, (1995) found that MLM triglycerides with EPA and DHA in the central bond were more readily absorbed sources of PUFA than triglycerides with similar composition but with a random distribution. The absorption improvement is due the pancreatic lipase regioselectivity towards positions 1 and 3 and typeselectivity toward medium-chain fatty acids (Bottino, Vandenburg, & Reiser, 1967). The released medium-chain FFA are rapidly absorbed into the portal vein while the 2-monoglycerides (which contain the long-chain polyunsaturated fatty acid) are absorbed *via* the lymphatic route (Small, 1991). Structured lipids (MLM) are used as a rapid energy food for patients suffering from malabsorption syndrome (Babayan, 1987), for infant formulas (Innis, 2011) and for the design of hypocaloric food.

Structured lipids are usually produced by (i) one-step process (acidolysis, interesterification) or by (ii) two-step processes which are the combination of alcoholysis or hydrolysis with esterification (Akoh & Kim, 2008).

1.2.2. Fish oil oxidation and stabilization

In all food system, the first mode of spoilage is microbial. Nevertheless, as this type of spoilage can be controlled by processing, oxidation becomes the set of chemical reactions responsible for the food quality deterioration (*i.e.* loss of organoleptic and nutritional properties) and the limitation of shelf-life (Schaich, Shahidi, Zhong, & Eskin, 2013; Shahidi & Zhong, 2008).

Oxidation rate is catalyzed by heat, light, metal ions, oxygen and lipoxygenases enzymes (Frankel, 2005). Hence, oxidation can be conducted by several pathways such as autoxidation, photooxidation, or by thermal or enzymatic processes, being autoxidation the most harmful for food systems (Shahidi, 2000). Additionally, oil composition highly determines the rate of oxidation. An increase of the availability of bis-allylic hydrogens results in a faster oxidation (Frankel, 2005). Hence, oils with high content of PUFA are more susceptible to oxidative spoilage. Additionally, the regiodistribution of the oil in the glycerol backbone exert some effect on the oxidation rate, those fatty acids located at the sn-2 position being more resistant to oxidation (Wijesundera et al., 2008).

Lipid autoxidation is a radical chain mechanism composed of three stages: initiation, propagation and termination. It is a self-propagating and self-accelerating process which usually begins at very slowly initial rate until reaching an exponential phase (Shahidi & Zhong, 2010). Firstly, unsaturated lipids lose a hydrogen atom and produce free radicals. Then, during propagation, lipid hydroperoxides are produced as primary product of oxidation. These compounds are highly unstable and break down resulting in a set of secondary compounds: aldehydes, ketones, alcohols, volatile organic acids and epoxy compounds, among others (Shahidi & Zhong, 2010). Hydroperoxides are tasteless and odorless whereas secondary volatile compounds are responsible for the rancidity and the off-flavors, some of them being detected at a low threshold (Jacobsen & Skall Nielsen, 2007). Finally, free radicals can interact with each other and form stable non-radical products by means of condensation reactions at low temperature (Frankel, 2005).

The stabilization of the oil against autoxidation is essential for its industrial use. There are several compounds able to partially inhibit oxidative reactions such as radical scanvengers, inactivators of peroxides (and other reactive oxygen species), singles oxygen quenchers, reducing agents or metal ion chelators (Shahidi & Zhong, 2006).

The direct addition of a combination of antioxidants to bulk oils (Serfert, Drusch, & Schwarz, 2009) has proved to be an efficient stabilization process. Besides, fish oil can enlarge its shelf life by emulsifying or microencapsulation. In these techniques, oil is dispersed in a continuous water phase and is stabilized employing emulsifiers and antioxidants. Regarding the use of antioxidants, it has been shown that some antioxidants which are efficient in stabilizing liquid systems do not necessarily increase the stability of an oil encapsulated by spry-drying (Serfert et al., 2009). Furthermore, the location of the antioxidants in the disperse system has an impact on its antioxidant activity, ideally being positioned at the oil/water-interface where autoxidation begins (Oehlke, Heins, Stöckmann, Sönnichsen, & Schwarz, 2011).

Apart from decreasing the rate of lipid oxidation, emulsions and microcapsules can be employed as delivery systems for the incorporation of Omega-3 PUFA into food. Microencapsulates are usually incorporated into solid products such as bread or powder products (Jacobsen & Skall Nielsen, 2007). Furthermore, emulsified Omega-3 oil is more suitable for liquid and semi-liquid foods (Let, Jacobsen, Sørensen, & Meyer, 2007).

1.3 Fish protein hydrolysates

Fish proteins can be classified in three groups: (i) myofibrillar proteins such as actin, myosin, tropomyosin and troponin (structural proteins soluble in salt solutions with high ionic strength accounting 66 to 77 wt% of the total), (ii) sarcoplasmic proteins such as myoglobin or enzymes (soluble in water and diluted buffers representing 20 to 30 wt%) and (iii) stroma proteins which are connective tissue, insoluble in concentrated saline solutions (2 to 3 wt% of the total protein) (Kristinsson & Rasco, 2000).

In the last decades, numerous studies have been focused on the production of fish protein hydrolysates (FPH) as potential added value ingredients for commercial food products (Halim, Yusof, & Sarbon, 2016). Several methods have been employed for the production of FPH as bacterial fermentation, autolysis and chemical, or enzymatic hydrolysis. Predominantly, enzymatic hydrolysis is regarded as the most adequate technique due to: (i) the mild conditions of pH and temperature which are required, (ii) the avoidance of side reactions due to the high specificity of enzymes and (iii) the high nutritional value of the final products.

Enzymatic hydrolysis of protein is catalyzed by proteases that cleave peptides bonds releasing carbonyl and amino groups, which participate in acid-base equilibria (Eq.1):

$$\begin{aligned} &P1-CO-NH-P2+\ H_2O \Rightarrow P1-COOH+P2-NH_2 \\ &P1-COOH\ \Leftrightarrow P1-COO^- + H^+ \\ &P2-NH_2 + H^+\ \Leftrightarrow P2-NH_3^+ \end{aligned} \qquad [1]$$

As a consequence of the continuous cleavage, proteins are broken down into products of lower molecular weight (Adler Nissen, 1986). FPH have demonstrated excellent physicochemical, technological and bioactive properties (Halim et al., 2016; Nalinanon, Benjakul, Kishimura, & Shahidi, 2011). Contrary, non-hydrolysed fish proteins do not possess these activities since the sequences which present bioactive potential are poorly or not accessible (Kim & Wijesekara, 2010). The technological and bioactive properties are determined by the enzymatic treatment, which is highly influenced by (i) the matrix employed as substrate (*e.g.* species, muscle, skin…), (ii) type and specificity of the selected protease, (iii) hydrolysis conditions (pH, temperature, enzyme : substrate ratio) and (iv) the extent of the hydrolysis reaction.

Technological properties are defined as “the physicochemical properties of proteins in the food systems during processing, storage and consumption” (Halim et al., 2016). Among these properties, solubility, emulsifying / foaming capacities and water / oil binding capacities are the most relevant for industrial applications. Effectively, FPH can be employed for the formulation of foam-based products since they have emulsifying and foaming properties (Nalinanon et al., 2011). Also, FPH improve the water holding, emulsification properties and texture when incorporated into food (Taheri, Anvar, Ahari, & Fogliano, 2013). Furthermore, due to their oil binding capacity, factor which influence the final product taste, FPH are employed in the meat and confectionery industries (Pires & Batista, 2013).

The production of fish protein hydrolysates exhibiting bioactive properties has gained an increasing interest in the last decade due to the added value of these products (Thorkelsson, Slizyte, Gildberg, & Kristinsson, 2009). Several studies have reported protein hydrolysates with antioxidant (Sathivel et al., 2003; Wu, Chen, & Shiau, 2003), antihypertensive (Jung et al., 2006; Ono, Hosokawa, Miyashita, & Takahashi, 2006), antimicrobial (Fleury, Defer, & Bourgougnon, 2008) or anticholesterolemic (Naqash & Nazeer, 2011) activities.

1.3.1 Antioxidant hydrolysates

Antioxidant compounds play a crucial role on the prevention of oxidation at a physiological level. Antioxidants can act by several pathways as by electronic transference or metal chelation. Metals catalyze lipid oxidation, being reduced metals considered more powerful than the oxidized ones (Shahidi & Zhong, 2008). Synthetic compounds such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) are synthetic compounds used as antioxidant. Despite their high antioxidant activity, their use in foods has begun to be restricted due to their potential hazardous effects (Ito, Fukushima, & Tsuda, 1985). Hence, several researches are focused on finding natural antioxidants with minimum side effects.

The antioxidant activity of a FPH hydrolysate depends on the molecular size of the peptides as well as on their amino acids composition and sequence. In the last decade, several authors have reported a strong antioxidant activity for FPH obtained from different species such as herring, (*Clupea harengus),* (Sathivel et al., 2003); hoki, (*Ohnius belengerii*) (Mendis, Rajapakse, & Kim, 2005) or saithe, (*Pollachius virens*) (Chabeaud, Vandanjon, Bourseau, Jaouen, & Guérard, 2009).

Several peptide sequences with variable size have been described as efficient antioxidant. For example, Asn-His-Arg-Tyr-Asp-Arg, Gly-Asn-Arg-Gly-Phe-Ala-Cys-Arg-His-Ala (0.856 kDa, 1.10 kDa, respectively) from horse mackerel and croaker skin (Sampath Kumar, Nazeer, & Jai Ganesh, 2011).

1.3.2 Antihypertensive hydrolysates

Hypertension is a risk factor which can cause cardiovascular diseases that affects around one third of the world population (Kearney et al., 2005). Blood pressure is commonly reduced by inhibiting the Angiotensin Converting Enzyme (ACE). This enzyme participates in the renin-angiotensin system, which regulates blood pressure. Numerous synthetic compounds (*i.e*. captopril, enalapril, alacepril and lisinopril) can, to some extent, inhibit ACE although they might present some side effects such as cough, taste disturbances or skin rashes (Kim & Wijesekara, 2010). Hence, numerous research works are being conducted to produce safer natural ACE inhibitors.

In this context, fish protein hydrolysates have been presented as a good alternative source (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). ACE-inhibitory peptides are usually constituted by 2-12 amino acids and contain hydrophobic residues (*e.g*. Phe, Trp, Tyr, Pro) in the C-terminal.

Antihypertensive peptides, with different amino acids composition and length, have been isolated from several hydrolysates. For instance, Ser-Pro-Arg-Cys-Arg from lizard fish hydrolysate (Wu et al., 2012) or Gly-Ile-Pro-Gly-Ala-Pro & Ala-Pro-Gly-Ala-Pro from thornback ray gelatin (Lassoued et al., 2015).

2. OBJECTIVES

The main objective of this PhD research is to develop new processes for the obtention of functional compounds from fish discards. To that end, this investigation has been focused in the two majority components of fish: lipids and proteins.

The following individual goals have been selected for the up-grading of lipids compounds:

- Review of the state of the art of the up-grading processes of fish discards with a focus on marine lipids (Chapter I).

- Seasonal characterization of the oil extracted from six fish discards species (*Sardina pilchardus, Trachurus mediterraneus, Pagellus Acarne, Micromesistius poutassou, Boops boops,* and *Scyliorhinus canicula*). Evaluation of their nutritional values and the regiosdistribution of the fatty acids within the glycerol backbone (Chapter II and III).
- Obtention of oil enriched with polyunsaturated fatty acids:
    - Concentration *via* low temperature winterization (Chapter IV).
    - Concentration by enzymatic processes in micro aqueous systems (Chapter V).
- Production of MLM structured lipids from sardine discards. Characterization of the oxidation, evaluation of the yield of each step of the process and regiodistribution of the fatty acids in the purified TAG (Chapter VI).

The following individual goals were selected for the up-grading of protein compounds:

- Review of the properties and applications of fish protein hydrolysates (Chapter VII).
- Seasonal characterization of the protein content from six fish discards species (*Sardina pilchardus, Trachurus mediterraneus, Pagellus Acarne, Micromesistius poutassou, Boops boops,* and *Scyliorhinus canicula*) (Chapter II and III).
- Kinetic study of the enzymatic hydrolysis of horse mackerel with bacterial and animal proteases, with a focus on:
    - Modelling by artificial neuronal networks (Chapter VIII).
    - Modelling of the hydrolysis by a crossed-mixture model (Chapter IX).
    - Optimization of the production of antihypertensive peptides (Chapter IX).
    - Optimization of the production of antioxidant peptides (Chapter X).
- Evaluation of the technological and antioxidant properties of sardine and horse mackerel hydrolysates as stabilizers for the microencapsulation of fish oil (Chapter XI).

Table 1. .Summary of the main techniques employed in this PhD research.

Analysis	Description	Chapter
Proximate composition	Protein, oil, ash and water content. (A.O.A.C., 2006)	II, III, VI, VIII-XI
Hydrolysis Degree	pH-stat method	VIII-XI
Fatty acid profile of oil	Esterification (Rodríguez-Ruiz, Belarbi, Sánchez, & Alonso, 1998) Direct Gas Chromatography (Camacho Paez, Robles Medina, Camacho Rubio, González Moreno, & Molina Grima, 2002)	II- VI
Lipid composition (MAG, DAG,TAG, FFA)	Thin Layer chromatography	II-VI
Lipid composition (1-MAG, 2-MAG)	Thin Layer chromatography	VI
Lipid composition of saturated compounds (MAG, DAG, TAG)	Ultra pressure liquid chromatography – Mass spectrometry (Moya-Ramírez, García-Román, & Fernández-Arteaga, 2016)	VI
Fatty acid content	Chemical valorisation (ISO 660:2009)	VI
Regiodistribution of fatty acids	Novozyme 435- mediated Alcoholysis (Shimada et al., 2003)	II-VI
Peroxide value	Spectrophotometric method (Drusch et al., 2012)	VI, XI
Anisidine value	Spectrophotometric determination (ISO 6885:2006)	VI
Fractionation of acylglycerols	Liquid column chromatography (Köse, Tüter, & Aksoy, 2002)	VI
Antihypertensive activity	Spectrophotometric method based on enzymatic reaction (Shalaby, Zakora, & Otte, 2006)	IX
Fe^{2+} Chelating activity	Spectrophotometric method (Decker & Welch, 1990)	X, XI
Fe^{3+} Reducing capacity	Spectrophotometric method (Oyaizu, 1986)	X,XI
DPPH radical scavenging activity	Spectrophotometric method based on the interaction of hydrolysate and 1,1-Diphenyl-2-picrylhydrazyl (Picot et al., 2010)	X, XI
In vitro digestion	Enzymatic hydrolysis (Garrett, Failla, & Sarama, 1999)	IX
Molecular weight distribution	Size Exclusion Chromatography employing fast protein liquid chromatography and SDS- PAGE	IX-XI
Amino acids composition	Reversed phase chromatography (Liu, Chang, Yan, Yu, & Liu, 1995)	XI

3. MATERIALS AND METHODS

Six fish species from the Alboran Sea were characterized and evaluated as potential substrates: sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), axillary seabream (*Pagellus Acarne)*, blue whiting (*Micromesistius poutassou)*, bogue *(Boops boops)* and small-spotted catshark (*Scyliorhinus canicula*). Other specific materials required for this work are described in each chapter.

The methods which have been employed in this PhD research are listed in Table 1.

4. RESULTS AND DISCUSSION

4.1. Fish oil up-grading

4.1.1. Substrate seasonal characterization

For the proper choice of an oil up-grading technique, it is required to previously characterize the oil, paying special attention to the fatty acid composition and regiodistribution within the glycerol backbone. Thus, the first step towards an efficient up-grading is the characterization of the oils employed as substrate, including the regiodistribution of the free fatty acids.

As stated in the objectives, six discarded species from the Alboran Sea have been selected as feasible raw material for the production of functional compounds. To determine their quality as potential substrate, for each species, it has been seasonally determined the lipid content, the oil composition (as triacylglycerols, diacylglyerols, monoacylglycerols and free fatty acids), the fatty acids global composition and their regiodistribution. Besides, nutritional indexes (atherogenicity and thrombogenic indexes and hypocholesterolaemic - hypercholesterolaemic ratio) have been estimated to evaluate the nutritional quality of the oil. Oil extraction has been conducted by hydraulic pressing, and crude oil was composed mainly by TAG.

According to their lipid content, species were classidied as fatty (sardine > 8 wt% of lipids), semi-fatty (axillary seabream, horse mackerel and bogue with a lipidic average content varying from 4 to 8 wt %) and lean (blue whiting and small-spotted catshark, < 4 wt%) (Ackman, 2005).

Lipid content varied considerably throughout the year and among species. Sardine was the species with the highest content of oil and with the highest seasonal variation (12.45 ± 7.11 wt%). Contrary, the lipid content of axillary seabream and small-spotted catshark hardly varied, presenting average values of 5.3±0.5wt% and 1.9±0.2wt%, respectively. The differences of the lipid content are mainly related to the species' feed intake, migratory behavior, sexual changes and spawning period (Brockerhoff et al., 1968). The influence of the spawning season is clearly observed in the case of sardine, which showed the lowest lipid amount (2.5 wt%) in spring after the spawning period.

Regarding their fatty acid profile, generally, PUFA were the main fraction (26.3 to 46.4 wt%), revealing the nutraceutical interest of these oils. Also, PUFA content reached a maximum in winter for all species but small-spotted catshark, in agreement with the well-established inverse correlation between temperature of a tissue and the degree of unsaturation of the fatty acids (Malins & Wekell, 1970). Among the fatty acids, palmitic (C16:0), EPA (C20:5n-3) and DHA (C22:6n-3) were the predominant compounds.

The values of the three indexes proved their nutritional quality. Indeed, atherogenicity indexes demonstrated that the extracted oils can be considered as healthy (Subhadra, Lochmann, Rawles, & Chen, 2006).

Concerning the fatty acids regiodistribution within the glycerine backbone, the central bond (sn-2) of the glycerol backbone was mainly esterified by PUFA. Indeed, an average 47.2±7.9 mol% of the total PUFA were esterified in this position. This relative percentage is greater than that expected stoichiometrically (33.33%), which demonstrates the sn-2 regioselectivity of PUFA. More specifically, this regioselectivity varied among individual polyunsaturated fatty acids. Effectively, EPA average content in the central bond corresponded to 17.1±5.1mol% of the total which implies a sn-1(3) regiospecifity. Contrary, DHA showed a strong sn-2 regiospecifity with more than 70 mol% of the total located in the sn-2 position.

Among all the species studied, bogue and small-spotted catshark presented a considerable high content of PUFA at the sn-2 position. On that account, they have been proposed as raw material for the synthesis of structured lipids by processes which preserve the composition of fatty acids at sn-2. Effectively, the use of alcoholysis (Munio et al., 2009) or a selective hydrolysis in micro aqueous systems might be feasible up-grading processes.

Oil extracted from sardine, axillary seabream, horse mackerel and blue whiting showed nutritional properties and high content of PUFA, although randomly distributed in the three positions of the glycerol backbone. Hence, these oils have been considered as interesting raw material for the production of PUFA concentrates by physical approaches, where the regiodistribution of the original oil has no relevance.

4.1.2. Obtention of oil enriched in PUFA

Based on the regiodistribution of PUFA in the oils extracted, two different PUFA concentration processes were studied: crystallization (winterization) at low temperature of free fatty acids and enzymatic hydrolysis of triacylglycerols in micro aqueous systems.

Winterization or fractional crystallization at low temperature consists in the removal of high melting point compounds (*i.e.* saturated fatty acids) by cooling (Gunstone, Harwood, & Dijkstra, 2012). For a given chain length, the melting point of the free fatty acids (FFA) decreases with the degree of unsaturation (Akoh, 2005; Gunstone et al., 2012). Therefore, at low temperatures, saturated fatty acids (SFA) crystallize and PUFA remain in the liquid phase (Wanasundara, & Shahidi, 2005). Finally, by filtration a liquid phase enriched in PUFA can be separated.

Lipase-mediated hydrolysis of triacylglycerols in micro aqueous systems is an enzymatic reaction in which DAG, MAG and FFA are released. It has been proposed that (by means of employing sn-1(3)-regiospecific lipases and fish oil whose PUFA present high sn-2 regioselectivity) a fraction of MAG and DAG enriched with PUFA at the sn-2 position might be produced.

In both studies, a refined sardine oil provided by Industrias Afines S.L. has been employed as a model system.

4.1.2.1. Crystallization (winterization) at low temperature of free fatty acids

As depicted in Fig. 2, the winterization was divided in the following steps: (i) production of FFA by chemical hydrolysis and purification, (ii) winterization and (iii) vacuum filtration.

The results showed that the highest PUFA concentrates (>80 wt%, 1.7-fold respect the original oil) were produced at -85 ºC and 24 h. It was found that lower temperatures increased the amount of the liquid phase recovered, while its concentration of PUFA decreased dramatically. Regarding the composition of the supernatant, SFA content was

deeply reduced in all cases (from 31.2 to <6 wt%) due to the selectivity of the crystallization. Contrary, MUFA only precipitated at -85 ºC. In the solid fraction, there were a residual amount of PUFA (0.057 ± 0.006 g) which remained constant independently of time and temperature. The presence of the PUFA in the solid phase could be explained by a number of phenomena such as intersolubilization, aggregation or occlusion during the crystallization of the SFA. Hence, mixed crystals might be formed at higher temperatures that expected for pure fatty acids (Wanasundara et al., 2005).

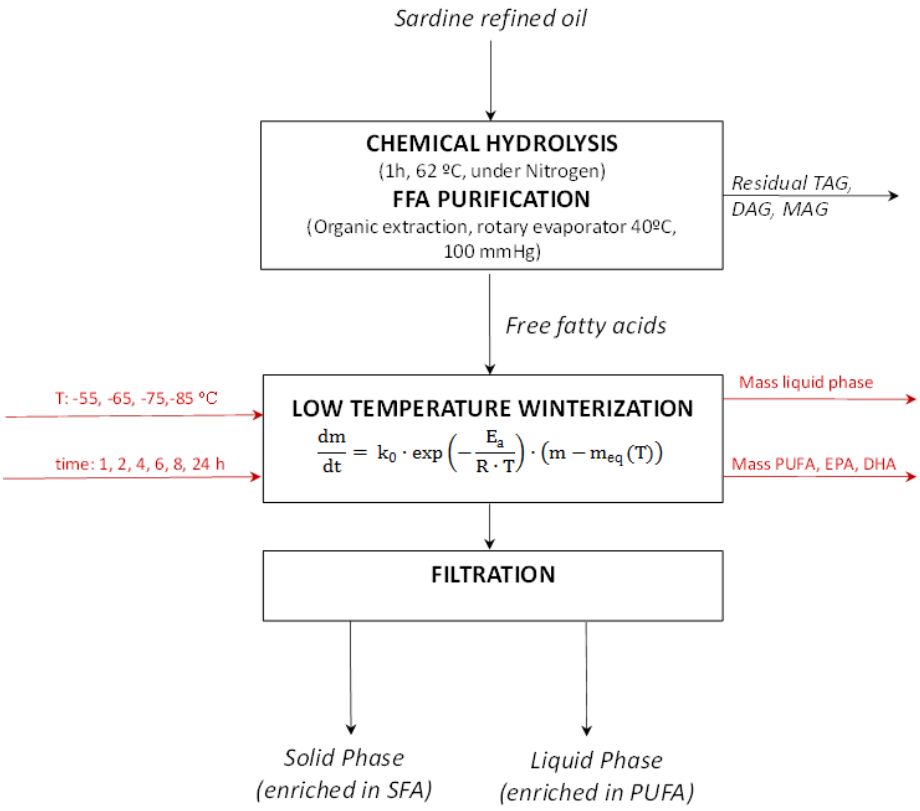


Figure 2. Schematic diagram of the experiment conducted for the modelling the winterization

In the solid fraction, there were a residual amount of PUFA (0.057 ± 0.006 g) which remained constant independently of time and temperature. The presence of the PUFA in the solid phase could be explained by a number of phenomena such as intersolubilization, aggregation or occlusion during the crystallization of the SFA. Hence, mixed crystals might be formed at higher temperatures that expected for pure fatty acids (Wanasundara et al., 2005).

The evolution of the masses of the supernatant, PUFA, EPA and DHA has been modeled as a function of temperature and time. The mass of supernatant was modeled by a diffusion-reaction model. This model explains the crystal surface grows by nucleation and

crystal growth (Marangoni & Wright, 2005; Mullin, 2001). That model was adapted to the current system resulting in Eq. 2:

$$\frac{dm}{dt} = K_{G\cdot A} \cdot (m - m_{eq}) \quad [2]$$

where m represents the mass (g) , t the time (h), , $K_{G\cdot A}$ a global coefficient (h^{-1}) and m_{eq} the equilibrium mass (g).

Equilibrium (m_{eq}) data have been calculated by adjusting and evaluating the experimental data at infinite time. The global coefficient, $K_{G\cdot A}$ (h^{-1}), has been calculated for each temperature and adjusted to an Arrhenius type equation (Chang, Wu, & Kimura, 2006; Mersmann, 2001).

Hence, the mass transfer model for the liquid phase can be expressed as a function of time and temperature (Eq. 3):

$$\frac{dm}{dt} = k_0 \cdot \exp\left(-\frac{E_a}{R \cdot T}\right) \cdot (m - m_{eq}(T)) \quad [3]$$

where, k_0 is the pre-exponential factor (h^{-1}), E_a the activation energy for crystallization ($kJ \cdot mol^{-1}$), R the gas constant ($kJ \cdot mol^{-1} \cdot K^{-1}$) and T the temperature (K).

The resultant equation (Eq. 4) was able to predict all data with errors minor than 10 %.

$$\frac{}{dt} = 5.23 \cdot \exp\left(-\frac{}{T}\right) \cdot (m - 0.022 \cdot T + 1.4029) \quad [4]$$

PUFA, EPA and DHA were concentrated as a result of the decrease of the liquid mass. Effectively, their masses remained virtually constant during the whole process and by decreasing the total mass, their concentration increased. Consequently, no mass transfer equations can be employed to model the increase of the concentration of PUFA, EPA or DHA in the liquid fraction. Hence, the following equation has been proposed for that calculation:

$$X_i = \frac{mass_{i,initial} - mass_{i,sol}}{mass_{L,t}} \quad [5]$$

where i refers to PUFA, EPA or DHA; X the mass fraction in the liquid phase, $mass_{i,initial}$ the initial mass in the oil, $mass_{i,sol}$ the average mass in the solid fraction and $mass_{L,t}$ the mass of the liquid fraction at a time t (calculated by integration of Eq. 4). The term $mass_{i,sol}$

has been estimated as the average mass in the solid, employing data of all the times and temperatures studied. By employing this model these data can be predicted with error minor than 10%.

4.1.2.2. Enzymatic hydrolysis of triacylglycerols in micro aqueous systems to maximize PUFA content in acylglycerols

In this case, the aim was to produce diacylglycerols and monoacylglycerols with high content of PUFA by *Rhizomucor miehei* lipase-mediated hydrolysis of sardine oil.

One of the most crucial variable to control in micro aqueous system is the water content. Effectively, water affects the enzyme structure and also the conditions of the system (*i.e.* by facilitation the reagent diffusion or influencing the equilibrium (Hari Krishna & Karanth, 2002). Water activity is the most convenient variable to characterize the content of water in microaqueous systems (Xia, 2009). Hence, three water activities (0.3, 0.6 and 0.8) have been tested to determine and model the water activity influence on the final composition of the sardine oil hydrolysis.

Sardine refined oil presented PUFA as main fraction (~ 43 mol%) followed by SFA (33.83 mol %). With respect to their regiodistribution, SFA was the major fraction esterified in the sn-2 position (47.84 mol%) followed by PUFA (30.25 mol%). DHA presented high sn-2 regiospecifity (70 mol% of the total was located at sn-2) while EPA showed the opposite behavior (7.21 mol% of the total was bonded at the sn-2 position).

For all the water activities tested, PUFA were concentrated to a maximum 1.2-fold under the form of DAG and TAG. Selectivity was much clear in the case of DHA. Indeed, this compound was concentrated as DAG and TAG, achieving concentration ratios of 1.94-fold. The mechanism underlying these results might depend on the regioselectivity of the enzyme, the regiodistribution of the fatty acids among the glycerol backbone and the own physical characteristics of the fatty acids (*i.e.* number of unsaturations or chain length).

The following second-order reversible reaction have been proposed for the kinetic model:

$$\mathrm{TAG} + \mathrm{W} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \mathrm{DAG} + \mathrm{FFA} \quad [6]$$

$$\mathrm{DAG} + \mathrm{W} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \mathrm{MAG} + \mathrm{FFA} \quad [7]$$

Where, TAG, DAG, MAG, FFA and W represent triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acids and water, respectively. These species are related stoichiometrically (*i.e.* FFA = 2·MAG + DAG). Considering the PUFA fraction, this stoichiometry was not followed (*i.e.* [DAG] > [FFA]). Consequently, the content of PUFA was modeled by doing a molar balance to the whole system as follows:

$$C_{i,GLOBAL} = C_{i,SFA} + C_{i,MUFA} + C_{i,PUFA} \quad [8]$$

The experimental data were satisfactorily modelled (SD < 16%) in all cases. The direct kinetic constant k_1 was similar in the three cases (0.031±0.006 min^{-1}). Hence, the release of the first fatty acid from the TAG might be independent on the type of fatty acid. Contrary, the kinetic constant k_2 varied for the three cases studied. For the global system it was smaller than in the SFA and MUFA fractions (0.42±0.04; 0.82±0.2 and 1.05 ± 0.17 min^{-1} respectively), showing the effect of the resistance that PUFA presented to be hydrolyzed.

One of the major limitations that selective lipid modification must face is the acyl migration, *i.e.* "the spontaneous movement of an acyl group from one hydroxyl group to an adjacent one" (Fureby, Virto, Adlercreutz, & Mattiasson, 1996). Hence, acyl migration involves acyl donors from sn-1(3) to sn-2 positions but it can also occur from the external bonds to the central one of the glycerol backbone (Xu, 2000).

Acyl migration kinetics of 2-MAG to 1(3)-MAG has been studied and modelled as a reversible first-order reaction as follows:

$$2-\mathrm{MAG} \underset{k_{-m}}{\overset{k_m}{\leftrightarrow}} 1-\mathrm{MAG} = 2-\mathrm{MAG} \underset{k_{-m}}{\overset{k_m}{\leftrightarrow}} 3-\mathrm{MAG} \quad [9]$$

Where, k_m and k_{-m} are the kinetic constants for the forward and reverse reactions.

The acyl migration pathway was independent on water activity and the enzyme support had no effect on the acyl migration. Furthermore, SFA, MUFA and PUFA fractions also presented similar acyl migration (k_m=0.018 ± 0.0018 min^{-1}). At thermodynamic equilibrium, the molar proportion between 1-MAG and 2-MAG were 86:14, values which are closed to those previously reported in the literature (9:1) (Boswinkel, Derksen, K, & Cuperus, 1996).

The enzymatic method proposed has led to concentration yields much lower than those obtained by the low temperature winterization. Hence, a process in which a concentrate in

free fatty acids was employed as substrate to produce MLM structured lipids as functional compounds was proposed for the up-grading of fish discards.

4.1.3. Production of MLM structured lipids from sardine discards

MLM structured lipids with caprylic acid as medium chain fatty acid (M) and concentrated polyunsaturated fatty acids (L) has been chosen as final functional compound due to their demand in the pharmaceutical industry.

For the production of MLM from sardine discards, the following steps have been conducted: (i) fish oil extraction, (ii) Omega-3 free fatty acids (FFA) concentration (low temperature winterization), (iii) enzymatic esterification (Eq. 10 and 11) and (iv) triacylglycerols purification (liquid column chromatography) (Fig. 3).

$$\text{Glycerine} + \frac{2}{3}\,\text{Caprylic acid} \xleftrightarrow{Rhizomucor\ \ mie\,hei} \text{TAG} + 1{,}3\ \text{DAG} + 1\text{MAG} + \text{FFA} + \text{H}_2\text{O} \quad [10]$$

$$1{,}3\ \text{DAG} + \frac{1}{3}\,\text{Concentrated FFA} \xleftrightarrow{Novozyme\ \ 435} \text{TAG} + 1{,}3\ \text{DAG} + \text{FFA} + \text{H}_2\text{O} \quad [11]$$

The oxidative state of the oil (peroxide value and anisidine index), the fatty acid composition and their regiodistribution in the glycerol backbone have been monitored throughout the whole process.

4.1.3.1. Process overview: yield and oxidative stability

Fish oil extraction, presented a yield of 39.5 wt% referred to the oil content of the sardine. Higher yields can be achieved by increasing the temperature and time of cooking. Nevertheless, the increase of the temperature (>50ºC) implies a more intense oxidation of the extracted oil and a loss of quality (García-Moreno, Morales-Medina, et al., 2014). Winterization presented a yield of 61.6 wt% when estimated as mass of concentrated fraction per mass of free fatty acids winterized. The loss is due to the SFA which are separated after precipitation and filtration. However, the loss of PUFA in the solid fraction was low (6.5 wt%).

With regards to the oxidative stability, the maximum values of peroxide and anisidine indexes for crude fish oil is 20 meq/kg oil and 60, respectively (Bimbo, 1998). On the other hand, refined fish oil must present better oxidative state (PV < 5 meq/kg oil and AI < 20), (Ackman, 2005). In the current process, the mild conditions of the extraction led to an extracted sardine oil with a low degree of oxidation. Also, the concentration step (winterization at -85ºC and solvent evaporation under vacuum) improved the oxidative

state of the concentrated fatty acids while it slightly increased the PV of the saturated fraction. Due to the low volatility of hydroperoxides (Jacobsen & Nielsen, 2007), they might have crystallized and remained in the solid phase. Despite having employed mild conditions for esterification (*i.e.* 37ºC, amber flasks, N_2 atmosphere, 24 h), the reaction mixture was considerably oxidized during esterification.

The separation of triglycerides by liquid chromatography followed by evaporation of solvent (40ºC, 100 mmHg) improved the oxidative stability of the oil until achieving values of refined oil. Since silica earth was employed as stationary phase, a partial adsorption of undesirable compound might happen resulting in a reduction of PV. Also, a considerable decrease of the anisidine value (*i.e.* content of volatile compounds) was observed, which could be related to the evaporation of solvent at high vacuum. To minimize the extent of the oxidation, further studies focused on the addition of antioxidants must be conducted.

4.1.3.2. Fatty acid composition and regiodistribution

The oil presented a high content of PUFA (41.67 mol %), DHA (16.7 mol %) and EPA (12.47 mol %) being the most abundant fatty acids of this fraction. After winterization at low temperature, the PUFA were concentrated 1.6-times in the liquid fraction. The concentrated FFA contained 610 mg of Omega-3 per g of oil, accomplishing with the minimum concentration required (*i.e.* 600 mg/ g of oil). With regard to the esterification step, TAG were the main fraction with around 60 mol% (90 wt%). The TAG presented a global composition closed to the theoretical expected (66 mol % SFA, 33 mol% of MUFA and PUFA). However, FFA and DAG composition differed from the expected one. Effectively, FFA had a high content of caprylic acid (59 mol%) in spite of the negligible amount expected. Also, in the case of the DAG, C16:1n-7, C18:1n-9, EPA and DHA were detected in a high proportion (theoretically DAG should be almost pure caprylic acid).

Additionally, produced TAG presented around 25 mol% of caprylic acid in the sn-2 position. These facts suggest that (i) the DAG employed as substrate was a mixture of 1,2-DAG and 1,3-DAG, as a result of acyl migration or (ii) parallel acidolysis was taken place. As a negative control, a direct esterification was also conducted. In that case, the TAG presented a 73.4 mol % of caprylic acid in the central bond. Hence, despite the two-step esterification process presented some limitation, it improved significantly the selectivity of the direct esterification process.

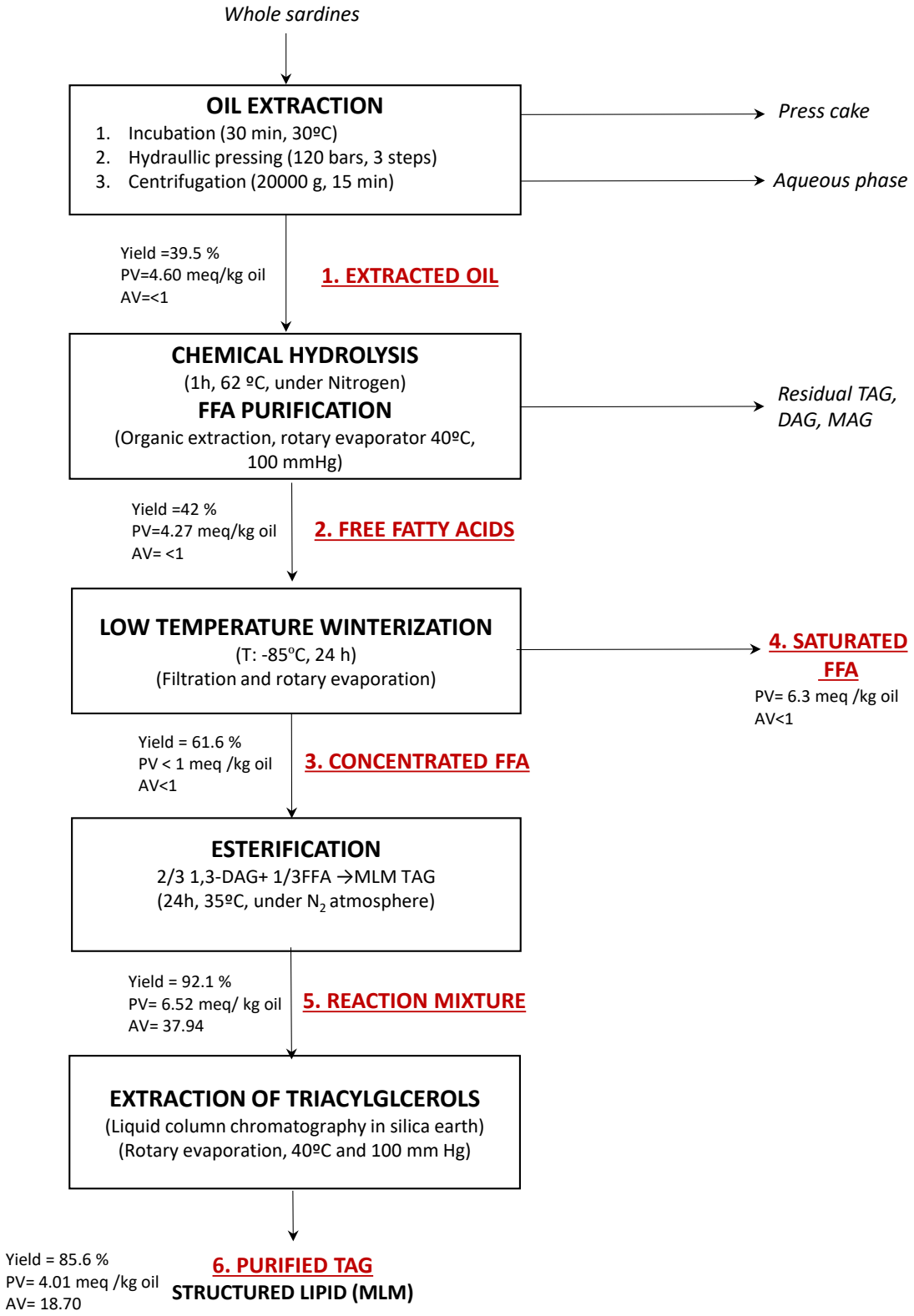


Figure. 3. Schematic diagram of the proposed process for the production of MLM structured lipids from sardine discards.

4.2. Fish protein up-grading

4.2.1. Substrate seasonal characterization

The seasonal variation of the protein content of the six discarded species listed in section 4.1.1. was evaluated. In contrast to the lipid fraction, the protein content remained virtually constant through the year. The highest protein content was found for small-spotted catshark (average value of 20.6±1.9 wt %) followed by axillary seabream, bogue, blue whiting, sardine and horse mackerel (average values of 18.5±1.6, 18.1±2.0, 18.1±0.6, 17.5±2.0 and 17.5±0.9 wt%, respectively).

The antioxidant and antihypertensive activities of these species have been previously studied in works of this group (García-Moreno, Batista, et al., 2014; García-Moreno, Espejo-Carpio, Guadix, & Guadix, 2015) employing subtilisin, trypsin and a combination of both. Horse mackerel presented the higher antihypertensive activity and high antioxidant activity (García-Moreno, Batista, et al., 2014; García-Moreno et al., 2015). Also, a synergic effect was observed when a mixture of proteases (*i.e.* subtilisin and trypsin) was employed. Consequently, horse mackerel was chosen as a substrate for the production of antihypertensive and antioxidant peptides employing mixtures of proteases.

On the other hand, sardine was the species with the highest antioxidant activity (García-Moreno, Batista, et al., 2014). Hence, these two species have been proposed, along with horse mackerel, as a raw material for the production of hydrolysates able to stabilize fish oil emulsions and microcapsules.

4.2.2. Kinetic study of the enzymatic hydrolysis of horse mackerel with a mixture of bacterial and animal proteases

In this section, a crossed mixture-process design was proposed involving the percentage of subtilisin in the enzyme mixture (0, 25, 50, 75 and 100%), the concentration of protein in the reaction volume (2.5 – 5 and 7.5 g/L) and the temperature (40 – 47.5 – 55ºC) as input variables. The response variables were the degree of hydrolysis, the antihypertensive and antioxidant activities (*i.e.* DPPH scavenging activity, Fe^{3+} reducing power and Fe^{2+} chelating activity). Additionally, in the case of artificial neuronal networks (ANN) modelling, time was also included as an input variable.

The hydrolysis of fish substrates is a heterogeneous reaction involving a number of simultaneous physical (*e.g.* substrate solubility, enzyme deactivation) and chemical

phenomena (*i.e.* cleavage of peptide bonds). Deterministic models, based on Michaelis-Menten theory, present a wide scope of applicability (Cavaille & Combes, 1995; Qian, Zhang, & Liao, 2011; Valencia, Pinto, & Almonacid, 2014; Zhou, Chen, & Li, 2003). However, the complexity of the model increases with the number of phenomena considered. In the current study, not only the degree of hydrolysis but also the bioactivities were correlated to both the process variables and the enzyme mixture. Hence, numerous factors such as molecular weight distribution or amino acids composition must be taken into account, resulting in an extremely complex model.

Therefore, empirical approaches such as response surface methodology (RSM) or artificial neural networks were preferred for modelling (Abakarov, Teixeira, Simpson, Pinto, & Almonacid, 2011; Baş, Dudak, & Boyaci, 2007).

RSM is commonly used for the modelling and optimization of protein hydrolysis (Valencia, Espinoza, Ceballos, Pinto, & Almonacid, 2015). The advantage of this technique is the small number of experimental data required (Keskin Gündoğdu et al., 2014). As main drawback, not all the biochemical processes are adequately modeled by the polynomial functions (*e.g.* quadratic or cubic) usually employed in RSM (Baş et al., 2007).

ANN arise as an alternative with high predicting capabilities, especially when involving a large volume of data (Baş et al., 2007; G. Zhang, Eddy Patuwo, & Y. Hu, 1998; Y. Zhang, Xu, & Yuan, 2009). ANN are empirical models inspired in human brain, where the neurons are arranged in layers and interconnected by mathematical functions.

4.2.2.1. Modelling employing ANN

The architecture of the assayed artificial neural network consisted in an input layer, a hidden layer and an output layer, as shown in Fig. 5. The four input variables (X_i) were temperature, concentration of protein in the reaction volume, percentage of subtilisin in the enzyme mixture and time. The number of neourons which composed the hidden layer varied from 1 to 10. As transfer function the sigmoid function was selected. Finally, the neuron of the output layer estimates the degree of hydrolysis.

The normalised output from such neural network was given by Eq.12

$$DH = \sum_{k=1}^{10} \omega_k \cdot \Phi\left(\sum_{i=1}^{4} w_{ki} \cdot X_i + b_k\right) + \beta \qquad [12]$$

To train the neural network, the input data were divided into three subsets. The first subset, which comprised 70% of the experimental data, was employed to fit the output responses to the experimental data. To that end, the Levenberg–Marquardt was chosen as training algorithm to minimize the mean squared error (MSE). The second subset, comprising 15% of the experimental data, was used to validate the model. The third subset (15% of experimental data) was employed to test the predictive capacity of the ANN.

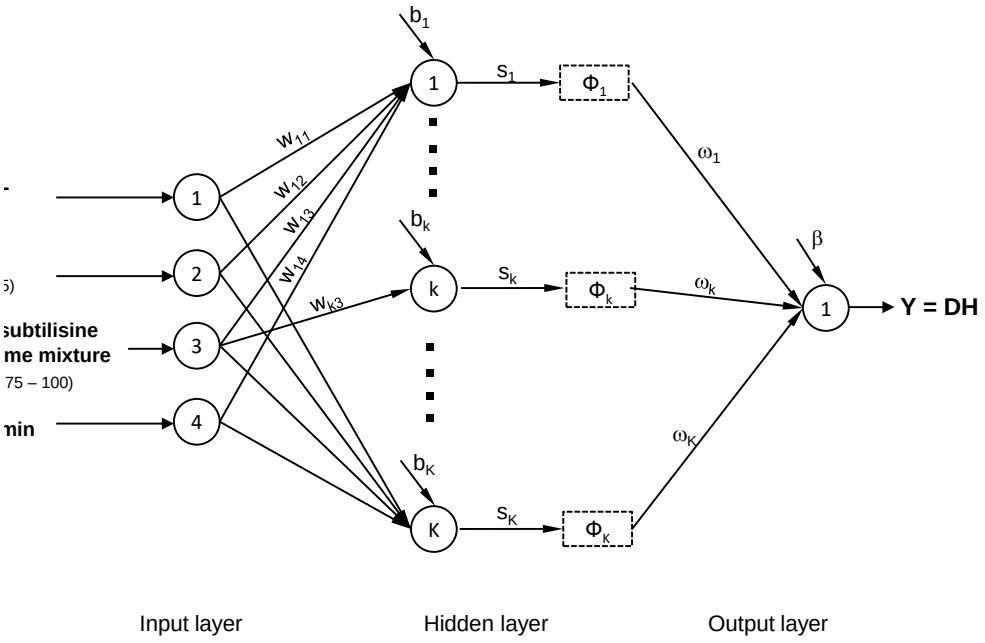


Figure. 5. Schematic diagram of the architecture and model parameters of the artificial neural network.

As result, an increase of the number of neurons in the hidden layer decreased the MSE of the test subset while improved the coefficient of determination. Consequently, the ANN composed of 10 neurons in the hidden layer was selected ($r^2 = 0.987$).

4.2.2.2. Modelling employing a crossed mixture-process model

A crossed mixture-process model was employed to model the final DH (*i.e.* after 4 hours of reaction), the percentage ACE inhibition and the antioxidant properties (*i.e.* DHHP scavenging, reducing power and chelating) of the resulting hydrolysate as a function of the process and mixture variables mentioned above. As shown in Fig. 6, process variables were modeled by a factorial design, while the enzyme composition followed a mixture model. The crossed mixture-process model was obtained (Cornell, 2002) by combination of both sub-models, as shown in Eq. 13:

$$Y(X_1, X_2, S, T) = (\alpha_0 + \alpha_1 \cdot S + \alpha_2 \cdot T + \alpha_{12} \cdot S \cdot T + \alpha_{11} \cdot S^2 + \alpha_{22} \cdot T^2) \cdot (\beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_{12} \cdot X_1 X_2) \quad [13]$$

where Y denotes any of the response variables (DH, antihypertensive or antioxidant activities) X_1 is the percentage of subtilisin in the enzyme mixture, X_2 is the percentage of trypsin in the enzyme mixture, S the substrate concentration and T the temperature.

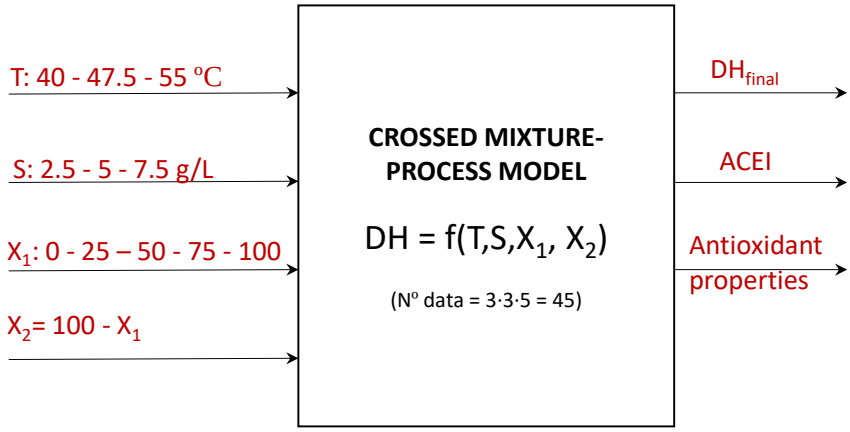


Figure 6. Schematic diagram of the design experiments proposed for the modelling of DH, ACEI an the antioxidant activity (DPPH scavenger activity, reducing power and metal chelating activity) as a function of temperature, substrate concentration and subtilisin and trypsin content by a crossed-mixture model.

Applying the aforementioned model and reducing it to the significant contributions, the DH can be expressed as Eq. 14 (r^2 =0.9913).

$$+ 4.66 \cdot 10^{-3} \cdot X_2 \cdot S^2 + 7.02 \cdot 10^{-5} \cdot X_1 \cdot X_2 \cdot T - 1.20 \cdot 10^{-6} \cdot X_1 \cdot X_2 \cdot T^2 \quad [14]$$

The optimization of the ANN led to the highest DH of 17.1% at an initial protein concentration of 2.54 g/L, 40 ºC, 4 h of hydrolysis and an enzyme mixture comprising 38.3% of subtilisin and 61.7% trypsin. On the other hand, the crossed mixture-process model predicted a maximum value of DH (15.94%), attained at 2.5 g/L, 40 ºC and 41.2% of subtilisin in the enzyme mixture. Both modelling approaches offered similar results. Hence, adding trypsin to the enzyme solution led to a higher final DH (17.1%), compared to the single use of subtilisin (maximum DH of 15.5%) allows to conduct hydrolysis at lower temperature (instead of 50 ºC). This fact presents both economical and nutritional advantages.

The experimental data showed higher DH at lower concentrations (2.5 g/L). This fact might be related to substrate inhibition or high protein solubility. Additionally, an increase of the reaction temperature played a detrimental effect on the final DH. This behavior

might be a result of a trade-off between the solubility of the substrate protein and the intervals of stability of the enzymes employed. Effectively, higher temperatures favor the solubility of the substrate, and hence, the availability of peptide bonds. By contrast, subtilisin is stable at high temperatures (maximum activity at 50ºC) while trypsin presents an optimal activity at 37 ºC (Najafian & Babji, 2012). Finally, the influence of the composition of the mixture of enzymes did not follow a clear tendency showing, in some cases, synergic effect when mixtures were employed. In fact, trypsin cleaves exclusively next to lysine and arginine residues (Olsen, Ong, & Mann, 2004) while subtilisin cleaves a wider range, preferably next to aromatic, acid and methionine residues (Adamson & Reynolds, 1996). Hence, their combination might increase the availability of potential cleavage peptide bonds.

4.2.2.3. Optimization of the production of antihypertensive peptides

As performed for the final DH, antihypertensive activity has been modelled following a crossed-mixture model. As a result, the following expression was obtained:

$$\begin{aligned} \mathrm{ACEI} = {} & 0.3708 \cdot X_1 - 2.61 \cdot 10^{-4} \cdot X_1 \cdot S \cdot T + 4.28 \cdot 10^{-5} \cdot X_1 \cdot T^2 + 0.0139 \cdot X_2 \cdot T \\ & - 1.77 \cdot 10^{-3} \cdot X_2 \cdot S \cdot T + 5.38 \cdot 10^{-3} \cdot X_2 \cdot S_2^2 + 0.0266 \cdot X_1 \cdot X_2 \\ & - 5.49 \cdot 10^{-3} \cdot X_1 \cdot X_2 \cdot S - 4.19 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot T + 3.71 \cdot 10^{-5} \cdot X_1 \cdot X_2 \cdot S \cdot T \\ & + 3.84 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot T^2 \end{aligned} \quad [15]$$

Where X_1 and X_2 are the percentages of subtilisin and trypsin in the enzymatic mixture, T the temperature (ºC) and S the protein concentration (g/L), the coefficient of determination being r^2=0.9972.

The highest experimental level of ACE inhibition (56.12%) was observed at 2.5 g/L of substrate, 55 ºC and only trypsin in the enzyme preparation. The maximum ACE inhibition predicted by the regression model (ACEI, 55.3%) was obtained from the same set of input variables. Employing trypsin at 55 ºC the extent of the proteolysis was limited, with a final DH around 9%. Levels of ACE inhibition above 50% were detected for tryptic hydrolysates processed above 48 ºC. Increasing amounts of subtilisin in the enzyme mixture allowed obtaining similar inhibitory levels, at the expense of higher processing temperatures (*e.g.* 50% ACE inhibition at 55ºC and 18.5% of subtilisin).

At low temperatures and concentrations, the inhibitory activity of the hydrolysate was favored by increasing levels of subtilisin in the enzyme preparation (*e.g.* 46% ACE inhibition at 40ºC and 21.6% of subtilisin). These conditions favored the extensive hydrolysis of the substrate, attaining values of final DH above 15.8%. Although the

potential ACE inhibitory effect of a given peptide depends on a range of factors (*e.g.* residue composition, hydrophobicity), most of the active peptides identified to date are di- and tripeptides (Li, Zhou, Huang, Sun, & Zeng, 2012; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). Additionally, the ACE inhibitory effect of a given hydrolysate is favored by the presence of peptides containing hydrophobic residues (*e.g.* Pro, Phe, Tyr) in the tripeptide sequence at the C-terminal end, since it facilitates the interaction with the active site of the Angiotensin I Converting Enzyme (Li, Le, Shi, & Shrestha, 2004).

The hydrolysates with the highest ACE inhibitory activity for each reaction temperature assayed were selected for assessing the effect of gastrointestinal enzymes on the ACE inhibitory activity. This effect has been measured by comparing the IC_{50} value of both the crude hydrolysates and their digests. The IC_{50} values ranged between 253 and 330 µg/mL, showing no significant differences before and after digestion. This is a very interesting feature, since gastrointestinal digestion is one of the main processes reducing the bioavailability of bioactive peptides.

4.2.2.4. Optimization of the production of antioxidant peptides

As oxidation can be conducted by several pathways, the measurement of antioxidant activity must be analyzed by several measurements based on different mechanisms (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002). Consequently, in this work three methods have been employed: DPPH scavenging activity, Fe^{3+} reducing power and iron (Fe^{2+}) chelating activity. DPPH scavenging assay, a reactive oxygen scavenging test, is commonly employed to determine the ability of a certain substrate to stabilize free radicals (*e.g.* by electron or hydrogen transfer). Reducing power assay, on the contrary, belongs to the redox potential tests. It measures the capacity of a compound to donate exclusively electrons to free radicals. Metals catalyze lipid oxidation, they decompose hydroperoxides, producing alkyl radicals and reactive oxygen species (Shahidi & Zhong, 2010). Hence, metal chelators can act as antioxidant by metal coordination preventing catalytic effect of metals.

In this chapter the three aforementioned antioxidant capacities were modelled resulting in Eq. 16-18 (r^2 >0.97).

$$\text{DPPH, \%} = 3.56 \cdot X_1 - 3.2 \cdot 10^{-1} \cdot X_1 \cdot S + 2.7 \cdot 10^{-2} \cdot X_1 \cdot S^2 - 9.0 \cdot 10^{-2} \cdot X_1 \cdot T + 8.9 \cdot 10^{-4} \cdot X_1 \cdot T^2 + 2.0 \cdot X_2 - 4.0 \cdot 10^{-1} \cdot X_2 \cdot S + 2.5 \cdot 10^{-2} \cdot X_2 \cdot S^2 - 1.4 \cdot 10^{-2} \cdot X_2 \cdot T + 2.3 \cdot 10^{-3} \cdot X_2 \cdot S \cdot T - 2.8 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot S \quad [18]$$

$$Fe^{3+}\text{reducting power} = 1.4 \cdot 10^{-2} \cdot X_1 - 2.2 \cdot 10^{-3} \cdot X_1 \cdot S + 1.9 \cdot 10^{-4} \cdot X_1 \cdot S^2 + 3.0 \cdot 10^{-4} \cdot X_2 \cdot T - 3.1 \cdot 10^{-6} \cdot X_2 \cdot T^2 + 1.2 \cdot 10^{-3} \cdot X_1X_2 - 2.7 \cdot 10^{-4} \cdot X_1X_2 \cdot S + 8.8 \cdot 10^{-6}X_1X_2 \cdot S^2 - 1.9 \cdot 10^{-5} \cdot X_1X_2 \cdot T + 3.5 \cdot 10^{-6} \cdot X_1 \cdot X_2 \cdot S \cdot T \quad [19]$$

$$Fe^{2+}\text{chelating activity} = 3.7 \cdot X_1 + 1.4 \cdot 10^{-1} \cdot X_1 \cdot S - 1.4 \cdot 10^{-2} \cdot X_1S^2 - 1.7 \cdot 10^{-1} \cdot X_1 \cdot T + 1.9 \cdot 10^{-3} \cdot X_1 \cdot T^2 + 1.02 \cdot X_2 - 3.0 \cdot 10^{-1}X_2 \cdot S + 1.4 \cdot 10^{-2} \cdot X_2 \cdot S^2 - 1.1 \cdot 10^{-2} \cdot X_2 \cdot T + 3.4 \cdot 10^{-3} \cdot X_2 \cdot S \cdot T + 7.2 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot S^2 + 3.1 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot T - 1.4 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot S \cdot T \quad [20]$$

Despite that DPPH scavenging activity and the Fe^{3+} reducing power act by different pathways, they both are related to electronic transfer and, generally, were influenced in a similar fashion by the operational variables. Effectively, both properties presented their global maximum when substrate concentration was 2.5 g/L and at 40 ºC. The effect of the enzymes composition was different since maximum DPPH scavenging activity (80.1% measured at a concentration of 3 mg of hydrolysate/mL) was produced when pure trypsin was employed. In the case of reducing power, its theoretical maximum (1.25 measured at 10 mg of hydrolysate/mL) was achieved with an enzyme mixture containing 58% subtilisin and 42% trypsin. As general a trend, it can be concluded that those hydrolysates produced at lower temperatures presented higher antioxidant properties, except for the reducing power of the hydrolysates at 7.5 g/L. High DPPH scavenging activity is related to the presence of hydrophobic amino acids and to the presence of Val, Leu, Ile, Ala, Phe or Lys at the N-terminal (Suetsuna et al., 2000). In the case of horse mackerel muscle, these residues represent 30 wt% of the amino acid composition (Morales-Medina, Tamm, Guadix, Guadix, & Drusch, 2015). By means of hydrolysis with subtilisin, Met, Phe, Tyr and Trp residues might be released. Furthermore, at low temperature (<45 ºC), trypsin presents enhanced proteolytic activity (*i.e.* it releases Tyr, Trp or Phe, apart from Lys and Arg), which leads to hydrolysates with improved scavenging properties.

Among the three antioxidant activities studied, chelating was the most powerful. As happened to the previous antioxidant activities, chelating activity of hydrolysates is mainly

determined by the amino acids composition and distribution. Glu, Asp, Lys or Arg at side chains can act as chelators (Liu et al., 2010). In the case of horse mackerel, these amino acids represented a ~54 wt% when including Lys and Arg (Morales-Medina et al., 2015). Hence, the high chelating activity is caused by the high content of amino acids able to present chelating activity.

Chelating activity presented a theoretical global maximum (47.44% measured at 0.6 mg of hydrolysate/mL) for the hydrolysate produced at 5 g/L, 55 ºC and employing pure subtilisin. A similar behavior was observed at the lowest substrate concentration where the local maximum was obtained in comparable temperature and enzyme mixture composition. Contrary, at increasing levels of substrate concentration, the local maximum was found at 40 ºC and employing around 55% of subtilisin.

Also, a bi-objective optimization was conducted with the following conditions:

$$
\begin{aligned}
&\textit{Maximize DPPH } (S,T,X_1, X_2) \\
&\qquad \textit{Reducing } (S, T, X_1, X_2) \\
&\textit{Subjected to:} \\
&\qquad 2.5 \leq S \leq 7.5 \\
&\qquad 40 \leq T \leq 55 \\
&\qquad 0 \leq X_1 \leq 100 \\
&\qquad X_2 = 100 - X_1 \\
&\qquad \textit{Chelating } (S, T, X_1, X_2) = \mathcal{E}
\end{aligned}
\qquad [19]
$$

Hence, for a fixed value of chelating activity, both DPPH scavenging capacity and reducing power have been maximized separately. As a result, the conditions that led to maximum DPPH scavenging activity and reducing power also conducted to the minimum chelating activity and *vice versa*. Presumably, this might be related to differences of amino acids involved in each activity.

4.2.3. Evaluation of technological and antioxidant properties of protein hydrolysates as stabilizers for the microencapsulation of fish oil

Microencapsulation of fish oil by spray-drying in presence of antioxidants can further extend the shelf life of fish oil (Serfert et al., 2009). Since oxidation begins at the interface, there is an increasing interest in the utilization of emulsifying compounds with antioxidant properties like proteins or protein hydrolysates. In this context, FPH have been proposed as

potential stabilizers for the microencapsulation of fish oil due to their technological and antioxidant properties.

Two discarded species of the Alboran Sea, namely sardine and horse mackerel were hydrolysed until degrees of hydrolysis 5 and 10 % (FPH(5) and FPH(10), respectively) with subtilisin and trypsin. FPH were used as emulsifiers and antioxidants in the production of emulsions (oil load 5%) and subsequent spray-drying to yield microcapsules with an oil load of 14.33%.

Additionally, the antioxidant properties of the FPH were analyzed (DPPH scavenging activity, Fe^{3+} reducing power and Fe^{2+} chelating activity) at several concentrations (1 to 30 mg/mL). All antioxidant activities showed a dose-dependent behavior. In the case of DPPH scavenging activity, it increased linearly (r^2> 0.99) with a high slope in the case of concentrations between 1 and 10 mg/mL, whereas, the slope was much lower for higher concentrations. On the contrary, for reducing and chelating capacities, the slope remained constant (r^2>0.95).

Prior to microencapsulation, it was conducted the selection of pH and FPH-fish oil ratio to produce physically stable emulsions. Several protein contents (0.5, 1 and 2 wt%) and pH values (2, 3 and 8) were tested. Emulsions stabilized with 2% of hydrolysates (DH5) at pH 2 were the most stable and, consequently, they were selected for the process of microencapsulation process. No stable emulsion was produced employing FPH(10).

The impact of the spray drying on the physical stability of the emulsion was monitored by measuring the oil droplet size distribution (ODSD). No significant variation of the ODSD was observed and, consequently, the process of encapsulation was considered as an innocuous step. Additionallly, high microencapsulation efficiency was obtained (98%). Hence, the integrity of the interface was maintained during spray-drying.

After 12 weeks of storage, the hydroperoxide content ranged from 114±18 to 136±17 mmol/kg oil for hydrolysates of horse mackerel produced with subtilisin and trypsin, respectively. The slope of the oxidation curves provide a rough value of the oxidation rate. Microcapsules stabilized with hydrolysates produced from sardine protein and subtilisin (SAH(5)) with showed the lowest value (1.42mmol/kg oil per day), followed by hydrolysates produced from horse mackerel protein and subtilisin (HAH(5), 1.48 mmol/kg oil per day).

Apart from the positive overall performance of the FPH-stabilised emulsions, no correlation between rate of oxidation and antioxidative properties of the FPH was found. SAH(5) and HAH(5) presented similar values of scavenging activity, whereas SAH(5) presented better reducing capacity. However the degree of oxidation of both microcapsule was similar. The absence of a clear correlation might be related to several factors, *i.e.* the narrow range of variation of the antioxidant properties. Also, *in vitro* tests are usually performed in the absence of lipids. The conformation of the protein may vary when located at the interface (Decker et al., 2005) and so the antioxidant properties. To determine whether the *in vitro* antioxidant tests can be a useful tool for the prediction of oxidation of microencapsulated oil, further comparisons between *in vivo* and *in vitro* test must be done.

5. REFERENCES

A.O.A.C. (2006). Official Methods of Analysis of the AOAC, Association of Official Analytical Chemists,. Washington DC.

Abakarov, A., Teixeira, A., Simpson, R., Pinto, M., & Almonacid, S. (2011). Modeling of squid protein hydrolysis: artificial neural network approach. *Journal of Food Process Engineering*, 34(6), 2026–2046. http://doi.org/10.1111/j.1745-4530.2009.00567.x

Ackman, R. G. (2005). Fish oils. In F. Shahidi (Ed.), Bailey's Industrial Oil and Fat Products (6th ed., pp. 279–317). Hoboken, NJ, USA: John Wiley & Sons.

Adamson, N. J., & Reynolds, E. C. (1996). Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. *Enzyme and Microbial Technology*, 19(3), 202–207. http://doi.org/10.1016/0141-0229(95)00232-4

Adler Nissen. (1986). Enzymic hydrolysis of food proteins. London: Elsevier Applied Science Publishers LTD.

Akoh, C. C. (2005). Handbook of Functional Lipids. CRC Press.

Akoh, C. C., & Kim, B. H. (2008). Structured lipids (pp. 841–872). Conference Proceedings, CRC Press.

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. (2002). Methods for testing antioxidant activity. *The Analyst*, 127(1), 183–198. http://doi.org/10.1039/b009171p

Babayan, V. K. (1987). Medium chain triglycerides and structured lipids. Lipids, 22(6), 417–420. http://doi.org/10.1007/BF02537271

Baş, D., Dudak, F. C., & Boyaci, I. H. (2007). Modeling and optimization IV: Investigation of reaction kinetics and kinetic constants using a program in which artificial neural network (ANN) was integrated. *Journal of Food Engineering*, 79(4), 1152–1158. http://doi.org/10.1016/j.jfoodeng.2006.04.004

Bimbo, A. P. (1998). Guidelines for Characterising Food Grade Fish Oil. INFORM - International News on Fats, Oils and Related Materials, 9(5), 473–483. Retrieved from http://www.iffo.net/system/files/Guidelines Food grade fish oil 1998-3.pdf

Bimbo, A. P. (2007). Processing of marine oils. In H. Breivik (Ed.), Long-Chain Omega-3 Specialty Oils (pp. 77–107). Bridgwater, England: The Oily Press.

Blanco, M., Sotelo, C. G., Chapela, M. J., & Pérez-Martín, R. I. (2007). Towards sustainable and efficient use of fishery resources: present and future trends. *Trends in Food Science & Technology Technology*, 18(1), 29–36. Journal Article. http://doi.org/10.1016/j.tifs.2006.07.015

Boswinkel, G., Derksen, J. T. P., K, van't R., & Cuperus, F. E. (1996). Kinetics of acyl migration in monoglycerides and dependence on acyl chainlength. *J. Am. Oil Chem. Soc*., 73(6), 707–711.

Bottino, N. R., Vandenburg, G. A., & Reiser, R. (1967). Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. *Lipids*, 2(6), 489–493. Journal Article. http://doi.org/10.1007/BF02533177

Bradbury, J. (2011). Docosahexaenoic Acid (DHA): An Ancient Nutrient for the Modern Human Brain. *Nutrients*, 3(5), 529–554. http://doi.org/10.3390/nu3050529

Brockerhoff, H., Hoyle, R. J., Hwang, P. C., & Litchfield, C. (1968). Positional distribution of fatty acids in depot triglycerides of aquatic animals. Lipids, 3(1), 24–9. http://doi.org/10.1007/BF02530964

Camacho Paez, B., Robles Medina, A., Camacho Rubio, F., González Moreno, P., & Molina Grima, E. (2002). Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by the acidolysis of cod liver oil and caprylic acid in a packed-bed reactor: Equilibrium and kinetics. *Chemical Engineering Science*, 57(8), 1237–1249. Journal Article.

Carbonell, A., Martín, P., Ranieri, S. de, & Team, W. (1998). Discards of the Western Mediterranean trawl fleets.

Cavaille, D., & Combes, D. (1995). Characterization of β-galactosidase from Kluyveromyces lactis. *Biotechnology and Applied Biochemistry*, 22(1), 55–64.

Chabeaud, A., Vandanjon, L., Bourseau, P., Jaouen, P., & Guérard, F. (2009). Fractionation by ultrafiltration of a saithe protein hydrolysate (Pollachius virens): Effect of material and molecular weight cut-off on the membrane performances. *Journal of Food Engineering*, 91(3), 408–414.

Chang, S.-H., Wu, S.-K., & Kimura, H. (2006). Crystallization Kinetics of Ti50Ni25Cu25 Melt-Spun Amorphous Ribbons. *Materials Transactions*, 47(10), 2489–2492. http://doi.org/10.2320/matertrans.47.2489

Chen, H., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative Properties of Histidine-Containing Peptides Designed from Peptide Fragments Found in the Digests of a Soybean Protein. *Journal of Agricultural and Food Chemistry*, 46(1), 49–53. http://doi.org/Doi 10.1021/Jf970649w

Christensen, M., Hoy, C., Becker, C., & Redgrave, T. (1995). Intestinal absorption and lymphatic transport of eicosapentaenoic (EPA), docosahexaenoic (DHA), and decanoic acids: dependence on intramolecular triacylglycerol structure. *Am J Clin Nutr*, 61(1), 56–61.

Cornell, J. A. (2002). Experiments with mixtures : designs, models, and the analysis of mixture data. New York: John Wiley & Sons, Inc.

Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring antioxidant effectiveness in food. *Journal of Agricultural and Food Chemistry*, 53(10), 4303–10. http://doi.org/10.1021/jf058012x

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, 38(3), 674–677. http://doi.org/10.1021/jf00093a019

Dillon, J. T., Aponte, J. C., Tarozo, R., & Huang, Y. (2013). Purification of omega-3 polyunsaturated fatty acids from fish oil using silver-thiolate chromatographic material and high performance liquid chromatography. *Journal of Chromatography A*, 1312, 18–25. http://doi.org/10.1016/j.chroma.2013.08.064

Drusch, S., Serfert, Y., Berger, A., Shaikh, M. Q. Q., Rätzke, K., Zaporojtchenko, V., & Schwarz, K. (2012). New insights into the microencapsulation properties of sodium caseinate and hydrolyzed casein. *Food Hydrocolloids*, 27(2), 332–338. http://doi.org/10.1016/j.foodhyd.2011.10.001

EU. (2011). Proposal for a regulation of the European Parliament and of the Council on the Common Fisheries Policy. COM, 425.

EU. (2013). Regulation (EU) no 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Official Journal of the European Union, 22–28.

Fleury, Y., Defer, D., & Bourgougnon, N. (2008). Antimicrobial peptides and antiviral activities from marine fished or farmed animals. In J. P. Bergé (Ed.), Added Value to Fisheries Waste (1st ed., pp. 163–196). Kerala: Transworld Research Network.

Frankel, E. (2005). Lipid oxidation (The Oily Press Lipid Library).

FROM-Fondo de Regulación y Organización del Mercado de los Productos de la Pesca y los, & cultivos Marinos. (2008). Asistencia técnica para la realización de una medida innovadora para la recuperación, gestión y valorización de los descartes generados por la flota española que faena en los caladeros atlántico y mediterráneo.

Fureby, A. M., Virto, C., Adlercreutz, P., & Mattiasson, B. (1996). Acyl Group Migrations in 2-Monoolein. *Biocatalysis and Biotransformation*, 14(2), 89–111. http://doi.org/10.3109/10242429609106879

García-Moreno, P. J., Batista, I., Pires, C., Bandarra, N. M., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2014). Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species. *Food Research International*, 65, Part C, 469–476. http://doi.org/10.1016/j.foodres.2014.03.061

García-Moreno, P. J., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2015). Production and identification of angiotensin I-converting enzyme (ACE) inhibitory peptides from Mediterranean fish discards. *Journal of Functional Foods*, 18, 95–105. http://doi.org/10.1016/j.jff.2015.06.062

García-Moreno, P. J., Morales-Medina, R., Pérez-Gálvez, R., Bandarra, N. M., Guadix, A., & Guadix, E. M. (2014). Optimisation of oil extraction from sardine (*Sardina pilchardus*) by hydraulic pressing. International *Journal of Food Science & Technology*, 49(10), 2167–2175. http://doi.org/10.1111/ijfs.12527

Garrett, D. A., Failla, M. L., & Sarama, R. J. (1999). Development of an *in Vitro* Digestion Method To Assess Carotenoid Bioavailability from Meals. *Journal of Agricultural and Food Chemistry*, 47(10), 4301–4309. http://doi.org/10.1021/jf9903298

Groenewold, S., Damalas, D., Maravelias, C. D., Osio, G. C., Maynou, F., Sbrana, M., Casey, J. (2000). Effects on benthic scavengers of discards and damaged benthos produced by the beam-trawl fishery in the southern North Sea. *ICES Journal of Marine Science*, 57(5), 1395–1406. http://doi.org/10.1006/jmsc.2000.0914

Gunstone, F. D., Harwood, J. L., & Dijkstra, A. J. (2012). The Lipid Handbook with CD-ROM, Third Edition. CRC Press.

Halim, N. R. A., Yusof, H. M., & Sarbon, N. M. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology*, 51, 24–33. http://doi.org/10.1016/j.tifs.2016.02.007

Hari Krishna, S., & Karanth, N. G. (2002). Lipases and Lipase-Catalyzed Esterification Reactions in Nonaqueous Media. Catalysis Reviews, 44(4), 499–591. http://doi.org/10.1081/CR-120015481

Innis, S. M. (2011). Dietary triacylglycerol structure and its role in infant nutrition. *Advances in Nutrition*, 2(3), 275–83. http://doi.org/10.3945/an.111.000448

Ito, N., Fukushima, S., & Tsuda, H. (1985). Carcinogenicity and Modification of the Carcinogenic Response by BHA, BHT, and Other Antioxidants. CRC *Critical Reviews in Toxicology*, 15(2), 109–150. http://doi.org/10.3109/10408448509029322

Jacobsen, C., Rustad, T., Nielsen, N. S., Falch, E., Jansson, & S. Storr, I. (2009). Processing of marine lipids and factors affecting their quality when used for functional foods. In J. B. Luten (Ed.), Marine functional food (pp. 89–114). Wageningen: Wagenigen Academic Publishers.

Jacobsen, C., & Skall Nielsen, N. (2007). Optimization of oxidative stability of omega-3 enriched food. In H. Breivik (Ed.), Long-Chain Omega-3 Specialty Oils (21st ed., pp. 197–219). Bridgwater, England: The Oily press Lipid Library.

Johnson, L. A. (2008). Recovery, refining, converting, and stabilizing edible fats and oils (pp. 205–243). Conference Proceedings, CRC Press.

Jump, D. B., Depner, C. M., & Tripathy, S. (2012). Omega-3 fatty acid supplementation and cardiovascular disease Thematic Review Series: New Lipid and Lipoprotein Targets for the Treatment of Cardiometabolic Diseases. *Journal of Lipid Research*, 53(12), 2525–2545. http://doi.org/10.1194/jlr.R027904

Jung, W.-K., Mendis, E., Je, J.-Y., Park, P.-J., Son, B. W., Kim, H. C. Kim, S.-K. (2006). Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (Limanda aspera) frame protein and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry*, 94(1), 26–32. http://doi.org/10.1016/j.foodchem.2004.09.048

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K., & He, J. (2005). Global burden of hypertension: analysis of worldwide data. *The Lancet*, 365(9455), 217–223. http://doi.org/10.1016/S0140-6736(05)17741-1

Kelleher, K. (2005). Discards in the world's marine fisheries. An update. Rome: FAO Fisheries and Aquaculture Department, Rome.

Keskin Gündoğdu, T., Deniz, I. İ., Calışkan, G., Sahin, E. S., Azbar, N., Çalışkan, G.,Azbar, N. (2014). Experimental design methods for bioengineering applications. *Critical Reviews in Biotechnology*, 8551(31), 1–21. http://doi.org/10.3109/07388551.2014.973014

Kim, B. H., & Akoh, C. C. (2015). Recent Research Trends on the Enzymatic Synthesis of Structured Lipids. *Journal of Food Science*, 80(8), C1713–C1724. http://doi.org/10.1111/1750-3841.12953

Kim, S.-K., & Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: A review. *Journal of Functional Foods*, 2(1), 1–9. http://doi.org/10.1016/j.jff.2010.01.003

Köse, Ö., Tüter, M., & Aksoy, H. A. (2002). Immobilized *Candida antarctica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresource Technology*, 83(2), 125–129. http://doi.org/10.1016/S0960-8524(01)00203-6

Kralovec, J. A., Zhang, S., Zhang, W., & Barrow, C. J. (2012). A review of the progress in enzymatic concentration and microencapsulation of omega-3 rich oil from fish and microbial sources. *Food Chemistry*, 131(2), 639–644. http://doi.org/10.1016/j.foodchem.2011.08.085

Kristinsson, H. G., & Rasco, B. A. (2000). Biochemical and functional properties of Atlantic salmon (Salmo salar) muscle proteins hydrolyzed with various alkaline proteases. *Journal of Agricultural and Food Chemistry*, 48(3), 657–666.

Kuratko, C. N., & Salem, N. (2013). Docosahexaenoic acid from algal oil. *European Journal of Lipid Science and Technology*, 115(9), 965–976. http://doi.org/10.1002/ejlt.201300060

Lassoued, I., Mora, L., Nasri, R., Jridi, M., Toldrá, F., Aristoy, M.-C.,Nasri, M. (2015). Characterization and comparative assessment of antioxidant and ACE inhibitory activities of thornback ray gelatin hydrolysates. *Journal of Functional Foods*, 13, 225–238. http://doi.org/10.1016/j.jff.2014.12.042

Lembke, P. (2013). Production Techniques for Omega-3 Concentrates. In F. De Meester, R. R. Watson, & S. Zibadi (Eds.), Omega-6/3 Fatty Acids (pp. 353–364). Humana Press.

Let, M. B., Jacobsen, C., Sørensen, A.-D. M., & Meyer, A. S. (2007). Homogenization conditions affect the oxidative stability of fish oil enriched milk emulsions: lipid oxidation. *Journal of Agricultural and Food Chemistry*, 55(5), 1773–80. http://doi.org/10.1021/jf062391s

Li, G.-H., Le, G.-W., Shi, Y.-H., & Shrestha, S. (2004). Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research*, 24(7), 469–486. http://doi.org/10.1016/j.nutres.2003.10.014

Li, Y., Zhou, J., Huang, K., Sun, Y., & Zeng, X. (2012). Purification of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide with an antihypertensive effect from loach (Misgurnus anguillicaudatus). *Journal of Agricultural and Food Chemistry*, 60(5), 1320–5. http://doi.org/10.1021/jf204118n

Liu, H., Chang, B., Yan, H., Yu, F., & Liu, X. (1995). Determination of amino acids in food and feed by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and reversed-phase liquid chromatographic separation. *Journal of AOAC International*, 78, 736–744.

Liu, Q., Kong, B., Xiong, Y. L., & Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry*, 118(2), 403–410. http://doi.org/10.1016/j.foodchem.2009.05.013

Malins, D. C., & Wekell, J. C. (1970). The lipid biochemistry of marine organisms. *Progress in the Chemistry of Fats and Other Lipids*, 10, 339–363. http://doi.org/10.1016/0079-6832(70)90011-X

Marangoni, A. G., & Wright, A. J. (2005). Physical Properties of Fats and Oils. In Handbook of Functional Lipids (pp. 135–162). CRC Press.

Martínez-Maqueda, D., Miralles, B., Recio, I., & Hernández-Ledesma, B. (2012). Antihypertensive peptides from food proteins: a review. *Food & Function*, 3(4), 350. http://doi.org/10.1039/c2fo10192k

Mendis, E., Rajapakse, N., & Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of Agricultural and Food Chemistry*, 53(3), 581–587.

Mersmann, A. (2001). Crystallization Technology Handbook. CRC Press.

Morales-Medina, R., Tamm, F., Guadix, A. M., Guadix, E. M., & Drusch, S. (2015). Functional and antioxidant properties of hydrolysates of sardine (S. pilchardus) and horse mackerel (T. mediterraneus) for the microencapsulation of fish oil by spray-drying. *Food Chemistry*, 194, 1208–1216. http://doi.org/10.1016/j.foodchem.2015.08.122

Moya-Ramírez, I., García-Román, M., & Fernández-Arteaga, A. (2016). Waste Frying Oil Hydrolysis in a Reverse Micellar System. ACS *Sustainable Chemistry and Engineering*, 4(3), 1025–1031.

Mullin, J. W. (2001). Crystallization. In J. W. Mullin (Ed.), Crystallization (4th Editio, pp. 216–288). Oxford: Butterworth Heinemann. http://doi.org/10.1016/B978-075064833-2/50008-5

Munio, M. del M., Robles, A., Esteban, L., Gonzalez, P. A., & Molina, E. (2009). Synthesis of structured lipids by two enzymatic steps: Ethanolysis of fish oils and esterification of 2-monoacylglycerols. *Process Biochem.*, 44, 723–730. Journal Article. http://doi.org/10.1016/j.procbio.2009.03.002

Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. *Peptides*. ttp://doi.org/10.1016/j.peptides.2011.11.013

Nalinanon, S., Benjakul, S., Kishimura, H., & Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry*, 124(4), 1354–1362. http://doi.org/10.1016/j.foodchem.2010.07.089

Naqash, S. Y., & Nazeer, R. A. (2011). Evaluation of bioactive properties of peptide isolated from Exocoetus volitans backbone. International *Journal of Food Science & Technology*, 46(1), 37–43. http://doi.org/10.1111/j.1365-2621.2010.02443.x

Oehlke, K., Heins, A., Stöckmann, H., Sönnichsen, F., & Schwarz, K. (2011). New insights into the antioxidant activity of Trolox in o/w emulsions. *Food Chemistry*, 124(3), 781–787. http://doi.org/10.1016/j.foodchem.2010.06.095

Olley, J., Dunstan, G. A., & Kolakowska, A. (2002). Fish Lipids. In Chemical and Functional Properties of Food Lipids. CRC Press.

Olsen, J. V, Ong, S. E., & Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular & Cellular Proteomics : MCP*, 3(6), 608–14. http://doi.org/10.1074/mcp.T400003-MCP200

Ono, S., Hosokawa, M., Miyashita, K., & Takahashi, K. (2006). Inhibition properties of dipeptides from salmon muscle hydrolysate on angiotensin I-converting enzyme. *International Journal of Food Science and Technology*, 41(4), 383–386.

Oyaizu, M. (1986). Studies on products of browning reactions: Antioxidative activities of browning products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, 44, 307–315. http://doi.org/http://dx.doi.org/10.5264/eiyogakuzashi.44.307

Picot, L., Ravallec, R., Fouchereau-Péron, M., Vandanjon, L., Jaouen, P., Chaplain-Derouiniot, M. Bourseau, P. (2010). Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. *Journal of the Science of Food and Agriculture*, 90(11), 1819–26. http://doi.org/10.1002/jsfa.4020

Pires, C., & Batista, I. (2013). Functional Properties of Fish Protein Hydrolysates. In R. Pérez-Gálvez & J. P. Bergé (Eds.), Utilization of fish waste (pp. 59–75). Boca Raton, Florida: CRC Press - Taylor & Francis Group.

Qian, J., Zhang, H., & Liao, Q. (2011). The properties and kinetics of enzymatic reaction in the process of the enzymatic extraction of fish oil. *Journal of Food Science and Technology*, 48(3), 280–284. http://doi.org/10.1007/s13197-010-0128-8

Rodríguez-Ruiz, J., Belarbi, E.-H. H., Sánchez, J. L. G., & Alonso, D. L. (1998). Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotechnology Techniques*, 12(9), 689–691. http://doi.org/10.1023/A:1008812904017

Rogalska, E., Cudrey, C., Ferrato, F., & Verger, R. (1993). Stereoselective hydrolysis of triglycerides by animal and microbial lipases. Chirality, 5(1), 24–30.

Rubio-Rodriguez, N., Beltran, S., Jaime, I., De, D. S. M., Sanz, M. T., & Carballido, J. R. (2010). Production of omega-3 polyunsaturated fatty acid concentrates: A review. *Innovative Food Sci. Emerging Technol*., 11, 1–12. Journal Article. http://doi.org/10.1016/j.ifset.2009.10.006

Ruxton, C. H. S., Reed, S. C., Simpson, J. A., & Millington, K. J. (2007). The health benefits of omega-3 polyunsaturated fatty acids: A review of the evidence. *Journal of Human Nutrition and Dietetics*, 20(3), 275–285. Journal Article. http://doi.org/10.1111/j.1365-277X.2007.00770.x

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: *Meat and fish. Nutrients*, 3(9), 765–791.

Sampath Kumar, N. S., Nazeer, R. A., & Jai Ganesh, R. (2011). Functional properties of protein hydrolysates from different body parts of horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*). Mediterranean *Journal of Nutrition and Metabolism*, 5(2), 105–110. http://doi.org/10.1007/s12349-011-0078-3

Sathivel, S., Bechtel, P. J., Babbitt, J., Smiley, S., Crapo, C., Reppond, K. D., & Prinyawiwatkul, W. (2003). Biochemical and functional properties of herring (*Clupea harengus*) byproduct hydrolysates. Journal of Food Science, 68(7), 2196–2200.

Schaich, K. M., Shahidi, F., Zhong, Y., & Eskin, N. A. M. (2013). Lipid Oxidation. Biochemistry of Foods (Third Edit). Elsevier. http://doi.org/10.1016/B978-0-08-091809-9.00011-X

Seafish. (2016). The Landing Obligation made simple.

Serfert, Y., Drusch, S., & Schwarz, K. (2009). Chemical stabilisation of oils rich in long-chain polyunsaturated fatty acids during homogenisation, microencapsulation and storage. *Food Chemistry*, 113(4), 1106–1112. http://doi.org/10.1016/j.foodchem.2008.08.079

Shahidi, F. (2000). Antioxidants in food and food antioxidants. *Die Nahrung*.

Shahidi, F., & Wanasundara, U. N. (1998). Omega-3 fatty acid concentrates: Nutritional aspects and production technologies. Trends in Food Science and Technology, 9(6), 230–240. Journal Article. http://doi.org/10.1016/s0924-2244(98)00044-2

Shahidi, F., & Zhong, Y. (2006). Antioxidants from marine by-products. In F. Shahidi (Ed.), Maximising the value of marine by-products (pp. 397–412). Cambridge: Woodhead Publishing. http://doi.org/10.1533/9781845692087.2.397

Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC International*, 91(4), 914–931.

Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews*, 39(11), 4067–79. http://doi.org/10.1039/b922183m

Shalaby, S. M., Zakora, M., & Otte, J. (2006). Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. *The Journal of Dairy Research*, 73(2), 178–86. http://doi.org/10.1017/S0022029905001639

Shimada, Y., Ogawa, J., Watanabe, Y., Nagao, T., Kawashima, A., Kobayashi, T., & Shimizu, S. (2003). Regiospecific analysis by ethanolysis of oil with immobilized *Candida antarctica* lipase. *Lipids*, 38(12), 1281–1286. http://doi.org/10.1007/s11745-003-1190-x

Small, D. M. (1991). The effects of glyceride structure on absorption and metabolism. *Annual Review of Nutrition*.

Subhadra, B., Lochmann, R., Rawles, S., & Chen, R. (2006). Effect of dietary lipid source on the growth, tissue composition and hematological parameters of largemouth bass (*Micropterus salmoides*). *Aquaculture*, 255(1–4), 210–222. http://doi.org/10.1016/j.aquaculture.2005.11.043

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry*, 11(3), 128–131. http://doi.org/10.1016/S0955-2863(99)00083-2

Sugihara, A., Shimada, Y., & Tominaga, Y. (1991). A novel Geotrichum candidum lipase with some preference for the 2-position on a triglyceride molecule. *Applied Microbiology and Biotechnology*, 35(6), 738–740. http://doi.org/10.1007/BF00169887

Taheri, A., Anvar, S. A. A., Ahari, H., & Fogliano, V. (2013). Comparison the functional properties of protein Hydrolysates from poultry byproducts and rainbow trout (*Onchorhynchus mykiss*) viscera, 12(1), 154–169.

Thorkelsson, G., Slizyte, R., Gildberg, A., & Kristinsson, H. G. (2009). Fish proteins and peptide products: processing methods, quality and functional properties. In J. B. Luten (Ed.), Marine functional Food (1st vol 2, pp. 115–133). Wageningen: Wageningen Academic Publishers.

Valencia, P., Espinoza, K., Ceballos, A., Pinto, M., & Almonacid, S. (2015). Novel modeling methodology for the characterization of enzymatic hydrolysis of proteins. *Process Biochemistry*, 50(4), 589–597. http://doi.org/10.1016/j.procbio.2014.12.028

Valencia, P., Pinto, M., & Almonacid, S. (2014). Identification of the key mechanisms involved in the hydrolysis of fish protein by Alcalase. *Process Biochemistry*, 49(2), 258–264. http://doi.org/10.1016/j.procbio.2013.11.012

Wanasundara, U. N., Wanasundara, P. K. J. P. D., & Shahidi, F. (2005). Novel Separation Techniques for Isolation and Purification of Fatty Acids and Oil By-Products. In Bailey's Industrial Oil and Fat Products. John Wiley & Sons, Inc.

Weichselbaum, E., Coe, S., Buttriss, J., & Stanner, S. (2013). Fish in the diet: A review. *Nutrition Bulletin*, 38(2), 128–177. http://doi.org/10.1111/nbu.12021

Wijesundera, C., Ceccato, C., Watkins, P., Fagan, P., Fraser, B., Thienthong, N., & Perlmutter, P. (2008). Docosahexaenoic acid is more stable to oxidation when located at the sn-2 position of triacylglycerol compared to sn-1(3). *JAOCS, Journal of the American Oil Chemists' Society*, 85(6), 543–548.

Wu, H.-C., Chen, H.-M., & Shiau, C.-Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, 36(9–10), 949–957. http://doi.org/10.1016/S0963-9969(03)00104-2

Wu, S., Sun, J., Tong, Z., Lan, X., Zhao, Z., & Liao, D. (2012). Optimization of Hydrolysis Conditions for the Production of Angiotensin-I Converting Enzyme-Inhibitory Peptides and Isolation of a Novel Peptide from Lizard Fish (*Saurida elongata*) Muscle Protein Hydrolysate. *Marine Drugs*, 10(12), 1066–1080. http://doi.org/10.3390/md10051066

Xia, X., Wang, C., Yang, B., Wang, Y.-H., Wang, X., 2009. Water activity dependence of lipases in non-aqueous biocatalysis. *Appl. Biochem. Biotechnol*. 159, 759–67. doi:10.1007/s12010-009-8618-8

Xu, X. (2000). Enzymatic production of structured lipids: Process reactions and acyl migration. *INFORM - International News on Fats, Oils and Related Materials*, 11(10), 1121–1130.

Zhang, G., Eddy Patuwo, B., & Y. Hu, M. (1998). Forecasting with artificial neural networks: *International Journal of Forecasting*. http://doi.org/10.1016/S0169-2070(97)00044-7

Zhang, Y., Xu, J.-L., & Yuan, Z.-H. (2009). Modeling and Prediction in the Enzymatic Hydrolysis of Cellulose Using Artificial Neural Networks. In Fifth International Conference on Natural Computation, 2009. ICNC '09 (Vol. 2, pp. 158–162). http://doi.org/10.1109/ICNC.2009.334

Zhou, Q. Z., Chen, X. D., & Li, X. (2003). Kinetics of lactose hydrolysis by beta-galactosidase of Kluyveromyces lactis immobilized on cotton fabric. *Biotechnology and Bioengineering*, 81(2), 127–33. http://doi.org/10.1002/bit.10414

I. Lipids from Marine Sources*

By-products of fish industry can account for up to 50% of the production depending on the industrial process. They contain a high level of lipids: mainly polyunsaturated fatty acids (PUFA) and a considerable fraction of EPA and DHA. These compounds present benefits for health such as: prevention of diabetes, allergy and some types of cancer, as well as anti-thrombotic, anti-arrhythmic and anti-inflammatory effects. This fact makes fish by-products an interesting raw material for the manufacture of PUFA concentrates and structured lipids. Indeed, the up-grading of these materials have received much attention for food, pharmaceutical and nutraceutical applications. This chapter reviews a set of techniques currently available to concentrate fish oils in PUFA, such as urea complexation, supercritical extraction, enzymatic processes or low temperature crystallization. Besides, this chapter presents structured lipids as a new approach to increase the therapeutic and nutritional value of the fish oils.

* BOOK CHAPTER: R. Morales-Medina, M. Mar Muñío, R. Pérez-Gálvez, A. Guadix, E. M. Guadix (2013). In "Utilization of Fish Waste", CRC Press. ISBN 9781466585799

1. FISHERIES WASTE REUTILIZATION: AN ECONOMIC AND ENVIRONMENTAL CHALLENGE TO FACE

The world production of aquaculture has remained virtually constant (~130 million tons) during the last decade. However, with the rise of the world's population, the demand of fish resources intended for human consumption has increased by around 10 million ton (FAO, 2012).

This imbalance can be alleviated, at some degree, by increasing the aquaculture production, which currently provides 25% of the world's total consumption. This figure is expected to increase up to 50% by 2030 (Tidwell & Allan, 2001). However, there is an evident need for developing more efficient technologies to supply human dietary requirements for lipids, proteins, polysaccharides and minerals, some of them present in a large amount of aquatic resources.

Nowadays, the recovery yields of mechanical processing are not higher than 30-40% of the entire weight of the fish (Torres et al., 2007). In the European Union, 3.17 million tons per year of by-products are generated by fish filleting, salting and smoking and, additionally, 1.5 million ton per year are produced from fish canning (AWARENET, 2004). Presumably, around 50% of the total fish capture ends up as waste or by-products. Further, fish farming has a detrimental effect on the marine environment and fish waste management involves a great ecological impact cost, mainly due to the high perishability of fish materials.

Although it is quite common to grind and discard by-products along with by-catches (Kristinsson and Rasco 2000), a considerably percentage are used as raw material in the production of (1) plant fertilizers, (2) livestock feeds and (3) added-value and specialty foods for humans. This up-grading is regarded as an opportunity for the fish processing industry to reduce disposal costs.

An approach to raise recovery yields involves a better usage of the by-products. By-products are a great source of protein, lipids and several nutrients; hence, their use as fertilizers or simply as waste implies a great loss of compounds which are difficult to obtain and which have a high commercial or nutraceutical importance. For instance, there are formulations or even pharmaceutical applications based on such inexpensive marine

feedstocks as the head, viscera, skin, tail or blood (Kim & Mendis, 2006). Moreover, when by-products are re-utilized for human food, the value of the product is multiplied five times (Gildberg et al., 2002). One of the most critical and important steps in order to achieve a better usage implies the development of new and economical technologies that allow the extraction and re-utilization of the protein or lipid fractions. Also, the attempt to develop new technologies for recovery and purification will reap benefits related to both, sustainability and human health (Ferraro et al., 2010).

The aim of this chapter is describing the oil content and lipid profile of fish species such as salmon and tuna. Also, the health benefits related to Omega-3 consumption will be discussed. Finally, two different ways of marine lipids up-grading: Omega-3 fatty acids concentrates and structured lipids will be explained.

2. MARINE LIPIDS

Regarding the lipid content, fish species can be classified into three different groups: lean, semi-fatty and fatty species. Lean fishes (as cod) store their lipid content mainly in the liver. On the other hand, the fatty species such as mackerel, present a higher amount of lipid which is distributed throughout the body, under the form of subcutaneous and visceral adipose tissue (Pearson et al. 1977). As far as semi-fatty species are concerned, their lipid content varies between 2 and 10% and it is stocked in organs and as subcutaneous deposits. Some authors have reported seasonal changes in the lipid content and the fatty acid composition for fatty fish species such as cod and haddock (Falch et al. 2006).

The most common marine lipids can be divided into two main groups: triglycerides (also named as triacylglycerides) which are built by fatty acids and phospholipids. Additionally, there are other smaller groups as wax esters (which are basically storing fats) and fat soluble vitamins.

2.1. Fatty Acids and Triglycerides

As triglycerides are composed of fatty acids, the description of this kind of lipids is the starting point. Fatty acids are carboxylic acids with long aliphatic tails which can be classified regarding their chain length, degree of unsaturation and the position of their double bonds.

Depending on the chain length, fatty acids can be classified as short when their aliphatic tail has less than six carbons; medium if the number of carbons ranges between 6 and 12 carbons and long-chain fatty acids composed of more than 12 carbons. The latter fatty acids are the most common in fish oils.

With regard to their degree of unsaturation, fatty acids are divided in two groups: saturated (with no double boundaries) and unsaturated. Furthermore, there are polyunsaturated fatty acids (PUFA) which contain several double bonds (C=C) conjugated and separated by a methylene group. Finally, depending on the position of the first double bond, fatty acids can be classified as Omega-3, Omega-6 and Omega-9 if the first double bond is in the third, sixth or ninth carbon atoms respectively from the methyl end.

Taking advantage of this common structure of PUFA, a shorthand nomenclature has been developed. It is composed of the number of carbons of the chain, a colon, the number of double bounds, the Greek symbol "ω" or the letter "n" and finally the position of the double bond which is nearest of the methyl side of the molecule. By this terminology, eicosapentaeonoic acid (EPA) which is represented in Fig.1a,b can be written as C20:5n-3.

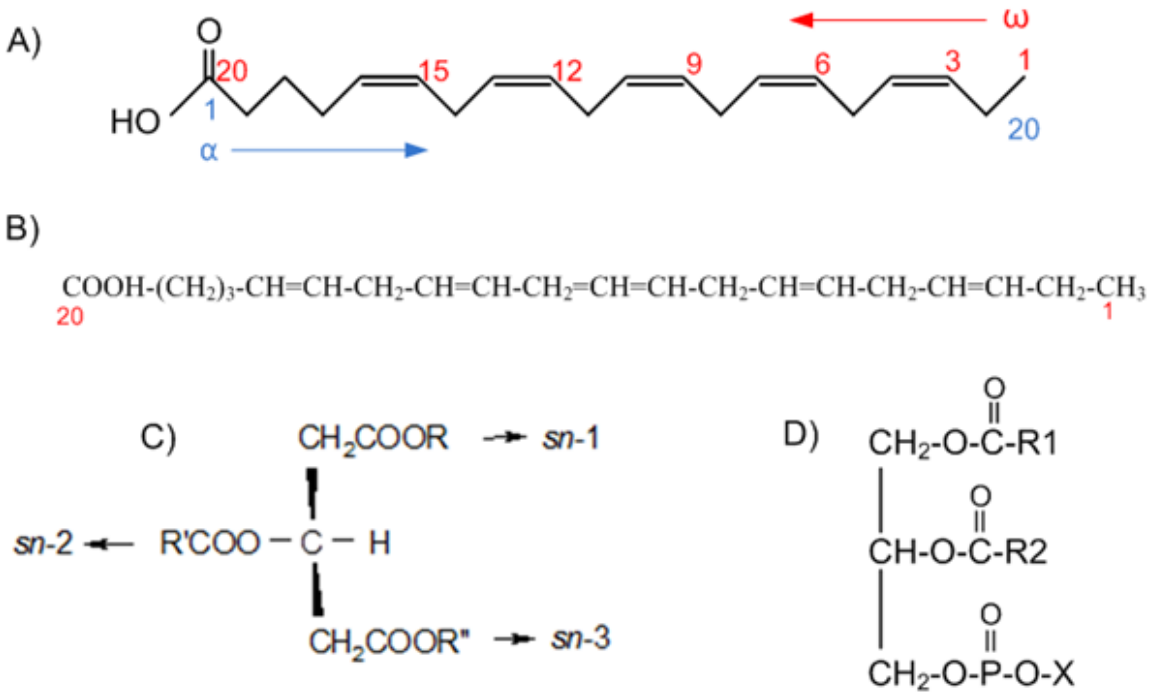


Figure 1. Schematic representation of marine lipids. A) and B) EPA. C) Generic triglyceride and D) Generic phospholipid.

The most common PUFA which are contained in fish oils are arachidonic (AA, C20:4n-6) and linoleic acid (LA, C18:2n-6), which is the AA metabolic precursor. In the case of Omega-3 PUFA, eicosapentaeonic acid (EPA, C20:5n-3), docosahexaenoic acid (DHA, C22:6n-3) and linolenic acid (LA, C18:2n-6) are the main ones. Regarding saturated fatty acids (SFA), myristic (C14:0), palmitic (C16:0) and stearic acid (C18:0) are the most abundant in fishes.

Triglycerides (Fig. 1c) are formed by a molecule of glycerol esterified in the three positions of the backbone with fatty acids. Generally, monounsaturated and saturated fatty acids are located in the sn-1 and sn-3 positions of glycerol while PUFA are situated in the sn-2 one. Nevertheless, there are a wide group of triglycerides which do not follow this rule, for instance, tridocosahexaenoylglycerol (tri-C22:6n-3), which is the main lipidic component of the eye lipids of some species.

2.2. Phospholipids

Phospholipids are the main component of cell membranes. Most of them are composed of a diglyceride bonded to a phosphate group linked to an organic molecule (X) (Fig. 1.d.). Their structure has a hydrophobic tail as well as a hydrophilic head.

In the case of phospholipids found in fish oil, monounsaturated and saturated fatty acids are usually esterified at the sn-1 position whereas the PUFA are esterified at position sn-2. As with the triglycerides, there are several exceptions such as didocosahexaenoyl-phosphoglycerides which are found in the eye epithelia of fish.

3. SOURCES OF MARINE LIPIDS

This section deals with recent data related to the lipid content and PUFA composition of five species: tuna, mackerel, sardine, cod, and salmon. Further, as the main part of the by-products composed by skin, head and viscera, a brief description of the lipid composition of these parts are given. Taking into account that the lipid profile varies depending on the season, some data related to its influence are described. These seasonal fluctuations are very relevant because they can cause several problems during the refining and standardization of oils. Finally, the lipid amount which can be obtained from the bones of different species is summarized.

3.1. Tuna

Tuna's migratory behavior and its feeding habits (Grigorakis et al., 2002) are the two main reasons that explain its high content of DHA (C22:6n-3), which accounts for more than 20% of the total fatty acids. Tuna's feed habit are based on small pelagic fishes and some crustaceans which have high levels of PUFA. In addition, although DHA content in the stomach - which basically depended on the diet - is low and inconstant, the DHA levels in muscle are consistent and high. This fact could be related to the influence of the migratory cicles (Saito et al., 2005).

Table 1. Total lipid content (% of fresh weight) in the different organs of *Euthynnus alletteratus*. Adapted from Salah and Saloua (2010).

	October	January	April	July
Liver	27.28	9.83	12.69	6.22
Red Muscle	12.2	6.46	5.71	4.38
White muscle	8.95	3.72	2.97	1.7
Viscera	12.15	3.57	5.73	7.37
Head	14.14	14.26	11	11.75
Gonad	1.56	1.49	2.49	2.69

As it can be seen in Table 1, the total lipid amount varies widely throughout the year (35-50%). The maximum levels appeared from July to October. It is remarkable that the head is one of the organs with the highest content, even more than the muscle; being its lipid content almost constant throughout all the seasons.

In Table 2, the lipid profile of the different organs in the month of January is shown (Salah and Saloua, 2010). PUFA percentage was the highest in winter whereas in autumn its value was minimum, contrary as happened for SFA. In all the organs the main fatty acid is DHA followed by C16:0 and EPA. Due to this kind of lipidic composition, tuna by-products are a suitable raw material for the obtention of nutraceutic products.

Table 2.Fatty acid content of different part of the body of tuna measured in January. Adapted from Salah and Saloua, (2008).

	Liver	Red Muscle	White Muscle	Viscera	Head	Gonad
C14:0	0.87	1.31	1.88	2.63	3.71	1.35
C16:0	20.82	17.41	17.96	19.19	13.69	21.10
C18:0	8.87	10.06	5.70	6.94	2.40	8.15
C18:1n-9	7.60	6.15	10.67	6.47	12.69	9.04
C18:2n-6	1.20	1.00	1.40	1.51	1.69	1.38
C20:5n-3	12.40	9.57	9.08	7.65	10.21	11.09
C22:6n-3	31.19	40.14	31.18	23.57	34.02	28.74
SFA	31.88	28.20	28.14	35.46	21.40	31.96
MUFA	11.05	9.39	15.48	10.02	22.23	13.53
PUFA	45.55	53.35	43.07	34.56	47.71	41.95

3.2. Salmon

The processing of red and pink salmon (*Oncorhynchus nerka* and *O.gorbuscha*) produces around 50,000 ton of salmon heads and 25,000 ton of viscera per year in Alaska (Oliveira & Bechtel, 2005).

All parts of the salmon are rich in PUFA, especially in Omega-3 fatty acids (25-36%) (Oliveira and Bechtel, 2005). A comparison of composition of the fatty acid of the whole salmon, head and viscera is shown in Table 3. The percentage of SFA did not vary significantly being palmitic acid the main individual fatty acid. PUFA fraction presented a high content in all parts studied (33-44%). In all cases, DHA was the predominant fatty acid, varying from 11.8% in the case of head to 17.3% in the viscera. Eicosapentaenoic acid (EPA, C20:5n-3) ranged from 7.6 % in heads to 10.9 % in the viscera.

Table 3. Pink salmon fatty acid percentage in the extracted oil. Adaptation from Oliveira and Bechtel, (2005).

	Wholefish	Head	Viscera
C14:0	3.6	4.12	3.44
C16:0	12.04	12.39	12.80
C18:0	3.25	2.87	4.45
C18:1n-9	12.74	11.76	12.17
C18:2n-6	1.73	1.67	1.38
C20:4n-6	0.75	0.56	2.59
C20:5n-3	7.70	7.56	10.93
C22:6n-3	15.78	11.77	17.32
SFA	16.51	17.47	16.59
MUFA	40.05	42.69	34.19
PUFA	38.50	33.26	44.46
Omega-3	32.31	27.84	35.15

3.3. Sardine

The lipid content of different parts of the Indian sardine (*Sardinella longiceps*) *i.e.* head, muscle and by-products (bones, viscera and fins) were compared (Table 4). The total lipid percentage (wet weight basis) varied from 11 - 15 %, being the fraction of by-products the one with the higher value. When focusing on the total percentage of PUFA, the amount did not vary significantly among the three samples. However, EPA and DHA percentage was predominantly located in the head and flesh.

Although PUFA amount was not as high as observed in tuna, sardine is a fatty fish with an adequate lipid profile for human consumption. Furthermore, the composition of sardine by-products makes of it a good choice for by-products up-grading.

Table 4. Percentage composition (wet weight basis) of different parts of Indian sardine (*Sardinella longiceps*). Adapted from Rai et al,.(2011).

	Head	Muscle	By-Products
C14:0	10.8	10.6	9.2
C16:0	25.5	25.2	35.3
C18:1n-9	6.8	7.4	10.9
C18:2n-6	1.2	1.2	1.0
C20:5n-3	13.4	15.0	5.0
C22:6n-3	8.2	9.2	5.6
SFA	44.1	42.7	57.4
MUFA	21.1	20.5	23.1
PUFA	26.0	28.0	28.0
Total Lipid	13.05	10.97	15.10

3.4. Mackerel

The case of mackerel is quite similar to that of the tuna; due to their migratory condition and feeding habits (crustaceans, mollusk and small fish) its level of DHA was quite high in the muscle.

Rai, (2011) conducted a research about the lipid composition of the Indian mackerel in the muscle, head and by-products (bones, viscera and fins). The highest lipid amount was found in the head 13.60%, followed by muscle (8.79%) and finally by-products (6.20%). As shown in Table 5, in all the cases the saturated fraction was higher than the unsaturated one. Furthermore, PUFA content were higher in the muscle followed by by-products and head. The predominant PUFA was in all cases DHA (C22:6n-3), whose content in the muscle fraction was double than in the other parts.

Table 5.Fatty acid composition of the muscle, head and by-products of the Indian mackerel (*Rastrelliger kanagurta*).Adapted from Rai et al., (2011).

	Muscle	Head	By-Products
C14:0	6.5	6.6	8.1
C16:0	25.0	33.4	34.3
C18:0	7.9	8.7	9.6
C18:2n-6	1.2	0.8	1.1
C20:4n-6	1.4	0.5	0.7
C20:5n-3	8.6	4.2	4.7
C22:5n-3	1.8	0.6	0.8
C22:6n-3	15.3	7.9	7.5
SFA	41.6	50.6	54.4
MUFA	22.1	21.5	22.4
PUFA	29	14.8	16.2

Table 6. Evolution of fatty acid profile. Total lipid expressed as mg/100 g edible fish of total lipids whereas the specific fractions of fatty acids SFA, MUFA and PUFA were expressed as mass percentage. Modification from Bandarra et al. (2001).

	Spring	Summer	Autumn	Winter
C14:0	58.4	102.4	169.6	135.8
C16:0	294.5	768.6	966.3	647.6
C18:0	101.4	255.2	291.6	204.7
C18:2n-6	31.0	41.3	56.4	38.8
C20:4n-6	19.2	49.2	56.5	35.3
C20:5n-3	10.1	28.2	38.5	25.5
C22:5n-3	133.5	394.6	570.7	329.6
C22:6n-3	318.0	759.4	858.5	598.3
SFA	489.1	1195.5	1526.3	1040.7
MUFA	490.0	1264.1	1610.3	1065.4
PUFA	618.7	1570.5	1947.6	1250.4
Lipid Total	1597.7	4030.1	5084.2	3082.1

In Table 6, it is shown the evolution throughout the year of the lipid content of mackerel fillets. The highest content was found in August while the minimum was observed in February. The saturated fraction ranged between 26.9% and 30.5% of total fatty acids, palmitic acid being the (16:0) the predominant one. Regarding the unsaturated fraction, its highest percentage was observed in July (43.4%).

3.5. Cod

Nowadays, liver and roe are the main parts of the cod dedicated to human consumption. Similar to other species, the lipid content of cod varied seasonally, in the case of liver from 58.5 to 76.1% (Falch et al., 2006).

Table 7. Levels of PUFA, MUFA, SFA, EPA and DHA (% of total fatty acids) and lipid content (% of wet weight) in the liver, viscera and trimmings of cod caught in Barents Sea. Adaptated from Falch et al., (2006).

	Liver			Viscera			Trimmings		
	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn
PUFA	41.0	35.5	36.0	41.3	41.2	41.7	36.8	38.2	47.3
MUFA	36.9	41.7	41.9	38.1	35.1	31.4	41.7	37.3	25.8
SFA	22.1	22.8	22.1	20.6	23.7	26.9	21.5	24.5	26.9
EPA	7.1	5.8	6.1	8.2	7.7	7.9	8.0	6.7	9.9
DHA	8.1	6.6	8.0	11.4	12.6	13.6	9.7	9.7	16.2

In Table 7, it is summarized, the composition of the oil extracted from the liver and from by-products (viscera and trimmings). In all cases, PUFA were the main fraction in the bulk, with a percentage of 35-41% for liver, around 41% for viscera and 37-47% for trimmings. EPA and DHA content was minimum in summer, fact which was related to the spawning period.

No significant differences were observed when the main fatty acid composition of by-products and liver oil were compared (Table 8). The energy value of by-products, calculated by Shahidi et al. (1997), presented an average value of 413.43 kJ/100g whereas the value estimated for the cod fillets was 344 kJ/100g. In the case of cod, its by-products presented nearly the same quality as the edible parts.

Table 8. Fatty acid composition of by-products and cod liver. Adaptated from Shahidi et al., (1997).

	By- Products	Liver
C14:0	2.0	3.7
C16:0	11.0	11.6
C18:0	4.0	2.3
C18:1n-9	20.3	21.7
C18:2n-6	3.8	0.8
C20:5n-3	8.9	7.7
C22:6n-3	13.3	11.4

3.6. Lipid composition of bones

The chemical composition of bones varied significantly among the species, varying the lipid content from 23 g/kg for cod to 509 g/kg for mackerel. Normally, fatty fish presents higher lipid levels in bones than lean fishes. Bones represent a significant part of the fish, around 10-15% of total fish biomass (Toppe et al., 2007). Although, bones applications for human health have been widely studied as a source of protein and collagen (Liaset et al., 2003; Kim & Mendis, 2006); its lipid content cannot be considered as negligible in some species. Nowadays several researches are conducted in order to extract the lipids from bones and use them as raw material for fishes diet (Toppe et al., 2007).

In Table 9, the lipid composition of the bones of several species are listed. Fatty species (trout, mackerel and salmon) presented almost the double lipid content than the lean ones (cod). In the case of cod, the low amount of lipid in the bones suggests that there might be a significant bulk of phospholipids.

When comparing the lipid composition of bones to the lipid profile of the whole fish similar profiles are observed.

Table 9. Fatty acids in fish bones in g/kg extracted lipid. Adapted from Toppe et al.,(2007).

	Cod	Salmon	Trout	Mackerel
C14:0	9	44	51	63
C16:0	87	119	130	157
C18:0	28	23	23	28
C18:1n-9	112	150	65	172
C20:5n-3	46	65	41	52
C22:6n-3	69	101	34	99
SFA	124	187	205	204
PUFA	148	284	255	221
Omega-3	126	233	213	195
Total Lipid	439	857	868	821

4. HEALTH BENEFITS ASSOCIATED TO MARINE LIPIDS

In human nutrition α-linolenic (ALA) and linoleic acid (LA), belonging to the Omega-3 and Omega-6 families, are assential fatty acids (*i.e.* they cannot be synthesized by the human body). These fatty acids play a very important role in the structure of cell membranes, and are precursors of eicosanoids, a group of compounds which are related to biological processes such as aggregation of blood platelets and metabolism of cholesterol.

The positive health effects of ALA are related to three main factors (i) ALA is a precursor of EPA and DHA (Fig. 2), (ii) ALA-rich diets increase the total Omega-3 fatty acid of cell membrane which modifies the behavior of the cell in a positive way (Nail et al., 1997, Tarpila et al., 2002) and iii) ALA can dampen inflammatory processes by affecting the production of eicosanoids. Nevertheless, depending on the eicosanoids precursor they can either cause damage or be beneficial to the human body.

ALA has a double influence on eicosanoids: firstly, as a precursor of EPA, which is by itself an eicosanoid, it does not promote inflammation and decreases the risk of diseases when compared to Omega-6 fatty acids. Secondly, since ALA is part of the cell membrane of phospholipids it can block the metabolic pathway of Omega-6 fatty acids (Magrum & Johnston, 1983, Garg et al., 1990, Healy et al., 2000) which, as it will be explained later, has a detrimental effect on the human health. However, ALA can minimize the production of cytokines, proteins which are liberated in response to infection or injury (Abbas et al., 1995) and which are responsible for chronic diseases such as rheumatoid arthritis (Caughey et al., 1996).

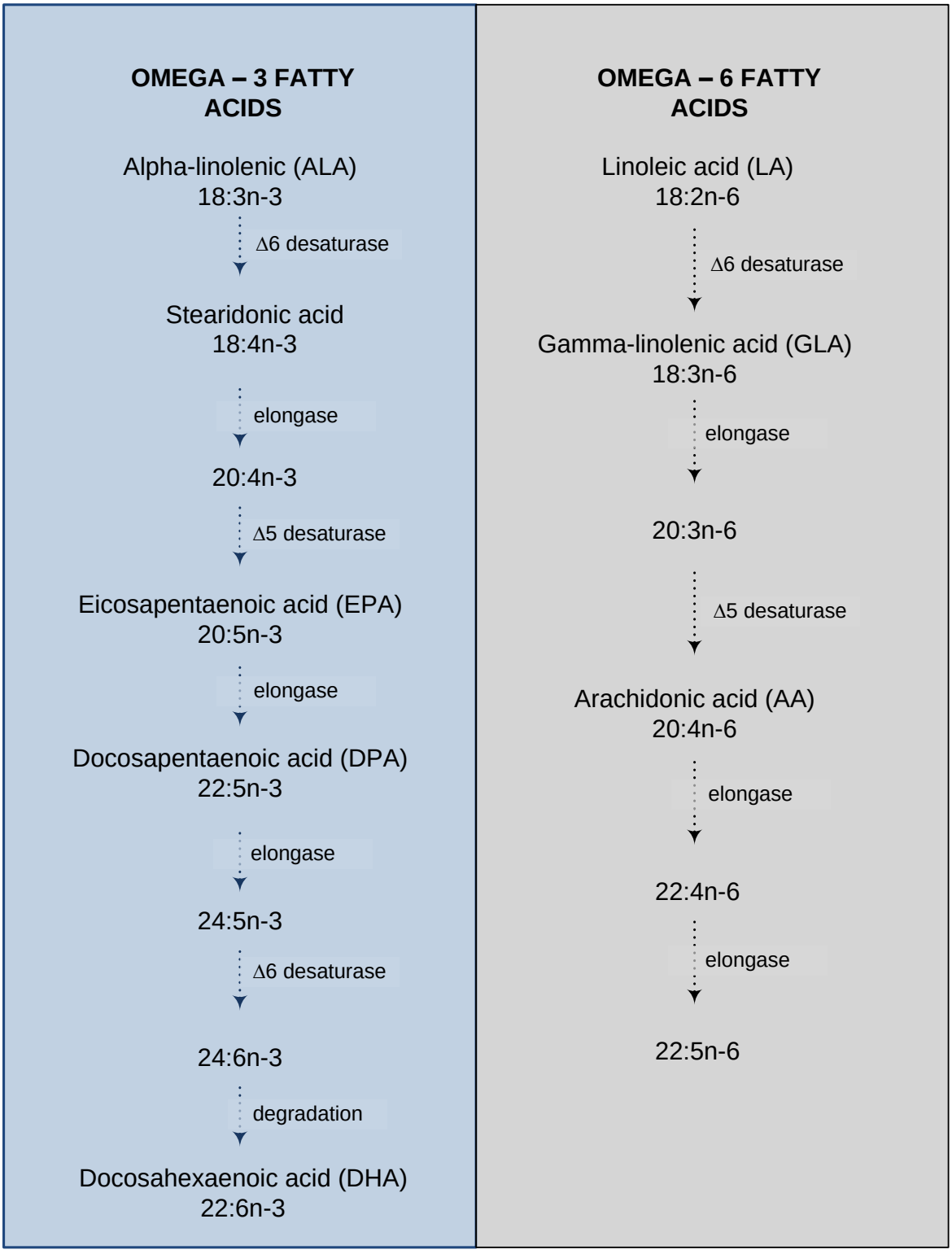


Figure 2.Omega- 3 and Omega-6 pathways. Adapted from Sahena et al., (2009).

As far as Omega-3 fatty acids are concerned, the two main PUFA obtained from LA are: gamma-linolenic acid (GLA) and arachidonic acid (AA). The first one is a precursor of some eicosanoids which have similar properties as those produced by ALA. However, the eicosanoids promoted by AA have negative properties as promoting the aggregation of

blood platelets and inflammatory reactions. As is shown in Fig. 2, the metabolic pathways of Omega-3 and Omega-6 depend on the same enzymes. Thus, an Omega-6 rich diet unbalances the equilibrium between Omega-3 and Omega-6, fact which over-activates the immune system and causes several chronic diseases. Hence, the influence of the dietary Omega-3 PUFA on several systems such as the cardiovascular and the immune ones has been deeply studied. PUFA might play a key role against diseases as cancer or diabetes. Furthermore, it has also been proved their positive effects related to mental health, cognition and male fertility.

4.1. Cardiovascular Benefits

Firstly, regarding cardiovascular and metabolic health, the consumption of EPA and DHA reduces the triglycerides levels as well as blood pressure. In addition, their ingestion increases the levels of cholesterol HDL and the stability of the plaques; as well as presents anti-thrombotic, anti-arrhythmic and anti-inflammatory effects. Also, EPA and DHA prevent from several coronary heart diseases (Malik et al., 1996, Ruxton et al., 2005, Fedacko et al., 2007, Harris et al., 2008). Although the mechanisms by which PUFA improve human health has not been totally elucidated, it is known that the anti-inflammatory properties are related to the reduction of the synthesis of arachidonic acid (Rallidis et al., 2003). Some of these beneficial effects have been proposed as claims to the European Food Safety Authority (EFSA). Those related to the maintenance of blood pressure and the triglyceride concentrations in normal levels have been approved. However, the claims associated to HDL and LDL cholesterol were declined.

4.2. Omega-3 Fatty Acids' Role in Mental Health

Omega-3 PUFA exerts a significant role on mental health and cognition, especially in: the early human development, depression and in the maintenance of the cognitive function in later life.

4.2.1. Early Human Development

In the course of the fetal development, the main effects related to EPA consumption are the improvement of both the fluidity and functionality of cell membranes (Bourre et al., 1991). Furthermore, by the intake of DHA and arachidonic acid supplementation, the brain and retinal function can be enhanced (Ruxton et al., 2005). Further benefits related to hand-eye

coordination, mental processing or lower risk of wheezing have been previously enumerated (Helland et al., 2003, Dunstan & Prescott ,2007, Miyake et al., 2009). However, every health claim related to these benefits has been so far rejected by EFSA.

4.2.2. Depression

Several authors have described the benefits of Omega-3 in neuropsychiatric disorders (Freeman et al., 2006, Parker et al., 2006, Schaefer et al., 2006). Despite the mechanism is not yet known, it is suspected that these fatty acids contribute to regulate the mood.

4.2.3. Maintenance of Cognitive Function: Alzheimer

Approximately 40 million people suffer from Alzheimer's disease and it is expected that this figure will be doubled by 2030. Since this disease is the main cause of dementia, most of the studies found in scientific literature are focused on studying the influence of Omega-3 fatty acids in the prevention of Alzheimer. It has been reported that the risk of suffering from Alzheimer can be decreased a 60% by the weekly consumption of two fillets of any oil-rich fish. (Morris et al., 2005).

In addition, a high content of Omega-3 fatty acid in plasma has been related to a reduction of the decline in sensorimotor and complex speed (Dullemeijer et al. 2007).

4.3. Prevention of Type 2 Diabetes

Preventing diabetes by means of Omega-3 fatty acids intake is a controversial theme. Balk et al., (2004) published a review whose main conclusion was the lack of improvement of glucose tolerance when the consumption of EPA/DHA increased. By contrast, Nkondjock and Receveur, (2003a) presented a study in which high intake of fatty fish reduced the presence of diabetes type 2, especially in people with obesity. Also, Terry et al., (2001), reported that high oily fish and seafood intake caused a deep reduction in diabetes type 2. Furthermore, positive effects in diabetes type 1 were found by Hu et al., (2003). Nowadays, the majority of the studies have concluded that there is not enough evidence in order to prove any significant effect (Mori et al., 2000; Browning et al., 2007).

4.4. Prevention of Allergy and Asthma

The connection between a high oily fish intake and a decrease in the risk of suffering from asthma or allergy was published by Schnappinger et al., (2009). What is more, the absence

of Omega-3 fatty acids during childhood was related to an enhancement of the risk of incurring in asthma (Laerum et al., 2007)

4.5. Prevention of Cancer

Due to the wide variety of cancers, it is difficult to establish a direct link between the intake of Omega-3 fatty acids and cancer prevention. Several reviews on breast, ovary, endometrial, stomach and pancreatic cancers have been so far published without obtaining enough evidence of the preventive activity of fish oil. (Rose & Connolly, 1999; Terry et al., 2001; Augustsson et al., 2003; Nkondjock et al., 2003b; Geelen et al., 2007).

5. LIPIDS OXIDATION: A SETBACK TO DEAL WITH

The tendency to oxidation of PUFA is the main factor responsible for limiting revalorization. Oxidation can cause a decrease of the nutritional value as well as exerts a detrimental effect on the flavor and taste.

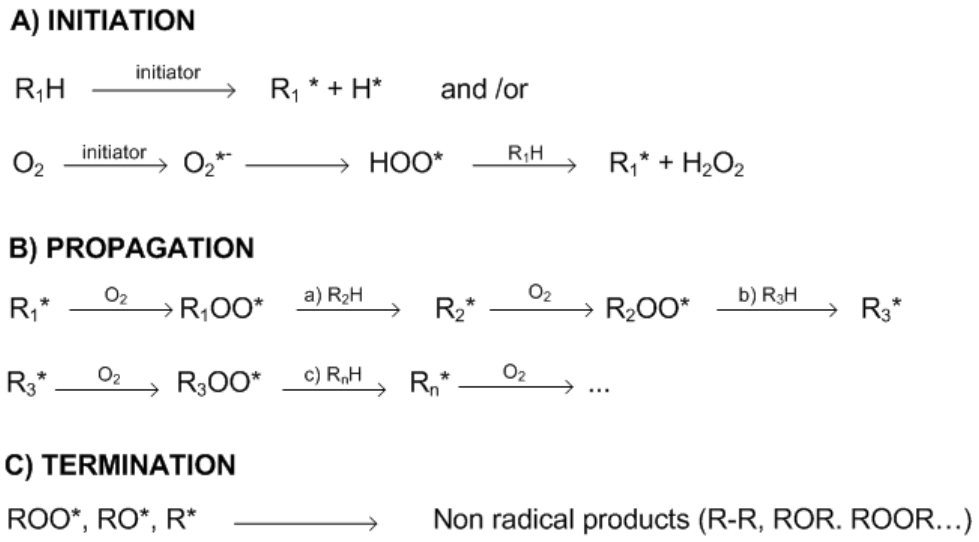


Figure 3. Steps of radical oxidation of oils: a) initiation, b) propagation and c) termination. Adapted from Shahidi et al. (2011)

There are several factors which catalyze lipid oxidation such as heat, light, metals, enzymes or microorganisms. Furthermore, depending on how the oxidation process is generated there are different types: autoxidation, photooxidation, thermal or enzymatic oxidation; most of them dominated by radical mechanism. The most usual oxidation process is autoxidation, a reaction between lipids and the atmospheric oxygen. In addition,

there are enzymes (lipoxygenases) which can accelerate the oxidation process. The usual oxidation targets are unsaturated fatty acids which can appear as free fatty acids, acylclycerols, phospholipids or alkyl esters (Shaidi & Zhong, 2010).

In this chapter, it is described the lipid autoxidation pathway as an example of a radical mechanism. This process involves three stages: initiation, propagation and termination. Firstly, in initiation, an unsaturated lipid molecule loses a hydrogen atom due to the presence of initiators: heat, light, metal or metalloproteins (Frankel, 2005). Because of this loss, the production of free radicals (R_1^*) begins (Fig. 3a). Depending on the nature of the element which causes the initiation of the oxidation process, the pathways can vary. Examples of different mechanisms are shown in Fig. 4 for initiation caused by UV light or heat (Fig. 4a), when originated by oxidizing metals (Fig. 4.b) and finally, when it is generated by reducing metals (Fig. 4c). All these variations can occur at the same time.

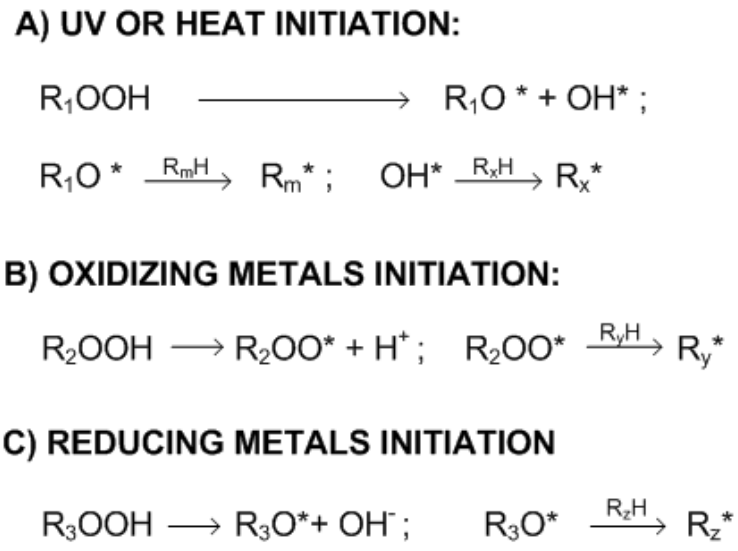


Figure 4.Different initiation step regarding the nature of the source which origins the oxidation: (a) UV ligh or heat, (b) Oxidizing metals or (c) reducing metals. Adapted from Shahidi et al. (2011)

Propagation consists of the repetition of these reactions several thousand times; this high number is achieved because of the creation of new radicals such as alkoxyl (RO*), peroxyl (ROO*), hydroxyl (*OH) and lipid radical (R*). Finally, the termination step (Fig. 3c) can be due to the absence of a cource of hydrogen, the interruption of the reaction by antioxidants or to the formation of non-radical compounds.

A wide variety of products is obtained from lipid oxidation. For instance, hydroperoxides are the primary products of oxidation. However, as they are very unstable they decompose and produce secondary products such as aldehydes, ketones, alcohols, volatile organic

acids, hydrocarbons or epoxy compounds. Normally, these secondary products are responsible for the undesirable odors as well as rancidity.

6. LIPIDS REVALORIZATION

The utilization of the lipid content of fish by-products is motivated by their availability and low cost. In this chapter, the main techniques to up-grade the lipid fraction are discussed with a special focus on PUFA concentrates. Also, marine lipids can be used as starting material to synthesize structured lipids which are referred as added value products (Muñío et al. 2008). Different ways to produce them are described.

6.1. Production of Omega-3 Concentrates

An Omega-3 concentrated oil contains, at least, 600 mg Omega-3/g oil. Omega-3 PUFA can be concentrated by physical or enzymatic methods. The yield of physical methods is increased by transforming the TAG into esters or free fatty acids. In this section the main techniques employed for the production of concentrates are summarized.

6.1.1. Low temperature crystallization (winterization)

Winterization of edible oils consists of the separation of the oils into several fractions by means of their melting points. The melting point of fatty acids depends on several factors, as the chain length and unsaturation degree. Saturated and monosaturated fatty acids (SFA and MUFA), have higher melting points than PUFA. Thus, the former crystallize while PUFA remain in the liquid phase. Oils which possess a higher melting point form crystals which can be filtrated and separated from the liquid fraction. The main objective of the winterization of fish oils is to separate the saturated and unsaturated fractions; to obtain an oil with high PUFA content. Organic solvents (hexane, acetone) are used in order to increase the rate of the mass transfer as well as the temperature of the process (Alasalvar et al., 2011).

Briefly, the main parameters of crystallization are: temperature, organic solvent, process time and the ratio organic solvent:oil. Wanasundara and Shahidi, (1999) reported that the recovery yields of PUFA were much higher by the usage of hexane rather than isopropanol. Besides, it has been proved that an increased of the solvent:oil ratio favors the concentration (López-Martínez et al., 2004) in spite of the economic disadvantages.

6.1.2. Urea complexation

This method achieves high yields and concentration of EPA + DHA of 85% (Haraldsson, 1983; Gámez-Meza et al., 2003; Liu et al., 2006). Urea complexation is based on the formation of solid complexes of straight-chain organic compounds with urea. These complexes are usually tetragonal-shaped, however, in the case of straight-chain aliphatic compounds the configuration varies, developing hexagonal prisms in whose inner channel several organic compounds (hydrocarbons, alcohols, esters and fatty acids) are fixed (Breivik, 2007). When a mixture of fatty acids or esters are added to urea, the more saturated compounds are trapped more easily. Consequently, the solid complexes can be removed, leaving a liquid fraction enriched in polyunsaturated acid or esters.

Briefly, this method begins with an alkaline hydrolysis of the triglycerides aiming to obtain free fatty acids. Subsequently, the free fatty acids are added to an ethanolic solution of urea. The complexation process then takes place by cooling and, finally, crystals are separated by filtration (Hayes et al., 1998; Crexi et al. 2012). One of the most relevant variables to control is the molar ratio between alcohol and triglycerides during the hydrolysis. Although the stoichiometry ratio is 3:1, the reversible behavior of the reaction makes necessary the use of an excess of alcohol (Meher et al., 2006).

Linko and Karinkanta, (1970) carried out experiments with herring oil and reached to the conclusion that the complexation process improved if (i) the length of the carbon chain increased, (ii) the number of double bonds decreased or (iii) the distance from the first double bond to the acid group increased. As a consequence, all PUFA were not concentrated in the same proportion and there were small fractions of EPA and DHA which were lost in the complexes.

Urea complexation presents several advantages as compared to winterization. The complexes are much more stable than crystals which are produced in winterization and the filtration step can be carried out at higher temperatures, which involves a higher economic yield. Further, since the separation is based on chemical properties (configuration of the organic compounds) rather than on physical properties (melting point or solubility) this method is preferred by many researchers (Wanasundara & Shahidi, 1999). However, urea complexation may lead to the production of ethyl carbamate (or urethane) which is carcinogen in animals. Hence, this method is not desirable in the food or pharmaceutical industry.

6.1.3. Distillation methods

This process, is widely employed to concentrate Omega-3 from fish oil on a large scale. The separation takes place due to differences in the boiling point and molecular weight of the compounds. Distillation consists of the separation of ethyl esters at low temperature and vacuum (from $10^{-3}$to 1 mbar). It must be carried out at low temperatures, otherwise undesirable reactions such as oxidation, hydrolysis, thermal oxidation, polymerization or isomerization may occur (Ackman et al., 1988, Wijesundara et al., 1989).

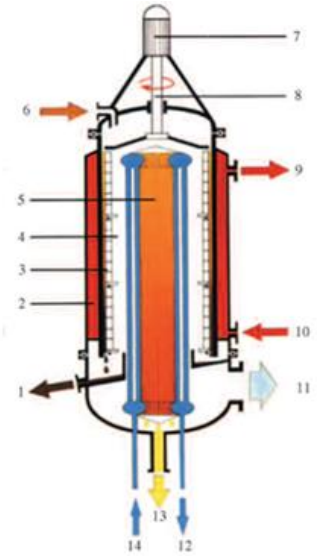


Figure 5.Section of a short path distillator. 1. Residue nozzle. 2. Heating jacket. 3. Wiper system. 4. Vacuum chamber. 5. Condenser. 6. Feed nozzle. 7. Motor. 8. Shaft with distribution plate for feed. 9. Heating oil (out). 10. Heating oil (in). 11. Vacuum connection. 12. Cooling liquid (out). 13. Distillate nozzle. 14. Cooling liquid (in). Figure reproduced from UIC GmbH, Alzenau-Hörstein, Germany. (http://www.dax-consult.dk/documents/fish-oil-industry.pdf).

PUFA are distillated by means of short-path distillation (molecular distillation), a technique which is characterized by short exposure time of the compounds and the short distance (20 to 70 mm) from the evaporator to the condenser.The main drawback of this process is the high equipment cost (high vacuum pump, distillator, condenser…). On the other hand, the production which can be achieved with short path distillation is 100-200 kg per square meter evaporator an hour (Martins et al., 2006).

For industrial production, there are two different operational conditions (centrifugal molecular distillation and thin film evaporator) depending on how the thin film of the mixture which is going to be evaporated is obtained. For centrifugal molecular distillation, the film is produced by the usage of a disk which rotates at high speed. As the disk is heated, the more volatile compounds are evaporated. The selective fractionation is controlled by changing the rotation speed, which varies the film thickness and the

residence time. Thin film evaporator distributes the compounds by the use of a mechanical agitator. The addition of wiping elements notably improves the heat transfer. In Fig. 5 it is showed a schematic diagram of a thin film evaporator.

By means of distillation, the EPA and DHA percentage of an initial sample with 25 % can be enhanced to 50 % (Skaliotis, 2011). Hence, high purity concentrates are obtained in a two-step process or in combination with selective enzymatic transesterification (Breivik et al., 1997).

6.1.4. Supercritical Extraction

In general, supercritical fluid extraction comprises two main steps: first, the extraction of the components of interest and second the separation of the compounds from the supercritical matrix. One of the most common supercritical solvents is carbon dioxide because of its properties such as non-reactivity, easy availability and low cost. Furthermore, when employed for the selective extraction of PUFA it can reduce oxidation and decomposition of oils.

As fish oil is composed of a wide variety of compounds with different chain lengths and saturation points, the fractionation of a specific compound is virtually impossible. However, by separating fatty acid methyl or ethyl esters the yield of the process (Eisenbach, 1984).

The most relevant variables which govern supercritical extraction are: pressure, temperature, solvent flow rate and process time. In addition, the study of the equilibrium phase of the mixtures is essential in order to design the fractionation process (Esquível et al., 1997; Dunforda et al.,1998). Solubility of the oil increases notably with pressure at a constant temperature due to the enhacement of the vapor pressure of the solute (Brunner 1994).

Perretti et al., (2007) conducted the optimization of the main variables of fractionation in a column with three sections. By modifying the temperature and pressure throught the column the composition required in the market can be obtained. In addition, fractionation of sardine oil achieved purity up to 95% of fatty acids methyl esters and yields around 45% (Létisse & Comeau, 2008). In this case, the extraction was carried out in four steps of 45 min each, at 60ºC, with a flow rate of 1 mL/min and the density of CO_2 was increased from 500 to 800 kg/m^3. Furthermore, a mathematical model for the supercritical extraction with

CO_2 has been developed and validated for fish oil ethyl esters mixtures (Martín & Cocero, 2007).

Although supercritical extraction results in high concentrates yield and is a more environmental- friendly process than the previously described. However, the main drawback is the economic costs. When scaling up, the amount of CO_2 which is consumed (solubility of oils is around 10 g/kg) (Sahena et al., 2011) it extremely high. This quantity of CO_2 can be minimized by the usage of the pressure swing technique (Zaidul et al., 2007), a method which, by combining cycles of pressurization and depressurization steps, can achieve the same yields as a continuous supercritical fractionation.

6.1.5. Enzymatic Processes

The production of Omega-3 concentrates by means of enzymatic processes is basically based on the usage of specific enzymes (lipases) which are responsible for reactions such as hydrolysis or esterification of triglycerides. The main difference between these processes is that, in case of hydrolysis, PUFA obtained from triglycerides might be concentrated as free fatty acids or acyglycerols, whereas when esterified they are concentrated in the form of esters (Haraldsson 1997, Lyberg and Adlercreutz 2008, Okada et al. 2008,Carvalho et al. 2009).

After the enzymatic reaction, a separation process is usually required; molecular distillation or urea complexation are commonly employed to increase the yield of concentration (Wanasundara & Shahidi, 1998; Carvalho et al., 2003).

The main limitation of enzymatic processes is related to the selectivity of lipases and the regiodistribution of PUFA in the glycerol backbone of the oil assayed as substrate. (Tanaka et al.,1993; Mbatia et al., 2010).As a result, sometimes, a lipase is useful in concentrating EPA and DHA in some species and not effective in others. This is the case of *Aspergillys niger* which was able to increase DHA amount in cod liver (Hoshino et al., 1990) but had no effect with tuna oil (Tanaka et al., 1992) or menhaden oil (Wanasundara & Shahidi, 1998).

As far as hydrolysis is concerned, several attempts have been conducted in order to improve the purity and yield of Omega-3 concentrates. For instance, a method in which sardine oil was concentrated by means of, firstly, hydrolysis with *Pseudomona cepacia* and, secondly, urea complexation achieved an Omega-3 purity of 86.58% and a yield of 78% (Gámez-Meza et al., 2003).Furthermore, Kojima et al., (2006) developed a process

able to concentrate DHA or EPA from a mixture of fish oil. The selectivity can vary by the choice of the enzyme: the usage of HU-lipases produced higher concentrates of EPA whereas the use of AK-lipase increased the DHA percentage. In both cases, hydrolysis was followed by urea complexation as a separation process.

Enzymatic esterification also requires the conversion of the concentrated esters into triglycerides or at least to a mixture of mono-, di- and triglycerides. This reconversion can be carried out in two different ways: (1) with a two-steps enzymatic reaction in which esters are hydrolysed and converted into fatty acids and then esterified with glycerol or (2) *via* a one-step reaction in which a direct transesterification of fatty acid esters takes place.

Enrichment of oil extracted from Nile perch viscera was conducted by esterification of free fatty acids by using *Pseudomonas cepacia*. This Lipase was able to discriminate between EPA and DHA with a recovery yield of EPA of 79% (Mbatia et al., 2011).

7. STRUCTURED LIPIDS

Generally, structured lipids (SL) are defined as triacylglycerides (TAG) obtained by chemical or enzymatic process that present a certain fatty acid composition to achieve a determinate purpose, taking into account the different positions of glycerol.

Provided that till the moment those aims are directed to human nutrition, the most important aspects are the nature of the fatty acids involved and their position in glycerol. Two different positions can be distinguished according to intestinal absorption, extreme or primary positions (sn-1 and sn-3) and intermediate or secondary position (sn-2). In Fig. 1c glycerol structure can be clearly appreciated, where R, R’ and R’’ are alquil radicals. Homogeneous triacylglycerides are obtained when the same fatty acid is located at three positions (R, R’ and R’’ are the same fatty acid) and heterogeneous triacylglycerides, when different fatty acids are located at three positions (R, R’ and R’’ are different fatty acids).

The aforementioned triacylglycerides can be transformed by chemical or enzymatic reactions into different species according to the position of the hydrolyzed fatty acid. Monoacylglycerides (MAG) are obtained by hydrolyzing two fatty acids from TAG molecule, diacylgycerides (1,2-DAG or 1,3-DAG) result from hydrolysis of just one of the fatty acids and, in both cases, hydrolyzed fatty acids remain as free fatty acids (FFA) in the media.

Since SL are focused at human nutrition, the raw material used in its production must be vegetal or animal unmodified TAG. Moreover, the usual synthesis process of TAG is enzymatic due to the delicate structure of fatty acids, especially PUFA.

Structured lipids began to be used in nutrition as medium chain triacylglycerides that were prepared from vegetable fatty acids: saturated fatty acids from caproic (C6:0) to lauric (C12:0) acids. Mentioned triacylglycerides are used to provide rapid energy food to patients with mal-absorption syndrome (Babayan, 1987) or to produce hypocaloric food for dietetic purposes. Currently these kinds of TAG are focused to be used as methods to provide functional fatty acids for treatment of disease or for certain deficiencies.

Therefore, structured lipids are currently defined as triacylglycerides containing short- or medium-chain fatty acids and long chain ones in the same glycerin (Akoh, 1995), even when they present any distribution (random structured lipids). The preferred distribution is the one that has medium chain fatty acids at sn-1(3) position and essential and functional fatty acids at sn-2 position of triacylgyceride (MLM structured).

In the last few years, enzymatic processes using lipases have firmly appeared to obtain specific lipids for each age and physiological state (Merolli, 1997; Fitch Haumann, 1997). But lipid's technological use is more complex than that for other macronutrients, mainly because of the poor solubility of their constituents in water.

Structured lipids synthesis can be developed basically by two kinds of processes: chemical and enzymatic. Occasionally, chemical processes include some kind of enzymatic reaction.

7.1. Digestion and absorption of lipids

The importance of developing structured lipids is due to the regioselectivity that enzymes involved in human digestions present.

As shown in Fig. 6, lipid digestion begins in the mouth with the usage of lingual lipase, an enzyme which hydrolyses TAG to diacylglycerides (DAG), monoacylglycerides (MAG) and FFA. This lipase shows a major specifity towards short and medium fatty acids as well as towards fatty acids in the sn-3 position; being the main products 1,2-DAG and FFA (Small, 1991; Christensen et al., 1995).

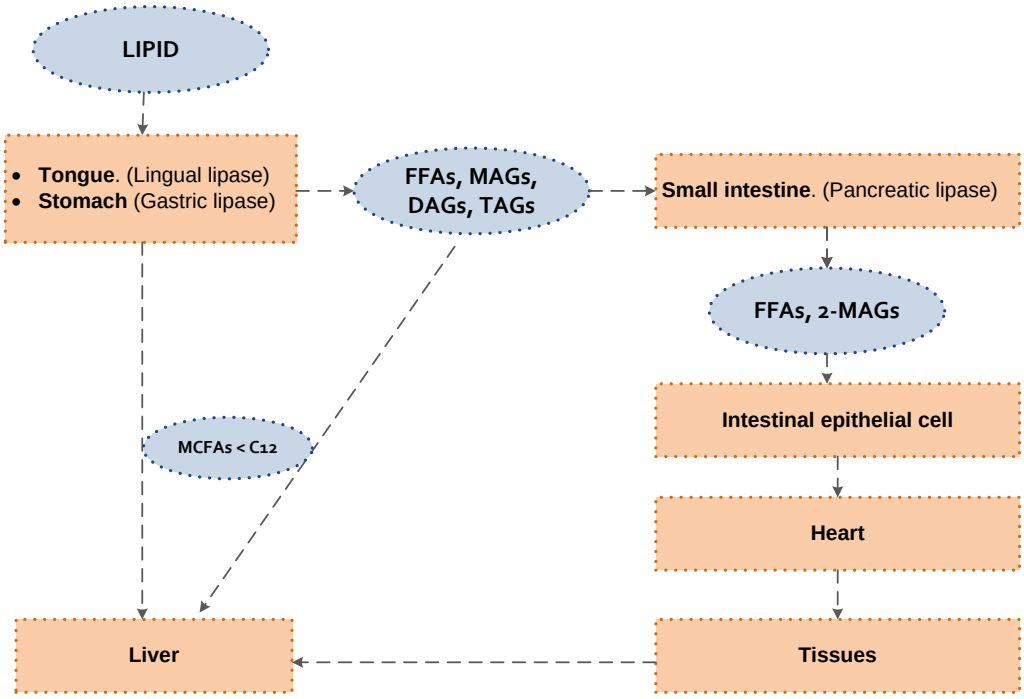


Figure 6. Digestion and absorption of lipids Adapted from Willis and Marangoni, (1999b).

In the stomach, digestion continues with the gastric lipase, an enzyme which is even more specific towards short and medium fatty acids than lingual lipase. In this case, positions 1 and 3 of TAG are hydrolyzed producing FFA, MAG and DAG. As short and medium free fatty acids have a high solubility in aqueous media they are absorbed through the stomach and transported to the liver. Mainly, they act as a rapid source of energy (Borum, 1992).

The compounds which are not soluble enough are finally hydrolyzed in the small intestine by means of the pancreatic lipase, a 1,3-specific enzyme (Small, 1991; Bernadier, 1995). However, this lipase presents a very low activity when reacting with sn-1 and sn-3 long PUFA (Christensen et al. 1995). Then, free fatty acids and 2-MAG formed micelles with bile salts and, subsequently, they are absorbed by the intestinal mucosa.

The position of long chain saturated fatty acids affects the solubility. For instance, palmitic acid is weakly absorbed because at body temperature it is solid and forms insoluble soaps in the intestine; however, as a 2-MAG, palmitic acid can be easily absorbed (Innis et al. 1995). By the placement of long chain PUFA at the sn-2 position, 2-MAG which are poorly hydrolyzed by pancreatic lipase are produced and the PUFA absorption is increased.

After intestinal absorption, new TAG are produced by the reaction of FFA and 2-MAG. Then, PUFA are combined with phospholipids and apolipoproteins in order to form chylomicrons and be transported through the lymphatic system to the general circulation.

7.2. Chemical Synthesis

In order to obtain structured lipids by chemical synthesis, reactions are developed at very high temperature. The time used for the synthesis is very long and reactions are catalyzed by alkaline metals or alkaline metal alkylates (Akoh, 1995). This kind of catalysis is cheaper and easily scalable but has no specificity and fatty acids distribution in the final product cannot be controlled (Willis & Marangoni, 1999a).

Halldorsson et al., (2003) proposed a chemical-enzymatic method to obtain structured TAG. The method has two steps: first, enzymatic esterification of glycerol with a medium chain fatty acid and Lipozyme lipase to obtain 1,3-DAG with 90% yield after purification. Later, chemical agents can be used to introduce in the molecule formed, pure EPA and DHA. With both reactions and after silica gel chromatography to remove pure fatty acids excess, final yields of around 90-95% can be achieved.

Lower yields and high purity can be obtained using just chemical synthesis of structured TAG with AA or DHA and lauric or palmitic acids (Fauconnot, 2005), reactions can be carried out in the presence of PUFAs, saturated MAG and a couple of chemical reagents. Structured lipids as APA and DPD yields could range between 63 and 68% with purity higher than 98% in each case (P: palmitic acid, A: arachidonic acid and D: docosahexaenoic acid).

7.3 Enzymatic Synthesis

Enzymatic processes present as great advantage that fatty acids distribution in the final product can be controlled because of lipases regiospecificity and specificity towards fatty acids.

Structured lipids can be synthetized by enzymatic catalysis in two ways:

- One-step processes: such as acidolyisis and interesterification.
- Two-step processes: like alcoholysis + esterification or hydrolysis + esterification.

Among the first kind of processes, acidolysis is the most used one while alcoholysis + esterification is the most commonly used among two step processes.

7.3.1. Acidolysis

Acidolysis reaction occurs between an ester and an acid with the substitution of acyl-ester by free fatty acids (Fig. 7a).

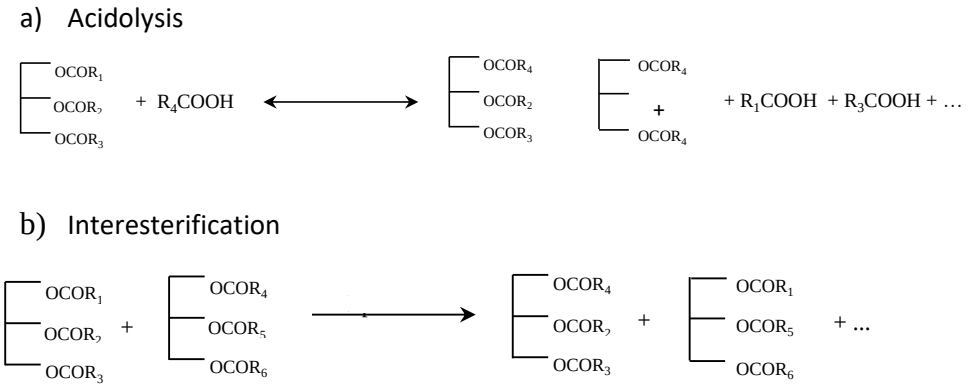


Figure 7.Generic reactions of (a) Acidolysis and (b) Interestification.

This reaction can be interpreted as a two-step process that requires a previous removal of a fatty acid from the TAG (hydrolysis) followed by an introduction of another fatty acid into the free position (esterification) (Ainsworth et al., 1996).

This kind of reaction has been used in EPA and DHA concentration from marine oils glycerides (Yamane et al., 1992, 1993) and in SLs production, type MLM (Hita, 2007).

Structured lipids synthesis by acidolysis has been carried out by several authors during last years. Due to the presence of just one step, it can be considered the easiest way to produce SL. Yields around 65% were obtained by Shimada, (1996a, b) when catalyzed acidolysis was developed with tuna oil rich in DHA and caprilic acid using Rhizopus delemar as the lipase. An important aspect of this process is that almost all DHA remained at the central position of TAG.

Xu et al., (1998) also synthetized structured lipids using fish oil, capric acid and Lipozyme IM® lipase in a batch reactor. After six hours of reaction, more than 60% incorporation of 10:0 was achieved but also 10% acilmigration from sn-1(3) to the central position. The same authors (Xu et al., 2000a) obtained 40% incorporation of 8:0 in a packed bed reactor with just 3% of acilmigration. The same incorporation around 40% of oleic acid was achieved when canola oil was used (Xu et al., 2000b).

Immobilized lipase IM60®, was used as a biocatalyst for the incorporation of capric acid (C10:0) into menhaden fish oil concentrate (34.7 mol% EPA and 34.4 mol% DHA), by means of an acidolysis reaction. It was found that the presence of hexane was beneficial for the incorporation of capric acid, while it was detrimental to the concentration of EPA and DHA. Nevertheless, this drawback can be alleviated by increasing the capric acid/oil molar ratio (Jennings & Akoh, 2001).

Structured lipids synthesis from cod liver oil, caprilic acid and Lipozyme IM® in different systems was studied by Camacho Páez et al., (2002). In the first system a batch reactor was used and temperature influence in the caprilic acid incorporation was analyzed. In the second system a packed bed reactor with recirculation was employed to study the kinetic process. Finally, the third system used was a packed bed reactor with no recirculation and MLM type TAG was obtained with 57% of 8:0 and 44% of EPA and DHA at the central position.

A similar study was developed by González et al., (2004) using caprilic acid, a commercial oil rich in EPA and Lipozyme IM®. In function of operating mode used TAG obtained had 51-64% of caprilic acid and 9.6-19.6% of EPA. When using the same conditions but with a packed bed reactor with recirculation, the structured lipids presented 59.5% caprilic acid, 9.6% EPA, 2.2% DHA and 11.8% oleic acid.

Acidolysis can also be carried out with tuna oil, caprilic acid and Rd lipase in a packed bed reactor with recirculation. In the absence of solvent structures TAG with 51% caprilic acid and 13% DHA were obtained after 73 h. The structure analysis showed that 91% of caprilic acid was located at extreme positions and 51% of DHA at central position. In order to purify SL, several extractions with water/ethanol mixture was used and TAG purity resulted to be 93-96% with a separation yield of 85% (Hita et al., 2007, 2009).

On the other hand, Camacho et al., (2007) proposed a kinetic model for acidolysis of different TAG and caprilic acid in a packed bed reactor with Lipozyme IM®. The data obtained was adjusted very well by the proposed model just when working with TAG concentration lesser than 0.1 mol/L. At higher concentrations a significant decrease in reaction rate was observed.

7.3.2. Interestification

Interesterification reaction occurs between two esters with an exchange of acyl group. If ester was a TAG the reaction is exemplified in Fig. 7.b. New TAG that can exhibit

different properties from the original TAG, are formed. For example, that is the way to produce n-3 PUFA-enriched TAG using Lipozyme lipase (Haraldsson et al., 1989).

Several authors obtained MLM-structured lipids using cotton seed oil, tricaprilin, tracaprin and trilinolein (Soumanou et al, 1998b, Fomuso & Akoh 1998).

Later, Han et al., (1999) used tricaprilin and EPA ethyl esters with several lipases and checked that water activity had no effect on the composition of the final product when specific lipases are employed. Also, the first EPA incorporation was faster than the later ones because of steric impediments.

Interesterification between triEPA and ethyl caprilate can be carried out with LipozymeTM at 40 ºC. Irimescu et al., (2000) reached a 91% molar of structured lipid (CEC, C: caprilic acid and E: EPA) in the final reaction mixture.

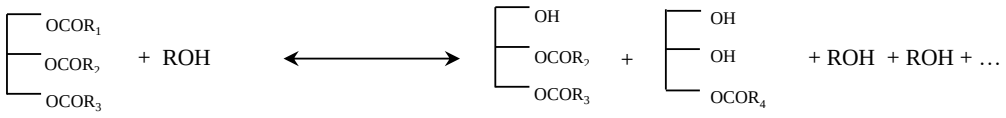
When using tripalmitin and saturated fatty acids (2 to 14 carbon atoms) ethyl esters in the presence of *Carica papaya* lipase act as catalyzer, Gandhi & Mukherjee, (2001) observed that incorporation was higher when increasing ethyl esters chain length.

7.3.3. Two Steps Process: Alcoholysis and Esterification

Alcoholysis reaction happens between an ester and an alcohol to produce alkyl group of the ester substitution by the alcohol one. If ester was a TAG the generic reaction is shown in Fig. 8a.

This reaction can be interpreted as a two-step process that requires a previous removal of a fatty acid from the TAG (hydrolysis) followed by the esterification of the alcohol and the fatty acid removed (Ainsworth et al. 1996). When alcohol used is glycerine, the final product obtained is a mixture of MAG, DAG and TAG.

a) Alcoholysis



b) Esterification

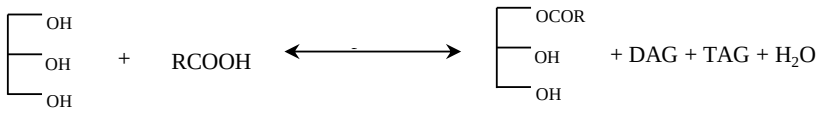


Figure 8.Generic reactions of the two-steps process (a) Alcoholysis and (b) Esterification.

Because water is not produced in alcoholysis, planning for water removal is not necessary. Nonetheless, main alcoholysis problem is that an excess of alcohol is needed to achieve a high yield and that excess should be recovered later.

Esterification reaction happens between a fatty acid and an alcohol to produce an ester. If alcohol was glycerin is used instead of alcohol, the reaction is shown in Fig. 8b. The typical reaction products are MAG, DAG, TAG and non-esterified free fatty acids. Using lipases acyl-selectivity towards some fatty acids, esterification can be applied for the GLA and DHA concentration (Shimada et al., 1997a, 2001a, b). For DHA concentration lauric alcohol and free fatty acids from tuna oil can be used to achieve an increase of 43% in the DHA mixture content. The same reaction can also be employed to obtain enriched TAG using glycerol and PUFAs (Esteban et al., 1998).

Several authors have carried out structured lipid synthesis using the two-step process (alcoholysis and esterification) and came to the conclusion that it has some advantages. Irimescu et al., (2001b) came to the conclusion that this process is faster and higher conversions can be achieved in the absence of toxic solvents.

Soumanou et al., (1998a) also came to the conclusion that higher concentration of MLM structured lipids were obtained using the above-mentioned two-step process as compared with acidolysis.

Alcoholysis was developed using several homogeneous oils and natural oils with Rhizopus delemar immobilized in a support and with organic solvents. After purification, 2-MAG were esterified with caprilic acid and Rhizopus miehei and the final product had more than 90% of caprilic acid at the extreme positions and more than 98% of PUFA at the centre.

Irimescu et al. (2001a) used two homogeneous TAG, triEPA and triDHA, to produce MLM structured lipids. *Candida antarctica* lipase was used in alcoholysis and after 2-MAG esterification with *Rhizopus miehei*, TAG were obtained with more than 85% yields. The whole process lasted less than 8 hours. No isomers were formed in the synthesis and yields slightly decreased when triEPA was used as a substrate. If heterogeneous oils (tuna oil) are used in the same process, after 3 hours of complete reaction, MLM structured lipids with purities higher than 85%, 45% of DHA content and a purification yield of 71% (Irimescu et al., 2001b) were obtained.

Torres et al., (2003a) synthetized structured TAG from fish oil. After the alcoholysis step and esterification with Pseudomonas cepacia, TAG with 55% of conjugated linoleic fatty acids and 18% of EPA and DHA were obtained.

The two-step process to obtain structured lipids was also developed using fish oil as the starting substrate and several lipases (*Candida antarctica, Rhizopus delemar* and *lipase D*) for the alcoholysis reaction. The yields achieved were more than 90% in 2-MAG production. Esterification of 2-MAG produced with caprilic acid and lipases D and DF produced MLM structured lipids with 95% yield and 98% of caprilic acid at the extreme positions (Muñío et al., 2008, 2009; Esteban et al., 2009)

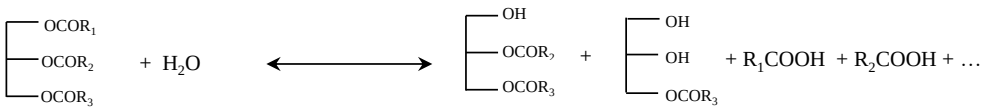
7.3.4. Two-Steps Process: Hydrolysis and Esterification

Hydrolysis reaction consists of converting a grease or ester decomposition into acids and alcohol in the presence of water. It is the inverse of esterification reaction (Fig. 9a).

Hydrolysis is the natural reaction catalyzed by lipases and it is used to obtain fatty acids from natural TAG like vegetal oils (Rooney & Weatherley, 2001), animal greases and, to a lesser extent, marine oils (Shimada et al., 1997b, 2001a).

As described earlier, esterification reaction happens between a fatty acid and an alcohol to produce an ester (Fig. 9b).

a) Hydrolysis



b) Esterification

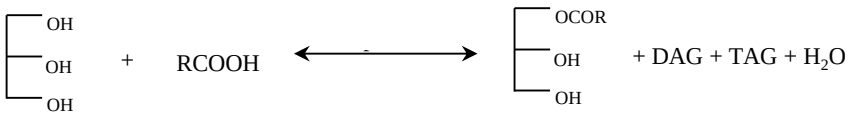


Figure 9.Generic reactions of the two steps process (a) Hydrolysis and (b) Esterification.

Because all reactions catalyzed by lipases consist in a hydrolysis reaction followed by an esterification reaction, it is possible to develope both reactions independently to optimize reaction conditions in each case (Willis & Marangoni, 1999b). The mentioned authors carried out oil hydrolysis and partial glycerides esterification with Lipozyme IM®. After hydrolysis final products mixture was distillated to reduce free fatty acids content lower

than 1%, so the composition was: 30% DAG, 2% MAG and 67% TAG. In this work, the yield obtained by four ways was compared: acidolysis, interesterifiaction and combined hydrolysis-acidolysis-esterification and hydrolysis-interesterification.

The highest yield was obtained by the last method (83%), followed by interesterification (74%). Two ways of acidolysis produced lesser yields (60% in one step and 66% in the second step). This was because of a decrease in enzymatic activity due to pH decrease in the aqueous phase round to lipase or because of free fatty acids accumulation in oil-water interphase that prevented new substrate access to lipase. The main disadvantage of the two-step processes studied by the authors is the high DAG content (around 17%) obtained with the final TAG.

8. REFERENCES

Abbas, A.K., A.H. Lichtman and J.S. Pober. Cellular and Molecular Immunology, W.B. Saunders Company, Philadelphia, USA.

Ackman, R.G., W.M.N. Ratnayake and B. Olsson. (1988). The "basic" fatty acid composition of Atlantic fish oils: Potential similarities useful for enrichment of polyunsaturated fatty acids by urea complexation. *J. Am. Oil Chem. Soc*. 65: 136−138.

Ainsworth, S., C. Versteeg, M. Plamer and M.B. Millikan. (1996). Enzymatic interesterification of fats. *Aust. J. Dairy Technol*. 51: 105−107.

Akoh. C.C. (1995). Structured lipids-enzymatic approach. Inform. 6: 1055−1061.

Arvanitoyannis, I.S. and A. Kassaveti. (2008). Fish industry waste: Treatments, environmental impacts, current and potential uses. *Int. J. Food Sci. Technol*. 43: 726−745.

Augustsson, K., D.S. Michaud, E.B. Rimm, M.F. Leitzmann, M.J. Stampfer, W.C. Willett and E. Giovannucci. (2003). A prospective study of intake of fish and marine fatty acids and prostate cancer. *Cancer Epidemiol. Biomarkers Prevent*. 12: 64−67.

AWARENET. (2004). Handbook for the prevention and minimization of waste and valorization of by-products in European agro-food industries. Agro-food waste minimization and reduction network (AWARENET). Grow Programme, European Commision 1−7.

Babayan, V.K. (1987). Medium chain triglycerides and structured lipids.*Lipids*. 22−6: 417−420.

Balk, E., M. Chung, A. Lichtenstein, P. Chew, B. Kupelnick, A. Lawrence, D. De Vine and J. Lau. (2004). Effects of Omega-3 fatty acids on cardiovascular risk factors and intermediate markers of cardiovascular disease. Evidence report/technology assessment (Summary) 93: 1−6.

Bernadier, C.D. (1995). Advanced Nutrition: Macronutrients.CRC Press, Inc. Boca Ratón, Florida. USA.

Borum, P.R. (1992). Medium-chain triglycerides in formula for preterm neonates: implications for hepatic and extrahepatic metabolism. *J. Pediatr*. 120: 139−145.

Bourre, J.M., O. Dumont, M. Piciotti, M. Clément, J. Chaudière, M. Bonneil, G. Nalbone, H. Lafont, G. Pascal and G.Durand. (1991). Essentiality of Omega 3 fatty acids for brain structure and function. *World Rev. Nutr. Diet*. 66: 103−117.

Breivik, H., G. G.Haraldsson and B. Kristinsson. (1997). Preparation of highly purified concentrates of eicosapentaenoic acid and docosahexaenoic acid. *J. Am. Oil Chem. Soc*. 74: 1425−1429.

Breivik, H. (2005). Concentrates. pp. 111−137. In: H. Breivik. [eds.]. Long-Chain Omega-3 specialty oils.The oily Press, Bridgwater, England.

Browning, L.M.,J.D. Krebs, C.S. Moore, G.D. Mishra, M.A. O'Connell and S.A. Jebb. (2007). The impact of long chain n-3 polyunsaturated fatty acid supplementation on inflammation, insulin sensitivity and CVD risk in a group of overweight women with an inflammatory phenotype. *Diabetes Obes.Metab*. 9: 70−80.

Brunner, G. (1994). Gas extraction. An Introduction to Fundamentals of Supercritical Fluids and the Application to Separation Processes.Steinkopff Darmstadt Springer, New York, USA.

Camacho, F., A. Robles, B. Camacho, P.A. González, L. Esteban and E. Molina. (2007). Modeling of the kinetic for the acidolisis of different triacylglycerols and caprylic acid catalyzed by Lipozyme IM immobilized in a packed bed reactor. *Chem. Eng. Sci*. 62: 3127−3141.

Camacho Páez, B., A. Robles Medina, F. Camacho Rubio, P. González Moreno and E. Molina Grima. (2002). Production of structured triacylglycerides rich in n−3 polyunsaturated fatty acids by the acidolisis of cod liver oil and caprylic acid in a packed bed reactor: equilibrium and kinetics. *Chem. Eng. Sci*. 57−8: 1237−1249.

Carvalho, P., P. Campos, M. Noffs and J.G. Oliveira. (2003). Aplicacoes de lipases microbianas na obtencao de concentrados de acidos graxos poliinsaturados. *Quim. Nova*. 26: 75−80.

Carvalho, P., P. Campos, M. Noffs, P. Fregolente and L. Fregolente. (2009). Enzymatic hydrolysis of salmon oil by native lipases: Optimization of process parameters. *J. Br. Chem. Soc*. 20: 117−124.

Caughey, G.E., E. Mantzioris and R.A. Gibson. (1996). The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n−3 fatty acids from vegetable oil or fish oil. *Am. J. Clin. Nutr*. 63: 116−122.

Christensen, M.S., C.E. Hoy, C.C. Becker and T.G. Redgrave. (1995). Intestinal absorption and lymphatic transport of eicosapentaeonic (EPA), docosahexaeonic (DHA); and decanoic acids: dependence on intramolecular triacylglycerol structure. *Am. J. Clin. Nutr*. 61: 56−61.

Crexi, V.T., M.L. Monte, L and A.A. Pinto. (2012). Polyunsaturated fatty acid concentrates of carp oil: Chemical hydrolysis and urea complexation. *J. Am. Oil Chem. Soc*. 89: 329−334.

Dullemeijer, C., J. Durga, I.A. Brouwer, O. Van De Rest, F.J. Kok, R.J.M. Brummer, M.P.J. Van Boxtel and P. Verhoef. (2007). N-3 Fatty acid proportions in plasma and cognitive performance in older adults. *Am. J. Clin. Nutr*. 86: 1479−1485.

Dunforda, N.T., M. Goto and F. Temelli. (1998). Modeling of oil extraction with supercritical CO2 from Atlantic mackerel (Scomber scombrus) at different moisture contents. J. Supercrit. Fluids. 13: 303−309.

Dunstan, J.A. And Prescott, S.L. (2007). Does fish oil supplementation in pregnancy reduce the risk of allergic disease in infants? *Curr. Opin. Allergy Clin.Immunol*. 5: 215−221.

Eisenbach, W. (1984). Supercritical fluid extraction: a film demonstration. *Berichte der Bunsengesellschaft/Phys. Chem. Chem. Phys*. 88: 882−887.

Erkkilä, A.T., N.R. Matthan, D.M. Herrington and A.H. Lichtenstein. (2006). Higher plasma docosahexaenoic acid is associated with reduced progression of coronary atherosclerosis in women with CAD. J. *Lipid Res*. 47(12): 2814−2819.

Esquível, M.M., N.M. Bandarra, I. Fontan, M.G. Bernardo-Gil, I. Batista, M.L. Nunes and J. A. Empis. (1997). Supercritical carbon dioxide extraction of sardine *Sardina pilchardu*s oil. *LWT - Food Sci. Technol*. 30: 715−720.

Esteban Cerdán, L, A. Robles Medina, A. Giménez Giménez, M.J. Ibáñez González and E. Molina Grima. (1998). Synthesis of polyunsaturated fatty acid-enriched triglycerides by lipase-catalized esterification. *J. Am. Oil Chem. Soc*. 75: 1329−1337.

Esteban, L., M.M. Muñío, A. Robles, E. Hita, M.J. Jiménez, P.A. González, B. Camacho and E. Molina. (2009). Synthesis of 2-monoacylglycerols (2-MAG) by enzymatic alcoholysis of fish oil using different reactor types. *Biochem. Eng. J*. 44: 271−279

Falch, E., T. Rustad, R. Jonsdottir, N. B. Shaw, J. Dumay, J. P. Berge, S. Arason, J. P. Kerry, M. Sandbakk and M. Aursand. (2006). Geographical and seasonal differences in lipid composition and relative weight of by-products from gadiform species. J. Food Compos.Anal. 19: 727−736.

FAO. (2012). Global Production Statistics 1950−2010. Available at: http://www.fao.org

Fauconnot, L., F. Robert, R. Villard and F. Dionisi. (2005). Chemical synthesis NMR characterization of structured polyunsaturated triacylglycerols. *Chem. Phys. Lipids* 139−2: 125−136.

Fedačko, J., D. Pella, V. Mechírová, P. Horvath, R. Rybár, P. Varjassyová and V. Vargová. (2007). N-3 PUFAs- From dietary supplements to medicines. *Pathophysiology*. 14: 127−132.

Ferraro, V., I.B. Cruz, R.F. Jorge, F.X. Malcata, M.E. Pintado and P.M.L. Castro. (2010). Valorisation of natural extracts from marine source focused on marine by-products: A review. *Food Res. Int*. 43: 2221−2233.

Fitch Haumann, B. (1997). Structured lipids allow fat. *Inform*. 8: 1004−1011.

Fomuso, L.B. and C.C. Akoh. (1998). Structured lipids: lipase-catalized interesterificaction of tricaproin and trilinolein. J. Am. Oil Chem. Soc. 75: 405−410.

Frankel, E.N. (2005). Free Radical Oxidation. In: E.N. Frankel. [eds.]. Lipid oxidation.The oily Press, Bridgwater, UK pp. 15−23.

Freeman, M.P., J.R. Hibbeln, K.L. Wisner, J.M. Davis, D. Mischoulon, M. Peet, P.E. Keck Jr., L.B., Marangell, A.J. Richardson, J. Lake and A.L. Stoll. (2006). Omega-3 fatty acids: Evidence basis for treatment and future research in psychiatry. *J. Clin. Psychiat*. 67(12): 1954−1967.

Gámez-Meza, N., J.A. Noriega-Rodríguez, L.A. Medina-Juárez, J. Ortega-García, J. Monroy-Rivera, F.J. Toro-Vázquez, H.S. García and O. Angulo-Guerrero. (2003). Concentration of eicosapentaenoic acid and docosahexaenoic acid from fish oil by hydrolysis and urea complexation. *Food Res. Int*. 36: 721−727.

Gandhi, N.N. and K.D. Mukherjee. (2001). Synthesis of designed lipids using papaya (*Carica papaya*) latex lipase. *J. Molecular Catalysis B: Enzymatic*. 11−4: 271−277.

Garg, M.L., A.B.R. Thomson and M. T. Clandinin. (1990). Interactions of saturated, n−6 and n−3 polyunsaturated fatty acids to modulate arachidonic acid metabolism. *J. Lipid Res*. 31: 271−277.

Geelen, A.,J.M. Schouten, C. Kamphuis, B. E. Stam, J. Burema, J.M.S. Renkema, E.J. Bakker, P. Van't Veer and E. Kampman. (2007). Fish consumption, n−3 fatty acids, and colorectal cancer: A meta-analysis of prospective cohort studies. *Am. J. Epidemiol*. 166: 1116−1125.

Gildberg, A., J.A. Arnesen and M. Carlehög. (2002). Utilisation of cod backbone by biochemical fractionation. *Process Biochem*.38, 475−480.

González Moreno, P.A., A. Robles Medina, F. Camacho Rubio, B. Camacho Páez and E. Molina Grima. (2004). Production of Structured Lipids by Acidolysis of an EPA-Enriched Fish Oil and Caprylic Acid in a Packed Bed Reactor: Analysis of Three Different Operation Modes. *Biotechnol. Progress*. 20–4: 1044–1052.

Grigorakis, K., M.N. Alexis, K.D.A. Taylor and M. Hole. (2002). Comparison of wild and cultured gilthead sea bream (Sparus aurata); composition, appearance and seasonal variations. Int. J. Food Sci. Technol. 37: 477–484.

Halldorsson, A., C.D. Magnusson and G.G. Haraldsson. (2003). Chemoenzymatic synthesis of structured triacylgycerols. *Tetrahedron Letters*. 42–43: 7675–7677.

Han, J.J., Y. Iwasaki and T. Yamane. (1999). Monitoring of lipase-catalyzed transesterification between eicosapentaenoic acid ethyl ester and tricaprylin by silver ion High-Performance Liquid Chromatography and High-Temperature Gas Chromatography.*J. Am. Oil Chem. Soc*. 76: 31–39.

Haraldsson, G. (1983). Separation of saturated/unsaturated fatty acids. *J. Am. Oil Chem. Soc*. 61: 219–222.

Haraldsson, G.C., P. A.Höskuldsson, S. Th. Sigurdsson, F. Thorsteinsson and S. Gudbjarnason. (1989). The preparation of triglycerides highly enriched with w–3 polyunsaturated fatty acids *via* lipase catalyzed interesterification. *Tethahedron Lett.* 30: 1671–16.74.

Haraldsson, G.G., B. Kristinsson, R. Sigurdardottir, G.G. Gudmundsson and H. Breivik. (1997). The preparation of concentrates of eicosapentaenoic acid and docosahexaenoic acid by lipase-catalyzed transesterification of fish oil with ethanol. *J. Am. Oil Chem*. 74: 1419–1424.

Harris, W.S., M. Miller, A. P. Tighe, M. H. Davidson and E. J. Schaefer. (2008). Omega-3 fatty acids and coronary heart disease risk: Clinical and mechanistic perspectives. *Atherosclerosis.* 197: 12–24.

Hayes, D. G., Y. C. Bengtsson, J.M. Van Alstine and F. Setterwall. (1998). Urea complexation for the rapid, ecologically responsible fractionation of fatty acids from seed oil. *J. Am. Oil Chem*. Soc. 75: 1403–1409.

Healy, D.A., F.A. Wallace and E. A. Miles. (2000). Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids* 35: 763–768.

Helland, I.B., L. Smith, K. Saarem, O.D. Saugstad and C.A. Drevon. (2003). Maternal supplementation with very-long.chain n.3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. *Pedriatric* 111: 39–44.

Hita, E., A. Robles, B. Camacho, A. Ramírez, L. Esteban, M.J. Jiménez, M.N. Muñío, P.A. González and E. Molina. (2007). Production of structured triacylglycerols (STAG) rich in docosahexaenoic acid (DHA) in position 2 by acidolisis of tuna oil catalyzed by lipases. *Process Biochem.* 42: 415–422.

Hita, E., A. Robles, B. Camacho, P.A. González, L. Esteban, M.J. Jiménez, M.J, M.M. Muñío and E. Molina. (2009). Production of structured triacylglycerols by acidolysis catalyzed by lipases immobilized in a packed bed reactor. *Biochem. Eng*. J. 46: 257–264.

Hu, F.B., E. Cho, K.M. Rexrode, C.M. Albert and J. E. Manson. (2003). Fish and long-chain ω-3 fatty acid intake and risk of coronary heart disease and total mortality in diabetic women. *Circulation*. 107(14): 1852–1857.

Innis, S. M. and R. Dyer, P. Quinlan and D. Diersen-Schade. (1995). Palmitic acid is absorbed as sn-2 monopalmitin from milk and formula with rearranged triacylglycerols and results in increased plasma triglyceride sn-2 and cholesterol ester palmitate in piglets. *J. Nutr*. Vol. 125:73-81.

Irimescu, R., M. Yasui, Y. Iwasaki, N. Shimidzu and T. Yamane. (2000). Enzymatic synthesis of 1,3-dicapryoyl-2-eicosapentaenoylglycerol. *J. Am. Oil Chem. Soc*. 77: 501–506.

Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki and T. Yamane. (2001a). Utilization of reaction medium-dependent regiospecificity of *Candida antarctica* (Novozym 435) for the synthesis of 1,3-dicapryloyl-2-docosahexaenoyl (or eicosapentaenoyl) glycerol. *J. Am. Oil Chem. Soc*. 78–3: 285–289.

Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki and T. Yamane. (2001b). Two-step enzymatic synthesis of docosahexaenoic acid-rich symmetrically structured triacylglycerols *via* 2-monoacylglycerols.J. *Am. Oil Chem. Soc.* 78–7: 743–748.

Jennings, B.H. and C.C. Akoh. (2001). Lipase catalyzed modification of fish oil to incorporate capric acid. *Food Chemistry* 72–3: 273–278.

Kim, S.K. and E. Mendis. (2006). Bioactive compounds from marine processing byproducts - A review. *Food Res. Int*. 39: 383–393.

Kojima, Y., E. Sakuradani and S. Shimizu. (2006). Different specificities of two types of Pseudomonas lipases for C20 fatty acids with a Δ5 unsaturated double and their application for selective concentration of fatty acids.Different specificities of two types of Pseudomonas lipases for C20 fatty acids with a Δ5 unsaturated double and their application for selective concentration of fatty acids. *J. Biosci. Bioeng*. 1: 490–500.

Kristinsson, H.G. and B.A. Rasco. (2000). Fish protein hydrolysates: Production, biochemical, and functional properties. Critical Reviews in Food Science and Nutrition. 40(1): 43–81.

Laerum, B.N., C. Svanes, T. Wentzel-Larsen, A. Gulsvik, K. Torén, E. Norrman, T. Gíslason, C. Janson and E. Omenaas. (2007). Young maternal age at delivery is associated with asthma in adult offspring. *Respiratory Medicine* 101: 1431–1438.

Létisse, M. and L. Comeau. (2008). Enrichment of eicosapentaenoic acid and docosahexaenoic acid from sardine by-products by supercritical fluid fractionation. *J. Sep. Sci.* 31: 1374–1380.

Liaset, B., E. Lied and M. Espe. (2000). Enzymatic hydrolysis of by-products from the fish-filleting industry; chemical characterisation and nutritional evaluation. *J. Sci. Food Agric*. 80: 581–589.

Linko, R.R. and H. Karinkanta. (1970). Fatty acids of long chain length in Baltic herring lipids. J. *Am. Oil Chem. Soc*. 47: 42–46.

Liu, S., C. Zhang, P. Hong and H. Ji. (2006). Concentration of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) of tuna oil by urea complexation: Optimization of process parameters. *J. Food Eng*. 73: 203–209.

López-Martínez, J.C., P. Campra-Madrid and J.L. Guil-Guerrero. (2004). γ-linolenic acid enrichment from Borago officinalis and Echium fastuosum seed oils and fatty acids by low temperature crystallization. *J. Biosci. Bioeng*. 97: 294–298.

Lyberg, A.M. and P. Adlercreutz. (2008). Lipase specificity towards eicosapentaenoic acid and docosahexaenoic acid depends on substrate structure. *Biochim.Biophys.* Acta. 1784: 343–350.

Magrum, L.J. and P. V.Johnston. (1983). Modulation of prostaglandin synthesis in rat peritoneal macrophages with n-3 fatty acids. *Lipids* 18: 514–521.

Malik, M., A.J. Camm, J.T. Bigger Jr., G. Breithardt, S. Cerutti, R.J. Cohen, P. Coumel,, E.L. Fallen, H.L. Kennedy, R.E. Kleiger, F. Lombardi, A. Malliani, A.J. Moss, J.N. Rottman, G. Schmidt, P.J. Schwartz and D.H. Singer. (1996). Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. *Eur. Heart* J. 17: 354–381.

Marchioli, R., R. M. Marfisi, G. Borrelli, C. Chieffo, M. G. Franzosi, G., Levantesi, A. P. Maggioni, G.L. Nicolosi, M. Scarano, M. G. Silletta, C. Schweiger, L. Tavazzi and G. Tognoni. (2007). Efficacy of n-3 polyunsaturated fatty acids according to clinical characteristics of patients with recent myocardial infarction: Insights from the GISSI-Prevenzione trial. *J. Cardiovasc. Med*. 8(SUPPL. 1): S34–S37.

Martín, A. and M.J. Cocero. (2007). Mathematical modeling of the fractionation of liquids with supercritical CO2 in a countercurrent packed column. J. Superc. Fluids. 39: 304–314.

Martins, P.F., C. B. Baristella, R. Maciel Filho and M.R. Wolf-Macie. (2006). Comparison of two different strategies for tocopherols enrichment using a molecular distillation process. *Ind. & Eng. Chem. Res*. 45: 753.

Mbatia, B., P. Adlercreutz, F. Mulaa and B. Mattiasson. (2010). Enzymatic enrichment of n-3 polyunsaturated fatty acids in Nile perch (Lates niloticus) viscera oil. Eur. J. Lipid Sci. Technol. 112: 977–984.

Mbatia, B., B. Mattiasson, F: Mulaa and P. Adlercreutz. (2011). Strategies for the enzymatic enrichment of PUFA from fish oil. *Eur. J. Lipid Sci. Technol*. 113: 717–723.

Meher, L.C., D.V. Sagar and S.N. Naik. (2006). Technical aspects of biodiesel production by transesterification–a review. Renew. *Sustain. Energy Rev*. 10: 248–268.

Merolli, A. (1997). Medium-chain lipids: new sources, uses. *Inform*. 8: 597–603.

Miyake, Y., S. Sasaki, M. Arakawa, K. Tanaka, K. Murakami and Y. Ohya. (2008). Fatty acid intake and asthma symptoms in Japanese children: The Ryukyus child health study. *Clin. Exp. Allergy* 38: 1644–1650.

Moore, C.S., S.P. Bryant, G.D. Mishra, J.D. Krebs, L.M. Browning, G.J. Miller and S.A. Jebb. (2006). Oily fish reduces plasma triacylglycerols: a primary prevention study in overweight men and women. *Nutr.* 22: 1012–1024.

Mori, T.A., V. Burke, I.B. Puddey, G.F. Watts, D.N. O'Neal, J.D. Best and L.J. Beilin. (2000). Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hypedipidemic men. *Am. J. Clin. Nutr*. 71: 1085–1094.

Morris, M.C., D.A. Evans, C.C. Tangney, J.L. Bienias and R.S. Wilson. (2005). Fish consumption and cognitive decline with age in a large community study. *Arch. Neurol*. 62: 1849–1853.

Muñío, M.M., L. Esteban, A. Robles, E. Hita, M.J. Jiménez, P.A. González, B. Camacho and E. Molina. (2008). Synthesis of 2-monoacylglycerols rich in polyunsaturated fatty acids by ethanolysis of fish oil catalyzed by 1,3 specific lipases. *Process Biochem*. 43: 1033–1039.

Muñío, M.M., A. Robles, L. Esteban, P.A. González and E. Molina. (2009). Synthesis of structured lipids by two enzymatic steps: ethanolysis of fish oils and esterification of 2-monoacylglycerols. Process Biochem. 44: 723–730.

Nair, S.S.D. J.W. Leitch, J. Falconer and M.L. Garg (1997). Prevention of cardiac arrhythmia by dietary (n-3) polyunsaturated fatty acids and their mechanism of action. *J. Nutr*. 127: 383–393.

Nkondjock, A. and O. Receveur. (2003a). Fish-seafood consumption, obesity, and risk of type 2 diabetes: An ecological study. *Diabetes Metab*. 29: 635–642.

Nkondjock, A. and B. Shatenstein, P. Maisonneuve and P. Ghadirian. (2003b). Specific fatty acids and human colorectal cancer: An overview. *Cancer Detect. Prev*. 27:55-66.

Okada, T. and M.T. Morrissey. (2008). Production of n-3 polyunsaturated fatty acid concentrate from sardine oil by immobilized Candida rugosa lipase. J. Am. Oil Chem. 73: C146–C150.

Oliveira, A.C.M. and P.J. Bechtel. (2005). Lipid composition of Alaska Pink Salmon (Oncorhynchus gorbuscha) and Alaska Walleye Pollock (*Theragra chalcogramma*) by-products. *J. Aquat. Food Prod. Technol.* 14: 73–91.

Parker, G. and N.A. Gibson, H. Brotchie, G. Heruc, A.M. Rees and D. Hadzi-Pavlovic. (2006). Omega-3 fatty acids and mood disorders. *Am. J. Psychiat*. 163: 969–978.

Pearson, A.M., J.D. Love, and F.B. Shorlan. (1977). "Warmed-Over" Flavor in Meat, Poultry and Fish. *Adv. Food Res*. 23: 1–74.

Perretti, G., A. Motori, E. Bravi, F. Favati, L. Montanari and P. Fantozzi. (2007). Supercritical carbon dioxide fractionation of fish oil fatty acid ethyl esters. *J. Supercr. Fluids* 40: 349–353.

Rai, A.K., H.C. Swapna, N. Bhaskar and V. Baskaran. (2011). Potential of seafood industry by-products as sources of recoverable lipids: fatty acid composition of meat and nonmeat component of selected Indian marine fishes. *J. Food Biochem*. 36. 441–448.

Rallidis, L.S., G. Paschos, G.K. Liakos, A. H. Velissaridou, G. Anastasiadis and A. Zampelas. (2003). Dietary α-linolenic acid decreases C-reactive protein, serum amyloid A and interleukin-6 in dyslipidaemic patients. *Atherosclerosis.* 167(2): 237–242.

Rooney, D. and L.R. Weatherley. (2001). The effect of reaction conditions upon lipase catalysed hydrolysis of high oleate sunflower oil in a stirred liquid-liquid reactor. *Process Biochem.* 36-10: 947–953.

Rose, D.P. and J.M. Connolly. (1999). Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol.Therapeut*. 83: 217–244.

Rubio, N. and S. Beltrán. (2011). Supercritical fluid technology for extraction, concentration & formulation of Omega-3 rich oils. A novel strategy for valorization of fish by-products. PhD Thesis, University of Burgos.

Ruxton, C H.S., Calder, S.C. Reed and M.J.A. Simpson. (2005). The impact of long-chain n-3 polyunsaturated fatty acids on human health. *Nutr. Res. Rev*. 18: 113–129.

Sahena, F., I.S.M. Zaidul, S. Jinap, N. Saari, H. A. Jahurul, K.A. Abbas and N.A. Norulaini. (2009). PUFAs in fish: Extraction, fractionation, importance in health. *Compr.Rev. Food Sci. Food Saf*. 8: 59–74.

Saito, H., Y. Seike, H. Ioka, K. Osako, M. Tanaka, A. Takashima, J. M. Keriko, S. Kose and J.C. Rodriguez Souza. (2005). High docosahexaenoic acid levels in both neutral and polar lipids of a highly migratory fish: *Thunnus tonggol* (Bleeker). *Lipids* 40: 941–953.

Salah, S. and S. Saloua. (2010). The influence of season on lipid content and fatty acids profile of Euthynnus alletteratus fillets and by-products. *J. Muscle Food*s 21: 365–378.

Sathivel, S. (2011). Fish oil extraction, purification, and its properties. In: C. Alasalvar, F. Shahidi, K. Miyashita and U. Wanasundara [eds.]. Handbook of Seafood Quality, Safety and Health Applications. Wiley-Blackwell, West-Sussex. United Kingdom pp. 423–430.

Schaefer, E.J., V. Bongard, A.S. Beiser, S. Lamon-Fava, S.J. Robins, R. Au, K.L. Tucker, D.J. Kyle, P.W.F. Wilson and P.A. Wolf. (2006). Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and alzheimer disease: The framingham heart study. *Arch. Neurol*. 63: 1545–1550.

Schnappinger, M., S. Sausenthaler, J. Linseisen, H. Hauner and J. Heinrich. (2009). Fish consumption, allergic sensitisation and allergic diseases in adults. *Ann. Nutr. Metab.* 54: 67–74.

Serhan, C.N. and J. Savill. (2005). Resolution of inflammation: The beginning programs the end. *Nature Immun*. 6(12): 1191–1197.

Shahidi, F. and Y. Zhong. (2010). Lipid oxidation and improving the oxidative stability. *Chem. Soc. Rev*. 39: 4067–4079.

Shimada, Y., A. Sugihara, H. Nakano, T. Yolota, T. Nagao, S. Komemushi and Y. Tominaga. (1996a). Production of structured lipids containing essential fatty acids by immobilized Rhizopus delemar lipase. *J. Am. Oil Chem. Soc*. 73: 1415–1420.

Shimada, Y., A. Sugihara, K. Maruyama, T. Nagao, S. Nakayama, H. Nakano and Y. Tominaga. (1996b). Production of structured lipids containing docosahexaenoic and caprylic acids using immobilized Rhizopus delemar lipase. *J. Ferment. Bioeng*. 81: 299–303.

Shimada, Y., A. Sugihara, H. Nakano, T. Kuramoto, T. Nagao, M. Gemba and Y. Tominaga. (1997a). Purification of docosahexaenoic acid by selective esterification of fatty acids from tuna oil with Rhizopus delemar lipase. *J. Am. Oil Chem. Soc*. 74: 97–101.

Shimada, Y., K. Maruyama, A. Sugihara, S. Moriyama and Y. Tominaga.(1997b). Purification of docosahexaenoic acid from tuna oil by two step enzymatic method: hydrolisys and selective esterification. *J. Am. Oil Chem. Soc*. 74: 1441–1446.

Shimada, Y., A. Sugihara and Y. Tominaga. (2001a). Enzymatic purification os polyunsaturated fatty acids. *J. Biosci. Bioeng*. 91: 529–538.

Shimada, Y., Y. Watanabe, A. Sugiara, T. Baba, T. Ooguri, S. Moriyama, T. Terai and Y. Tominaga. (2001b). Ethyl esterification of docosahexaenoic acid in an organic solvent.free system with immobilized *Candida antarctica* lipase. *J. Biosci. Bioeng*. 92: 19–23.

Skaliotis, L. (2011). Short path evaporation for the production of premium quality oils. *Lipid Technol.* 23: 204–206.

Small, D.M. (1991). The effects of glyceride structure on absorption and metabolism. *Annu. Rev. Nutr*. 11: 413–434.

Soumanou, M.M., U. T. Bornscheuer and R.D. Schmid. (1998a). Two-step enzymatic reaction for the synthesis of pure structured triacylglycerides. *J. Am. Oil Chem. Soc*. 75–6: 703–710.

Soumanou, M.M., U.T. Bornscheuer, U. Schmid and R.D. Schmid. (1998b). Synthesis of structured triglycerides by lipase catalysis.*Fett/Lipid*. 100-4-5: 156–160.

Tanaka, Y., J. Hirano and T. Funada. (1992). Concentration of docosahexaenoic acid in glyceride by hydrolysis of fish oil with Candida cylindricae lipase. *J. Am. Oil Chem. Soc*. 69: 1210–1214.

Tanaka, Y., T. Funada, J. Hirano and R. Hashizume. (1993). Triglyceride specificity of Candida cylindracea lipase-effect of docosahexaenoic acid on resistance of triglycerides to lipase. *J. Am. Oil Chem.* 70: 1031–1034.

Tarpila, S., A. Aro and I. Salminen. (2002). The effect of flaxseed supplementation in processed foods on serum fatty acids and enterolactone. *Eur. J. Clin. Nutr.* 56: 157–165.

Terry, P., P. Lichtenstein, M. Feychting, A. Ahlbom and A.Wolk. (2001). Fatty fish consumption and risk of prostate cancer. Lancet. 357(9270): 1764–1766.

Thorsdottir, I., H. Tomasson, I. Gunnarsdottir, E. Gisladottir, M. Kiely, M.D. Parra, N.M. Bandarra, G. Schaafsma and J.A. Martinéz. (2007). Randomized trial of weight-loss-diets for young adults varying in fish and fish oil content. *Int. J. Obesity.* 31(10): 1560–1566.

Tidwell, J.H. and G.L. Allan. (2001). Fish as food: Aquaculture's contribution. Ecological and economic impacts and contributions of fish farming and capture fisheries. EMBO Reports 2: 958–963.

Torres, C.F., T.J. Nettekoven and C.G. HillJr. (2003). Preparation of purified acylglycerols of eicosapentaenoic acid and docosahexaenoic acid and their re-esterification with conjugated linolenic acid.Enzyme *Microb. Technol*. 32: 49–58.

Torres, J.A., Y.C. Chen, J. Rodrigo-García and J. Jaczyznski. (2007). Recovery of by-products from seafood processins streams. pp 65–90. In: F. Shahidi [Ed.]. Maximising the value of marine by-products. Woodhead Publishing Ltd, Cambridge, UK.

Wanasundara, U.N. and F. Shahidi. (1998). Lipase-assisted concentration of n–3 polyunsaturated fatty acids in acylglycerols from marine oils. *J. Am. Oil Chem. Soc*. 75: 945–951.

Wijesundera, R.C. and W.M.N. Ratnayake and R.G. Ackman. (1989). Eicosapentaenoic acid geometrical isomer artifacts in heated fish oil esters. *J. Am. Oil Chem. Soc*. 66: 1822–1830.

Willis, W.M. and A.G. Marangoni. (1999a). Assessment of lipase and chemically catalyzed lipid modification strategies for the production of structured lipids. *J. Am. Oil Chem. Soc*. 76: 443–450.

Willis, W.M. and A.G. Marangoni. (1999b). Biotechnological strategies for the modification of food lipids.*Biotechnol.Gen. Eng. Rev*. 16: 141–175.

Xu, X., S. Balchen, C.E. Høy and J. Adler-Niessen. (1998). Pilot batch production of specific-structured lipids by lipase catalyzed interesterification: preliminary study on incorporation and acyl migration. *J. Am. Oil Chem. Soc*. 75: 301–308.

Xu, X., L.B. Fomuso and C.C. Akoh. (2000a). Synthesis of structured triacylglycerols by lipase catalyzed acidolisis in a packed bed bioreactor. *J. Agric. Food Chem.* 48-1: 3–10.

Xu, X., L.B. Fomuso and C.C. Akoh. 2000b. Modification of menhaden oil by enzymatic acidolisis to produce structured lipids: optimization by response surface design in a packed bed reactor. *J. Am. Oil Chem. Soc.* 77-2: 171–176.

Yamane, T., S. Tomomasa, Y. Sahashi, L. Vikersveen and T Hocino. (1992). Production of n-3 PUFA-enriched fish oil by lipase-catalyzed acidolysis without solvent. *J. Am. Oil Chem. Soc.* 69: 1104–1107.

Yamane, T., T. Suzuki, and T. Hocino. (1993). Increasing n-3 polyunsaturated fatty acid content of fish oil by temperature control of lipase-catalyzed acidolysis. *J. Am. Oil Chem. Soc*. 70: 1285–1287.

Zaidul, I.S.M., N.A.N. Norulaini, A.K.M. Omar, Y. Sato and R.L. Smith Jr. (2007). Separation of palm kernel oil from palm kernel with supercritical carbon dioxide using pressure swing technique. *J. Food Eng.* 81: 419–428.

II. Seasonal Variations in the Regiodistribution of Oil Extracted from Small-Spotted Catshark and Bogue*

The aim of this work was to seasonally characterize the nutritional quality of oil extracted from small-spotted catshark (*Scyliorhinus canicula*) and bogue (*Boops boops*). The proximate composition, lipid profile and regiodistribution of the fatty acid in the glycerol backbone were analyzed. In addition, three nutritional indexes were calculated (atherogenicity and thrombogenicity indexes and the hypocholesterolaemic -hypercholesterolaemic ratio). Both species presented PUFA as the predominant fraction, the most abundant being DHA. Healthy values of the aforementioned indexes were maintained throughout the year. Moreover, the relative composition of Omega-3 fatty acids at the sn-2 position ranged from 47.3 to 66.8 mol%, attracting the interest in the employment of these oils as the raw source for the production of 2-monoacylglycerols. Regarding the individual behavior of each fatty acid, DHA presented a high tendency to occupy the sn-2 bond, whereas EPA presented the opposite behavior.

* JOURNAL PAPER: R. Morales-Medina, P.J. García-Moreno, R. Pérez-Gálvez, M. Muñío, A. Guadix, E.M. Guadix (2015). Seasonal variations in the regiodistribution of oil extracted from small-spotted catshark and bogue. *Food and Function* 6:2646-2652. http://dx.doi.org/10.1039/c5fo00448a

1. INTRODUCTION

Discards are defined as the fraction of the fish catch which is not retained on board but rejected to the sea for any reason. Discards are composed by non-target species (*e.g.* marine sponges, echinoderms, fish species of low commercial value, seals), juvenile individuals below minimum landing size or target species over fishing quota. The last FAO report (Kelleher, 2005) estimated a yearly tonnage of discards around 7.3 million tons, representing 8% of worldwide catches. Discarding not only has a negative impact on future fishing productivity, but also poses a number of environmental problems since it alters marine's trophic chains and contribute to the dissemination of toxic compounds and parasites present in fish viscera (Blanco, Sotelo, Chapela, & Pérez-Martín, 2007) .

The discard rate (*i.e*. ratio of discarded fish related to the total catch) of a given fishery depends on a number of factors such as fishing gear, local markets or fishing regulations, among others. In the case of southwest Mediterranean Sea (Alboran Sea), discard rates arise up to 23% for trawling and 10% for purse seine fisheries. This represents an underutilization of fishing stocks, especially in an area where fish catches have been reduced to a half during the past decade (FROM, 2008). Most of discards in this area comprise non-target species such as bogue or small-spotted catshark, which are considered in this work.

International organisms have warned against the adverse effects of fishing discards, and their recommendations have so far been incorporated into the fishing regulations of some countries such as Iceland or Norway, which have adopted policies minimizing discards. In the case of the European Union, the new Common Fisheries Policy (EU, 2013) introduces a progressive discard ban in European fisheries. In application of this policy, all catches from pelagic fisheries, such as mackerel, horse mackerel or sardine, must be brought ashore since the 1st January 2015. As for the rest of species, discard prohibition will come into force from 2017 on, while some specific fisheries such as hake, Norway lobster, common sole or plaice will be exempt of these measures until 2019.

As a consequence of discard bans, a supplementary amount of fish (mainly composed of non-target species) will be landed, which will be difficulty put into marked without an adequate commercial promotion. An alternative solution is the conversion of these underutilized materials into added-value products of interest in nutraceutical and

pharmaceutical applications. For instance, some studies have explored the nutritional properties of the lipid fraction of some Mediterranean discarded species such as bogue or horse mackerel (Orban et al., 2011). Fish oils have a high content of polyunsaturated fatty acids (PUFA), which play a beneficial role for the human health (Ruxton, Reed, Simpson, & Millington, 2007). More specifically, eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic (DHA C22:6n-3), belonging to the Omega-3 family, can prevent cardiovascular diseases due to their anti-thrombotic, anti-arrhythmic and anti-inflammatory activities (Ruxton et al., 2007).

A key feature when studying the nutraceutical value of a given compound is its bioavailability (*i.e.* the fraction of the ingested dose which is absorbed and exerts its biological activity after digestion). Several enzymes are involved in the lipid digestion, being gastric and pancreatic lipase the most important. Both of them are specific towards external carbons (position sn-1 and sn-3) and hydrolyze triacylglycerides (TAG) to 2-monoacylglycerides (2-MAG), (*i.e.* the fatty acid is bonded in the central position) and free fatty acids. PUFA located in the central bond of the glycerol backbone (sn-2 position) present much better absorption than those released as free fatty acids which precipitate in the intestine. Hence, the human digestion and metabolism of lipids present different efficiency depending on the position of the fatty acid within the glycerol backbone; being those located in sn-2 position more easily absorbed (Koletzko, Shamir, & Phillip, 2014). Therefore, during last decades, there is a growing interest in the production of structured lipids, where DHA and EPA are located in the sn-2 and short chain fatty acids in sn-1(3).

Due to the regioselectivity of lipases involved in human digestion, the characterization of the relative lipid profile of fatty acids occupying sn-2 bond should be considered as a useful tool aiming at selecting the most appropriate up-grading technique. Those fish oils with high content of PUFA in sn-2 might be considered as a source for the production of 2-MAG which can be enriched in PUFA by physical methods as low temperature fractionation (Wang, Li, Wang, Jin, & Wang, 2014). Monoacylglycerols and diacylglycerols account around the 75% of the total production of emulsifiers (Li, Du, Li, Li, & Liu, 2010), their applications and production technique has been recently reviewed (Feltes, de Oliveira, Block, & Ninow, 2013). Moreover, 2-MAG with a high content of PUFA can be esterified aiming to produce structured lipids with a medium chain fatty acids in the sn-1(3) bonds (Munio, Robles, Esteban, Gonzalez, & Molina, 2009). These structured lipids present a faster absorption than the original oil and their daily intake

might result in a less accumulation of fats. Additionally, due to the role that DHA plays on the development of brain and eye of infant (Ruxton et al., 2007), they are being employed for the production of ready-to-feed infant formula.

Seasonal variations of the proximal composition and the lipid profile have been previously described for several species (sardine, bogue, horse mackerel, small-spotted catshark, axillary seabream) (Esquível et al., 1997; García Moreno et al., 2013; Orban et al., 2011). Additionally, the nutritional value of these oils has been evaluated employing indexes as the thrombogenic (TI) or the atherogenicity (AI) ones (Dal Bosco, Mugnai, Mourvaki, & Castellini, 2012; Santos-Silva, Bessa, & Santos-Silva, 2002; Šimat, Bogdanović, Poljak, & Petričević, 2015; Ulbricht & Southgate, 1991). The regioselectivity of fatty acids in fish oils was firstly described by Brockerhoff, Hoyle, Hwang, & Litchfield (1968) in a study which aimed to globally describe the lipid of marine sources. During last decades, the regiodistribution of fish oils have been analyzed as an initial characterization of the oils prior to the production of structured lipids (Munio et al., 2009). However, no systematically study of the seasonal variations of the regiodistribution of the fatty acids of oils extracted from bogue and small-spotted catshark has been yet described in the literature.

The aim of this study was to evaluate the seasonal variations of the nutraceutical quality of oil extracted from small-spotted catshark (*Scyliorhinus canicula*) and bogue (*Boops boops*). To this end, proximate composition, lipid profile and fatty acid regiodistribution were analyzed during the year. This characterization is the first approach to the selection of the most adequate technique for the up-grading of these oils.

2. MATERIALS AND METHODS

2.1. Raw materials

Fish samples from small-spotted catshark (*Scyliorhinus canicula*) and bogue (*Boops boops*) were supplied every season by the fishing harbor of Motril (Spain). Both species are discarded in Alboran Sea due to their low commercial value. They were kept in ice during transportation and pressed the same day to avoid microbial spoilage. Three individuals were chosen for the somatometric measurements shown in Table 1.

Table 1. Seasonal somatometric data of discarded species of the Alboran Sea: means ± standard deviation

	Autumn		Winter		Spring		Summer	
	weight, g	size, cm	weight, g	size, cm	weight, g	size, cm	weight, g	size, cm
Small-spotted catshark	218.4±56.6	38.2±4.1	230.3±39.3	40.1±0.8	281.8±43.8	43.8±1.9	253.4±34.7	41.0±1.0
Bogue	78.7±2.6	15.0±0.1	93.8±4.8	21.3±0.8	84.0±12.4	20.7±1.5	86.2±10.4	21.0±2.0

2.2. Proximate composition and oil extraction

The samples were analyzed for their proximate composition according to the official methods recognized by the Association of Official Analytical Chemist, (2006). Fish oil was extracted by hydraulic pressing, according to the method described elsewhere (García Moreno et al., 2013). To this end, two kilograms of whole fish were immersed in a water bath at 40ºC for 30 min. The preheated material was then fed to a hydraulic press (model ESP-K, Sanahuja, Spain), where it was pressed stepwise until attaining a final pressure of 120 bar. The press liquor released from the press chamber was collected and centrifuged at 20,000g, from which the upper oily phase was recovered. The analysis were done in duplicate.

2.3. Fatty acid profile and lipid composition

Oil samples were converted into fatty acid methyl esters prior to their analysis. To that end, methylation was conducted following the method described by Rodríguez-Ruiz, Belarbi, Sánchez, & Alonso, (1998) with minor variations. Firstly, a solution of oil in hexane (1 mg/mL) was prepared. An aliquot of 1 mL was extracted and mixed with 1 mL of the freshly prepared transesterification reagent (methanol/acetyl chloride, 20:1, v/v) and 50 µL of standard solution of nonadecanoic acid (Sigma Aldrich) in hexane (2 mg/mL). Then, samples were heated at 90ºC for 1 hour, being shaken every 15 min. After methylation, 1 mL of distilled water was added and the organic phase was manually extracted.

Fatty acid methyl esters were analyzed according to Camacho Paez, Robles Medina, Camacho Rubio, González Moreno, & Molina Grima (2002) by means of a chromatograph (Agilent 7890A, Agilent Technologies S.A.) equipped with a capillary column of fused silica Omegawax (0.25 mm × 30 m, 0.25 µm standard film; Supelco, Bellefonte, PA). Results were reported as the average value of three replicates.

The lipid sample was fractionated into monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG) by thin layer chromatography. To this end, 2 mg of oil were

spotted on silica-gel plates (Precoated TLC plates, SIL G-25; Macherey-Nagel, Sigma–Aldrich). A mobile phase consisting of a mixture of chloroform/acetone/methanol (95:4.5:0.5, v/v/v) was employed to separate the different lipid species. After separation, each fraction was recovered and methylated as described before.

2.4. Oxidative and nutritional indices

Fatty acid content was referred to the mass of fish by means of a conversion factor as described by Weihrauch, Posati, Anderson, & Exler, (1977). Furthermore, the intrinsic peroxidability index (PI, %) was computed for all samples according to Arakawa & Sagai, (1986).

Subsequently, the lipid profile was employed to estimate the indices of atherogenicity (AI), thrombogenicty (TI) (Ulbricht & Southgate, 1991) and the hypocholesterolaemic/ hypercholesterolaemic ratio (HH) (Santos-Silva et al., 2002).

2.5. Determination of the positional distribution of fatty acids in TAG

An ethanolysis with the lipase Novozym 435 from *Candida antarctica* was conducted to study the regiodistribution of fatty acids in the TAG, adapted from the method described by Shimada et al (2003). By this approach, all the monoacylglycerols produced are esterified in the second position (2-MAG) so they can be easily separated by thin layer chromatography, as previously described.

The percentage of a given fatty acid in sn-2 position was related to the total content of that fatty acid as follows:

$$\%\mathrm{FA_i}\ \text{in sn2 position} = \frac{\text{content of } \mathrm{FA_i}\ \text{in sn2 position}}{3 \cdot \text{total content of } \mathrm{FA_i}\ \text{in TAG}} \cdot 100 \qquad [1]$$

The total percentage of each fatty acid located in sn-2 was calculated by multiplying the aforementioned percentage by the global fatty acid percentage, both in molar basis.

2.6. Statistical analysis

Data were presented as an average value ± standard deviation. Additionally, a coefficient of variation, defined as the ratio between standard deviation and mean value, was chosen to evaluate the seasonal variations among each species.

3. RESULTS AND DISCUSSION

3.1. Proximate composition

The seasonal proximate composition of both species is shown in Table 2. The ash content remained practically constant along the year with an average value of 3.34±0.55wt%. Similarly, the protein content did not deeply vary throughout the year (average value: 19.34 ± 2.29 wt%) being the percentage of small-spotted catshark higher than that of bogue in all seasons (20.6 ± 1.9 and 18.1 ± 2.0 wt%, respectively). This difference might be related to the high level of non-protein nitrogen compounds (*i.e.* ammonia, trimethylamine oxide or urea) which are presented in elasmobranchs species (Økland, Stoknes, Remme, Kjerstad, & Synnes, 2005). Protein content was similar to the values previously described in the literature (García Moreno et al., 2013; Orban et al., 2011; Šimat et al., 2015).

Table 2. Seasonal proximate compositions of discarded species of the Alboran Sea.

[wt, %]	Small-spotted catshark				Bogue			
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
Moisture	75.5	75.5	76.2	76.2	70.4	73.2	78.3	77.8
Ash	2.8	2.9	2.7	3.2	4.2	3.5	4.0	3.4
Protein	18.1	21.5	20.3	22.6	15.8	20.0	16.9	19.5
Lipid	1.8	1.8	2.0	2.2	6.1	5.4	1.0	1.1

Moisture and lipid content showed the highest seasonal variations in the case of bogue (average: 74.9 ± 3.8 and 3.4 ± 2.7 wt%, respectively) whereas for small-spotted catshark these values remained relatively constant (average: 75.9 ± 0.4 and 2.0 ± 0.9 wt%, respectively) (Table 2). For bogue, the fat content correlated inversely with water content (r^2 = -0.958), trend which has been described for a wide group of fishes (García Moreno et al., 2013; Orban et al., 2011). Contrary, small-spotted catshark presented a direct correlation (r^2 = 0.818). Taking into account Ackman's classification for fish species regarding their lipid content, bogue is considered a semi-fatty fish (<8 wt%) while small-spotted catshark belongs to lean fish category (Ackman, 1989). Moreover, these species

store lipids in different sites being the liver the main location for the small-spotted catshark (Vannuccini, 1999) and muscles and/or subcutaneous depots in the case of bogue.

The variations of lipid content among species and seasons are related to feed intake, spawning period or migratory habits (Brockerhoff et al., 1968). It is a common behavior that the minimum content of lipid coincides with the end of the spawning period, because lipids are employed as the main energy source (Huss, 1995). The reproductive behavior of species located in the Alboran Sea has been studied, being the spawning season of bogue spring while small-spotted catshark has a wider range: from November to July (Pérez-Martín, 2001). Bogue has considerable variations of the lipid content thorough the year (Table 2), achieving the maximum and minimum content in autumn (6.0 wt%) and spring (1.0 wt%) respectively. The maximum content is similar to that reported by Prato & Biandolino, (2012) and higher than that described by García Moreno et al (2013). In the case of small-spotted catshark, the lipid content remained practically constant along the year with an average value of 1.9 ± 0.2 wt% (Table 2), datum which agrees with previous works of this group (García Moreno et al., 2013).

3.2. Fatty acids profile and nutritional indices

Fatty acid profile of fish oil depends on a number of factors: reproductive status, age, species, sex or food availability (Brockerhoff et al., 1968). Among polar fractions, TAG was the only group detected by thin layer chromatography, hence the global lipid profile corresponds uniquely to that fraction.

Table 3 summarizes the fatty acid profile mass distribution during the year. PUFA fraction was the most abundant one (34.4 to 47.1 wt%) in both species, followed by saturated fatty acids (20.8 to 31.8 wt%) in the case on bogue and by monounsaturated fatty acids (22.1 to 20.5 wt%) in the case of small-spotted catshark. Main fatty acids of saturated, monounsaturated and polyunsaturated fatty acids were palmitic (C16:0), oleic (C18:1n-9) and docosahexanoic acid (C22:6n-3) accounting each one more than 60 wt% of their respective fraction. EPA was the second most abundant PUFA: representing a 16.9±1.5 and 21.0±1.2 wt% of the total PUFA for small-spotted catshark and bogue respectively.

Table 3. (A) Small-spotted catshark Seasonal fatty acid profiles (weight %) of oils extracted from discarded species of the Alboran Sea. Data are means of triplicate determinations. SD < 5%. Average values within a row followed by different letter mean significant differences (p<0.05).

	Small-spotted catshark					
	Autumn	**Winter**	**Spring**	**Summer**	**Average**	**CV%**
C14:0	1.9	5.0	2.0	1.7	2.7	59.3
C16:0	15.1	17.0	19.1	16.3	16.9	9.9
C16:1n-7	4.7	6.4	5.5	5.2	5.5	13.1
C16:2n-4	0.7	1.0	0.8	0.7	0.8	17.7
C16:3n-4	0.6	0.7	0.0	0.6	0.5	67.4
C16:4n-1	0.0	0.0	0.0	0.5	0.1	200.0
C18:0	3.8	5.4	4.3	3.9	4.4	16.8
C18:1n-7	3.9	2.7	4.3	4.1	3.8	19.2
C18:1n-9	14.6	13.1	18.3	18.5	16.1	16.7
C18:2n-6	1.5	1.5	1.0	1.0	1.3	23.1
C18:3n-3	0.7	1.3	0.0	0.5	0.6	86.0
C18:4n-3	0.2	0.9	0.0	0.5	0.4	97.9
C20:1n-9	2.4	2.3	2.9	2.9	2.6	12.2
C20:3n-6	0.5	1.1	0.0	1.3	0.7	81.5
C20:4n-3	0.6	0.9	0.0	0.7	0.6	70.4
C20:5n-3	7.2^{B}	7.8^{C}	7.5^{C}	6.8^{A}	7.3	5.8
C22:1n-9	1.5	0.6	1.3	1.6	1.3	36.1
C22:5n-3	3.3	2.4	2.9	2.8	2.9	13.0
C22:6n-3	31.2^{F}	25.6^{C}	26.2^{D}	27.1^{E}	27.5	9.2
Others	5.8	4.3	3.6	3.4	4.3	25.4
SFA	20.8^{A}	27.3^{C}	25.5^{B}	21.8^{EF}	23.9	12.8
MUFA	27.1^{F}	23.8^{G}	32.4^{H}	32.4^{H}	28.9	14.6
PUFA	46.4^{D}	44.5^{E}	38.5^{F}	42.4^{G}	43.0	7.9
n-6	2.0	2.5	1.0	2.3	2.0	34.1
n-3	43.1	40.3	36.7	38.3	39.6	7.0
n-3/n-6	21.6	15.8	36.1	16.4	22.5	42.0
EPA+DHA	38.4	33.4	33.8	33.9	34.9	6.8

Table 3. (B) Bogue Seasonal fatty acid profiles (weight %) of oils extracted from discarded species of the Alboran Sea. Data are means of triplicate determinations. SD < 5%. Average values within a row followed by different letter mean significant differences ($p<0.05$).

	Bogue					
	Autumn	**Winter**	**Spring**	**Summer**	**Average**	**CV%**
C14:0	5.1	4.9	5.9	5.9	5.5	9.7
C16:0	19.3	16.9	19.5	15.2	17.7	11.6
C16:1n-7	6.6	6.4	6.3	6.7	6.5	2.8
C16:2n-4	1.1	1.0	1.2	1.6	1.2	21.5
C16:3n-4	0.0	0.2	0.0	0.9	0.3	155.3
C16:4n-1	0.0	0.0	0.0	0.3	0.1	200.0
C18:0	5.8	5.3	6.4	5.6	5.8	8.0
C18:1n-7	2.8	2.7	2.5	2.7	2.7	4.7
C18:1n-9	14.4	13.0	14.5	13.1	13.8	5.9
C18:2n-6	1.6	1.5	1.4	1.3	1.5	8.9
C18:3n-3	2.3	1.3	0.9	0.7	1.3	54.8
C18:4n-3	0.0	0.9	1.8	1.6	1.1	75.7
C20:1n-9	1.2	2.3	2.1	2.0	1.9	25.4
C20:3n-6	0.0	0.0	0.0	1.1	0.3	200.0
C20:4n-3	0.0	0.9	0.8	0.7	0.6	68.0
C20:5n-3	8.2^{D}	8.4^{DE}	7.8^{C}	8.6^{E}	8.3	4.1
C22:1n-9	0.0	0.0	0.8	1.2	0.5	120.0
C22:5n-3	2.4	2.4	2.6	3.3	2.7	16.0
C22:6n-3	25.2^{B}	25.5^{A}	18.0^{B}	20.4^{B}	22.3	16.5
Others	3.8	6.4	7.6	6.9	6.2	26.9
SFA	30.3^{D}	27.2^{G}	31.8^{DE}	26.7^{H}	29.0	8.5
MUFA	25.0^{A}	23.0^{BC}	26.2^{D}	25.7^{CE}	25.0	5.6
PUFA	41.0^{A}	43.5^{B}	34.4^{C}	40.7^{C}	39.9	9.7
n-6	1.6	1.5	1.4	2.4	1.7	26.5
n-3	38.2	40.8	31.8	35.4	36.6	10.6
n-3/n-6	23.3	28.1	22.5	14.7	22.2	25.0
EPA+DHA	33.4	33.8	25.8	29.1	30.5	12.5

Table 4.(A) Small spotted-catshark seasonal fatty acids profiles (g/100g fish). Nutritional indexes and oxidative status of oils extracted from discarded species of the Alboran Sea. Average values within a row followed by different letter mean significant differences ($p<0.05$).

	Small-spotted catshark					
	Autumn	**Winter**	**Spring**	**Summer**	**Average**	**CV%**
SFA	0.46^{C}	0.24^{A}	0.61^{D}	0.36^{B}	0.41	37.24
MUFA	0.59^{E}	0.21^{F}	0.77^{G}	0.53^{H}	0.53	44.48
PUFA	1.02^{E}	0.39^{B}	0.91^{D}	0.69^{C}	0.75	36.59
EPA	0.16^{F}	0.07^{BCE}	0.18^{G}	0.11^{H}	0.13	38.25
DHA	0.68^{E}	0.23^{F}	0.62^{G}	0.44^{H}	0.49	41.66
EPA+DHA	0.84	0.30	0.80	0.55	0.62	40.67
PI/100	3.22^{E}	2.88^{D}	2.75^{C}	2.88^{D}	2.93	6.86
AI	0.26^{B}	0.46^{D}	0.31^{C}	0.25^{A}	0.32	30.42
TI	0.12^{A}	0.17^{E}	0.15^{C}	0.14^{B}	0.15	15.62
HH	3.43^{H}	2.35^{D}	2.65^{F}	3.16^{G}	2.90	16.86
SFA	0.46^{C}	0.24^{A}	0.61^{D}	0.36^{B}	0.41	37.24
MUFA	0.59^{E}	0.21^{F}	0.77^{G}	0.53^{H}	0.53	44.48
PUFA	1.02^{E}	0.39^{B}	0.91^{D}	0.69^{C}	0.75	36.59

Table 4.(B) Bogue seasonal fatty acids profiles (g/100g fish). Nutritional indexes and oxidative status of oils extracted from discarded species of the Alboran Sea. Average values within a row followed by different letter mean significant differences ($p<0.05$).

	Bogue					
	Autumn	**Winter**	**Spring**	**Summer**	**Average**	**CV%**
SFA	1.67^{E}	1.33^{A}	0.26^{F}	0.24^{A}	0.87	83.86
MUFA	1.37^{A}	1.13^{B}	0.21^{C}	0.23^{D}	0.74	81.65
PUFA	2.25^{F}	2.13^{A}	0.28^{G}	0.37^{B}	1.26	85.86
EPA	0.45^{A}	0.41^{BC}	0.06^{D}	0.08^{CE}	0.25	83.26
DHA	1.39^{A}	1.25^{B}	0.15^{C}	0.18^{D}	0.74	90.00
EPA+DHA	1.84	1.66	0.21	0.26	0.99	88.30
PI/100	2.74^{D}	2.88^{A}	2.21^{C}	2.55^{B}	2.59	11.06
AI	0.50^{D}	0.47^{F}	0.58^{E}	0.50^{E}	0.51	9.34
TI	0.19^{D}	0.16^{G}	0.23^{F}	0.19^{F}	0.19	14.61
HH	2.22^{E}	2.39^{A}	1.78^{B}	2.25^{C}	2.16	12.23
SFA	1.67^{E}	1.33^{A}	0.26^{F}	0.24^{A}	0.87	83.86
MUFA	1.37^{A}	1.13^{B}	0.21^{C}	0.23^{D}	0.74	81.65
PUFA	2.25^{F}	2.13^{A}	0.28^{G}	0.37^{B}	1.26	85.86

For small-spotted catshark, MUFA showed the highest CV (14.6%) followed by SFA (12.8%) while for bogue the major variations happened in the PUFA fraction (CV 9.7 wt%). A negative correlation was found between the percentage of SFA and the fat content for bogue but no correlation was found for small-spotted catshark.

From a nutritional point of view, the proportion n-3/n-6 (*i.e.* Omega-3/Omega-6) could be regarded as an index referring the quality of the oil. These groups present opposite behaviors being Omega-3 anti-inflammatory and anti-aggregatory (Fedačko et al., 2007). EPA and arachidonic acid (C20:4n-6) might compete for some enzymes as cyclooxygenase or lipoxygenase for the production of eicosanoids. Although the recommended n-3:n-6 ratio is 1:2-4, the average real intake in western diet is 1:25 (Simopoulos, 2002). In the studied oils, the Omega-3 PUFA content was much higher than the Omega-6 PUFA one, resulting in ratios varying from 14.7 to 43.1. Hence, the consumption of these oils could balance the excess of n-6 in human diets. Additionally, it has been reported that n-3:n-6 ratios higher than 3.5 might reduce cholesterol levels and improve the plasma lipid profile (Riediger et al., 2008). The differences observed among species and seasons could be related to the diet habit (Brockerhoff et al., 1968). Effectively, small-spotted catshark has a diet based mainly on crustaceans, decapods, fishes and mollusks while bogue is herbivorous (Pérez-Martín, 2001).

In Table 4, it is shown the composition of fatty acids in g/100g fish basis. The influence of seasonality is noticeably higher than in the global profile due to the influence of the fluctuations of the lipid content during the year.

As a result of the high content of PUFA, the current oils are extremely prone to oxidation and, consequently to spoilage. Peroxidability index (PI), is an intrinsic indicator of the tendency of oils to be oxidized. PI values ranged from 221 and 322%, these high values were closely related to the content of PUFA and, more specifically, DHA which was the most unsaturated fatty acid.

Three nutritional indexes (AI, TI and HH ratio) were estimated so as to quantify the quality of the oil. Thrombosis and atherosclerosis are closely related to coronary heart diseases. It has been reported that SFA promote cardiovascular diseases while PUFA and MUFA play a protective role (Ulbricht & Southgate, 1991). In this sense, the studied fish oils showed AI and TI minor than 1 (Table 4), and, hence, they can be described as healthy (Subhadra, Lochmann, Rawles, & Chen, 2006). Averages AI values were 0.32±0.1 and 0.51±0.05 for

small-spotted catshark and bogue respectively, the lower values of small-spotted catshark are related to the higher content of DHA. On the other hand, both species presented similar values of TI, being the average value 0.17±0.03. The current data are in the same range as the values estimated for goldfish (Dal Bosco et al., 2012); moreover the current values were slightly lower than those described for bogue by Šimat et al.,(2015). Additionally, AI and TI values were lower than those calculated for lamb, beef, pork or palm oil (Ulbricht & Southgate, 1991). HH ratio is a parameter corresponding to the coefficient between the total percentage of hypocholesterolemic and hypercholesterolemic fatty acids. From a nutritional point of view, higher HH values are considered more beneficial for the human health. The values of the hypocholesterolaemic/ hypercholesterolaemic values ranged from 1.78 (bogue, in spring) to 3.43 (small-spotted catshark, in autumn), data which are similar to those reported for black needle or mackerel (Fernandes et al., 2014). The values of these three nutritional indexes show the optimal nutritional quality of oils extracted from bogue and small-spotted catshark.

3.3. Regiospecific distribution of fatty acids in TAG

Table 5 shows the mass profile of the 2-MAG produced after the specific alcoholysis. These data were further employed, together with the global profile, for the calculation of the regioselectivity of fatty acids (Eq. 1). In both species, the CV was higher in the case of the sn-2 position than in the global profile.

Table 5. Seasonal composition of fatty acids in sn-2 position (% of total fatty acid weight) of oils extracted from discarded species of the Alboran Sea. Data are means of triplicate determination being SD <5%. For each specie values within a row with different superscript letters indicate significant differences ($p<0.05$).

	Small-spotted catshark					Bogue			
	Aut.	Win.	Spr.	Sum.	CV. %	Aut.	Win.	Spr.	CV. %
EPA	3.6^{C}	4.2^{F}	3.0^{AB}	2.7^{A}	19.6	3.2^{B}	3.9^{D}	4.5^{F}	16.5
DHA	43.1^{B}	43.1^{B}	63.4^{F}	55.8^{E}	19.5	48.1^{C}	36.9^{A}	51.2^{D}	16.5
SFA	18.5^{B}	20.9^{C}	12.9^{A}	14.4^{A}	22.1	22.7^{D}	31.8^{E}	22.5^{D}	20.7
MUFA	20.1^{CD}	20.8^{D}	16.0^{B}	18.9^{C}	11.2	10.6^{A}	16.0^{B}	11.7^{A}	22.6
PUFA	56.2^{B}	57.5^{B}	71.1^{E}	65.9^{D}	11.3	60.9^{C}	50.6^{A}	65.8^{D}	13.2

Furthermore, in Table 6 it is shown for the main fatty acids and fractions: (i) the percentage with respect to the total oil content which is located in sn-2 (marked with symbol α) and (ii) the relative composition of the central bond (symbol β). First calculations follow the stoichiometric proportion, being their maximum value 33.3 mol%

Due to the specificity of human lipases enzymes, the relative composition of the sn-2 position might be considered as the most effective amount of PUFA which will be properly metabolized and it should be considered when evaluating the nutritional value of these oils.

Table 6. Seasonal composition (%, molar basis) of main fatty acids and fractions at sn-2 position. Relative composition of the fractions at sn-2 position. Data are means of triplicate determination with SD <5%.

	Small-spotted catshark						Bogue[1]				
	Aut.	Win.	Spr.	Sum.	Aver.	CV, %	Aut.	Win.	Spr.	Aver.	CV.%
C16:0$^{\alpha}$	4.8	6.4	4.4	4.6	5.1	18.1	6.2	7.5	5.9	6.5	13.0
C18:1n-9$^{\alpha}$	3.1	3.2	0.9	4.0	2.8	47.5	1.2	1.9	1.8	1.6	23.2
C20:5n-3$^{\alpha}$	1.1	1.4	1.0	0.9	1.1	19.6	1.1	1.3	1.4	1.3	12.1
C22:6n-3$^{\alpha}$	17.0	13.0	19.7	17.3	16.8	16.6	15.2	10.8	15.3	13.8	18.7
SFA$^{\alpha}$	6.3	8.6	5.2	5.8	6.5	23.0	9.5	12.0	8.9	10.1	16.2
MUFA$^{\alpha}$	6.2	7.8	5.9	6.9	6.7	12.6	4.1	5.6	4.3	4.7	17.5
PUFA$^{\alpha}$	21.0	17.3	22.3	20.7	20.3	10.5	19.8	15.8	20.1	18.6	12.9
	Relative percentage at sn-2										
SFA$^{\beta}$	18.9	25.4	15.5	17.3	19.3	22.4	28.4	36.0	26.7	30.4	16.3
MUFA$^{\beta}$	18.5	23.1	17.7	20.7	20.0	12.1	12.3	16.7	12.9	14.0	17.1
PUFA$^{\beta}$	62.6	51.4	66.8	62.0	60.7	10.8	59.3	47.3	60.4	55.7	13.1
DHA$^{\beta}$	51.1	38.9	59.1	51.8	50.2	16.7	45.7	32.4	46.0	41.4	18.8
DHA + EPA$^{\beta}$	54.4	43.0	62.1	54.5	53.5	14.7	49.1	36.2	50.3	45.2	17.3

[1]Data of the sn-2 profile are not available for summer. $^{\alpha}$ Data are the product of percentage of fatty acids located at sn- 2 position and the percentage of the fatty acid in the total profile divided by 100. Maximum value 33.3%. $^{\beta}$ Relative data are calculated by dividing data calculated in β by 33.33 and multiplying by 100. These data refer to the composition of sn-2 expressed as mole percentage.

Small-spotted catshark and bogue contained an average content of PUFA in the central position of 60.7 ± 6.6 and 55.7 ± 7.3 mol% respectively. In both cases this value was much higher than the global one (40.8 ± 3.9 and 39.8 ± 3.6 mol%). Thus, the total amount of PUFA presented high regiospecifity towards the central position. On the other hand, MUFA presented the opposite behavior, being 1(3)-specific. The regioselectivity of SFA differed between species: in the case of bogue the global and sn-2 relative lipid profile were similar (32.9±2.7 and 30.4±5.0mol% respectively), which implies the absence of regioselectivity. However, as for small-spotted catshark, this fraction presented selectivity towards 1(3) bonds.

DHA showed a high 2-regiospecificity with relative average values of 50.2 ± 8.4 (small-spotted catshark) and 41.4 ± 7.8 mol% (bogue). The percentage of the total DHA in the central bond varied from ~50 mol% (winter) to ~80 mol% (spring). Palmitic was the second most abundant fatty acid of the central position. In the case of bogue, it presented 2-regiospecifity with an average content of 49.9 ± 10.8 mol%. However, for small-spotted catshark, this percentage was closed to the stoichiometric one (33.2 ± 8.4 mol%), and no specificity to any bond of the glycerol backbone was showed. In the case of EPA and oleic acid, they presented 1(3) specificity, with only a ~15mol% of their global amount situated in sn-2.

These oils presented a high content of PUFA in the central position of the glycerol backbone, being the percentage of DHA greater than 75% during the whole year. Hence, concentrations techniques which preserve the fatty acid esterified in the central position should be employed, as for instance: alcoholysis, hydrolysis or acidolysis. Alcoholysis and hydrolysis could be employed for the production of 2-MAG which might be lately esterified so as to produce structured lipids. Acidolysis, a one-step process, could be considered as one of the simplest techniques for the production of structured lipids; however PUFA located in positions 1(3) are resistant to be displaced by medium-chain fatty acids resulting in a decrease of the yield of the desired structured lipids (Soumanou, Bornscheuer, Menge, & Schmid, 1997). Acyl migration is one of the main difficulties of the 2-MAG production; this non-desired process can be minimized by selecting the most suitable solvent, immobilization carrier and enzyme. Munio et al (2009), by combining alcoholysis and esterification, produced 63% of 2-MAG a high yield of recovery (90%). The global production of structured lipids yielded 80% and no acyl-migration was detected. Additionally, 2-MAG were synthesized by enzymatic ethanolysis employing Novozym 435 (Wang et al., 2014). After solvent fractionation they produced 2-MAG with a purity of 99% and no acyl-migration was detected. As a concentration step, the 2-MAG were crystallized in hexane resulting in an 80% of PUFA, which represented a yield of 50%. Munio et al.,(2009) produced structured lipids from cod liver and tuna oil, whose global DHA content was much lower than the oils studied in this work. Moreover, the proportion of the DHA located in the sn-2 bond was a half less than that obtained in the present study. On the other hand, Wang et al.,(2014) employed randomized arachidonic acid-rich oil with 46.2% of arachidonic acid and described that the main drawback of their research was the loss of 66.7% of the target PUFA. Based on the results of these studies, it could be a good approach to conduct an alcoholysis of hydrolysis of the small-spotted catshark or bogue oils followed by isolation of MAG and a concentration step. Due to the high relative content of DHA in the central bond which is followed by palmitic and oleic acid, the efficiency and yield of concentration of DHA 2-MAG should be higher than those referred in the literature. However, since the EPA is mainly bonded in 1(3) positions, the remaining free fatty acids or esters might content a considerable percentage of EPA.

4. CONCLUSIONS

The nutritional quality of these oils has been proved not only with nutritional indexes (AI and TI < 1 and HH ratio >1.5) and global profile (PUFA content > 35 wt%) but also by measuring the relative composition of the fatty acids esterified in the sn-2 position (PUFA content >47mol%). In the case of small spotted catshark the lipid content did not deeply vary throughout the year and PUFA percentage presented CV of 7.9 (global profile) and 10.8 (sn-2 relative profile). For bogue, the amount of lipid varied more dramatically from 1.0 to 6.1wt%, however, the PUFA content in both, global and relative sn-2, was similar to those obtained for small-spotted catshark (9.7 and 13.1 respectively).

The study of the regiodistribution of the fatty acids might be considered a useful tool prior to the selection of the up-grading technique. Regarding the relative sn-2 profile, these oils might be considered as a raw source for the production of 2-MAG by means of alcoholysis or hydrolysis where the acylmigration should be minimized. Since the DHA is the most abundant PUFA (>80%), physical concentration as low temperature crystallization might be a good technique to produce DHA 2-MAG with high purity.

5. REFERENCES

A.O.A.C. (Association of Official Analytical Chemist). (2006). Official Methods of Analysis of the AOAC, Association of Official Analytical Chemists,. Washington DC.

Ackman, R. G. (1989). Nutritional composition of fats in seafoods. Progress in Food and Nutrition Science.

Arakawa, K., & Sagai, M. (1986). Species differences in lipid peroxide levels in lung tissue and investigation of their determining factors. Lipids, 21(12), 769–775. http://doi.org/10.1007/BF02535410

Blanco, M., Sotelo, C. G., Chapela, M. J., & Pérez-Martín, R. I. (2007). Towards sustainable and efficient use of fishery resources: present and future trends. *Trends in Food Science & Technology Technology*, 18(1), 29–36. http://doi.org/10.1016/j.tifs.2006.07.015

Brockerhoff, H., Hoyle, R. J., Hwang, P. C., & Litchfield, C. (1968). Positional distribution of fatty acids in depot triglycerides of aquatic animals. *Lipids*, 3(1), 24–9. http://doi.org/10.1007/BF02530964

Camacho Paez, B., Robles Medina, A., Camacho Rubio, F., González Moreno, P., & Molina Grima, E. (2002). Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by the acidolysis of cod liver oil and caprylic acid in a packed-bed reactor: Equilibrium and kinetics. *Chemical Engineering Science*, 57(8), 1237–1249.

Dal Bosco, A., Mugnai, C., Mourvaki, E., & Castellini, C. (2012). Seasonal changes in the fillet fatty acid profile and nutritional characteristics of wild Trasimeno Lake goldfish (*Carassius auratus*). *Food Chemistry*, 132(2), 830–834. http://doi.org/10.1016/j.foodchem.2011.11.043

Esquível, M. M., Bandarra, N. M., Fontan, I., Bernardo-Gil, M. G., Batista, I., Nunes, M. L., & Empis, J. A. (1997). Supercritical carbon dioxide extraction of sardine *Sardina pilchardus* oil. *LWT - Food Science and Technology*, 30(7), 715–720.

EU. (2013). Regulation (EU) nº 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Official Journal of the European Union, 22–28.

Fedačko, J., Pella, D., Mechírová, V., Horvath, P., Rybár, R., Varjassyová, P., & Vargová, V. (2007). n - 3 PUFAs-From dietary supplements to medicines. *Pathophysiology*, 14(2), 127–132. http://doi.org/10.1016/j.pathophys.2007.04.001

Feltes, M. M. C., de Oliveira, D., Block, J. M., & Ninow, J. L. (2013). The Production, Benefits, and Applications of Monoacylglycerols and Diacylglycerols of Nutritional Interest. *Food and Bioprocess Technology*, 6(1), 17–35.

Fernandes, C. E., Vasconcelos, M. A. da S., Ribeiro, M. de A., Sarubbo, L. A., Andrade, S. A. C., & Filho, A. B. de M. (2014). Nutritional and lipid profiles in marine fish species from Brazil. *Food Chemistry*, 160, 67–71. http://doi.org/10.1016/j.foodchem.2014.03.055

FROM-Fondo de Regulación y Organización del Mercado de los Productos de la Pesca y los, & cultivos Marinos. (2008). Asistencia técnica para la realización de una medida innovadora para la recuperación, gestión y valorización de los descartes generados por la flota española que faena en los caladeros atlántico y mediterráneo.

García Moreno, P. J., Pérez-Gálvez, R., Morales-Medina, R., Guadix, A., Guadix, E. M., (2013). Discarded species in the west Mediterranean Sea as sources of Omega-3 PUFA. European *Journal of Lipid Science and Technology*, 115(9), 982–989. http://doi.org/10.1002 ejlt.201300021

Huss, H. H. (1995). Quality and quality changes in fresh fish. FAO Fisheries Technical Paper.

Kelleher, K. (2005). Discards in the world's marine fisheries: An update. FAO. Fisheries Technical Paper.

Koletzko, B., Shamir, R., & Phillip, M. (2014). World Review of Nutrition and Dietetics. Nutrition and growth. Preface. World Review of Nutrition and Dietetics, 109.

Li, W., Du, W., Li, Q., Li, R., & Liu, D. (2010). Dependence on the properties of organic solvent: study on acyl migration kinetics of partial glycerides. *Bioresource Technology*, 101(15), 5737–42. http://doi.org/10.1016/j.biortech.2010.03.018

Munio, M. del M., Robles, A., Esteban, L., Gonzalez, P. A., & Molina, E. (2009). Synthesis of structured lipids by two enzymatic steps: Ethanolysis of fish oils and esterification of 2-monoacylglycerols. *Process Biochem.*, 44, 723–730. http://doi.org/10.1016/j.procbio. 2009.03.002

Økland, H. M. W., Stoknes, I. S., Remme, J. F., Kjerstad, M., & Synnes, M. (2005). Proximate composition, fatty acid and lipid class composition of the muscle from deep-sea teleosts and elasmobranchs. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 140(3), 437–443. http://doi.org/10.1016/j.cbpc.2004.11.008

Orban, E., Di Lena, G., Nevigato, T., Masci, M., Casini, I., & Caproni, R. (2011). Proximate, unsaponifiable lipid and fatty acid composition of bogue (*Boops boops*) and horse mackerel (*Trachurus trachurus*) from the Italian trawl fishery. *Journal of Food Composition and Analysis*, 24(8), 1110–1116. http://doi.org/10.1016/j.jfca.2011.03.009

Pérez-Martín, M. (2001). Especies de Interés Pesquero en el Litoral de Andalucía. (Junta de Andalucía. Consejería de Agricultura, Ed.). Sevilla: Viceconsejería. Servicio de Publicaciones y Divulgacion.

Prato, E., & Biandolino, F. (2012). Total lipid content and fatty acid composition of commercially important fish species from the Mediterranean, Mar Grande Sea. *Food Chemistry*, 131(4), 1233–1239. Journal Article. http://doi.org/10.1016/j.foodchem.2011.09.110

Riediger, N. D., Othman, R., Fitz, E., Pierce, G. N., Suh, M., & Moghadasian, M. H. (2008). Low n-6:n-3 fatty acid ratio, with fish- or flaxseed oil, in a high fat diet improves plasma lipids and beneficially alters tissue fatty acid composition in mice. *European Journal of Nutrition*, 47(3), 153–160. http://doi.org/10.1007/s00394-008-0709-8

Rodríguez-Ruiz, J., Belarbi, E.-H. H., Sánchez, J. L. G., & Alonso, D. L. (1998). Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotechnology Techniques*, 12(9), 689–691. http://doi.org/10.1023/A:1008812904017

Ruxton, C. H. S., Reed, S. C., Simpson, J. A., & Millington, K. J. (2007). The health benefits of Omega-3 polyunsaturated fatty acids: A review of the evidence. *Journal of Human Nutrition and Dietetics*, 20(3), 275–285. http://doi.org/10.1111/j.1365-277X.2007.00770.x

Santos-Silva, J., Bessa, R. & Santos-Silva, F. (2002). Effect of genotype, feeding system and slaughter weight on the quality of light lambs. *Livestock Production Science*, 77(2–3), 187–194. http://doi.org/10.1016/S0301-6226(02)00059-3

Shimada, Y., Ogawa, J., Watanabe, Y., Nagao, T., Kawashima, A., Kobayashi, T., & Shimizu, S. (2003). Regiospecific analysis by ethanolysis of oil with immobilized *Candida antarctica* lipase. *Lipids*, 38(12), 1281–1286. http://doi.org/10.1007/s11745-003-1190-x

Šimat, V., Bogdanović, T., Poljak, V., & Petričević, S. (2015). Changes in fatty acid composition, atherogenic and thrombogenic health lipid indices and lipid stability of bogue (*Boops boops*) during storage on ice: Effect of fish farming activities. *Journal of Food Composition and Analysis*, 40, 120–125. http://doi.org/10.1016/j.jfca.2014.12.026

Simopoulos, A. P. (2002). The importance of the ratio of Omega-6/Omega-3 essential fatty acids. Biomedicine & Pharmacotherapy, 56(8), 365–379. http://doi.org/10.1016/S0753-3322(02)00253-6

Soumanou, M. M., Bornscheuer, U. T., Menge, U., & Schmid, R. D. (1997). Synthesis of Structured Triglycerides from Peanut Oil with Immobilized Lipase. *Journal of the American Oil Chemists' Society*, 74(4), 427–433.

Subhadra, B., Lochmann, R., Rawles, S., & Chen, R. (2006). Effect of dietary lipid source on the growth, tissue composition and hematological parameters of largemouth bass (*Micropterus salmoides*). *Aquaculture*, 255(1–4), 210–222. http://doi.org/10.1016/j.aquaculture.2005.11.043

Ulbricht, T. L. V, & Southgate, D. A. T. (1991). Coronary heart disease: Seven dietary factors. Lancet.

Vannuccini, S. (1999). Shark utilization, marketing and trade. FAO Fisheries Technical Paper, 389.

Wang, X., Li, M., Wang, T., Jin, Q., & Wang, X. (2014). An improved method for the synthesis of 2-arachidonoylglycerol. *Process Biochemistry*, 49(9), 1415–1421. http://doi.org/10.1016/j.procbio.2014.05.021

Weihrauch, J. L., Posati, L. P., Anderson, B. A., & Exler, J. (1977). Lipid conversion factors for calculating fatty acid contents of foods. *Journal of the American Oil Chemists' Society*, 54(1), 36–40. http://doi.org/10.1007/BF02671370

III. Nutritional Indexes, Fatty Acids Profile and Regiodistribution of Oil Extracted from Four Discarded Species of the Alboran Sea: Seasonal Effects*

The new EU fisheries regulations state that all catches from pelagic fisheries must be landed from the 1st January 2015 on. Consequently, the development of new techniques for the up-grading of these discards is required. The aim of this work was to study the variations of the lipid profile and nutritional value of the oil extracted from four discarded species: axillary seabream (*Pagellus acarne*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), and blue whiting (*Micromesistius poutassou*). To that end, the proximate composition, the lipid profile, the regiospecific distribution of fatty acids within the glycerol backbone, the nutritional parameters (thrombogenic and atherogenicity indexes and hypocholesterolemic/ hypercholesterolemic ratio) and the peroxidability index were determined in each season. All species presented polyunsaturated fatty acids (PUFA) as the major fraction in both the global and the sn-2 profile. DHA presented highly regiospecifity to position sn-2 whereas the tendency of EPA was to occupy sn-1(3) positions. The relative concentration of PUFA occupying sn-2 position varied from 30 to 60 % (molar basis).

This work proposes an up-grading strategy for the fish oils extracted from four discarded pelagic species. In the last decades, fish oils have been widely employed as source for the production of structured lipids or PUFA concentrates. The choice between both applications depends on the fatty acids regiodistribution within the glycerol backbone. Oils with high PUFA content at the central bond may be selected for the production of structured lipids by using sn-1(3) lipases. In this case, the central position remains unmodified. To obtain concentrates it is necessary to release the free fatty acids and subsequently re-esterified them. Consequently, oils with lower PUFA content in the central position are preferred for the production of concentrates by urea complexation or winterization.

* JOURNAL SHORT COMMUNICATION: R. Morales-Medina, P.J. García-Moreno, R. Pérez-Gálvez, M. Muñío, A. Guadix and E. M. Guadix. Nutritional indexes, fatty acids profile and regiodistribution of oil extracted from four discarded species of the Alboran Sea: seasonal effects. *European Journal of Lipid Science and Technology* 118:1409-1415. http://dx.doi.org/10.1002/ejlt.201500486

1. INTRODUCTION

Extractive fishing generates variable amounts of discards, so called the fraction of organic material in the catch which is not retained for sale but dumped to the sea (Kelleher, 2005). Besides the impact on future catches, fish discards also involve ecological and environmental concerns. The last FAO assessment has estimated global discards to be 7.3 million tones, with a global discard rate (*i.e.* amount of discards as a percentage of the total catch) of 8% (Kelleher, 2005). As for the Alboran Sea (southern Mediterranean coast of Spain), recent studies reported average discard rates of 23% for trawling and 10% for purse seine fisheries, accompanied by a depletion of 50% in fish stocks during the past decade (FROM., 2008). Discards in this area mostly comprise target species such as blue whiting, horse mackerel and sardine, discarded by minimum landing-size requirements, quota restrictions or market considerations. Other species, such as axillary seabream, are discarded due to their lack of commercial value.

The new Common Fisheries Policy promotes the progressive prohibition of discard practices in European fisheries. In accordance to new EU fisheries regulations (EU, 2013), all catches from small and large pelagic fisheries (*e.g.* mackerel, horse mackerel, anchovy, sardine, tuna, swordfish, among other species) should be landed from the 1st January 2015 on. Landing obligations should be accompanied by the commercial promotion of non-target species, as well as technical solutions able to convert these underutilized biomasses into added-value products of interest in food and nutraceutical applications. In this framework, fish by-catches and by-products from fish processing (*e.g.* canning industries, fish auctions) have become a highly available source for the extraction of fish oil.

The beneficial role of fish oils in human health has been widely described (Ruxton, Reed, Simpson, & Millington, 2007). Polyunsaturated fatty acids (PUFA) are the main contributors to these properties, more specifically, those belonging to the Omega-3 family such as eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids. Omega-3 long chain fatty acids play an important role in the prevention of cardiovascular diseases owing to their anti-thrombotic, anti-arrhythmic and anti-inflammatory properties (Ruxton et al., 2007). However, the efficiency of the human digestibility and metabolism of lipids is sensitive to the position of the fatty acids (FA) within the glycerol backbone; being those FA located in the sn-2 position more easily absorbed (Koletzko, Shamir, &

Phillip, 2014). Hence, the analysis of both, the fatty acids profile and their regiospecific distribution would be of interest when selecting an appropriate up-grading process: *e.g.* concentrates or structured lipids. In a previous work, oil extracted from two discarded species: bogue (*Boops boops*) and small-spotted catshark (*Scyliorhinus canicula*) was characterized employing the aforementioned techniques (Morales-Medina et al., 2015). Due to the high relative percentage of EPA and DHA placed in the central bond, these oils were considered as a useful raw material for the production of structured lipids. In contrast, some oils might present a randomly distribution of PUFA and they could be up-graded by producing PUFA concentrates. Concentration techniques are commonly based in a number of physical approaches such as supercritical fluid extraction, low temperature crystallization or molecular distillation (Rubio-Rodriguez et al., 2010). These processes are based on the difference of physical properties (*i.e.* melting point) that fatty acids present depending on their number of carbon or unsaturations. As a previous step for the production of concentrates, acylglycerols are converted into methyl esters to enhance the yield of the concentration (Lembke, 2013). Consequently, a reesterification of the concentrated methyl esters must be conducted aiming, at producing acylglycerols which are better digested and absorbed. Furthermore, enzymatic methods have been employed for the concentration of oils taking advantage on the specificity of lipases (Rubio-Rodriguez et al., 2010).

Several studies have been conducted to characterize the seasonal variation of lipid content and composition of different species such as sardine (*Sardina pilchardus*) (Esquível et al., 1997; García-Moreno et al., 2013) or horse mackerel (*Trachurus trachurus* and *Trachurus mediterraneus*) (Bandarra, Batista, Nunes, & Empis, 2001; García-Moreno et al., 2013). Other studies have monitored the seasonal variations of the nutritional value of fish oil throughout a year, by means of nutritional indexes such as the thrombogenic (TI) and the atherogenicity (AI) indexes or the hypocholesterolemic/hypercholesterolemic (HH) ratio (Dal Bosco, Mugnai, Mourvaki, & Castellini, 2012). However, no study of the seasonal fluctuation of the regiodistribution of these pelagic species has been yet reported in literature.

The aim of this work is to evaluate the seasonal variations of the lipid composition and nutritional value of the oil extracted from four pelagic discarded species of the Alboran Sea: axillary seabream (*Pagellus acarne*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), and blue whiting (*Micromesistius poutassou*). To that end, the

proximate composition, the lipid profile and the regiospecific distribution of fatty acids within the glycerol backbone was analyzed. Additionally, the fatty acids profile was employed to determine the major nutritional parameters, namely thrombogenic and atherogenicity indexes and hypocholesterolemic/ hypercholesterolemic ratio and the peroxidability index.

2. MATERIALS AND METHODS

2.1 Materials

Axillary seabream (*Pagellus acarne*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), and blue whiting (*Micromesistius poutassou*) were the fish species chosen for this study. During 2013, once per season, fish samples were provided by the fishing harbor of Motril (Spain). They were kept in ice and pressed the same day. Somatometric measurements were taken on three individuals (Table 1).

Table 1. Seasonal somatometric data of discarded species of the Alboran Sea: means ± standard deviation.

Species	Autumn			Winter		
	Nº fishes	weight, g	size, cm	Nº fishes	weight, g	size, cm
Axillary seabream	19	118.2±15.5	14.4±0.3	26	77.4±14.5	21.5±0.9
Sardine	45	56.5±24.7	15.2±2.8	42	50.3±24.6	17.8±3.2
Horse mackerel	35	62.1±13.2	16.5±0.8	25	78.7±40.8	20.6±2.6
Blue whiting	28	79.37±17	22.00±1.73	27	77.4±14.6	21.5±0.9

Species	Spring			Summer		
	Nº fishes	weight, g	size, cm	Nº fishes	weight, g	size, cm
Axillary seabream	28	79.3±4.8	18.7±0.6	20	126.7±16.2	20.7±0.7
Sardine	41	52.2±7.7	17.8±1.3	27	71.4±15.0	19.7±2.1
Horse mackerel	48	45.3±11.0	17.5±1.8	59	30.0±2.1	15.7±0.3
Blue whiting	36	61.8±13.2	20.2±1.1	63	28.7±4.7	16.7±1.0

2.2. Proximate composition and oil extraction

The proximate composition of the fish species was determined following the official methods of the A.O.A.C.,(2006). To extract the oil, ~2 kg of the whole fish were preheated at 40ºC for 30 min and then pressed stepwise at 120 bar by means of a hydraulic press

(model ESP-K Sanahuja, Spain). Finally, the press liquor was collected and then centrifuged at 20,000×g in order to recover the oily phase (Morales-Medina et al., 2015).

2.3. Fatty acid profile and lipid composition

The extracted oil samples were methylated by direct transesterification prior to their identification by gas chromatography.

A solution of oil in hexane (1mg/mL) was mixed with a reagent mixture containing methanol and acetyl chloride (20:1 v/v). Nonadecanoic acid (C19:0) (Sigma-Aldrich) was used as internal standard for quantitative determination. The samples were heated up to 90ºC for 1 hour with intermediate shaking. The fatty acids methyl esters were analyzed following the protocol described by Camacho Paez, Robles Medina, Camacho Rubio, González Moreno, & Molina Grima, (2002) by means of an Agilent 7890A chromatograph (Agilent Technologies, S.A.) coupled to a capillary column of fused silica Omegawax (0.25 mm × 30 m, 0.25 µm standard film; Supelco, Bellefonte, PA), equipped with a flame-ionization detector. Results were expressed as the average weight percentage of three replicates.

Thin layer chromatography (TLC) was employed to fractionate the lipid sample monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG). A mass of 2 mg of oil was spotted on silica-gel plates (Precoated TLC plates, SIL G-25; Macherey-Nagel, Sigma–Aldrich). The plates were developed in a mixture of chloroform/acetone/methanol (95:4.5:0.5, v/v/v) and the spots of each species were visualized by using iodine vapor in a nitrogen stream. Each fraction was scraped from the plates and methylated as previously described.

2.4. Oxidative and nutritional indexes

The composition of fatty acids was related to the mass of fish by using the conversion factor reported by Weihrauch, Posati, Anderson, & Exler, (1977) and considering the lipid content and fatty acid profile determined for each species. The intrinsic peroxidability index (PI, %) was calculated according to Arakawa & Sagai, (1986). Furthermore, the amount of each fatty acid (g/100 g of fish) was employed to calculate the indexes of atherogenicity (AI), thrombogenicity (TI) (Ulbricht & Southgate, 1991) and the

hypocholesterolemic/ hypercholesterolemic ratio (HH) (Santos-Silva, Bessa, & Santos-Silva, 2002).

2.5. Determination of the positional distribution of fatty acids in TAG

The regiospecific distribution of fatty acids in the TAG was analyzed by ethanolysis with Novozym 435 from *Candida antarctica* lipase (Novozymes A/S, Bagsvaerd, Denmark) (Shimada et al., 2003). A mixture of 750 mg of sample, 3 mL of dry absolute ethanol and 375 mg of Novozyme 435 was located in a batch reactor (300 rpm, 4 h and 35 ºC). The fraction corresponding to 2-monoacylglycerols (2-MAG) produced in the sn-1(3) specific alcoholysis was separated by TLC. This analysis allowed the calculation of the relative content of fatty acid in sn-2 position as follows:

$$\%\mathrm{FA_i}\ \text{in sn2 position} = \frac{\text{content of FA}_\mathrm{i}\ \text{in sn2 position}}{3 \cdot \text{total content of FA}_\mathrm{i}\ \text{in TAG}} \cdot 100 \quad [1]$$

The total percentage of each fatty acid located in sn-2 was calculated by multiplying the aforementioned percentage and the fatty acid percentage, both in molar basis.

3. RESULTS AND DISCUSSION

3.1. Proximate composition

Table 2 shows the seasonal variations in the proximate composition for the discarded species studied. For a given species, the ash and the protein content remained constant along the year. However, differences were found among species, with values ranging from 2.5 to 5.0 wt% in the case of ashes and from 5.5 to 20.6 wt% for protein content (Table 2). These results are in line with previous works conducted on the same species (García-Moreno et al., 2013; Zotos & Vouzanidou, 2012).

Table 2. Seasonal proximate compositions of four discarded species of the Alboran Sea.

[%]	Axillary seabream				Sardine				Horse mackerel				Blue whiting			
	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.	Aut.	Win.	Spr.	Sum.
Moisture	72.1	70.4	71.5	72.2	61.4	71.4	75.5	59.4	72.2	74.1	78.4	70.8	76.4	78.8	77.7	77.2
Ash	3.9	4.8	4.8	5.0	2.8	3.0	3.3	3.3	3.6	3.3	3.6	3.1	2.5	3.3	2.9	3.0
Protein	17.2	20.6	17.4	18.8	17.0	17.1	15.5	20.2	17.3	17.8	16.5	18.6	18.1	17.5	19.0	18.0
Lipid	5.2	5.7	5.8	4.7	16.0	12.5	2.5	18.8	4.9	5.4	1.0	6.2	3.2	1.1	2.5	1.5

Lipid and moisture content showed high variations within seasons and among species. Moisture ranged from 59.4 to 78.8 wt%, while lipid content varied between 1.0 and 18.8 wt% (Table 2). As a general trend, fat and moisture content correlated inversely for axillary seabream (r^2= 0.524), sardine (0.850), horse mackerel (0.879) and blue whiting (0.640) respectively. These differences might be closely related to the feed intake, migratory swimming habits and sexual changes in connection with spawning (Brockerhoff, Hoyle, Hwang, & Litchfield, 1968). The reproductive behavior of the species of the Alboran Sea has been described by Pérez-Martín, (2001). The spawning season of axillary seabream occurs in summer; horse mackerel and blue whiting during winter and/or spring and sardine in autumn or winter (Pérez-Martín, 2001). In this case, the influence of the spawning season was clearly observed in the case of sardine, which showed the highest lipid content (18.8 wt%) in summer and the lowest (2.5 wt%) in spring after the spawning period (Table 2).

3.2. Fatty acids profile and nutritional indexes

The present study was focused on the polar fraction of the lipids being triacylglycerols the only group detected by TLC (data not shown). The fatty acid profile depends on several factors such as species, age, season, reproductive status, food availability and sex among others (Brockerhoff et al., 1968).

Table 3 summarizes the seasonal variations of fatty acid profiles for the species studied. Polyunsaturated fatty acids (PUFA) were the major fraction except for blue whiting, where the monounsaturated fatty acids (MUFA) were predominant. PUFA ranged between 26.3 wt% (spring, blue whiting) and 44.7 wt% (winter, axillary seabream) being DHA (C22:6n-3) the major fatty acid (10.8 – 22.8 wt%). PUFA content reached a maximum in winter for all species in agreement with the well-established inverse correlation between temperature of a tissue and the degree of unsaturation of the fatty acids (Malins & Wekell, 1970). MUFA were the second main fraction (23.1 to 43.1 wt%) with oleic acid (C18:1n-9) as the main component (5.8 to 25.4 wt%). SFA varied between 19.3 and 35 wt%. This fraction was composed by palmitic acid (C16:0), myristic (C14:0) and stearic acid (C18:0), being palmitic the most abundant fatty acid (15.4 to 21.7wt%). Although fish oils presented a wide variety of fatty acid whose length varied from C14 to C22, in all cases more than 50% of the fatty acids profile corresponded to C16:0, C18:1n-9, EPA and DHA. All the

species studied presented excellent n-3/n-6 (*i.e.* Omega-3/Omega-6) ratio ranging from 8.9 to 39.8 they might be included in the human diet so to balance the n-3:n-6 intake.

Table 4 shows the composition of fatty acids represented as g/100g fish. In this case, the influence of seasonality was noticeably higher than in the global profile due to the considerable variations of the lipid content among the year.

The peroxidability index (PI) is an indicator of the tendency of fish oil spoilage. It is directly related to the amount of secondary oxidation compounds (malondialdehyde) but no relation has been found to the primary ones (Yun & Surh, 2012). PI values were considerably high due to the high percentage of PUFA (Table 4).

Atherogenicity and thrombogenic index (AI, TI, respectively) refer quantitatively to the tendency of an oil to prevent cardiovascular diseases (Ulbricht & Southgate, 1991). Atherogenicity indexes were, in all cases, below one, value which is considered as healthy (Table 4) (Subhadra, Lochmann, Rawles, & Chen, 2006). TI values (<0.3) were lower than

those commonly reported for animal sources and similar to that of goldfish (Dal Bosco et al., 2012; Ulbricht & Southgate, 1991). The hypocholesterolemic/ hypercholesterolemic ratios ranged from 1.07 to 2.42 corresponding to blue whiting and in spring and axillary seabream in winter. According to the values of AI, TI and HH ratio, the extracted oils present an interesting composition for their application as nutraceutical compounds.

3.3. Regiospecific distribution of fatty acids in TAG.

Table 5 shows the composition (molar basis) of the three fractions and their main fatty acids located in sn-2 referred as: (i) the percentage when compared to the global value of the three positions (indicated with symbol a) and (ii) the percentage focused exclusively on those located in sn-2 position (indicated with symbol b and referred as relative). In the first case, the data have been calculated by multiplying the percentage of fatty acids located in sn-2 and the total fatty acid percentage (molar basis). These data follows the stoichiometric ratio and consequently their maximum value is 33.3%.

In average 45.4±7.0 mol% of the total PUFA of oils were located in the sn-2 position. The average content of the total SFA and MUFA occupying the central bond were 31.7 ± 3.0 and 15.3 ± 0.6 mol%, respectively. Hence, SFA presented a slight tendency to sn-1(3) bonds whilst MUFA showed a larger 1-3 regiospecificity.

Table 3. (A) Seasonal fatty acid profiles (weight %) of oils extracted from discarded species of the Alboran Sea. Data are means of triplicate determinations. SD < 5%. .

	Axillary seabream				Sardine			
	Autumn	**Winter**	**Spring**	**Summer**	**Autumn**	**Winter**	**Spring**	**Summer**
C14:0	4.1	3.9	4.3	4.7	6.4	6.4	9.1	7.0
C16:0	16.4	15.4	18.6	17.0	20.4	19.2	21.1	17.7
C16:1n-7	5.9	6.8	6.4	6.9	6.7	5.8	8.7	8.2
C16:2n-4	1.4	1.3	0.9	1.1	1.2	1.3	1.6	1.5
C16:3n-4	1.0	0.3	0.8	0.8	0.0	0.0	1.2	0.9
C16:4n-1	0.0	0.0	0.0	0.3	0.0	0.0	1.8	1.3
C18:0	7.3	7.2	7.6	6.6	4.2	4.8	3.8	3.9
C18:1n-7	4.9	5.1	4.2	4.7	3.2	3.1	3.5	3.2
C18:1n-9	13.2	14.2	12.4	13.9	11.1	11.3	5.8	7.8
C18:2n-6	1.8	2.1	1.2	0.2	1.4	2.1	0.9	1.0
C18:3n-3	0.3	0.3	0.0	0.4	2.3	2.2	2.8	2.2
C18:4n-3	1.2	1.3	1.3	0.9	0.0	0.0	0.0	0.7
C20:1n-9	1.1	1.2	1.2	1.2	3.0	3.5	2.8	2.0
C20:3n-6	1.4	1.5	1.6	1.8	1.0	0.0	0.0	0.7
C20:4n-3	0.5	0.6	0.6	0.6	0.9	0.0	1.1	0.9
C20:5n-3	10.9	8.9	13.0	10.2	13.8	11.9	17.2	15.1
C22:1n-9	0.0	0.0	0.0	0.0	2.8	0.0	4.2	1.9
C22:5n-3	4.1	4.5	3.7	4.8	2.5	2.7	2.2	2.4
C22:6n-3	17.2	16.7	16.5	16.1	18.1	21.8	10.8	15.9
Others	7.7	10.0	7.5	7.9	0.9	3.9	1.5	5.9
SFA	27.8	19.3	30.5	28.3	31.1	30.4	33.9	28.6
MUFA	25.1	27.2	24.2	26.7	26.8	23.8	25.0	23.1
PUFA	39.7	44.7	39.6	37.1	41.3	42.0	39.6	42.6
n-6	3.2	3.6	2.9	2.0	2.5	2.1	0.9	1.8
n-3	34.2	32.3	35.1	33.0	37.6	38.6	34.1	37.0
n-3/n-6	10.8	8.9	12.3	16.5	15.3	18.3	39.8	20.8
EPA+DHA	28.1	25.5	29.5	26.3	31.9	33.7	28.0	30.9

Table 3 (B). Seasonal fatty acid profiles (weight %) of oils extracted from discarded species of the Alboran Sea. Data are means of triplicate determinations. SD < 5%. .

	Horse mackerel				Blue whiting			
	Autumn	**Winter**	**Spring**	**Summer**	**Autumn**	**Winter**	**Spring**	**Summer**
C14:0	7.6	7.3	7.1	4.7	3.0	3.1	2.9	2.7
C16:0	20.5	18.2	21.7	19.2	20.2	19.9	21.0	19.8
C16:1n-7	6.8	6.4	6.3	7.1	5.9	5.9	6.8	6.3
C16:2n-4	1.5	1.6	0.0	1.0	1.2	1.6	1.4	1.2
C16:3n-4	1.1	0.7	0.0	0.7	0.0	0.0	0.7	0.7
C16:4n-1	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.1
C18:0	4.7	4.3	6.2	5.4	5.3	4.7	4.6	4.6
C18:1n-7	2.5	2.5	3.0	3.3	3.2	3.4	3.1	2.8
C18:1n-9	10.6	9.8	11.2	19.4	21.3	19.7	25.4	23.6
C18:2n-6	1.6	1.7	0.0	1.1	1.3	1.4	1.0	1.0
C18:3n-3	2.2	2.3	2.1	1.0	1.5	0.0	0.6	0.7
C18:4n-3	0.0	0.6	0.0	0.6	0.0	1.2	1.1	1.3
C20:1n-9	3.5	3.3	3.0	1.7	2.7	2.8	3.4	2.5
C20:3n-6	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.7
C20:4n-3	0.0	0.9	0.0	0.6	1.0	0.0	0.7	0.6
C20:5n-3	11.2	9.5	11.6	9.7	7.1	6.2	5.9	6.6
C22:1n-9	6.0	5.8	5.1	1.8	2.9	2.1	4.5	2.1
C22:5n-3	2.0	2.1	2.2	2.2	1.6	1.0	1.1	1.6
C22:6n-3	16.7	19.5	20.6	16.9	19.7	22.8	13.8	18.0
Others	1.4	3.4	0.0	2.4	2.1	4.3	2.1	3.1
SFA	32.8	25.5	35.0	29.3	28.5	23.0	28.5	27.1
MUFA	29.6	27.9	28.6	33.3	36.0	33.8	43.1	37.3
PUFA	36.2	43.1	36.4	35.0	33.4	38.9	26.3	32.5
n-6	1.6	1.7	0.0	1.9	1.3	1.4	1.0	1.6
n-3	32.1	34.8	36.4	31.1	30.9	31.2	23.3	28.9
n-3/n-6	20.6	20.6	--	16.5	23.3	21.6	24.1	17.8
EPA+DHA	27.9	29.0	32.2	26.7	26.8	29.0	19.7	24.6

Table 4.A Seasonal fatty acids profiles (g/100g fish), nutritional indexes and oxidative status of oils extracted from axillary seabream and sardine of the Alboran Sea.

	Axillary seabream				Sardine			
	Aut.	**Win.**	**Spr.**	**Sum.**	**Aut.**	**Win.**	**Spr.**	**Sum.**
SFA	1.30	1.00	1.61	1.20	4.60	3.50	0.75	4.99
MUFA	1.18	1.41	1.28	1.13	3.97	2.74	0.55	4.02
PUFA	1.86	2.31	2.10	1.57	6.11	4.84	0.87	7.42
EPA	0.51	0.46	0.69	0.43	2.04	1.37	0.38	2.63
DHA	0.80	0.86	0.87	0.68	2.68	2.51	0.24	2.77
PI/100	2.42	2.58	2.47	2.34	2.56	2.71	2.26	2.54
AI	0.43	0.40	0.48	0.47	0.59	0.59	0.87	0.66
TI	0.21	0.15	0.22	0.21	0.21	0.20	0.21	0.19
HH	2.36	2.42	2.05	2.10	1.84	2.04	1.32	1.79

Table 5.A. Seasonal composition (%, molar basis) of main fatty acids and fractions in sn-2 position and relative composition of the fractions in sn-2 position of axillary seabream and sardine. Data are means of triplicate determination with SD <5%.

	Axillary seabream				Sardine			
	Aut.	**Win.**	**Spr.**	**Sum.**	**Aut.**	**Win.**	**Spr.**	**Sum.**
C16:0[a]	6.8	7.5	6.7	6.4	7.2	7.2	7.1	5.7
C18:1n-9[a]	1.7	2.3	1.3	1.6	1.6	2.2	1.3	1.6
C20:5n-3[a]	1.8	1.5	1.9	1.3	2.6	2.1	2.9	2.6
C22:6n-3[a]	11.5	7.5	13.8	12.4	11.3	12.4	9.2	12.5
SFA[a]	10.3	10.7	10.0	9.6	10.8	11.6	10.7	9.2
MUFA[a]	5.3	7.5	4.7	5.5	4.9	5.0	5.3	5.1
PUFA[a]	17.7	14.3	18.6	18.3	17.7	16.8	17.3	19.0
Relative percentage in sn-2								
SFA[b]	31.0	33.0	29.9	28.8	32.2	34.7	32.1	27.6
MUFA[b]	15.8	23.0	14.1	16.4	14.7	15.0	16.0	15.3
PUFA[b]	53.2	44.0	55.9	54.8	53.1	50.3	51.8	57.1
DHA[b]	34.5	22.3	41.5	37.3	33.8	37.3	27.7	37.6
DHA+EPA[b]	39.8	27.0	47.3	41.3	41.5	43.5	36.5	45.3

[a]Data are the product of percentage of fatty acid located in sn-2 position and the percentage of the fatty acids in the total profile divided by 100. Maximum value 33.3%

[b]Relative data are calculated by dividing data calculated in [a] by 33.33 and multiplying by 100. These data refer to the composition of sn-2 expressed as mole percentage.

Table 4.A. Seasonal fatty acids profiles (g/100g fish), nutritional indexes and oxidative status of oils extracted from horse mackerel and blue whiting of the Alboran Sea.

	Horse mackerel				Blue whiting			
	Aut.	**Win.**	**Spr.**	**Sum.**	**Aut.**	**Win.**	**Spr.**	**Sum.**
SFA	1.45	1.26	0.28	1.65	0.80	0.20	0.61	0.34
MUFA	1.31	1.38	0.23	1.87	1.01	0.29	0.93	0.47
PUFA	1.60	2.13	0.29	1.97	0.94	0.34	0.57	0.41
EPA	0.49	0.47	0.09	0.55	0.20	0.05	0.13	0.08
DHA	0.74	0.96	0.16	0.95	0.76	0.25	0.42	0.31
PI/100	2.23	2.59	2.56	2.22	2.2	2.53	1.66	2.08
AI	0.69	0.64	0.66	0.44	0.36	0.38	0.46	0.34
TI	0.24	0.18	0.27	0.21	0.20	0.16	0.24	0.20
HH	1.58	1.76	1.58	2.09	2.27	2.22	1.07	2.28

Table 5.A. Seasonal composition (%, molar basis) of main fatty acids and fractions in sn-2 position and relative composition of the fractions in sn-2 position of horse mackerel and blue whiting. Data are means of triplicate determination with SD <5%.

	Horse mackerel				Blue whiting			
	Aut.	**Win.**	**Spr.**	**Sum.**	**Aut.**	**Win.**	**Spr.**	**Sum.**
C16:0[a]	8.5	7.6	5.8	5.2	6.3	6.4	11.0	6.3
C18:1n-9[a]	2.3	3.0	4.3	8.0	3.3	4.6	5.1	5.7
C20:5n-3[a]	1.2	1.3	2.0	1.2	1.5	1.2	2.1	1.5
C22:6n-3[a]	7.7	8.4	14.4	11.9	12.4	12.1	5.6	14.4
SFA[a]	14.1	12.6	9.2	7.5	7.1	8.0	13.8	7.6
MUFA[a]	5.6	7.2	6.9	10.6	6.9	9.1	11.4	7.8
PUFA[a]	13.6	13.8	17.2	15.3	18.7	16.4	10.4	17.9
Relative percentage in sn-2								
SFA[b]	42.4	37.4	27.7	22.4	21.8	23.9	38.8	22.9
MUFA[b]	16.9	21.5	20.6	31.8	21.1	27.1	32.1	23.5
PUFA[b]	40.7	41.1	51.7	45.8	57.2	49.0	29.1	53.6
DHA[b]	23.0	25.1	43.1	35.8	37.2	36.3	16.9	43.3
DHA+EPA[b]	26.5	29.1	49.0	39.5	41.7	39.9	23.1	47.9

[a] Data are the product of percentage of fatty acid located in sn-2 position and the percentage of the fatty acids in the total profile divided by 100. Maximum value 33.3%

[b] Relative data are calculated by dividing data calculated in a by 33.33 and multiplying by 100. These data refer to the composition of sn-2 expressed as mole percentage.

The main pathway for the bio-synthesis of TAG is by acylation of the exogenous 2-MAG and the tendency to accumulate specific fatty acid in sn-2 position is related to the specific conditions of the initial substrate (Malins & Wekell, 1970). Therefore, the diet appears to be a critical factor of lipid composition. Among all species, sardine is the only vegetarian and one with the highest content of PUFA, which could be related to the high content of EPA and DHA in plankton (Brockerhoff et al., 1968). Contrary, the diet of horse mackerel is based on small pelagic fishes and zooplankton, factor which might justify that its average PUFA content located in sn-2 position was the lowest.

Regarding fatty acids, DHA occupied preferably the sn-2 bond. On average a 73.0±17.5 mol% of the total DHA was located in position 2, whereas in the case of EPA this value was much lower: 17.7 ± 5.9 mol%, which implies a tendency to sn-1(3) positions. On the other hand, palmitic and oleic acid presented 1(3)-regiospecificity with a relative percentage in position 2 of 20.9 ± 4.0 mol% and 9.0 ± 5.7 mol%, respectively. Palmitic acid and EPA have been described as 2-regiospecific (Brockerhoff et al., 1968; Malins & Wekell, 1970; Wijesundera, Kitessa, Abeywardena, Bignell, & Nichols, 2011), which differs from the current results. Contrary, Suárez et al. (Suárez et al., 2010) described the regiospecificity of sardine oil fatty acids presenting similar results to those described in the current work.

The main techniques employed for the concentration of PUFA are: supercritical fluid extraction, low temperature crystallization and molecular distillation (Rubio-Rodriguez et al., 2010). These techniques are based on the difference of physical properties (*i.e.* melting point) that fatty acids or esters present depending on their number of carbon or unsaturations. Since the yield of the processes is much higher when concentrating free fatty acids or methyl esters instead of the whole TAG, a chemical hydrolysis or esterification is the first step of the concentration process (Lembke, 2013). Hence, oils with low relative content of PUFA in position sn-2 are a raw material of interest for the production of PUFA concentrates.

4. CONCLUSIONS

The up-grading of the oil extracted from four discarded species of the Alboran Sea may be a good alternative for their valorization. All of them presented appropriate TI, AI and HH indexes for nutraceutical applications. Due to their high average content of PUFA in the

global profile they could be employed as raw sources for the production of concentrates or even, in specific seasons for the production of structured lipids with processes where the fatty acids located in sn-2 position will remain unmodified. Oils with a high content of PUFA in the sn-2 position could be employed as source of structured lipids or concentrated by techniques where the central position of the TAG remained unchangeable *i.e.* selective hydrolysis with lipases. On the other hand, oils with lower content, as blue whiting in spring, could be concentrated by the aforementioned techniques which involve treatments of esterification and re-esterification.

5. REFERENCES

A.O.A.C. (2006). Official Methods of Analysis of the A.O.A.C. Washington DC: Association of Official Analytical Chemists.

Arakawa, K., & Sagai, M. (1986). Species differences in lipid peroxide levels in lung tissue and investigation of their determining factors. *Lipids*, 21(12), 769–775. http://doi.org/10.1007/BF02535410

Bandarra, N., Batista, I., Nunes, M., & Empis, J. (2001). Seasonal variation in the chemical composition of horse-mackerel (*Trachurus trachurus*). *European Food Research and Technology*, 212(5), 535–539. http://doi.org/10.1007/s002170100299

Brockerhoff, H., Hoyle, R. J., Hwang, P. C., & Litchfield, C. (1968). Positional distribution of fatty acids in depot triglycerides of aquatic animals. *Lipids*, 3(1), 24–9. http://doi.org/10.1007/BF02530964

Camacho Paez, B., Robles Medina, A., Camacho Rubio, F., González Moreno, P., & Molina Grima, E. (2002). Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by the acidolysis of cod liver oil and caprylic acid in a packed-bed reactor: Equilibrium and kinetics. *Chemical Engineering Science*, 57(8), 1237–1249. Journal Article.

Dal Bosco, A., Mugnai, C., Mourvaki, E., & Castellini, C. (2012). Seasonal changes in the fillet fatty acid profile and nutritional characteristics of wild Trasimeno Lake goldfish (*Carassius auratus L.*). *Food Chemistry*, 132(2), 830–834. http://doi.org/10.1016/j.foodchem.2011.11.043

Esquível, M. M., Bandarra, N. M., Fontan, I., Bernardo-Gil, M. G., Batista, I., Nunes, M. L., & Empis, J. A. (1997). Supercritical carbon dioxide extraction of sardine *Sardina pilchardus* oil. *LWT - Food Science and Technology*, 30(7), 715–720.

EU. (2013). Regulation (EU) no 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Official Journal of the European Union, 22–28.

FROM-Fondo de Regulación y Organización del Mercado de los Productos de la Pesca y los, & cultivos Marinos. (2008). Asistencia técnica para la realización de una medida innovadora para la recuperación, gestión y valorización de los descartes generados por la flota española que faena en los caladeros atlántico y mediterráneo.

García-Moreno, P.J., Pérez-Gálvez, R., Morales-Medina, R., Guadix, A., Guadix, E. M. (2013). Discarded species in the west Mediterranean Sea as sources of Omega-3 PUFA. *European Journal of Lipid Science and Technology*, 115(9), 982–989. http://doi.org/10.1002/ejlt.201300021

Kelleher, K. (2005). Discards in the world's marine fisheries: An update. FAO. Fisheries Technical Paper.

Koletzko, B., Shamir, R., & Phillip, M. (2014). World Review of Nutrition and Dietetics. Nutrition and growth. Preface. World Review of Nutrition and Dietetics, 109.

Lembke, P. (2013). Production Techniques for Omega-3 Concentrates. In F. De Meester, R. R. Watson, & S. Zibadi (Eds.), Omega-6/3 Fatty Acids (pp. 353–364). Humana Press.

Malins, D. C., & Wekell, J. C. (1970). The lipid biochemistry of marine organisms. *Progress in the Chemistry of Fats and Other Lipids*, 10, 339–363. http://doi.org/10.1016/0079-6832(70)90011-X

Morales-Medina, R., García-Moreno, P. J., Pérez-Gálvez, R., Muñío, M., Guadix, A., & Guadix, E. M. (2015). Seasonal variations in the regiodistribution of oil extracted from small-spotted catshark and bogue. *Food Funct*., 6(8), 2646–2652. http://doi.org/10.1039/C5FO00448A

Pérez-Martín, M. (2001). Especies de Interés Pesquero en el Litoral de Andalucía. (Junta de Andalucía. Consejería de Agricultura, Ed.). Sevilla: Viceconsejería. Servicio de Publicaciones y Divulgacion.

Rubio-Rodriguez, N., Beltran, S., Jaime, I., De, D. S. M., Sanz, M. T., & Carballido, J. R. (2010). Production of Omega-3 polyunsaturated fatty acid concentrates: A review. *Innovative Food Sci. Emerging Technol.*, 11, 1–12. Journal Article. http://doi.org/10.1016/j.ifset.2009.10.006

Ruxton, C. H. S., Reed, S. C., Simpson, J. A., & Millington, K. J. (2007). The health benefits of Omega-3 polyunsaturated fatty acids: A review of the evidence. *Journal of Human Nutrition and Dietetics*, 20(3), 275–285. http://doi.org/10.1111/j.1365-277X.2007.00770.x

Santos-Silva, J., Bessa, R. J., & Santos-Silva, F. (2002). Effect of genotype, feeding system and slaughter weight on the quality of light lambs. *Livestock Production Science*, 77(2–3), 187–194. http://doi.org/10.1016/S0301-6226(02)00059-3

Shimada, Y., Ogawa, J., Watanabe, Y., Nagao, T., Kawashima, A., Kobayashi, T., & Shimizu, S. (2003). Regiospecific analysis by ethanolysis of oil with immobilized *Candida antarctica* lipase. *Lipids*, 38(12), 1281–1286. http://doi.org/10.1007/s11745-003-1190-x

Suárez, E. R., Mugford, P. F., Rolle, A. J., Burton, I. W., Walter, J. A., & Kralovec, J. A. (2010). 13C-NMR regioisomeric analysis of EPA and DHA in fish oil derived triacylglycerol concentrates. *Journal of the American Oil Chemists' Society*, 87(12), 1425–1433. http://doi.org/10.1007/s11746-010-1638-2

Subhadra, B., Lochmann, R., Rawles, S., & Chen, R. (2006). Effect of dietary lipid source on the growth, tissue composition and hematological parameters of largemouth bass (*Micropterus salmoides*). *Aquaculture*, 255(1–4), 210–222. http://doi.org/10.1016/j.aquaculture.2005.11.043

Ulbricht, T. L. V, & Southgate, D. A. T. (1991). Coronary heart disease: Seven dietary factors. *Lancet*. 1

Weihrauch, J. L., Posati, L. P., Anderson, B. A., & Exler, J. (1977). Lipid conversion factors for calculating fatty acid contents of foods. *Journal of the American Oil Chemists' Society*, 54(1), 36–40. http://doi.org/10.1007/BF02671370

Wijesundera, C., Kitessa, S., Abeywardena, M., Bignell, W., & Nichols, P. D. (2011). Long-chain Omega-3 oils: Current and future supplies, food and feed applications, and stability. *Lipid Technology*, 23(3), 55–58. http://doi.org/10.1002/lite.201100091

Yun, J.-M., & Surh, J. (2012). Fatty Acid Composition as a Predictor for the Oxidation Stability of Korean Vegetable Oils with or without Induced Oxidative Stress. *Preventive Nutrition and Food Science*, 17(2), 158–65. http://doi.org/10.3746/pnf.2012.17.2.158

Zotos, A., & Vouzanidou, M. (2012). Seasonal changes in composition, fatty acid, cholesterol and mineral content of six highly commercial fish species of Greece. *Food Science and Technology International*, 18(2), 139–49. http://doi.org/10.1177/1082013211414785

IV. Mass Transfer Model of Sardine Oil Polyunsaturated Fatty Acids (PUFA) Concentration by Crystallization at Low Temperature [*]

Low temperature crystallization of oil is employed in the concentration of Omega-3 fatty acids which are products of interest in the food industry. The aim of this work was to model the influence of temperature and time in the mass of the solid and liquid phases. The model developed enabled the estimation of polyunstarated fatty acids (PUFA), eicosapentaenoic acid and docosahexaenoic acids (EPA and DHA) contents in the liquid phase. Solutions of sardine oil in hexane were crystallized at several temperatures (-55, -65, -75 and -85°C) and times (1 to 24 hours). The transient variation of liquid phase mass at a given temperature was modelled employing two parameters: the equilibrium mass of the phase (m_{eq}, g) and the overall mass transfer coefficient ($K_{G \cdot A}$, h^{-1}). The results showed that the highest PUFA concentrates (>80%) were produced at -85ºC and 24h. The model proposed is a useful tool for estimating the composition and total mass of winterized oils ($r^2 > 0.83$).

[*] JOURNAL PAPER: R. Morales-Medina, G. De León, M. Munio, A. Guadix and E. Guadix. (2016). Mass Transfer Model of Sardine Oil Polyunsaturated Fatty Acids (PUFA) Concentration by Crystallization at Low Temperature. *Journal of Food Engineering* 183:16-23. http://dx.doi.org/10.1016/j.jfoodeng.2016.03.009

1. INTRODUCTION

Fish discards are defined as the fraction of organic material in the catch which is not retained for sale but dumped back to the sea (Kelleher, 2005). Discarding presents a negative impact on the future fishing productivity and also alters the marine trophic chains causing an environmental problem. To prevent this situation, the new Common Fisheries Policy has introduced new EU fisheries regulations aimed to ban the discard practices in Europe (EU, 2013). From the 1st January 2015 on, all catches from pelagic species (mackerel, horse mackerel or sardines, among others) must be landed. In the case of the rest of the fish species this new regulation will come into force from 2017 on.

Therefore, technical solutions able to convert the fish discards into added-value products must be found. In this framework, the extraction of fish oil for the production of polyunsaturated fatty acids (PUFA) concentrates has become an interesting up-grading process. Specifically, in the Mediterranean Sea, it has been studied the oil content and composition of main pelagic discarded species. Among them, sardines presented the highest oil content (18 wt%) with a high content of polyunsaturated fatty acids (~ 40 wt%) (Morales-Medina et al., 2015). Hence, employing oil sardine as raw material for the production of concentrates could be an interesting up-grading proposal.

Polyunsaturated fatty acids, especially eicosapentaenoic and docosahexaenoic acids (EPA and DHA) have been described to exert a positive effect on the cardiovascular system (Jump, Depner, & Tripathy, 2012). Additionally they present positive influence on the visual development of neonates (Weichselbaum, Coe, Buttriss, & Stanner, 2013), on the brain system functions (Bradbury, 2011), and on some types of cancer (Bougnoux, Hajjaji, Maheo, Couet, & Chevalier, 2010). Consequently, refined oil products, with high concentration of polyunsaturated fatty acids, have increased their demand in the last years since they are used as food supplements and as nutraceutical products (Dillon, Aponte, Tarozo, & Huang, 2013; Kralovec, Zhang, Zhang, & Barrow, 2012; Kuratko & Salem, 2013; Lembke, 2013).

Polyunsaturated fatty acids concentration techniques can be conducted by several physical approaches such as supercritical fluid extraction, low temperature crystallization, molecular distillation or urea complexation (Rubio-Rodriguez et al., 2010). Prior to concentration, triacylglycerols are converted into methyl esters or fatty acids to enhance

the yield of the concentration (Lembke, 2013). Once the PUFA has been concentrated, those esters or fatty acids must be re-esterified into triacylglycerols which are better digested and absorbed by human digestion (Small, 1991).

Winterization or fractional crystallization consists in the removal of high melting point compounds (*i.e.* saturated fatty acids) by cooling (Gunstone, Harwood, & Dijkstra, 2012). For a given chain length, the melting point of the free fatty acids (FFA) decreases with the degree of unsaturation (Akoh, 2005; Gunstone et al., 2012). Therefore, at low temperatures, saturated fatty acids (SFA) crystallize and PUFA remain in the liquid phase (Wanasundara, Wanasundara, & Shahidi, 2005). Among the several types of winterization, solvent fractional crystallization is the most commonly employed (Cunha, Crexi, & Pinto, 2009; Haraldsson, 1983). The usage of solvents promotes crystal formation (Lee & Foglia, 2001) and increases the yield and purity of the crystals (Cunha et al., 2009).

The main variables controlling solvent fractional crystallization are: (i) oil composition, (ii) temperature of crystallization, (iii) mobility of molecular species in the oil (influenced by the solvent polarity), (iv) oil:solvent ratio and (v) rate of cooling (López-Martínez, Campra-Madrid, & Guil-Guerrero, 2004). The oil composition refers not only to degree of unsaturation (*i.e.* saturated, monounsaturated of polyunsaturated fatty acids) but also the class of lipids, i.e triacylglycerols, free fatty acids or esters. The high variety of fatty acids present in fish oil results into a heterogeneous composition of triglycerides; fact which hinders the concentration process (Lembke, 2013). Hence, it is preferable to hydrolyze or esterify triacylglycerols to produce free fatty acids or fatty acids methyl/ethyl esters which can be more easily separated by physical approaches. In this point, Vázquez and Akoh (2011) described that, for the concentration of stearidonic acid (C18:4n-3) the employment of free fatty acids was more efficient than the use of esters. Regarding the temperature of crystallization, it has been widely described that: the lower the temperature, the higher the concentration of PUFA in the liquid fraction (Rubio-Rodriguez et al., 2010; Shahidi & Wanasundara, 1998; Wanasundara, 1996). Several authors (López-Martínez et al., 2004; Vázquez & Akoh, 2011; Wanasundara, 1996) have studied the influence of the organic solvent (hexane, acetone, diethyl ether or isobutanol among others), being the hexane the solvent that presented higher concentrations. Vázquez and Akoh (2011) also analyzed the employment of mixtures of hexane and acetone in several proportions; however, higher concentrations were achieved employing solely hexane. Additionally, the lower the solvent:oil ratio, the higher the purity and yield of the PUFA concentrates in the liquid

phase (López-Martínez et al., 2004; Vázquez & Akoh, 2011). However, the usage of high volume of organic solvents presents an economical drawback. Hence, the selection of the oil:solvent ratio should be taken by balancing the fixed cost and the desired enrichment of PUFA.

As reviewed before, several studies have been conducted aiming to qualitatively describe the influence of several variables (*i.e.* temperature, time, type of solvent) on the production of PUFA concentrates. However, all those studies were focused on the composition of the liquid phase at a fixed time without considering the mass evolution of each phase. Additionally, from an industrial point of view, the knowledge of the evolution of the liquid and solid mass is fundamental for a proper design, control and optimization.

The aim of this work was to model the influence of temperature and time in the mass of the liquid phase obtained *via* crystallization at low temperature by following the theory of the mass transference. Moreover, the composition of the liquid phase (wt%) of polyunsaturated fatty acids, EPA and DHA was calculated employing the aforementioned model.

2. MATERIALS AND METHODS

2.1. Materials

Refined sardine oil with a total amount of EPA and DHA of 31.9% (21.5% of EPA and 10.4% of DHA) was provided by Industrias Afines S.L.

2.2. Free fatty acids production

Free fatty acids were released from the triacylglycerols by means of basic hydrolysis following the method described by Wanasundara and Shahidi, (1999). Refined sardine oil was mixed with KOH (85%), aqueous ethanol (96%, v/v) and distilled water at 62ºC during 1 hour under nitrogen atmosphere. The FFA produced were purified by subsequent extractions with distilled water (175 mL) and hexane. The remaining aqueous phase was acidified to pH 1 with 3 M HCl and the FFA were recovered by solvent extraction using hexane. Then, anhydrous sodium sulphate was added to the mixture of hexane and FFA aimed to extract the remaining water content. Finally, the mixture was filtered and the

solvent was removed at 40 °C and 100 mmHg. Free fatty acids were stored at -80 °C until use.

2.3. Fatty acid profile analysis and lipid class composition

Direct transesterification was conducted to determine the fatty acid profile of samples following a method described by Rodríguez-Ruiz et al. (1998). To that end, 1 mL of the sample in hexane (1 mg/mL) was mixed with 1 mL of reagent mixture (methanol and acetyl chloride, 20:1, v/v). Then, samples were heated up to 90ºC for 1 hour. As internal standard for quantitative determination nonadecanoic acid (C19:0) was employed. The fatty acids methyl esters were analyzed with an Agilent 7890A chromatograph (Agilent Technologies, S.A.) connected to a capillary column of fused silica Omegawax (0.25 mm × 30 m, 0.25 µm standard film; Supelco, Bellefonte, PA), and a flame-ionization detector (Camacho Paez, Robles Medina, Camacho Rubio, González Moreno, & Molina Grima, 2002). All measurements were done in triplicate and the results were expressed on a weight basis.

Thin layer chromatography (TLC) was employed to determine the polar lipid fraction composition of the samples. To that end, plates of silica-gel (Precoated TLC plates, SIL G-25; Macherey-Nagel, Sigma–Aldrich) were activated at 100ºC for 1 hour. A mass of 1 mg of sample was spotted directly on the plate. The mobile phase was composed of chloroform/acetone/methanol (95:4.5:0.5, v:v:v) and the spots of each lipid were visualized by spraying the plate with iodine vapor in a nitrogen stream. Each fraction was scraped, methylated and analyzed as previously described.

2.4. Winterization

100 mL of a solution (5 wt%) of free fatty acids in hexane were located in amber flasks (125 mL) and stored in a Panasonic ultra-low temperature freezer MDF-U3386S (Panasonic Healthcare Co, Ltd) at several temperatures (-55, -65, -75 and -85ºC) during 1, 2, 3, 4, 6, 8 and 24 hours. The selection of both, the solvent and the oil:solvent ratio, was conducted considering results described in literature. Effectively, hexane has been referred as the solvent with whom higher purity and yield were obtained (López-Martínez et al., 2004; Vázquez & Akoh, 2011; Wanasundara & Shahidi, 1999). The oil:solvent ratio was fixed to 5% (wt %) to obtain high concentrates in PUFA with an economical assumable volume of hexane (López-Martínez et al., 2004; Vázquez & Akoh, 2011).

The crystalized fatty acids obtained after each trial were recovered by filtering samples, at the same temperature as winterized, through a 25 µm filter paper using a vacuum filter pump. The solvent was removed from the liquid fraction with a Büchi Rotavapor R-210 (Büchi Labortechnik AG) during 15 min at 40°C and 100 mmHg. The mass of the solid and the liquid fractions was determined for each trial. All crystallizations were done in duplicate. Finally, the fatty acid profile of each phase was analyzed as described before.

3. THEORY: MODEL OF THE LIQUID FRACTION MASS EVOLUTION

Crystallization process consists in two major events, nucleation and crystal growth. (Marangoni & Wright, 2005; Mullin, 2001). One of the most accepted mechanism for crystallization is the diffusion-reaction model. This model suggests that crystal surface grows in a two-stage process. Firstly, in a supersaturated solution, molecules are transported by diffusion and convection. Then, they are built into the surface of the crystal by integration or an integration reaction (Mersmann, 2001). Hence, winterization implies, initially, diffusion and convection of the free fatty acids and, secondly, the integration of those free fatty acids in a solid phase (Mersmann, 2001). These steps are controlled by a gradient of concentration which is the driving force of the process (Appendix 1). The most common mechanistic model employed for describing these steps is the diffusion-reaction one (Eq. 1):

$$\frac{dm}{dt} = K_G A(c - c_{eq})^g \quad [1]$$

where m represents the mass (g) , t the time (h), K_G ($kg \cdot m^{-2} \cdot s^{-1}$) the global crystal growth coefficient, A (m^2) the crystal's superficial area, g the global order of crystal growth and C_{eq} is the concentration achieved at the equilibrium (Mullin, 2001).

In crystallization, the global order of crystal growth (g) has no fundamental significance and it is comprised between 1 and 2. It has been described that, generally, g is equal to 1 (Mullin, 2001), value which has been employed in this work. Additionally, as the volume of solvent remained constant, the concentration gradient was replaced by the mass one. Finally, it was introduced a global coefficient $K_{G \cdot A}$ (h^{-1}) which englobes the solvent volume and the area of crystals (Eq. 2):

$$\frac{dm}{dt} = K_{G\cdot A} \cdot (m - m_{eq}) \quad [2]$$

Equilibrium data were calculated by adjusting and evaluating the experimental data at infinite time. To that end, experimental data were adjusted to an asymptotic equation (Eq 3) using CurveExpert Version 1.4. To solve the nonlinear regression, the Levenberg-Marquardt method (which combines the steepest-descent method and a Taylor series based method) was employed. The quality of the correlation was evaluated calculating the coefficient of determination (r^2).

$$m = \frac{A + B \cdot t}{C + D \cdot t} \quad [3]$$

The limit of the aforementioned function at infinite time was calculated by dividing B/D (Eq 4).

$$\lim_{t\to\infty} m = \frac{B}{D} \quad [4]$$

The influence of the temperature on the equilibrium mass was linearly correlated. The global coefficient, $K_{G\cdot A}$ (h^{-1}), was estimated for each temperature by analytically solving the differential equation. To determine the influence that the temperature exerted on the global coefficient $K_{G\cdot A}$ an Arrhenius type equation (Eq. 5) was employed (Chang, Wu, & Kimura, 2006; Mersmann, 2001)

$$K_{G\cdot A} = k_0 \cdot \exp\left(-\frac{E_a}{R \cdot T}\right) \quad [5]$$

where, $K_{G\cdot A}$ is the global coefficient (h^{-1}), k_0 the pre-exponential factor (h^{-1}), E_a the activation energy for crystallization ($kJ\cdot mol^{-1}$), R the gas constant ($kJ\cdot mol^{-1}\cdot K^{-1}$) and T the temperature (K).

Hence, the mass transfer model for the liquid phase can be expressed as a function of time and temperature (Eq. 6):

$$\frac{dm}{dt} = k_0 \cdot \exp\left(-\frac{E_a}{R \cdot T}\right) \cdot (m - m_{eq}(T)) \quad [6]$$

where m is the mass (g), t is the time (h), k_0 the pre-exponential factor (h_{-1}), E_a the activation energy for crystallization ($kJ\cdot mol^{-1}$), R the gas constant ($kJ\cdot mol^{-1}\cdot K^{-1}$), T the temperature (K) and m_{eq} the equilibrium mass (g).

4. RESULTS AND DISCUSSION

4.1. Experimental data

The original refined sardine oil was mainly composed by PUFA (45.5%) followed by the saturated fatty acids (SFA) (31.2%) and monounsaturated fatty acids (MUFA) (Table 1). Among the polyunsaturated fatty acids, EPA and DHA represented a 70% of the fraction. With regard to saturated fatty acids, palmitic (C16:0) and myristic acid (C14:0) were the majoritarian with 18.9 and 8.7%, respectively. Finally, the oleic acid (C18:1n-9) was the most abundant among the monounsaturated fatty acids (9%).

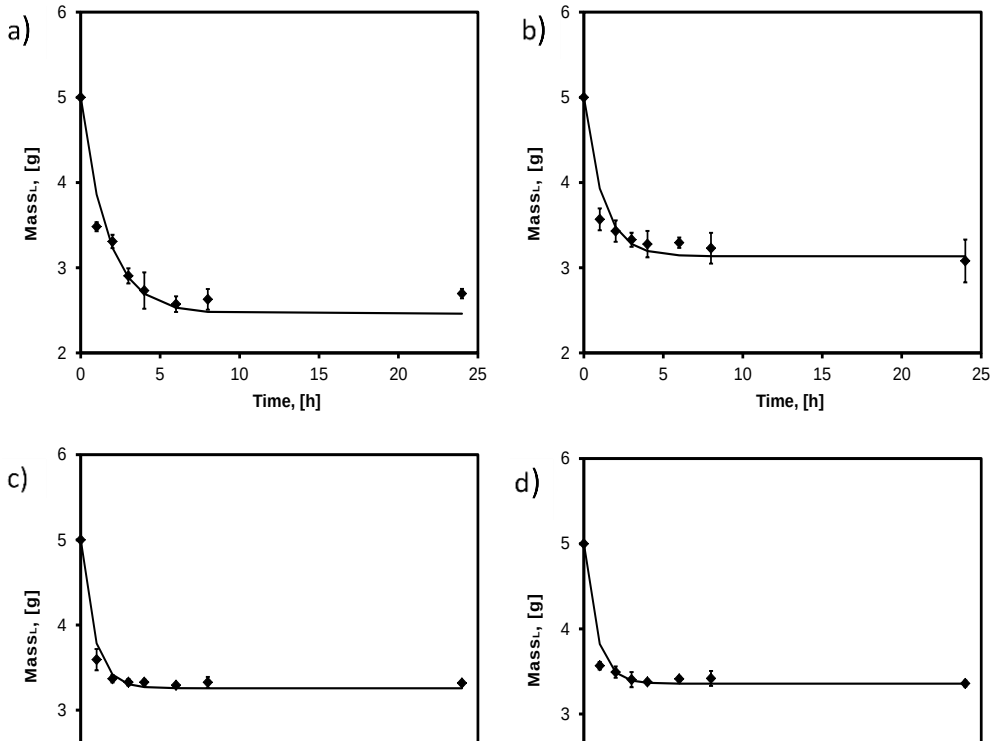


Figure 1. Liquid mass recovered after crystallization at different temperatures (a) -85 °C, (b) -75 °C, (c) -65 °C and (d) -55 °C. Symbols represent experimental data. Continuous line corresponds to the liquid mass recovered predicted by the model evaluated.

The increase of PUFA in the liquid fraction is mainly due to the selective crystallization of fatty acids with lower fusion temperature. Consequently, as the liquid phase was concentrated the liquid mass decreased (Fig. 1). At all tested temperature, the system virtually achieved the equilibrium after 4 hours varying the final mass significantly with temperature. At the cooler assayed temperature (-85ºC), the recovered mass in the liquid

fraction represented the 54% of the initial one, whereas the PUFA content was almost duplicated. In Fig. 1, it can be seen that the final liquid mass increased with the effect of temperature. At the higher temperatures (-55 and -65ºC), the percentage of the recovered liquid mass was similar (~66%) whereas at -75ºC it was slightly lower (61.6%).

Table 1 Mass percentage of fatty acid classes in liquid phase obtained after simple crystallization experiments at 1, 2, 3, 4 and 24 hours. Data are means of duplicate analysis. SD < 6%.

T, ºC	Time, h	SFA	MUFA	PUFA	EPA	DHA
RSO	0	31.2	22.3	45.5	21.5	10.3
-85	1	1.5	29.9	64.1	29.5	14.0
	2	1.25	19.7	74.8	33.4	15.9
	3	1.05	18.8	75.8	36.0	17.1
	4	0.7	15.1	80.7	38.7	18.4
	24	0.7	12.3	83.4	38.6	18.3
-75	1	2.6	29.8	63.2	29.0	13.5
	2	1.6	31.0	64.3	30.1	14.2
	3	1.4	30.7	64.1	30.1	14.0
	4	1.6	30.4	63.8	30.2	14.3
	24	1.5	19.9	74.5	34.4	16.2
-65	1	3.3	30.3	62.4	29.3	13.6
	2	1.8	31.1	64.1	30.0	13.9
	3	2.1	30.7	63.3	29.7	13.8
	4	2.3	30.5	62.6	29.8	14.1
	24	2.0	30.8	64.1	29.9	14.1
-55	1	5.5	29.9	61.6	29.0	13.7
	2	4.1	30.6	62.2	29.5	13.9
	3	2.9	30.5	63.4	29.5	14.2
	4	3.3	29.6	63.1	29.5	13.9
	24	3.4	30.2	62.5	29.3	13.8

The opposite tendency was observed in the solid fraction, remaining the total content of oil constant in all experiments (data not shown). Although the mass of the liquid fraction decreased significantly during winterization, this decreased was mainly due to the crystallization of SFA and MUFA. Effectively, the recovery yields of PUFA, EPA and DHA in the liquid fraction were higher than 90% (data not shown).

Table 1 shows the fatty acid profile of the liquid mixture recovered after winterization. Compared to initial concentration values of the sardine oil, significant reductions of SFA percentage along with an increment of PUFA concentration in the liquid phase were obtained in all tested temperatures. The highest decrease of SFA and increase of PUFA was observed after 24 h at -85 °C. These conditions yielded to an increase over 80% of PUFA with respect to their initial concentration in fish oil. This yield decreased with

temperature, at -75ºC the PUFA increase was 63% while at -65 and -55ºC it was dramatically reduced to 40%.

Table 2 Mass percentage of fatty acid classes in solid phase obtained after simple crystallization experiments at 1, 2, 3, 4 and 24 hours. Data are means of duplicate analysis. SD < 6%.

T, ºC	Time, h	SFA	MUFA	PUFA	EPA	DHA
-85	1	84.5	5.6	8.3	4.23	2.3
	2	53.4	20.7	13.9	3.2	2.4
	3	64.7	26.1	6.8	3.2	1.8
	4	61.7	28.1	6.5	3.6	2.1
	24	48.3	40.5	9.8	1.5	2.6
-75	1	86.6	4.6	6.6	3.4	2.0
	2	86.9	4.4	7.5	3.4	1.7
	3	86.2	4.2	7.3	3.0	1.7
	4	82.7	5.3	9.3	3.6	1.8
	24	79.8	11.4	7.1	3.2	1.7
-65	1	85.5	4.7	7.5	3.6	1.80
	2	86.6	4.5	6.7	3.3	2.25
	3	86.5	4.8	7.5	3.5	1.7
	4	85.1	5.3	7.0	3.7	2.09
	24	86.7	4.6	6.1	3.0	1.76
-55	1	88.4	3.8	5.5	2.84	1.48
	2	87.2	4.4	6.1	3.3	1.6
	3	85.8	4.7	7.1	3.4	1.7
	4	86.1	4.8	6.8	3.7	1.9
	24	86.2	4.8	6.6	3.5	2.0

This significant variation in PUFA content and in liquid mass might be explained in terms of the difference in total amount of MUFA at different temperatures (Table 1). The difference of polarity between the solvent and the FFA is crucial during the winterization process.

The employment of hexane, a solvent with very low polarity, with a mixture of FFA decreased the solubility of certain FFA in the liquid fraction (*i.e.* saturated fatty acids) and enhanced the selectivity of the process (Vázquez & Akoh, 2011). Since solubility decreases with temperature, presumably, at temperatures below -75ºC the MUFA fraction became virtually insoluble. Hence, a significant decrease of the mass of MUFA in the liquid fraction was only observed at the lowest temperature tested.

With regard to the composition of the solid phase, two trends can be observed depending on the temperature (Table 2). At cooler temperatures (-85 and -75ºC), a decrease of SFA percentage corresponding to an increase of MUFA is observed with time. This behavior was favored with decrease of temperature. This reduction of SFA content might be

explained by the selectivity of the crystallization because SFA precipitated faster than MUFA. Initially, the solid fraction was mainly composed by SFA, once MUFA began to crystallize the SFA percentage decreased. By contrast, at higher temperatures (-55.0 ºC and -65.0 ºC), the composition of the solid phase remained virtually constant within time. Concerning the PUFA percentage in the solid fraction, it may be explained by occlusion during crystallization, among other factors that will be discussed in the following section. Presumably, the rate of crystal growth was significantly higher than that of the PUFA diffusion through the crystal. EPA and DHA concentration increased at lower temperatures, as happened to the PUFA fraction (Table 1). Effectively, at -85 °C and 24 hours their concentration was the highest: 56.7%, value that represents a 1.78-fold increase when compared to the raw sardine oil. As happened to the liquid mass evolution, after the first four hours the rate of concentration was intensely reduced.

As reviewed before, several studies have been conducted in the field of PUFA concentration by low temperature winterization. One of the first attempts was done employing menhaden oil in acetone at -18 and -35ºC (Kinsella, 1990). At the lower temperature, Kinsella, (1990) obtained an Omega-3 concentrate (30%) with a recovering yield of 70 %. Wanasundara (1996) concentrated sea blubber oil employing acetone and hexane as solvents (2.5 g/mL). The highest concentration was obtained at -70ºC, the coldest temperature assayed, and after 24h. The contents of EPA and DHA were higher in acetone than in hexane (2.1-1.8 fold and 2.5-2.4 fold, respectively). However, the recovery yields were around three times higher when hexane was employed. Additionally, concentration of stearidonic acid from soybean oil (Vázquez & Akoh, 2011) or γ-linoleic acid from seed oils (López-Martínez et al., 2004) has been conducted by low temperature crystallization.

4.2. Model of the global mass evolution of the liquid fraction

The required parameters to predict the mass evolution of crystallization employing the model based on diffusion-reaction growth mechanism are: the transfer rate coefficient ($K_{G\cdot A}$) and the equilibrium mass of the studied phase (m_{eq}).

Table 3 Coefficients of empirical correlation of liquid and solid mass evolution (Eq 3).

T, ºC	A	B	C	D	R^2
			Liquid		
-85	2.00	1.41	0.40	0.57	0.989
-75	1.14	2.12	0.23	0.68	0.998
-65	0.75	2.44	0.15	0.75	0.998
-55	0.57	2.61	0.11	0.78	0.999
			Solid		
-85	-1.50E-3	1.52	0.42	0.60	0.989
-75	3.42E-4	1.34	0.24	0.72	0.998
-65	-1.03E-4	1.30	0.15	0.75	0.998
-55	-4.63E-6	1.29	0.12	0.79	0.999

The equilibrium masses were estimated employing Eq. 4 and the respective coefficients values showed in Table 3. To extent the usage of the model to any temperature comprised between -55 and -85ºC, equilibrium masses were linearly correlated to temperature (r^2 =0.89). The calculation of $K_{G\cdot A}$ was done for each tested temperature and employing the aforementioned correlation between liquid equilibrium mass and temperature. Effectively, equation 6 was analytically integrated for times up to four hours, where equilibrium was not yet reached, and values of $K_{G\cdot A}$ were calculated (Table 4).

Table 4 Calculated $K_{G\cdot A}$ values and R^2 coefficients of the model and its comparison with experimental data. (R^2 coefficients refer to the linear regressions employed for calculating $K_{G\cdot A}$ (Eq 5))

T (°C)	$K_{G\cdot A}$ (h^{-1})	M_{eqL} (g)	M_{eqS} (g)	R^{2*}
-85	0.596	2.46	2.54	0.929
-75	0.851	3.13	1.87	0.837
-65	1.195	3.26	1.74	0.922
-55	1.262	3.36	1.64	0.897

These constants ranged between 1.262 h^{-1} at -55°C to 0.596 h^{-1} at -85°C ($r^2 > 0.94$). The influence that the temperature exerted on $K_{G\cdot A}$ was described employing Eq. 5, being the values of k_0 and $-E_a/R$: 5.23 h^{-1} and 1072 K, respectively ($r^2 = 0.95$).

Consequently, the liquid mass evolution can be modelled as a function of temperature as shown in Eq. 7. In Fig. 1 the continuous lines correspond to the liquid mass recovered predicted by this model.

$$\frac{}{\mathrm{dt}} = 5.23 \cdot \exp\left(-\frac{}{\mathrm{T}}\right) \cdot (\mathrm{m} - 0.022 \cdot \mathrm{T} + 1.4029) \qquad [7]$$

A double influence of the temperature on the mass evolution is expressed in the current

model. Effectively, $K_{G \cdot A}$ and the equilibrium mass are directly affected by the temperature in opposite tendencies. On one hand, $K_{G \cdot A}$, which represent the velocity of the mass transfer, decreased with the temperature. On the other hand, the driven force increased with lower temperatures, due to a decrease of the equilibrium mass.

In the Fig. 2 it is shown how for all tested temperatures the error was minor than 10% with a high correlation ($r^2 = 0.94$).

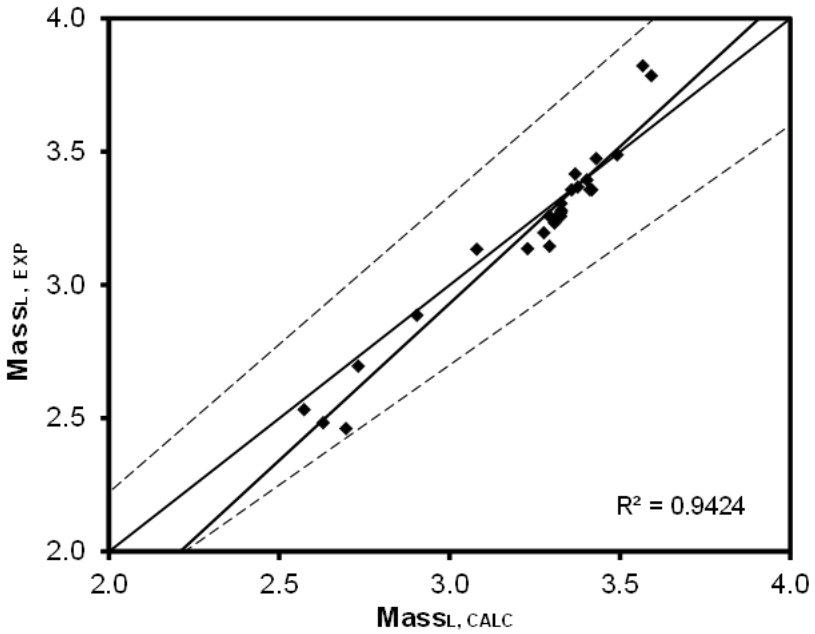


Figure 2. Experimental vs calculated mass of the liquid phase. Dashed lines represent an interval of ±10% of error.

4.3. Model of PUFA, EPA and DHA concentration for each fraction

The concentration of the PUFA in the liquid phase is due to the selective crystallization of the MUFA and SFA fractions. Since the total mass of the liquid phase selectively decreased, the concentration of PUFA in the liquid phase increased. In Table 2, it is shown the percentage of EPA, DHA and PUFA in the solid fraction. For a given time, their individual masses in the solid fraction can be calculated by multiplying the aforementioned percentages and the total mass of the solid fraction. The masses of EPA, DHA and PUFA of the solid fraction remained constant independently of time and temperature being the average values: 0.057±0.006 g, 0.0313±0.04 g and 0.11±0.02 g, respectively. Consequently, the processes by which PUFA are transported into the solid phase happened at the initial times in a relatively fast process. The influence of the composition of fatty acids has been widely described in the case of triacylglycerols where the heterogeneity of fatty acid affects not only the melting point but also the crystalline structure (Himawan,

Starov, & Stapley, 2006). As for free fatty acids crystallization, the effect of the mixture of fatty acids has also influence, although in a minor extent. Takiguchi et al., (1998) described that interactions between molecules in the crystals and in the solution were observed when the number of carbons did not differ by more than four carbons independently of the degree of unsaturation. Additionally, the presence of the PUFA in the solid phase could be due to occlusion during the crystallization of the SFA. Also, since nucleation is a heterogeneous mechanism, external catalytic sites or surfaces can reduce the energy barrier and, consequently, PUFA can be incorporated in some crystalline structure. These external sites can be impurities (minor compounds) which were presented in the oil as traces of monoacylglycerols, diacylglycerols, or phospholipids, among others (Ribeiro et al., 2015). Additionally, it could be possible that the nucleus and crystal of SFA may act as seeds favoring some minor crystallization of PUFA. Also, although the solubility of a given fatty acid in a mixture is quite similar to the one in the pure state inter-solubilization might occur. Hence, mixed crystals might be formed at higher temperatures that expected for pure fatty acids (Wanasundara et al., 2005).

As stated before, the mass of EPA, DHA and PUFA in the solid fraction remained constant independently of time and temperature. Due to these constant values, no mass transfer equations can be employed to model the increase of the concentration of PUFA in the liquid fraction. Consequently, the following equation is proposed for that calculation:

$$X_i = \frac{mass_{i,initial} - mass_{i,sol}}{mass_{L,t}} \quad [8]$$

where i refers to PUFA, EPA or DHA; X the mass fraction in the liquid phase, $mass_{i,initial}$ the initial mass in the oil, $mass_{i,sol}$ the average mass in the solid fraction and $mass_{L,t}$ the mass of the liquid fraction at a time t (calculated by integration of Eq. 7). The term $mass_{i,sol}$ has been estimated as the average mass in the solid, employing data of all the times and temperatures studied.

By employing this model these data can be predicted with error minor than 10% as it is shown in Fig. 3 and acceptable correlation (r^2>0.83).

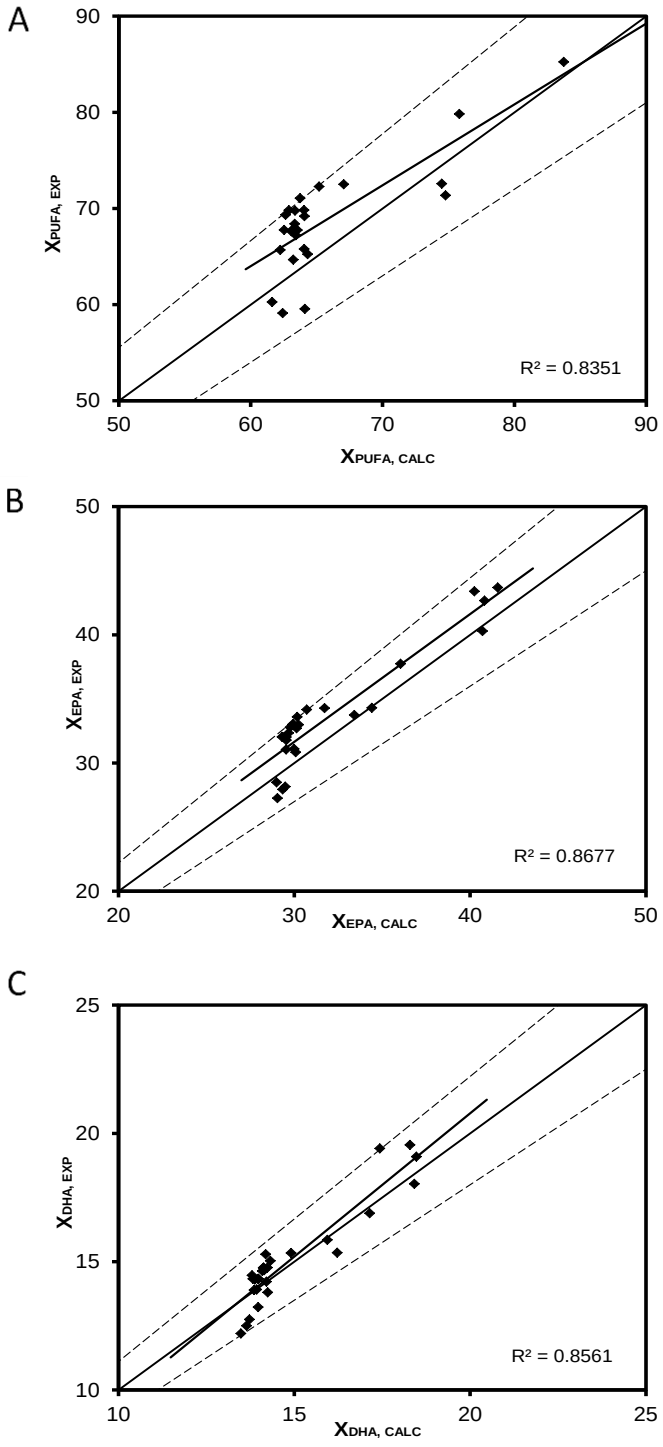


Figure. 3. (a)PUFA ,(b), EPA and (c) DHA experimental vs calculated mass fraction of the liquid phase. Dashed lines represent an interval of ±10% of error

5. CONCLUSIONS

In this study, PUFA concentrates were produced from refined sardine oil by means of solvent fractional crystallization at several temperatures (-55, -65, .75 and -85ºC) and times (1, 2, 3, 4, 6, 8, 24h). The PUFA concentration was favored at cooler temperatures and long times. A significant increased (1.7-fold) of the PUFA content was obtained when winterization was conducted at -85ºC during 24 hours. Total liquid mass recovered decreased at lower temperatures mainly because of the crystallization of SFA and MUFA. The liquid mass evolution with time was modeled following the theory of mass transference. Effectively, a function of temperature and time with two constants ($K_{G\cdot A}$ and meq) efficiently predicted the behavior of the system (r^2>0.94). The mass fraction of PUFA, EPA and DHA was also modeled by employing the aforementioned model and solid mass loss coefficients (r^2>0.83). In the case of refined sardine oil, this model is able to predict both, the mass and composition, of PUFA, EPA and DHA at any temperature comprised between -55 and -85ºC. This methodology can be employed for modelling winterization of other types of oils previous estimation of the parameters: transfer rate coefficient, equilibrium masses, $mass_{i,initial}$ and $mass_{i,sol}$.

APPENDIX. DIFUSSION-REACTION THEORY

Crystal growth is a process where two stages occur: diffusion and reaction. Both stages are governed by different concentration driving forces (Mullin, 2001). In the first one, solute molecules are transported from the bulk of the fluid phase to the solid surface (Eq A.1):

$$\frac{dm}{dt} = k_d \cdot A \cdot (c - c_i) \qquad [A1]$$

where m is mass, t represents time, k_d is a coefficient of mass transfer by diffusion, A is the surface are of the crystal, c is the solute concentration in the bulk phase and c_i is the solute concentration in the solution at the crystal-solution interface.

In the second step, the reaction, the solute molecules arrange themselves into the crystal lattice (Eq A.2):

$$\frac{dm}{dt} = k_r \cdot A \cdot (c_i - c_{eq}) \qquad [A2]$$

where m is mass, t represents time, k_r is a rate constant for the surface reaction process, A

is the surface are of the crystal, c_i is the solute concentration in the solution at the crystal-solution interface and C_{eq} the equilibrium saturation concentration.

These equations employ interfacial concentrations which are difficult to measure. Hence, they are combined resulting in (Eq A.3)

$$\frac{dm}{dt} = K_G A(c - c_{eq})^g \quad [A3]$$

where m represents the mass , t the time , K_G is the global crystal growth coefficient, A is the crystal's superficial area and g is the global order of crystal growth, C_{eq} is the concentration achieved at the equilibrium. The value of the growth rate order, g, is comprised in the range 1 to 2; being generally 1 (Mullin, 2001).

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Meaning
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
TAG	Triacylglycerols
FFA	Free fatty acids
TLC	Thin layer chromatography
m	Mass, g
t	Time, h
K_G	Global crystal growth coefficient, $g \cdot m^{-2} \cdot h^{-1}$
A	Crystal's superficial area, m^2
g	Global order of crystal growth, -
C	Concentration, g/m^3
C_{eq}	Equilibrium concentration, g/m^3
m_{eq}	Equilibrium mass, g
$K_{G \cdot A}$	Transfer rate coefficient, h^{-1}
A, C	Coefficients empirical correlation (m, t), g
B, D	Coefficients empirical correlation (m, t), $g \cdot h^{-1}$
k_0	Pre-exponential factor, h^{-1}
E_a	Activation energy for crystallization, $kJ \cdot mol^{-1}$
R	gas constant, $kJ \cdot mol^{-1} \cdot K^{-1}$
T	Temperature, K
X_i	Solid mass fraction, -
$mass_{i,initial}$	Initial mass of a given fatty acid, g
$mass_{i,sol}$	Average mass of a given fatty acid in the solid fraction, g
$mass_{L,t}$	Mass of the liquid fraction for a given time, g

6. REFERENCES

Akoh, C. C. (2005). Handbook of Functional Lipids. CRC Press.

Bougnoux, P., Hajjaji, N., Maheo, K., Couet, C., & Chevalier, S. (2010). Fatty acids and breast cancer: Sensitization to treatments and prevention of metastatic re-growth. *Progress in Lipid Research*, 49(1), 76–86. http://doi.org/10.1016/j.plipres.2009.08.003

Bradbury, J. (2011). Docosahexaenoic Acid (DHA): An Ancient Nutrient for the Modern Human Brain. *Nutrients*, 3(5), 529–554. http://doi.org/10.3390/nu3050529

Camacho Paez, B., Robles Medina, A., Camacho Rubio, F., González Moreno, P., & Molina Grima, E. (2002). Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by the acidolysis of cod liver oil and caprylic acid in a packed-bed reactor: Equilibrium and kinetics. *Chemical Engineering Science*, 57(8), 1237–1249.

Chang, S.-H., Wu, S.-K., & Kimura, H. (2006). Crystallization Kinetics of $Ti_{50}Ni_{25}Cu_{25}$ Melt-Spun Amorphous Ribbons. *Materials Transactions*, 47(10), 2489–2492. http://doi.org/10.2320/matertrans.47.2489

Cunha, D. C., Crexi, V. T., & Pinto, L. A. de A. (2009). "Winterização" de óleo de pescado *via* solvente. *Ciencia E Tecnologia de Alimentos*, 29(1), 207–213.

Dillon, J. T., Aponte, J. C., Tarozo, R., & Huang, Y. (2013). Purification of Omega-3 polyunsaturated fatty acids from fish oil using silver-thiolate chromatographic material and high performance liquid chromatography. *Journal of Chromatography A*, 1312, 18–25. http://doi.org/10.1016/j.chroma.2013.08.064

EU. (2013). Regulation (EU) no 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Official Journal of the European Union, 22–28.

Gunstone, F. D., Harwood, J. L., & Dijkstra, A. J. (2012). The Lipid Handbook with CD-ROM, Third Edition. CRC Press.

Haraldsson, G. (1983). Separation of Saturated/Unsaturated fatty acids. *Journal of the American Oil Chemists' Society*, 61(2), 219–222.. http://doi.org/10.1007/BF02678772

Himawan, C., Starov, V. M., & Stapley, A. G. F. (2006). Thermodynamic and kinetic aspects of fat crystallization. *Advances in Colloid and Interface Science*, 122(1–3), 3–33. http://doi.org/10.1016/j.cis.2006.06.016

Jump, D. B., Depner, C. M., & Tripathy, S. (2012). Omega-3 fatty acid supplementation and cardiovascular disease Thematic Review Series: New Lipid and Lipoprotein Targets for the Treatment of Cardiometabolic Diseases. *Journal of Lipid Research*, 53(12), 2525–2545. http://doi.org/10.1194/jlr.R027904

Kelleher, K. (2005). Discards in the world's marine fisheries: An update. FAO. Fisheries Technical Paper.

Kinsella, J. E. (1990). Sources of Omega-3 fay acids in human diets. In R.. Lees & M. Karel (Eds.), Omega-3 Fatty Acids in Health and Disease (pp. 157–200). New York: Marcel Dekker Inc.

Kralovec, J. A., Zhang, S., Zhang, W., & Barrow, C. J. (2012). A review of the progress in enzymatic concentration and microencapsulation of Omega-3 rich oil from fish and microbial sources. *Food Chemistry*, 131(2), 639–644. http://doi.org/10.1016/j.foodchem.2011.08.085

Kuratko, C. N., & Salem, N. (2013). Docosahexaenoic acid from algal oil. *European Journal of Lipid Science and Technology*, 115(9), 965–976. http://doi.org/10.1002/ejlt.201300060

Lee, K.-T., & Foglia, T. A. (2001). Fractionation of menhaden oil and partially hydrogenated menhaden oil: Characterization of triacylglycerol fractions. *Journal of the American Oil Chemists' Society*, 78(3), 297–304. http://doi.org/10.1007/s11746-001-0260-9

Lembke, P. (2013). Production Techniques for Omega-3 Concentrates. In F. De Meester, R. R. Watson, & S. Zibadi (Eds.), Omega-6/3 Fatty Acids (pp. 353–364). Humana Press.

López-Martínez, J. C., Campra-Madrid, P., & Guil-Guerrero, J. L. (2004). γ-Linolenic acid enrichment from Borago officinalis and Echium fastuosum seed oils and fatty acids by low temperature crystallization. *Journal of Bioscience and Bioengineering*, 97(5), 294–298. http://doi.org/10.1016/S1389-1723(04)70208-X

Marangoni, A. G., & Wright, A. J. (2005). Physical Properties of Fats and Oils. In Handbook of Functional Lipids (pp. 135–162). CRC Press.

Mersmann, A. (2001). Crystallization Technology Handbook. CRC Press.

Morales-Medina, R., García-Moreno, P. J., Pérez-Gálvez, R., Muñío, M. M., Guadix, A., & Guadix, E. M. (2015). Nutritional indexes, fatty acids profile and regiodistribution of oil extracted from four discarded species of the Alboran Sea: Seasonal effects. *European Journal of Lipid Science and Technology, In Press*. http://doi.org/10.1002/ejlt.201500486

Mullin, J. W. (2001). Crystallization. In J. W. Mullin (Ed.), Crystallization (4th Editio, pp. 216–288). Oxford: Butterworth Heinemann. http://doi.org/10.1016/B978-075064833-2/50008-5

Ribeiro, A. P. B., Masuchi, M. H., Miyasaki, E. K., Domingues, M. A. F., Stroppa, V. L. Z., de Oliveira, G. M., & Kieckbusch, T. G. (2015). Crystallization modifiers in lipid systems. *Journal of Food Science and Technology*, 52(7), 3925–46. http://doi.org/10.1007/s13197-014-1587-0

Rodríguez-Ruiz, J., Belarbi, E.-H. H., Sánchez, J. L. G., & Alonso, D. L. (1998). Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotechnology Techniques*, 12(9), 689–691. http://doi.org/10.1023/A:1008812904017

Rubio-Rodriguez, N., Beltran, S., Jaime, I., De, D. S. M., Sanz, M. T., & Carballido, J. R. (2010). Production of Omega-3 polyunsaturated fatty acid concentrates: A review. *Innovative Food Sci. Emerging Technol.*, 11, 1–12. http://doi.org/10.1016/j.ifset.2009.10.006

Shahidi, F., & Wanasundara, U. N. (1998). Omega-3 fatty acid concentrates: Nutritional aspects and production technologies. *Trends in Food Science and Technology*, 9(6), 230–240. http://doi.org/10.1016/s0924-2244(98)00044-2

Small, D. M. (1991). The effects of glyceride structure on absorption and metabolism. Annual Review of Nutrition.

Takiguchi, H., Iida, K., Ueno, S., Yano, J., & Sato, K. (1998). Heterogeneous nucleation of n-alcohol crystals from solution assisted by vapor-deposited thin films of fatty acids. *Journal of Crystal Growth*, 193(4), 641–647. http://doi.org/10.1016/S0022-0248(98)00610-1

Vázquez, L., & Akoh, C. C. (2011). Concentration of Stearidonic Acid in Free Fatty Acid and Fatty Acid Ethyl Ester Forms from Modified Soybean Oil by Winterization. *Journal of the American Oil Chemists' Society,* 88(11), 1775–1785. http://doi.org/10.1007/s11746-011-1857-1

Wanasundara, U. N. (1996). Marine oils : stabilization, structural characterization and Omega-3 fatty acid concentration (doctoral). Memorial University of Newfoundland.

Wanasundara, U. N., & Shahidi, F. (1999). Concentration of Omega 3-polyunsaturated fatty acids of seal blubber oil by urea complexation: Optimization of reaction conditions. *Food Chemistry*, 65(1), 41–49. http://doi.org/10.1016/s0308-8146(98)00153-8

Wanasundara, U. N., Wanasundara, P. K. J. P. D., & Shahidi, F. (2005). Novel Separation Techniques for Isolation and Purification of Fatty Acids and Oil By-Products. In Bailey's Industrial Oil and Fat Products. John Wiley & Sons, Inc.

Weichselbaum, E., Coe, S., Buttriss, J., & Stanner, S. (2013). Fish in the diet: A review. Nutrition Bulletin, 38(2), 128–177. http://doi.org/10.1111/nbu.12021

V. Lumped model of the lipase catalyzed hydrolysis of sardine oil to maximize polyunsaturated fatty acids content in acylglycerols [*]

The aim of this work was to produce diacylglycerols and monoacylglycerols with high content of polyunsaturated fatty acids (PUFA) (more specifically, EPA and DHA). To that end, *Rhizomucor miehei* lipase mediated-hydrolysis of sardine oil was conducted at several constant water activities. The system was mechanistically modelled to predict the evolution of the concentration of triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids (FFA) along the time. Additionally the concentration of saturated (SFA), monounsaturated (MUFA) and PUFA in the acylglycerols and FFA were modelled for each fraction. The release of the first fatty acid from the triacylglycerol was independent on the unsaturation degree, while the hydrolysis of the second was highly affected by the degree of unsaturation being the PUFA the fatty acids that presented the lowest rate. This fact was related to the regiodistribution of the fatty acids in the original sardine oil. MAG produced was maximum at lower water activities and presented lower PUFA content than the original oil. DAG content was maximized at high water activity (35 mol%) achieving a 2-fold concentration of DHA.

[*] MANUSCRIPT SUBMITTED FOR PUBLICATION: R. Morales-Medina, M. Munio, A. Guadix, E.M. Guadix and F. Camacho. Lumped model of the lipase catalyzed hydrolysis of sardine oil to maximize polyunsaturated fatty acids content in acylglycerols *Journal of Food Engineering.* Under review.

1. INTRODUCTION

Fish discards are defined as the fraction of organic material in the catch which is not retained for sale but dumped back to the sea (Kelleher, 2005). Discarding presents an environmental problem since it modifies the trophic chains of the sea. Additionally, it implies the underutilization of the marines' sources. Consequently, the new Common Fisheries Policy has banned the discard practices in Europe (EU, 2013), since January 2015 catches from pelagic species (*e.g.* sardines or horse mackerel) must be landed. This new regulation will be applied from 2017 on, for the rest of species.

Accordingly, new techniques able to convert fish discards into added-value product are required. In this context, the production of oil with high polyunsaturated fatty acids (PUFA) content from fish oil has been considered as an interesting up-grading technique. PUFA, more specifically, eicosapentaenoic and docosahexaenoic acids (EPA and DHA) plays a beneficial role on the cardiovascular system (Jump et al., 2012) as well as on the brain functions and on some types of cancer (Bradbury, 2011). A detailed description of the benefit that PUFA exert on the human health has been recently conducted elsewhere (Morales-Medina, R., Munio, M Muñio, Pérez-Gálvez, R., Guadix, 2013). Due to all these beneficial properties, there is an increasing demand of modified oils with high PUFA content to produce food supplements or nutraceutical products (Dillon et al., 2013; Lembke, 2013; Rubio-Rodriguez et al., 2010; Shahidi and Wanasundara, 1998).

Concentration of PUFA can be conducted by enzymatic or physical approaches (Lembke, 2013; Morales-Medina et al., 2015; Rubio-Rodriguez et al., 2010). Physical approaches (i.e supercritical fluid extraction or low temperature winterization) can lead to high purity when PUFA are concentrated as esters of fatty acids instead of in the triacylglycerol form (Lembke, 2013). Hence, a pretreatment to convert triacylglycerols is required. Also, after concentration, a re-esterification must be conducted to improve the PUFA digestibility and absorption in the human digestion (Lawson and Hughes, 1988; Small, 1991). Enzymatic methods are based on the use of lipases, which are highly selective. Indeed, lipases are classified in two groups: (i) random lipases which indistinctly react with the three positions of the glycerol backbone and (ii) 1,(3)-specific lipases which preferably react with the external bonds of the glycerol backbone (Hari Krishna and Karanth, 2002). The enzymatic tecniques present as main advantage that the PUFA can be concentrated as acylglycerols.

In this context, there is special interest on the monoacylglycerols and diacylglycerols fractions. Monoacylglycerols (MAG) and diacylglycerols (DAG) account around 75% of the total production of emulsifiers that are applied in the food industry (Feltes et al., 2013). They are also widely employed in the pharmaceutical and cosmetic industries because the present no side effect when ingested (Feltes et al., 2013). Hence, the production of MAG or DAG with high content of PUFA (mainly EPA or DHA) would imply an added nutritional value to these product which are usually employed as food emulsifiers. Additionally, in the case of the 2-MAG with high content of PUFA, they can be employed as substrate for the production of structured lipids. Effectively, 2-MAG can be re-esterified with a medium chain fatty acid resulting in a MLM structured lipid (Munio et al., 2009). These structured lipids present a faster absorption than the original oil and their daily intake might result in a less accumulation of fats (Ruxton et al., 2007).

The most used enzymatic methods employed to produce 2-MAG are alcoholysis, glycerolisis and hydrolysis. Alcoholysis is a reaction between an ester (i.e a triacylglycerol) and an alcohol such as methanol or ethanol. It has been widely studied and proposed as a good technique for the production of 2-MAG with a maximum theoretical yield of 33 mol% (Esteban et al., 2009). Glycerolysis is a variation of alcoholysis in which the alcohol employed is glycerine. In this case, the yield is much higher than in alcoholysis. Effectively, processes with a yield of MAG higher than 90 % has been reported, resulting in the conversion of almost all triacylglycerols into MAG (Pawongrat et al., 2007). Consequently, to increase the PUFA content a second step consisted of concentration with acetone at low temperature was required (Pawongrat et al., 2007). Hydrolysis is a reaction between an ester and water. In the presence of excess of water, it leads to high content of PUFA in the DAG and TAG fractions with negligible amount of MAG (Hoshino et al., 1990; Kahveci and Xu, 2011). Hence, the control of water content of the system might be an interesting option to increase the MAG production.

Water affects the enzyme structure and also the conditions of the system (*i.e.* by facilitation the reagent diffusion or influencing the equilibrium (Hari Krishna and Karanth, 2002)). The water content is measured as water activity, especially in micro aqueous systems (Xu, 2000). Water activity is a dynamic parameter which measures the energy status of the water in a system and it is defined as the vapor pressure of water divided by that of the pure water at the same temperature. It is considered the most convenient way of characterizing the water level of micro aqueous systems, because it determines the

distribution of the water among the phases (Xia et al., 2009). Several works have been conducted for studying the effect of hydrolysis and the distribution of the fatty acids among the species produced in excess of water (Hoshino et al., 1990; Kahveci and Xu, 2011). Despite several works have focused on the kinetic study of alcoholysis or acydolysis to produce modified triacylglycerols (Carrín and Crapiste, 2008; Pacheco et al., 2010; Zhao et al., 2007), there is no studies focus on the kinetic behavior of fish oil hydrolysis in microaqueous systems. Additionally, the partition of the fatty acids among the acylglycerols and free fatty acids fractions has not been modelled. Also, further research is required to better understand the effect of water activity on the kinetic of the hydrolysis and on the selectivity of lipases.

The aim of this work was to produce diacylglycerols and monoacylglycerols with high content of polyunsaturated fatty acids (PUFA) (more specifically, EPA and DHA).To that end, we studied the influence of water activity on the *Rhizomucor miehei* lipase mediated-hydrolysis of sardine oil. A mechanistic model composed of two second-order reversible reactions was proposed. Also, since 2-MAG are of special interest for the synthesis of structured lipids, the acyl migration that MAG underwent was also modelled.

2. MATERIALS AND METHODS

2.1. Oil, lipases and chemicals

Refined sardine oil with 21.5 wt% of EPA and 10.4 wt% of DHA was provided by Industrias Afines S.L (Mos, Spain).

The lipases employed were Novozyme 435 from *C. antarctica* kindly donated by Novozymes (Denmark) and immobilized lipase *Rhizomucor miehei* from Sigma-Aldrich (St Louis, MO, USA). Nonadecanoic acid employed as standard was purchased from Sigma-Aldrich (St. Louis, MO). All chemicals and solvents were of analytical grade.

2.2. Enzymatic hydrolysis of triacylglycerols

Hydrolysis was conducted at three different water activities (a_w: 0.33, 0.6 and 0.8). Water activity (a_w) of the media was controlled employing mixtures of salts with different degree of hydration (Zacharis et al., 1997). A mixture of sodium acetate anhydrous and tri-hydrate (0.5g:0.5g) was employed to fix the water activity to 0.3 . In the case of a_w 0.6, the mixture

of salts were $NaHPO_4*2{\cdot}H_2O$ and $NaHPO_4*7{\cdot}H_2O$ (0.5g:0.5g) and for a_w 0.8 a mixture of $NaHPO_4*7{\cdot}H_2O$ and $NaHPO_4*12{\cdot}H_2O$ (0.5g:0.5g) was used. Additionally, to ensure that water activity of the media was effectively controlled, it was monitored throughout the whole reaction time employing a LabMaster a_w (Novasina, Lachen, Switzerland).

Each pair of salts were mixed with 25 mL of hexane and stored in 50mL Erlenmeyer amber flaskfor 48 h at 40ºC and 300 rpm until equilibrium was obtained. Then, 2.5 g of refined oil and 0.25 g immobilized lipase Rhizomucor miehei were added to the media. Rhizomucor miehei is a sn-1(3) specific lipase, *i.e.* it is a regiospecific lipase with high selectivity through the external bonds of the glycerol backbone (Rodrigues and Fernandez-Lafuente, 2010). Lipozyme RM IM was immobilized in a Duolite ES 562 macroporous support, a weak anion-exchange resin based on phenol-formaldehyde copolymers (Rodrigues and Fernandez-Lafuente, 2010). In the Appendix 1, the main physical characteristics of the immobilized enzyme are listed.

The reaction mixture was stirred at 300 rpm in an orbital shaker (Heidolph, Unimax 1010, Germany) and kept at 40ºC under nitrogen atmosphere. Samples were taken at several times: 0, 5, 10, 15, 30, 60, 90, 120, 180, 240 and 480 min. Each sample was filtered and stored in 50 mL of hexane under nitrogen atmosphere and at -80ºC until analysis.

2.3. Study of the acyl migration of monoacylglycerols

2-MAG fraction was individualy studied, due to their valuable application for the production of structured lipids. Hence, the extent of the acyl migration (*i.e.* migration of acyl groups from the sn-2 to the sn-1(3) positions by electronic rearrangement) was individually studied. To that end, 2-MAG were produced by ethanolysis and incubated in the same conditions as the hydrolysis with minor variations. Additionally, the influence of the enzyme support and water activity was evaluated.

To produce the required mass of 2-MAG the method described by Munio et al., (2009) was scaled up: 50 g of oil, 200 g of analytical grade absolute ethanol (99.5%, v/v) and 23.3 g of the lipase Novozyme 435. This lipase is a non-specific one that, in the presence of high content of ethanol, becames highly 1,3 selective (Shimada et al., 2003). The reaction was incubated in 500 mL Erlenmeyer flasks covered with aluminum foil and under nitrogen atmosphere in an orbital shaker at 35ºC for 2 h and stirred at 300 rpm. This reaction was conducted four times. Finally, the reaction was stopped by removing the lipase by filtration

and the solvent was extracted by rotary evaporation (40ºC, 110 mm Hg). The oil mixture (2-MAG, TAG and ethyl esters) was stored under inert atmosphere and at -80ºC until analysis and separation.

The recovery of 2-MAG was conducted by solvent extraction following the protocol described by Munio et al., (2009). The mixture of 2-MAG, TAG and ethyl esters was dissolved in an ethanol/water solution (90:10, v/v) in a proportion of 1:9 (v/v). To remove ethyl esters, the mixture was extracted with an equal volume of hexane three times. Organic phase was then mixed with anhydrous Na_2SO_4 to remove water traces. The purified 2-MAG was stored at -80ºC and inert atmosphere. As a result, a mixture composed of 96.41 mol % of 2-MAG and 3.59 mol % of 1-MAG was obtained with a negligible amount of triacylglycerols, whose fatty acid composition is listed in Table 1.

The influence of the acyl migration on the MAG distribution was studied (i) at water activity of 0.3 in the presence and absence of deactivated lipase (*Rhizomucor miehei*) and (ii) at water activity of 0.8 in the presence of deactivated lipase. These experiments were conducted following exactly the same conditions of the hydrolysis; however, the produced MAG were employed as substrate instead of the refined sardine oil.

The enzyme was thermally deactivated by autoclaving (15 min, 120ºC) and then dried (24 h, 110ºC), 0.25 g of deactivated enzyme were added to the reaction mixture in the test done in the presence of enzyme.

2.4. Determination of the fatty acids profile and lipid class composition

The fatty acid profile of samples was determined by direct transesterification following the method described by Rodríguez-Ruiz et al. (1998). An aliquot of 1mL of a solution of 1mg/mL of the sample in hexane was mixed with 1 mL of reagent mixture (methanol and acetyl chloride, 20:1, v/v). For quantitative determination, nonadecanoic acid (C19:0) was employed as internal standard. Transesterification was conducted at 90ºC for 1 hour with intermittent agitation. To analyze the fatty acids methyl esters, an Agilent 7890A chromatograph (Agilent Technologies, S.A.) connected to a capillary column of fused silica Omegawax (0.25 mm × 30 m, 0.25 µm standard film; Supelco, Bellefonte, PA), and a flame-ionization detector (Camacho Paez et al., 2002) was employed. All analysis were done in triplicate and the results were expressed on molar basis.

Thin layer chromatography (TLC) was employed to separate the following polar lipid fractions: monoacylglycerols (MAG), free fatty acids (FFA), diacylglycerols (DAG) and triacylglycerols (TAG). Firstly, plates of silica-gel (Precoated TLC plates, SIL G-25; Macherey-Nagel, Sigma–Aldrich) were activated at 100ºC for 1 hour. Then, a mass of 2 mg of the reaction mixture was spotted on the plate. A mixture composed of chloroform/acetone/methanol (95:4.5:0.5, v:v:v) was used as mobile phase and the spot of each lipid group was visualized by spraying the plate with iodine vapor in a nitrogen stream. Each fraction was scraped, methylated and analyzed as previously described.

For the separation of 1-MAG and 2-MAG, prior to analysis, plates were immersed in a hydroethanolic solution (1:1, v/v ethanol 99% and distilled water) of boric acid at 1.2 wt% and dried under Nitrogen flow (Henderson and Tocher, 1992).

2.4.1. Positional distribution of fatty acids in TAG

To determine the regiodistribution of the fatty acids in the TAG an ethanolysis of the refined sardine oil with Novozyme 435 was conducted. In previous works of our group, the whole process is described in detail (R. Morales-Medina et al., 2015a). By this highly selective alcoholysis, produced monoacylglycerols are mainly 2-MAG and they can be easily separated by thin layer chromatography, as previously described.

The percentage of a given fatty acid in sn-2 position can be related to the total content of that fatty acid by the following equation:

$$\text{Relative content of FA}_i \text{ in sn2 position} = \frac{\text{content of FA}_i \text{ in sn2 position}}{3 \cdot \text{total content of FA}_i \text{ in TAG}} \cdot 100 \quad [1]$$

The total percentage of each fatty acid located in sn-2 can be estimated by multiplying the aforementioned percentage and the global fatty acid percentage, both in molar basis.

2.5. Kinetic model of the triacylglycerols hydrolysis

The following assumptions are adopted for the development of the kinetic model: firstly, external mass transfer limitations were negligible since proper stirring speed was employed. Secondly, internal mass transport was negligible due to the small size of the immobilized enzyme and to their macroporous characteristics (Appendix 1). Hence, the enzyme concentration was included in the kinetic constant and the system was studied as a pseudohomegeneous one. Thirdly, the immobilized lipase has a rigid 1(3)-regiospecifity,

consequently, the direct formation of 1,3-DAG or 1(3)-MAG was neglected. Consequently, the model describes the behavior of TAG, DAG, MAG and FFA. Finally, the hydrolysis reactions belonged to second-order reversible reactions as shown in Eq 2 and Eq 3.

$$\mathrm{TAG} + \mathrm{W} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \mathrm{DAG} + \mathrm{FFA} \quad [2]$$

$$\mathrm{DAG} + \mathrm{W} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \mathrm{MAG} + \mathrm{FFA} \quad [3]$$

Where, TAG, DAG, MAG, FFA and W represent triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acids and water, respectively.

The set of ordinary nonlinear differential equations derived from the aforementioned reaction mechanism is:

$$\frac{d[\mathrm{TAG}]}{dt} = -k_1 \cdot a_w [\mathrm{TAG}] + k_{-1} \cdot [\mathrm{DAG}] \cdot [\mathrm{FFA}] \quad [4]$$

$$\frac{d[\mathrm{DAG}]}{dt} = k_1 \cdot a_w \cdot [\mathrm{TAG}] - k_{-1} \cdot [\mathrm{DAG}] \cdot [\mathrm{FFA}] + k_{-2} \cdot [\mathrm{MAG}] \cdot [\mathrm{FFA}] - k_2 \cdot a_w \cdot [\mathrm{DAG}] \quad [5]$$

$$\frac{d[\mathrm{MAG}]}{dt} = k_2 \cdot a_w [\mathrm{DAG}] - k_{-2} \cdot [\mathrm{MAG}] \cdot [\mathrm{FFA}] \quad [6]$$

$$\frac{d[\mathrm{FFA}]}{dt} = k_1 \cdot a_w [\mathrm{TAG}] - k_{-1} \cdot [\mathrm{DAG}] \cdot [\mathrm{FFA}] + k_2 \cdot a_w \cdot [\mathrm{DAG}] - k_{-2} \cdot [\mathrm{MAG}] \cdot [\mathrm{FFA}] \quad [7]$$

Where, k_i and k_{-i} are the kinetic constants for the forward and reverse in "i" reaction (Eq, 2 and 3) and t is the reaction time.

The concentration of the water is assumed to be equivalent to the water activity of the system.

Additionally, the equilibrium constant of each reaction were calculated as follows:

$$K_{eq1} = \frac{[\mathrm{DAG}] \cdot [\mathrm{FFA}]}{[\mathrm{TAG}] \cdot a_w} = \frac{k_1}{k_{-1}} \quad [8]$$

$$K_{eq2} = \frac{[\mathrm{MAG}] \cdot [\mathrm{FFA}]}{[\mathrm{DAG}] \cdot a_w} = \frac{k_2}{k_{-2}} \quad [9]$$

This set of ordinary nonlinear differential equations was employed for modelling the global evolution of the system (*i.e.* total content of TAG, DAG, MAG and FFA). Also, fatty acids were divided into three groups: (i) saturated (SFA), (ii) monounsaturated (MUFA) and (iii) polyunsaturated fatty acids (PUFA). SFA and MUFA were modelled following equations

[4] to [7]. Contrary, PUFA was modelled by doing a molar balance to the whole system as follows:

$$C_{i,GLOBAL} = C_{i,SFA} + C_{i,MUFA} + C_{i,PUFA} \quad [10]$$

C_i is the concentration of a given specie (TAG, DAG, MAG or FFA) and global, SFA, MUFA and PUFA refer to the global system and to the fractions of saturated, monounsaturated or polyunsaturated fatty acid fractions, respectively.

Kinetic model of the acyl migration of monoacylglycerides

Acyl migration kinetics of 2-MAG to 1(3)-MAG was studied and modelled as a reversible first-order reaction as follows:

$$2-\mathrm{MAG} \underset{k_{-m}}{\overset{k_m}{\leftrightarrow}} 1-\mathrm{MAG} = 2-\mathrm{MAG} \underset{k_{-m}}{\overset{k_m}{\leftrightarrow}} 3-\mathrm{MAG} \quad [11]$$

Where, k_m and k_{-m} are the kinetic constants for the forward and reverse reactions. It is assumed that the migration to 1-MAG and 3-MAG is equivalent, therefore, in the text 1-MAG will represent the total content of 1(3)-MAG. Hence, the differential equation characterizing the acyl migration was:

$$\frac{d[2-\mathrm{MAG}]}{dt} = -k_m \cdot [2-\mathrm{MAG}] + k_{-m} \cdot [1-\mathrm{MAG}] \quad [12]$$

$$\frac{d[1-\mathrm{MAG}]}{dt} = k_m \cdot [2-\mathrm{MAG}] - k_{-m} \cdot [1-\mathrm{MAG}] \quad [13]$$

Where, 2-MAG and 1-MAG are the 2 and 1(3)-monoacylglycerols, t is the reaction time and k_m and k_{-m} are the kinetic constants for the forward and reverse reactions.

The constant of equilibrium can be expressed as:

$$K_{eqm} = \frac{[1-\mathrm{MAG}]}{[2-\mathrm{MAG}]} = \frac{k_m}{k_{-m}} \quad [14]$$

2.6. Statistical analysis

Reactions were done by duplicate and each sample was analyzed twice (four values per sample). Kinetic equations of the proposed models (Eqs. 4-7, 12 and 13) were specified in MatLab 7.0 and the parameters were obtained by minimizing the square error between the predicted and real values by the "fmeansearch" function. The kinetic constants are

expressed as an average value ± SD of the three values obtained at each fixed water activity. Additionally, for a given water activity, the equilibrium constants were estimated employing the equilibrium concentrations of each specie. As done with the kinetic constants, the equilibrium constants are expressed an average values ± SD of the three values obtained.

3. RESULTS AND DISCUSSION

3.1. Degree of concentration of PUFA in each acylglycerol and FFA fractions

The fatty acid profile of the refined sardine oil is listed in Table 1. The predominant fatty acids were palmitic (C16:0) and eicosapentanoic fatty acid (EPA, C20:5n-3) accounting, each one, ~ 19 mol% of the total. In the global profile, the PUFA fraction had the highest portion (~ 43 mol%) followed by SFA (33.83 mol %) and MUFA (23.21 mol%). Docosahexanoic acid (DHA, C22:6n-3) content was 8.3 mol%. The absolute composition of the fatty acids esterified at the central bond of the glycerol backbone is listed in Table 1 (sn-2, mol %). Palmitic acid was also the predominant fatty acid (31.14, mol%), DHA content was the second most abundant (18.02 mol %). Effectively, DHA presented high regioselectivity towards the central position with 72 mol% of the total DHA located in the sn-2 bond. Contrary, EPA content at the sn-2 was much lower than the observed in global profile (4.19 and 19.37 mol%, respectively) with only 7 mol% bonded at sn-2. This profile agrees with the previously reported for sardine oil (R. Morales-Medina et al., 2015b).

As a result of the hydrolysis a mixture of MAG, DAG, FFA and residual TAG were obtained. No glycerin was produced since the aforementioned fractions followed the reaction stoichiometry (*i.e.* [FFA] = 2·[MAG] + [DAG]). In Fig. 1 it is depicted, for each water activity, the composition as SFA, MUFA and PUFA content. Additionally, the EPA and DHA content is shown. In all water activities tested, PUFA were slightly concentrated in the form of TAG and DAG, with a maximum increase of 1.2-fold in the DAG produced at a_w 0.6 (Fig. 1.b) while SFA was concentrated in the MAG.

Table 1. Refined sardine oil fatty acid profile (total, mol%) and its regiodistribution (sn-2, mol%). Fatty acid profile of 2-MAG produced for the acil-migration study.

Fatty Acid	Sardine oil			2-MAG
	Global, mol%	sn-2, mol %	Relative sn-2, mol %	mol, %
C14:0	10.49	16.18	51.41	16.31
C16:0	19.98	31.14	51.95	27.83
C16:1n-7	10.35	16.36	52.69	16.64
C16:2n-4	1.89	1.72	30.34	2.72
C16:3n-4	2.04	1.95	31.86	2.78
C16:4n-1	2.81	1.54	18.27	1.87
C18:0	3.37	0.49	4.85	0.84
C18:1n-7	3.41	0.64	6.26	0.91
C18:1n-9	8.53	3.98	15.55	3.94
C18:2n-6	1.44	0.30	6.94	1.29
C18:3n-3	0.80	0.94	39.17	0.69
C18:4n-3	2.56	0.00	0.00	0.00
C20:1n-9	0.70	0.97	46.19	1.43
C20:3n-6	0.24	0.00	0.00	0.00
C20:4n-3	0.63	0.00	0.00	0.00
C20:4n-6	1.30	0.00	0.00	0.00
C20:5n-3	19.37	4.19	7.21	3.98
C22:1n-9	0.21	0.00	0.00	0.00
C22:5n-3	1.57	1.60	33.97	2.37
C22:6n-3	8.30	18.02	72.37	16.40
SFA	33.83	47.81		44.98
MUFA	23.21	21.94		22.92
PUFA	42.96	30.25		32.10

The influence of the selectivity of the enzyme is much clear when comparing EPA and DHA. While DHA was concentrated as DAG and TAG (achieving concentration ratios of 1.94-fold in DAG at a_w 0.6, Fig. 1.b), it content in the FFA fraction was low (<0.4-fold in all cases). Contrary, EPA was distributed in a similar proportion among TAG, DAG and FFA while in MAG it content decreased (>0.6-fold in all cases) (Fig. 1). The mechanism underlying these results might depend of the regioselectivity of the enzyme, the regiodistribution of the fatty acids among the glycerol backbone and the own physical characteristics of the fatty acids (*i.e.* number of unsaturations or chain length).

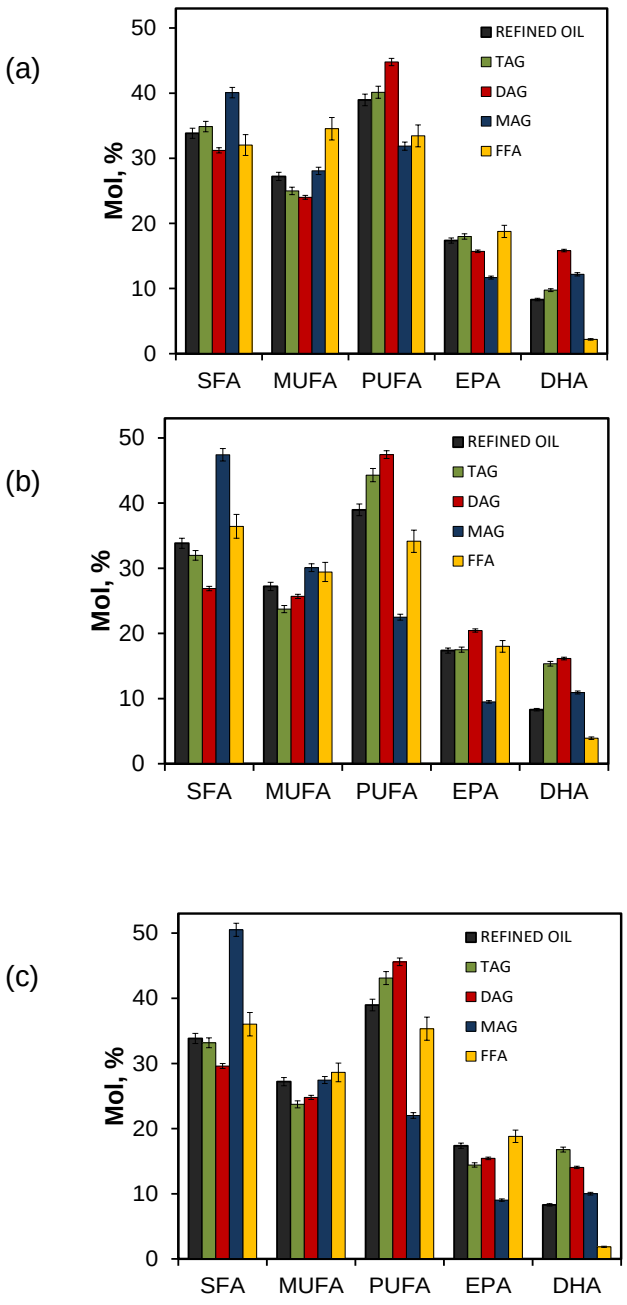


Figure.1. Molar composition of the total species (MAG, DAG, TAG, FFA) of the oil produced in the Rhizomucor miehei hydrolysis at water activity of 0.3; 0.6 and 0.8 (A, B, C) respectively.

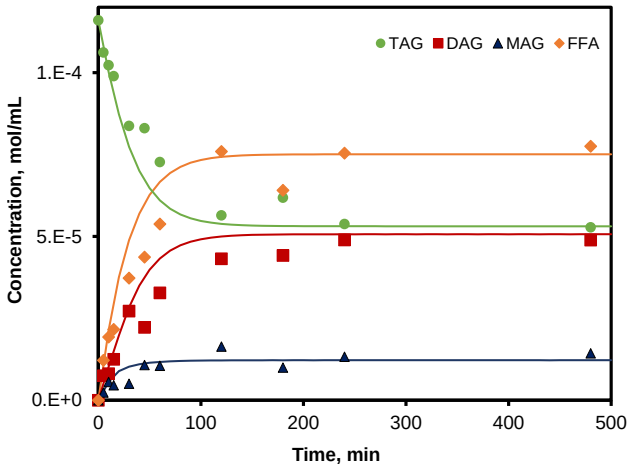
DHA presented a high regioselectivity towards the central position, oppositely, EPA presented high sn-1(3) regiospecificity (Table 1). *Rhizomucor miehe*i is a sn-1(3) specific lipase with a high tendency to react with fatty acids located at the extreme bonds of the glycerol backbond. The concentration of PUFA in the form of TAG and DAG has been previously described for hydrolysis with lipases (Kahveci and Xu, 2011). The resistance of the PUFA to be hydrolyzed is related to the molecular conformation of cis carbon double bond. Long PUFA (as EPA and DHA) causes steric hindrance. The double bonds results into the bending of the fatty acids and the approaching of the terminal methyl groups to the ester bonds. Consequently, the enzyme active sites cannot reach the ester bonds of the long PUFA and the glycerol backbone. Hence, long PUFA might be protected from hydrolysis, this steric hindrance is increased with the number of carbon double bonds (Bottino et al., 1967; Okada and Morrissey, 2007). However, this tendency is not common for all lipases, for instance lipases from *Pseudomonas* showed higher activity towards DHA than EPA (Haraldsson and Kristinsson, 1998). In a recent review (Rodrigues and Fernandez-Lafuente, 2010), the use of *Rhizomucor miehei* to improve the content of polyunsaturated fatty acid in the glyceride fraction was described. Indeed, the authors remarked that this lipase released PUFA in an extremely slowly rate.

During 1,3-specific lipase-catalyzed hydrolysis, the theoretical 2-MAG yield is 33%. Without taking into account the effect of acyl migration, the fatty acid composition of the 2-MAG might be similar to the one of the central position of the original triglycerides.

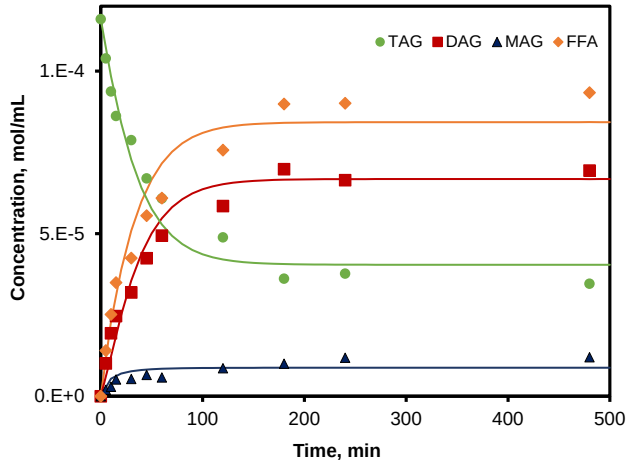
3.2. Hydrolysis kinetic model

Two second-order reversible reactions (Eq. 2 and 3) were proposed as the mechanism of the current hydrolysis. A comparison of the modeling and experimental results of the global system are shown in Fig. 2.A (1 to 3) for water activity of 0.3, 0.6 and 0.8, respectively.

(A.1)



(A.2)



(A.3)

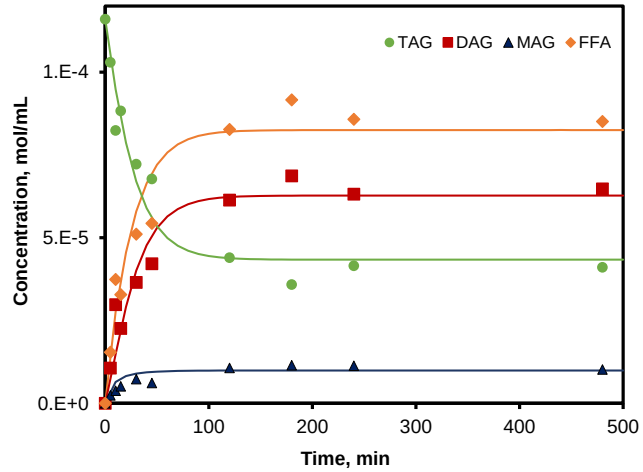
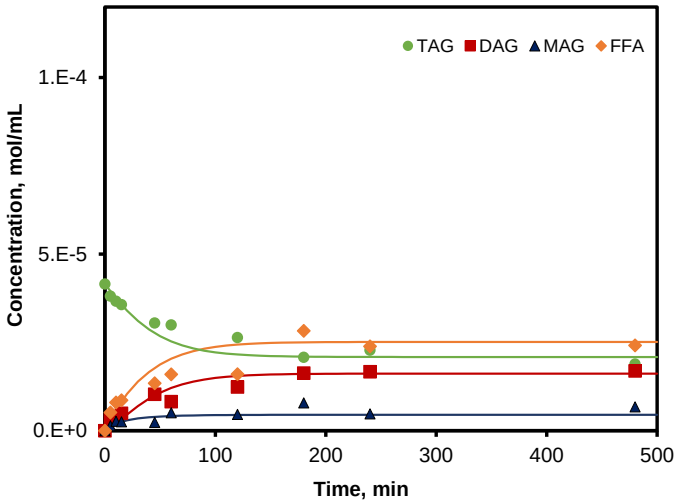
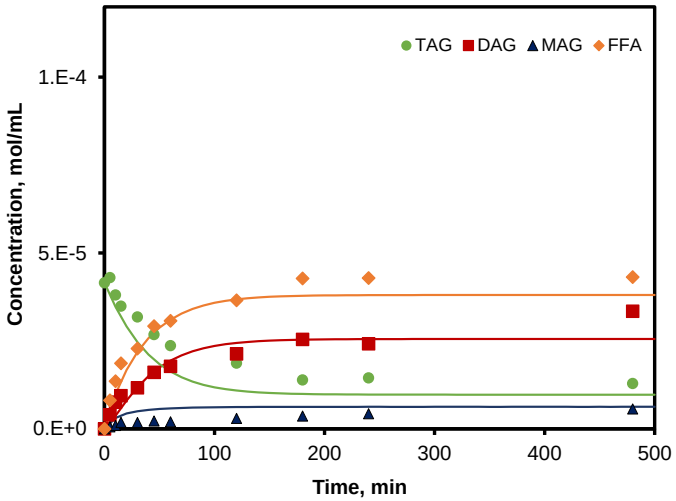


Figure 2.A. Comparison of modeling and experimental results for the hydrolysis of refined sardine oil. (A) Global system, The numbers 1,2 and 3 refer to the constant water activity tested: 0.3; 0.6 and 0.8 respectively.

(B.1)



(B.2)



(B.3)

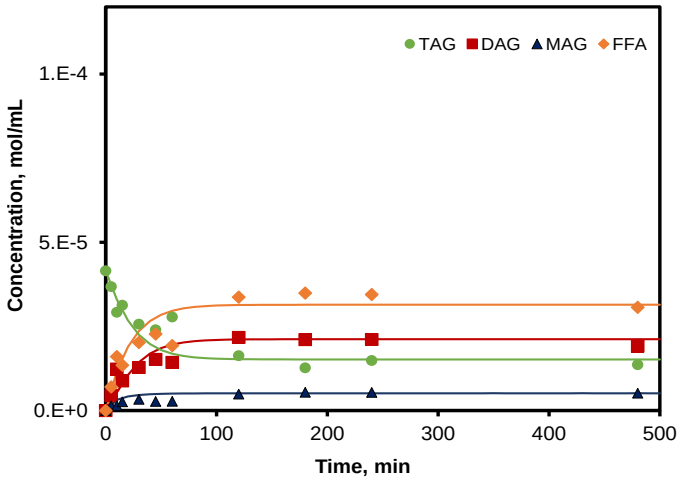
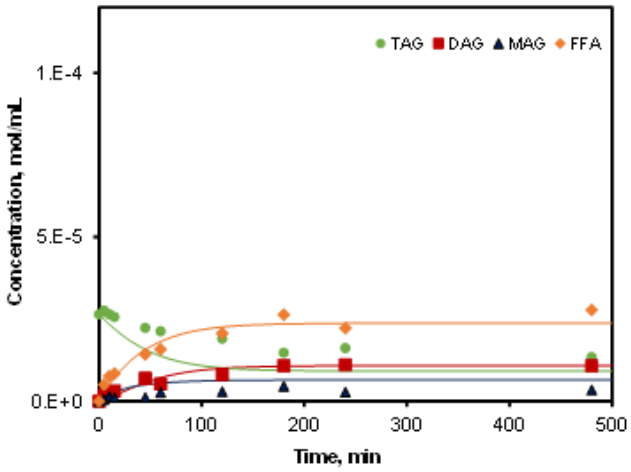
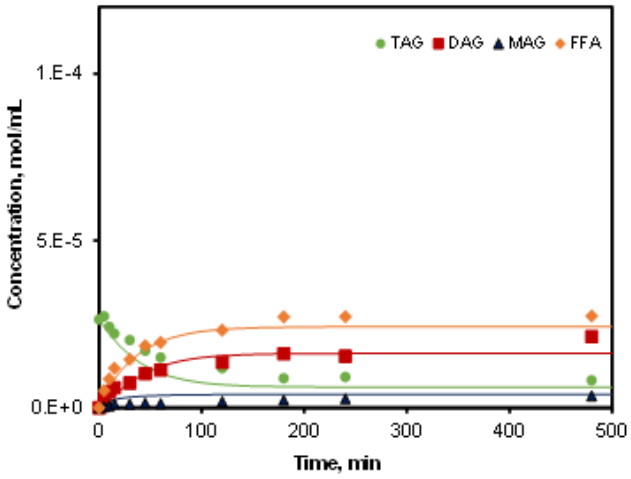


Figure 2.B Comparison of modeling and experimental results for the hydrolysis of refined sardine oil. (B) Saturated fatty acids fraction, (The numbers 1,2 and 3 refer to the constant water activity tested: 0.3; 0.6 and 0.8 respectively.

(C.1)



(C.2)



(C.3)

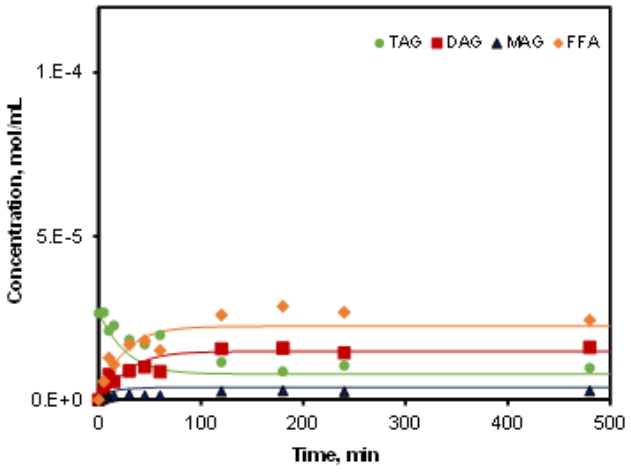
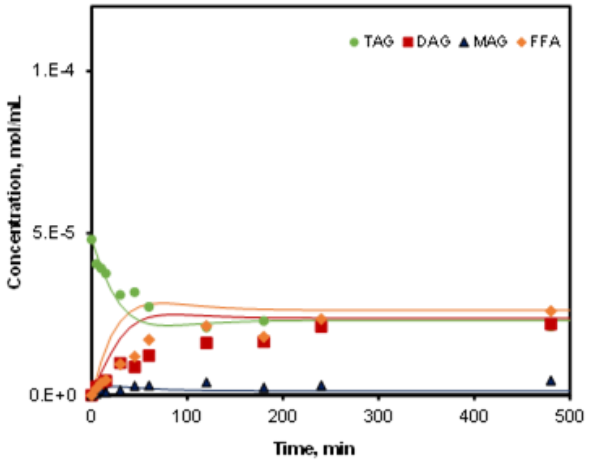
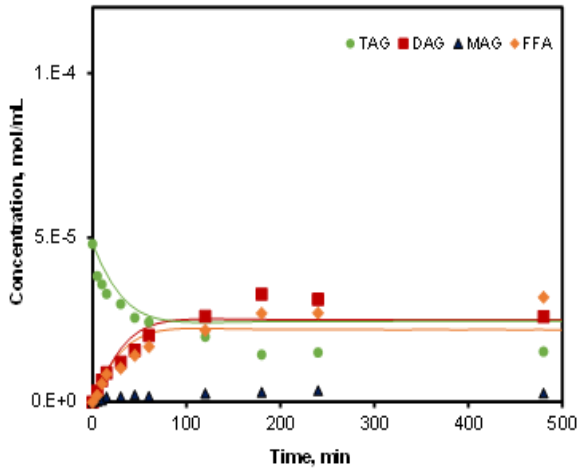


Figure 2.C Comparison of modeling and experimental results for the hydrolysis of refined sardine oil. (C) Monounsaturated fatty acids fraction, (The numbers 1,2 and 3 refer to the constant water activity tested: 0.3; 0.6 and 0.8 respectively.

(D.1)



(D.2)



(D.3)

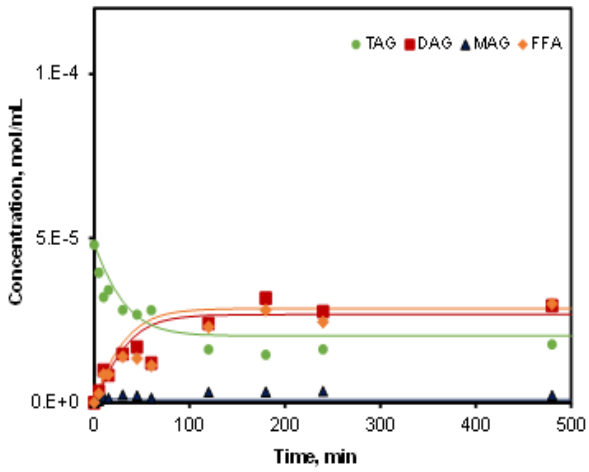


Figure 2.D Comparison of modeling and experimental results for the hydrolysis of refined sardine oil. (D) Polyunsaturated fatty acids fraction, (The numbers 1,2 and 3 refer to the constant water activity tested: 0.3; 0.6 and 0.8 respectively.

In the equilibrium, the lower water activity (0.3) led to the higher residual TAG (~27 mol%) and also the higher MAG content (~7 mol%) (Fig. 2A.1). At higher water activities (0.6 and 0.8) the residual the residual TAG was lower than 20 mol% while DAG were more abundant (Fig. 2.A.2 and Fig. 2.A.3). This effect of water activity was also observed for the SFA and MUFA fractions (Fig. 2.B and Fig. 2.C).

Water activity exerted an important influence on the final products, being a considerable difference of the final composition of reactions conducted at a_w 0.3 and at 0.6 and 0.8. The influence of the water activity has been previously described; for instance in the case of esterification mediated by immobilized *R. miehei*, the initial reaction rate was higher at a_w ~ 0.69 (Lee and Parkin, 2001), while for alcoholysis the optimal water activity was 0.55 (Adlercreutz, 2013). Mainly, the water activity modifies the hydration level of the enzyme and the flexibility of the protein and, consequently the catalytic activity (Xia et al., 2009). Despite water acts as a substrate, it can also inhibited the enzyme activity. Effectively, multilayers of water molecules can be present between the bulk organic solution and the active site of the enzyme, forming a diffusion barrier for hydrophobic substrates and decreasing the conversion rate (Adlercreutz, 2013). Contrary, a moderate water activity might activate the lipase. Effectively, an increase of water around the active site increases the local polarity and stabilizes polar transition states (Adlercreutz, 2013).

PUFA fraction showed no stoichiometric ratio being the concentration in DAG higher than in the FFA as it can be seen in Fig. 2.D.2 and Fig. 2.D.3. Consequently, adapting the mechanistic model, as done for SFA and MUFA fractions, was not a feasible option. Hence, the PUFA content of the acylglycerols and the FFA was calculated through a molar balance to the global system (Eq. 15).

The experimental data were satisfactorily modelled in the case of the global model (Fig.2.A, SD < 8 %), and for the SFA and MUFA fractions (Fig. 2.B and Fig. 2.C, SD < 12%). The major deviations were observed for the PUFA fraction (Fig. 2.D, SD < 16 %) as a result of an arithmetic combination of the previous models.

The kinetic and equilibrium constant of the global system, SFA and MUFA fractions are summarized in Table 2. The direct kinetic constant, k_{d1} was similar in the three cases. Hence, the release of the first fatty acid from the TAG might be independent on the type of fatty acid. *R. miehei* has been described as 1(3)-regiospecific enzyme (Rodrigues and Fernandez-Lafuente, 2010), this similarity might be related to the regiodistribution of the

original fatty acids within the glycerol backbone. Contrary, k_{d2} varied for the three cases studied. For the global system it was smaller than in the SFA and MUFA fractions (Table 2), showing the effect of the resistance that PUFA presented to be hydrolyzed.

Table 2. Rate and equilibrium constants of the *R.miehei* lipase mediated-hydrolysis reaction for the global systems and the saturated and monounsaturated fatty acids.

	k_{d1}, min^{-1}	K_{eq1}, mol/mL	k_{d2}, min^{-1}	K_{eq2}, mol/mL
GLOBAL	0.033 ± 0.006	1.1e-4 ± 0.2e-4	0.42 ± 0.04	1.4e-5 ± 0.2e-5
SFA	0.030 ± 0.0006	3.2e-5 ± 0.9e-5	0.82 ± 0.20	7.9e-6 ± 0.7e-6
MUFA	0.032 ± 0.002	3.7e-5 ± 0.7e-5	1.05 ± 0.17	5.9e-6 ± 0.04e-6

Furthermore, in all cases, the equilibrium constants of the hydrolysis of DAG (K_{eq2}) was an order of magnitude smaller than the equilibrium constant of the hydrolysis of TAG K_{eq1}) (Table 2). Presumably, a fatty acid is more easily release from a TAG than from a DAG. This fact could explain the low content of MAG. This resistance might be due to the steric hindrance that PUFA present or to the configuration of the DAG. Effectively, without taking into account the acyl-migration (which is discussed in the next section), the DAG produced should be the isomer 1,2-DAG while the lipase employed is 1(3) regioselective. Consequently, the enzyme might be less efficient hydrolyzing DAG which only present one ester to be hydrolyzed (in case acyl migration is negligible) compared with the two esters that TAG have.

3.3. Kinetic model of the acylmigracion in monoacilglycerols

One of the major limitations that selective lipid modification must face is the acyl migration, *i.e.* "the spontaneous movement of an acyl group from one hydroxyl group to an adjacent one" (Fureby et al., 1996). Hence, acyl migration involves acyl donors from sn-1(3) to sn-2 positions but it can also occur from the external bonds to the central one of the glycerol backbone (Xu, 2000). This non enzymatic reaction is catalyzed by heat, acids, bases, ion exchange resins and even organic solvent might exert some influence. Several works have been conducted to elucidate the effect of the organic solvents (Compton et al., 2007; Li et al., 2010a), temperature, water activity (Li et al., 2010b) and chain length (Boswinkel et al., 1996).

In the Fig. 3 the evolution of the molar fraction of the 2-MAG in a mixture of MAG isomers is depicted for three cases: at a water activity of 0.3 and 0.8 in the presence of the deactivated enzyme; and at a water activity of 0.3 without enzyme. In the three cases, the kinetic and equilibrium constants were similar proving that (i) the acyl migration pathway

was independent on water activity and that (ii) the enzyme support had no effect on the acyl migration. Furthermore, SFA, MUFA and PUFA fractions also presented similar acyl migration (data not shown). Compton, Laszlo, Appell, Vermillion, & Evans, (2012) also reported that an increase of the unsaturation degree after the C9 carbon of 2-MAG had no appreciable effect on the acyl migration rate.

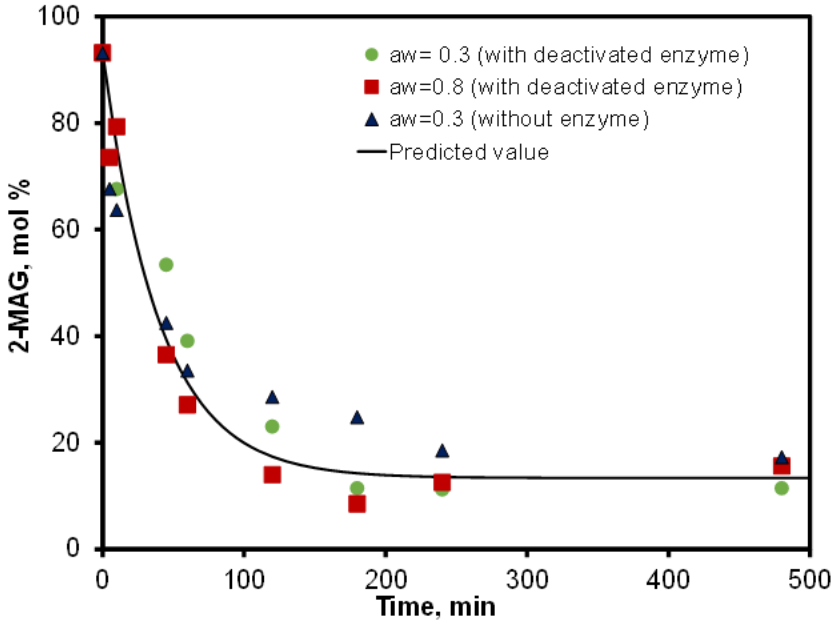


Figure 3. Modeling and experimental data of acyl migration of fatty acids between 2-MAG and 1-MAG at water activities 0.3 and 0.8 and in the presence and absence of deactivated enzyme.

At thermodynamic equilibrium, the molar proportion between 1-MAG and 2-MAG were 86:14, values which are closed to those previously reported in the literature (9:1) (Boswinkel et al., 1996). Acyl migration is initiated by the nucleophilic attack of a lone pair of electrons of the free hydroxyl oxygen, consequently the initial driving force is the efficiency of the nucleophilic attack, among others. Since the primary hydroxyl oxygen is a better nucleophile than the secondary one, the acyl migration from a secondary to a primary hydroxyl oxygen is favored (Kodali et al., 1990).

The rate and equilibrium constants of the reversible first-order reaction proposed as mechanism for the acyl migration were 0.018 ± 0.0018 1/min (k_m) and 3.24 ± 0.82 (K_m), respectively, resulting in a model with low deviation (SD < 7%). Both constants were independent of water activity. Regarding the equilibrium constant, if the three positions were thermodynamically equivalent, the theoretically equilibrium constant should be ~2. The higher value obtained implies that the acyl-migration is thermodynamically favored to the extreme positions of the glycerol.

The rate of acyl migration is affected by several variables as temperature, type of solvent, acid/base impurities, time, fatty acid chain length (Boswinkel et al., 1996; Compton et al., 2007; Fureby et al., 1996). Also, Li, Du, Li, Sun, & Liu, (2010) described that the rate of acyl migration was slightly influence by water activity when conducted a methanolysis of triolein employing tert-butanol as a solvent. In the current work, the rate of acyl migration is considerably faster than that reported in others research (Compton et al., 2007; Li et al., 2010c). This variation might be related to reaction medium, the acyl migration rate is high for non-polar solvents and low in polar alcohols (Li et al., 2010a). We employed hexane as solvent, while in the aforementioned studies reactions were conducted in tert-butanol or in a solvent free system. Furthermore, an increase of the concentration of DAG is described to accelerate the rate of acyl migration (Xu, 2000), hence the high concentration of MAG might also affect.

4. CONCLUSIONS

In the current work, *R.miehei* lipase-.mediated hydrolysis of sardine oil was conducted at several fixed water activities (0.3, 0.6 and 0.8) aimed to produce MAG and DAG enriched of DHA and EPA. The hydrolysis was modelled following a two reversible second-order mechanism at constant water activities. The rate and conversion slightly increased at water activities higher than 0.6. Contrary, acyl migration underwent in monoacylglycerols was independent on the water activity and the enzyme support. The hydrolysis was also modelled for the fractions of SFA, MUFA and PUFA resulting that the release of the first fatty acid from the triacylglycerol was independent on the unsaturation degree, while the hydrolysis of the second was highly affected by the degree of unsaturaturation being the PUFA the fatty acids that presented the lowest rate

The current method allows to increase the DHA concentration (up to 2-fold) in the DAG and TAG fraction, while it decreases in the MAG and FFA species. However, the global PUFA content of the system remained virtually constant in all the systems tested. New susbtrates with a high content of PUFA in the central bond of the glycerol backbone might be tested to improve the concentration ratio. Due to the low production of MAG (7 mol% at a_w 0.33) others methods as glycerolysis or alcoholysis might be considered in order to enhance the productivity of MAG. Hence, the current method is proposed as an easy

process to produce DAG with high content of DHA which can be employed as food emulsifiers with nutritional value.

TABLE OF ABBREVIATIONS

Symbol	Meaning
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
TAG	Triacylglycerols
FFA	Free fatty acids
DAG	Diacylglycerols
MAG	Monoacylglycerols
1-MAG	1-Monoacylglyerol
2-MAG	2-Monoacylglycerol
TLC	Thin layer chromatography
a_w	Water activity
t	Time, min
k_1, k_2	Kinetic constant for the forward reactions of hydrolysis, 1/min
k_{-1}, k_{-2}	Kinetic constant for the reverse reactions of hydrolysis, 1/(min·mol/mL)
K_{eq1}, K_{eq2}	Equilibrium constant of the hydrolysis reactions, mol/mL
$C_{i,\ global}$	Concentration of the total i fraction (MAG, DAG, TAG or FFA), mol/mL
$C_{i,\ SFA}$	SFA concentration of the total i fraction (MAG, DAG, TAG or FFA), mol/mL
$C_{i,\ MUFA}$	MUFA concentration of the total i fraction (MAG, DAG, TAG or FFA), mol/mL
$C_{i,\ PUFA}$	PUFA concentration of the total i fraction (MAG, DAG, TAG or FFA), mol/mL
k_m	Kinetic constant for the forward reaction of the acyl migrationhydrolysis, 1/min
k_{-m}	Kinetic constant for the reverse reaction of the acyl migrationhydrolysis, 1/min
K_{eqm}	Equilibrium constant of the acyl migration reaction

APPENDIX. CHARACTERISTIC OF THE PARTICLES OF IMMOBILIZED ENZYME

Parameter	Value
Bulk density, g/cm^3	0.668
Specific pore volume, cm^3/g	0.679
Solid density, g/cm^3	1.221
Specific surface area, m^2/g	104.7
Average particle diameter, mm	0.4
Average pore radii, nm	13.0
Porosity	0.453

Data obtained from (Camacho Paez et al, 2002)

5. REFERENCES

Adlercreutz, P., 2013. Immobilisation and application of lipases in organic media. doi:10.1039/c3cs35446f

Boswinkel, G., Derksen, J.T.P., K, van't R., Cuperus, F.E., 1996. Kinetics of acyl migration in monoglycerides and dependence on acyl chainlength. *J. Am. Oil Chem. Soc*. 73, 707–711.

Bottino, N.R., Vandenburg, G.A., Reiser, R., 1967. Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. *Lipids* 2, 489–493. doi:10.1007/BF02533177

Bradbury, J., 2011. Docosahexaenoic Acid (DHA): An Ancient Nutrient for the Modern Human Brain. *Nutrients* 3, 529–554. doi:10.3390/nu3050529

Camacho Paez, B., Robles Medina, A., Camacho Rubio, F., González Moreno, P., Molina Grima, E., 2002. Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by the acidolysis of cod liver oil and caprylic acid in a packed-bed reactor: Equilibrium and kinetics. *Chem. Eng. Sci*. 57, 1237–1249.

Carrín, M.E., Crapiste, G.H., 2008. Enzymatic acidolysis of sunflower oil with a palmitic-stearic acid mixture *Journal of Food Engineering* 84, 243–249. doi:10.1016/j.jfoodeng.2007.05.016

Compton, D.L., Laszlo, J.A., Appell, M., Vermillion, K.E., Evans, K.O., 2012. Influence of fatty acid desaturation on spontaneous acyl migration in 2-monoacylglycerols. *J. Am. Oil Chem. Soc*. 89, 2259–2267. doi:10.1007/s11746-012-2113-z

Compton, D.L., Vermillion, K.E., Laszlo, J. a., 2007. Acyl Migration Kinetics of 2-Monoacylglycerols from Soybean Oil *via* 1H NMR. *J. Am. Oil Chem. Soc*. 84, 343–348. doi:10.1007/s11746-007-1049-1

Dillon, J.T., Aponte, J.C., Tarozo, R., Huang, Y., 2013. Purification of Omega-3 polyunsaturated fatty acids from fish oil using silver-thiolate chromatographic material and high performance liquid chromatography. *J. Chromatogr.* A 1312, 18–25. doi:10.1016/j.chroma.2013.08.064

Esteban, L., Muñío, M. d. M., Robles, A., Hita, E., Jiménez, M.J., González, P.A., Camacho, B., Molina, E., 2009. Synthesis of 2-monoacylglycerols (2-MAG) by enzymatic alcoholysis of fish oils using different reactor types. *Biochem. Eng. J*. 44, 271–279. doi:10.1016/j.bej.2009.01.004

EU, 2013. Regulation (EU) no 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Off. J. Eur. Union 22–28.

Feltes, M.M.C., de Oliveira, D., Block, J.M., Ninow, J.L., 2013. The Production, Benefits, and Applications of Monoacylglycerols and Diacylglycerols of Nutritional Interest. *Food Bioprocess Technol*. 6, 17–35.

Fureby, A.M., Virto, C., Adlercreutz, P., Mattiasson, B., 1996. Acyl Group Migrations in 2-Monoolein. *Biocatal. Biotransformation* 14, 89–111. doi:10.3109/10242429609106879

Haraldsson, G.G., Kristinsson, B., 1998. Separation of eicosapentaenoic acid and docosahexaenoic acid in fish oil by kinetic resolution using lipase. *J. Am. Oil Chem. Soc*. 75, 1551–1556.

Hari Krishna, S., Karanth, N.G., 2002. Lipases and Lipase-Catalyzed Esterification Reactions in Nonaqueous Media. Catal. Rev. 44, 499–591. doi:10.1081/CR-120015481

Henderson, R.J., Tocher, D.R., 1992. Thin-layer chromatography, in: Hamilton, R.J., Hamilton, S. (Eds.), Lipids Analysis: A Practical Approach. Oxford University Press: IRL Press.

Hoshino, T., Yamane, T., Shimizu, S., 1990. Selective Hydrolysis of Fish Oil by Lipase to Concentrate n-3 Polyunsaturated. *Agric. Biol. Chem*. 54, 1459–1467. doi:10.1080/00021369.1990.10870149

Jump, D.B., Depner, C.M., Tripathy, S., 2012. Omega-3 fatty acid supplementation and cardiovascular disease Thematic Review Series: New Lipid and Lipoprotein Targets for the Treatment of Cardiometabolic Diseases. *J. Lipid Res.* 53, 2525–2545. doi:10.1194/jlr.R027904

Kahveci, D., Xu, X., 2011. Repeated hydrolysis process is effective for enrichment of Omega 3 polyunsaturated fatty acids in salmon oil by *Candida rugosa* lipase. *Food Chem*. 129, 1552–1558.

Kelleher, K., 2005. Discards in the world's marine fisheries: An update. FAO. Fish. Tech. Pap.

Kodali, D.R., Tercyak, A., Fahey, D.A., Small, D.M., 1990. Acyl migration in 1,2-dipalmitoyl-sn-glycerol. Chem. Phys. *Lipids* 52, 163–170. doi:10.1016/0009-3084(90)90111-4

Lawson, L.D., Hughes, B.G., 1988. Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters. *Biochem. Biophys. Res*. Commun. 152, 328–335. doi:10.1016/S0006-291X(88)80718-6

Lee, C.H., Parkin, K.L., 2001. Effect of water activity and immobilization on fatty acid selectivity for esterification reactions mediated by lipases. *Biotechnol. Bioeng*. 75, 219–227. doi:10.1002/bit.10009

Lembke, P., 2013. Production Techniques for Omega-3 Concentrates, in: Meester, F. De, Watson, R.R., Zibadi, S. (Eds.), Omega-6/3 Fatty Acids, Nutrition and Health. Humana Press, pp. 353–364.

Li, W., Du, W., Li, Q., Li, R., Liu, D., 2010a. Dependence on the properties of organic solvent: study on acyl migration kinetics of partial glycerides. *Bioresour. Technol*. 101, 5737–42. doi:10.1016/j.biortech.2010.03.018

Li, W., Du, W., Li, Q., Sun, T., Liu, D., 2010b. Study on acyl migration kinetics of partial glycerides: Dependence on temperature and water activity. *J. Mol. Catal. B Enzym*. 63, 17–22. doi:10.1016/j.molcatb.2009.11.012

Li, W., Li, R., Li, Q., Du, W., Liu, D., 2010c. Acyl migration and kinetics study of 1(3)-positional specific lipase of Rhizopus oryzae-catalyzed methanolysis of triglyceride for biodiesel production. *Process Biochem*. 45, 1888–1893. doi:10.1016/j.procbio.2010.03.034

Morales-Medina, R., Munio, Maria del Mar, Pérez-Gálvez, R., Guadix, A. and G.E., 2013. Lipids from Marine Sources, in: Pérez-Gálvez, R., Bergé, J.-P. (Eds.), Utilization of Fish Waste. CRC Press, Boca Raton.

Morales-Medina, R., De León, G., Almécija, M. del C., Guadix, A.M., Guadix, E.M., Munio, M., 2015. Omega-3 Concentrates from Fish Oil: A Comparison among Physical and enzymatic treatments, in: Burton, F. (Ed.), Fish Oil: Uses, Properties and Role in Human Health. Nova Science Publishers, New York, pp. 29–73.

Morales-Medina, R., García-Moreno, P.J., Pérez-Gálvez, R., Muñío, M., Guadix, A., Guadix, E.M., 2015a. Seasonal variations in the regiodistribution of oil extracted from small-spotted catshark and bogue. *Food Funct*. 6, 2646–2652. doi:10.1039/C5FO00448A

Morales-Medina, R., García-Moreno, P.J., Pérez-Gálvez, R., Muñío, M.M., Guadix, A., Guadix, E.M., 2015b. Nutritional indexes, fatty acids profile and regiodistribution of oil extracted from four discarded species of the Alboran Sea: Seasonal effects. *Eur. J. Lipid Sci. Technol*. In Press. doi:10.1002/ejlt.201500486

Munio, M. del M., Robles, A., Esteban, L., Gonzalez, P.A., Molina, E., 2009. Synthesis of structured lipids by two enzymatic steps: Ethanolysis of fish oils and esterification of 2-monoacylglycerols. *Process Biochem*. 44, 723–730. doi:10.1016/j.procbio.2009.03.002

Okada, T., Morrissey, M.T., 2007. Production of n-3 polyunsaturated fatty acid concentrate from sardine oil by lipase-catalyzed hydrolysis. Food Chem. 103, 1411–1419. doi:10.1016/j.foodchem.2006.10.057

Pacheco, C., Crapiste, G.H., Carrín, M.E., 2010. Lipase-catalyzed acidolysis of sunflower oil: Kinetic behavior *Journal of Food Engineering* 98, 492–497. doi:10.1016/j.jfoodeng. 2010.01.032

Pawongrat, R., Xu, X., H-Kittikun, A., 2007. Synthesis of monoacylglycerol rich in polyunsaturated fatty acids from tuna oil with immobilized lipase AK. *Food Chem*. 104, 251–258.

Rodrigues, R.C., Fernandez-Lafuente, R., 2010. Lipase from Rhizomucor miehei as a biocatalyst in fats and oils modification. *J. Mol. Catal. B Enzym*. 66, 15–32. doi:10.1016/ j.molcatb.2010.03.008

Rodríguez-Ruiz, J., Belarbi, E.-H.H., Sánchez, J.L.G., Alonso, D.L., 1998. Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotechnol. Tech*. 12, 689–691. doi:10.1023/A:1008812904017

Rubio-Rodriguez, N., Beltran, S., Jaime, I., De, D.S.M., Sanz, M.T., Carballido, J.R., 2010. Production of Omega-3 polyunsaturated fatty acid concentrates: A review. *Innov. Food Sci. Emerg. Technol*. 11, 1–12. doi:10.1016/j.ifset.2009.10.006

Ruxton, C.H.S., Reed, S.C., Simpson, J.A., Millington, K.J., 2007. The health benefits of Omega-3 polyunsaturated fatty acids: A review of the evidence. J. Hum. Nutr. Diet. 20, 275–285. doi:10.1111/j.1365-277X.2007.00770.x

Shahidi, F., Wanasundara, U.N., 1998. Omega-3 fatty acid concentrates: Nutritional aspects and production technologies. *Trends Food Sci. Technol*. 9, 230–240. doi:10.1016/s0924-2244(98)00044-2

Shimada, Y., Ogawa, J., Watanabe, Y., Nagao, T., Kawashima, A., Kobayashi, T., Shimizu, S., 2003. Regiospecific analysis by ethanolysis of oil with immobilized *Candida antarctica* lipase. *Lipids* 38, 1281–1286. doi:10.1007/s11745-003-1190-x

Small, D.M., 1991. The effects of glyceride structure on absorption and metabolism. Annu. Rev. Nutr.

Xia, X., Wang, C., Yang, B., Wang, Y.-H., Wang, X., 2009. Water activity dependence of lipases in non-aqueous biocatalysis. *Appl. Biochem. Biotechnol*. 159, 759–67. doi:10.1007/s12010-009-8618-8

Xu, X., 2000. Enzymatic production of structured lipids: Process reactions and acyl migration. *Inf. - Int. News Fats, Oils Relat. Mater*. 11, 1121–1130.

Zacharis, E., Omar, I.C., Partridge, J., Robb, D.A., Halling, P.J., 1997. Selection of salt hydrate pairs for use in water control in enzyme catalysis in organic solvents. *Biotechnol. Bioeng*. 55, 367–374.

Zhao, H., Lu, Z., Bie, X., Lu, F., Liu, Z., 2007. Lipase catalyzed acidolysis of lard with capric acid in organic solvent *Journal of Food Engineering* 78, 41–46. doi:10.1016/j.jfoodeng.2005.08.049

VI. Development of an Up-grading Process to Produce MLM Structured Lipids from Sardine Discards [*]

The aim of the work was to produce MLM structured lipids with caprylic acid as medium chain fatty acid (M) and concentrated polyunsaturated fatty acids (L) from sardine discards (*Sardine pilchardus*) in the central bond of the glycerol. To that end, the following steps were conducted: (i) fish oil extraction, (ii) Omega-3 free fatty acids (FFA) concentration (low temperature winterization), (iii) two-steps enzymatic esterification and (iv) triacylglycerols purification (liquid column chromatography). The resultant purified triacylglycerol accomplished with the oxidative state (peroxide and anisidine value) required for refined oils. The enzymatic process consisted of the esterification of the concentrate FFA and dicaprylic glycerol employing Novozyme 435. This process presented high regioselectivity with ~ 80 mol% of concentrated fatty acids esterified at the sn-2 position.

[*] MANUSCRIPT SUBMITTED FOR PUBLICATION: R. Morales-Medina, M. Munio, A.M. Guadix and E.M. Guadix. Development of an Up-grading Process to Produce MLM Structured Lipids from Sardine Submitted to *Food Chemistry*. Under review

1. INTRODUCTION

Fish discards are defined as the fraction of organic material in the catch which is dumped back to the sea (Kelleher, 2005). Discarding is causing an environmental problem due to the negative impact that this practice exerts on the fishing productivity and on the marine trophic chains. To minimize this problematic situation, the new European Common Fisheries Policy has introduced new fisheries regulations aimed at banning the discard practices in Europe (EU, 2013). Consequently, there is an increasing interest in developing technical solutions able to convert the fish discards into added-value compounds. In this context, the extraction and modification of fish oil have been widely studied as an up-grading option (Bimbo, 2012; Lembke, 2013; Sathivel, 2010).

Fish oil presents a high content of Omega-3 polyunsaturated fatty acids (PUFA), more specifically eicosapentanoic and docosahexanoic acids (EPA and DHA). These fatty acids play a positive role on the cardiovascular and nervous system and on the development of the neonates' brain (Morales-Medina, R., Munio, M.M., Pérez-Gálvez, R., Guadix, 2013).

Industrially, fish oil extraction is commonly conducted by the wet reduction method. This method consists of three steps (i) cooking of raw materials (85 to 95ºC), (ii) pressing stage and (iii) centrifugation (Bimbo, 2007; Rubio-Rodríguez et al., 2012). As a result, three products are obtained: (i) a solid phase with high content of protein, (ii) a liquid phase with impurities and (iii) crude fish oil (Pérez-Gálvez et al., 2009). The solid phase, has been widely employed to produce fish protein hydrolysates with technological and biological activities (Halim, Yusof, & Sarbon, 2016). Crude fish oil must be refined to accomplish with the quality standards (Johnson, 2008). The conventional steps of refining are: degumming (removal of phospholipids), neutralization of free fatty acids, bleaching with activated clays (removal of oxidation products and pigments) and deodorization by vacuum distillation (elimination of the volatile compounds) (Bimbo, 2012; Rubio-Rodriguez et al., 2010). There are a considerable number of publications focused on the improvement of the extraction and refining of oils (Bimbo, 2007; Kim & Akoh, 2015). However, most of these publications are focused on specific steps, without offering a global view and description of the whole process.

Up-grading of fish oil is focused on the production of Omega-3 concentrates, *i.e.* increasing the Omega-3 content until, at least, 600 mg/ g oil (Rubio-Rodriguez et al.,

2010). Physical and enzymatic methods have been employed for the production of Omega 3 concentrates (Lembke, 2013). Enzymatic methods are based on the use of lipases and usually presents low enrichment yield (Rubio-Rodriguez et al., 2010). Physical methods are based on the difference of physical properties (*e.g.* melting point) that fatty acids present depending on their length or unsaturation degree.

In the physical methods, to enhance the efficiency of the concentration triacylglycerols are converted into esters of FFA prior to the concentration (Lembke, 2013). Then, the concentrated products should be re-esterified because Omega-3 fatty acids are better absorbed by human organisms as acylglycerides rather than esters or free fatty acids (Lawson & Hughes, 1988). These concentrated FFA or esters are an interesting substrate for the production of structured lipids, *i.e.* modified or synthetic oils and fats containing long-chain fatty acids (L) and medium-chain fatty acids (M), in which each group is located specifically at the sn-2 or sn-1(3) positions of the glycerol backbone (Xu, 2000). These modifications include changes in the composition and /or positional distribution of the fatty acids (Kim & Akoh, 2015). For clinical nutrition purposes, MLM structured lipids are of special interest since the long-chain fatty acids are most efficiently metabolized when located at the sn-2 position of the acylglycerol (Xu, 2000).

Structured lipids are mainly produced by enzymatic methods. The use of lipases presents several advantages as high selectivity or reduction of oxidation and spoilage, since mild conditions are required for enzymatic reactions. Structured lipids have been produced by acidolysis or by interesterification (Akoh & Kim, 2008; Kim & Akoh, 2015). Also, two steps enzymatic treatments have been tested as the combination of alcoholysis and esterification (Munio, Robles, Esteban, Gonzalez, & Molina, 2009), where 2-MAG were produced and then, after purification, they were re-esterified to produce MLM. The selection of the original oil (the substrate) will determine the composition and regiodistribution of the strucutured lipid. Indeed, special attention should be paid to the fatty acid composition of the central position of the original TAG, as the structured lipid will present a similar sn-2 composition (Morales-Medina et al., 2015a). This limitation could be overcome by producing structured lipids employing concentrates of FFA which can be esterified with diacylglycerols composed of fatty acids with medium length chain.

The aim of the work was to produce MLM structured lipids with caprylic acid as medium chain fatty acid (M) and concentrated polyunsaturated fatty acids (L) from sardine discards (Sardine pilchardus) in the central bond of the glycerol. To that end, the following steps

were conducted: (i) fish oil extraction, (ii) Omega-3 free fatty acids (FFA) concentration (low temperature winterization), (iii) enzymatic esterification and (iv) triacylglycerols purification (liquid column chromatography). Furthermore, the oxidative state of the oil (peroxide value and anisidine index) was monitored throughout the whole process. Additionally, two enzymatic treatments were compared: (i) one step esterification of glycerol and FFA with the desired composition and (ii) two steps esterification which involves, firstly, the production of dicaprylic diglycerides and, secondly, their esterification with the concentrated free fatty acids.

2. MATERIALS AND METHODS

2.1. Materials

Sardines (*Sardine pilchardus*) were selected due to the high lipid content and the high nutritional value of its oil with ~45 mol% of polyunsaturated fatty acids (Morales-Medina et al., 2015b). Sardines were supplied in summer, when their lipid content is maximum, by the fishing harbor of Motril (Spain). They were kept in ice during transportation and pressed the same day to avoid microbial spoilage.

The lipases employed were Novozyme 435 from *C. antarctica* kindly donated by Novozymes (Denmark) and immobilized lipase *Rhizomucor miehei* from Sigma-Aldrich (St Louis, MO, USA). Glycerine (99 %, Sigma–Aldrich, St Louis, MO, USA), caprylic acid (98% purity, Sigma–Aldrich) and n-hexane (Panreac S.A., Barcelona, Spain) were employed as substrates and solvents of the enzymatic esterifications. Also, molecular sieves of 3 Å (pellets, 3.2 mm) were obtained from Sigma-Aldrich. Finally, silica gel for column chromatography (ultrapure, 60-220 μm) was acquired from Acros Organics, (Madrid, Spain).

2.2. Production Process

The process can be divided into three main stages: (i) fish oil extraction, (ii) Omega-3 concentration and (iii) enzymatic treatment and purification *via* chromatography column (Fig. 1). More specifically, the oil was extracted from the sardines by combining hydraulic

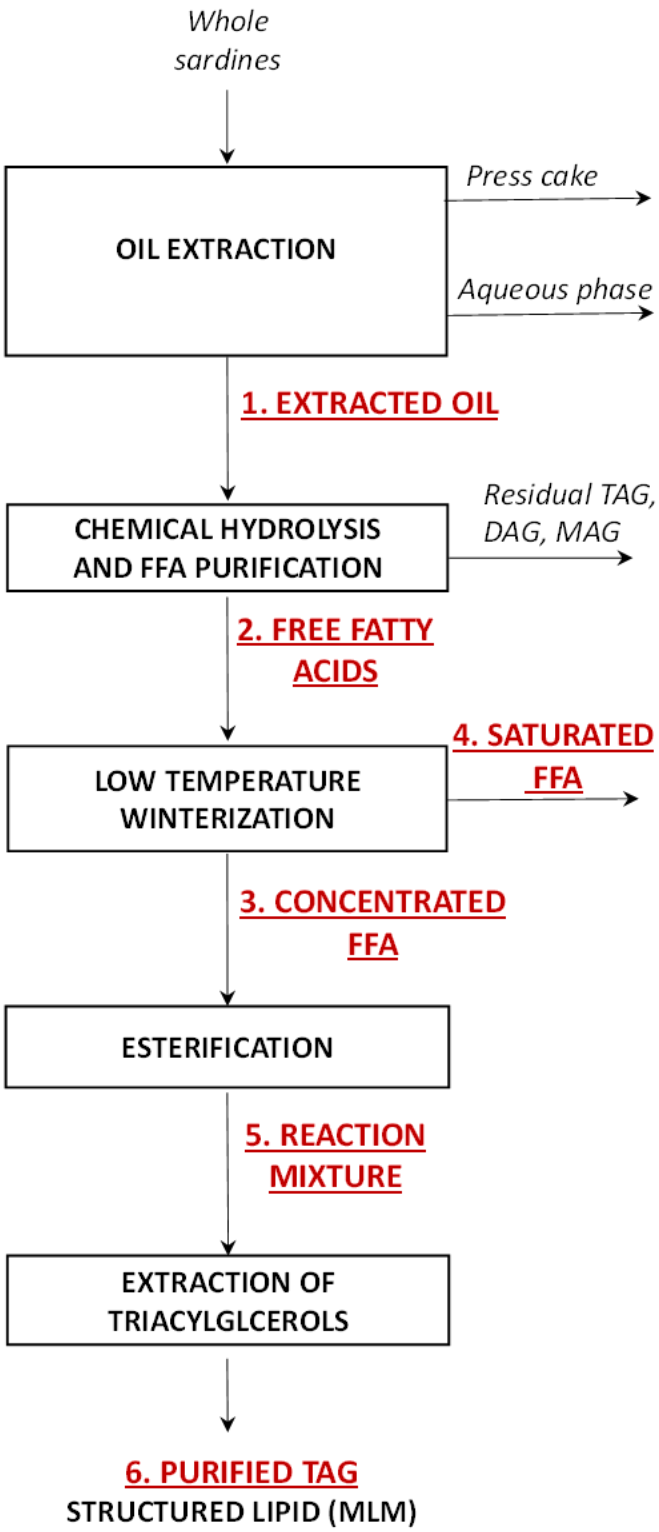


Figure 1. Schematic diagram of the whole process described for the production of structured lipids from sardines.

press and centrifugation, as a result three phases were obtained: press cake, aqueous phase and extracted oil. Then, the extracted oil was chemically hydrolyzed to produce free fatty acids (FFA) which were extracted and purified with organic solvents. Consequently, the extracted FFA were winterized at -85°C for 24 h, producing two phases: (i) a liquid one concentrated in polyunsaturated fatty acids (PUFA) and (ii) a solid one with high content of saturated fatty acids (SFA). Subsequently, an enzymatic esterification was conducted and the produced triacylglycerols were purified by means of liquid chromatography and kept in amber flasks at -85°C until analysis.

In all cases the yield of each step was calculated as follows:

$$\text{Yield} = \frac{\text{Recovered mass of the desired compound, g}}{\text{Initial mass of substrate or raw material, g}} \cdot 100 \quad [1]$$

Two enzymatic esterification processes were evaluated for this process a simple and direct esterification (Fig. 2.A):

$$\text{Glycerine} + \frac{}{3}\,\text{Caprylic acid} + \frac{}{3}\,\text{concentrated FFA} \xleftrightarrow{\qquad} \text{TAG} + \text{DAG} + \text{MAG} + \text{FFA} + \text{H}_2\text{O} \quad [2]$$

And a two steps esterification (Fig. 2.B):

$$\text{Glycerine} + \frac{2}{3}\,\text{Caprylic acid} \xleftrightarrow{Rhizomucor\ \ mie\,hei} \text{TAG} + 1{,}3\ \text{DAG} + 1\text{MAG} + \text{FFA} + \text{H}_2\text{O} \quad [3]$$

$$1{,}3\ \text{DAG} + \frac{1}{3}\text{Concentrated FFA} \xleftrightarrow{Novozyme\ \ 435} \text{TAG} + 1{,}3\ \text{DAG} + \text{FFA} + \text{H}_2\text{O} \quad [4]$$

In the direct esterification (Eq. 2) a stoichiometric mixture of concentrates FFA, caprylic acid and glycerin were esterified with a non-specific lipase (Novozyme 435) (Fig. 2b). In this case, the FFA were randomly esterified. This direct reaction was conducted as a reference value to evaluate the improvement that a two steps enzymatic treatment might exert. The two steps esterification consisted of, firstly, the production of dicaprylic glycerols with a 1,3-specific lipase (*Rhizomucor miehei*) (Eq. 3).

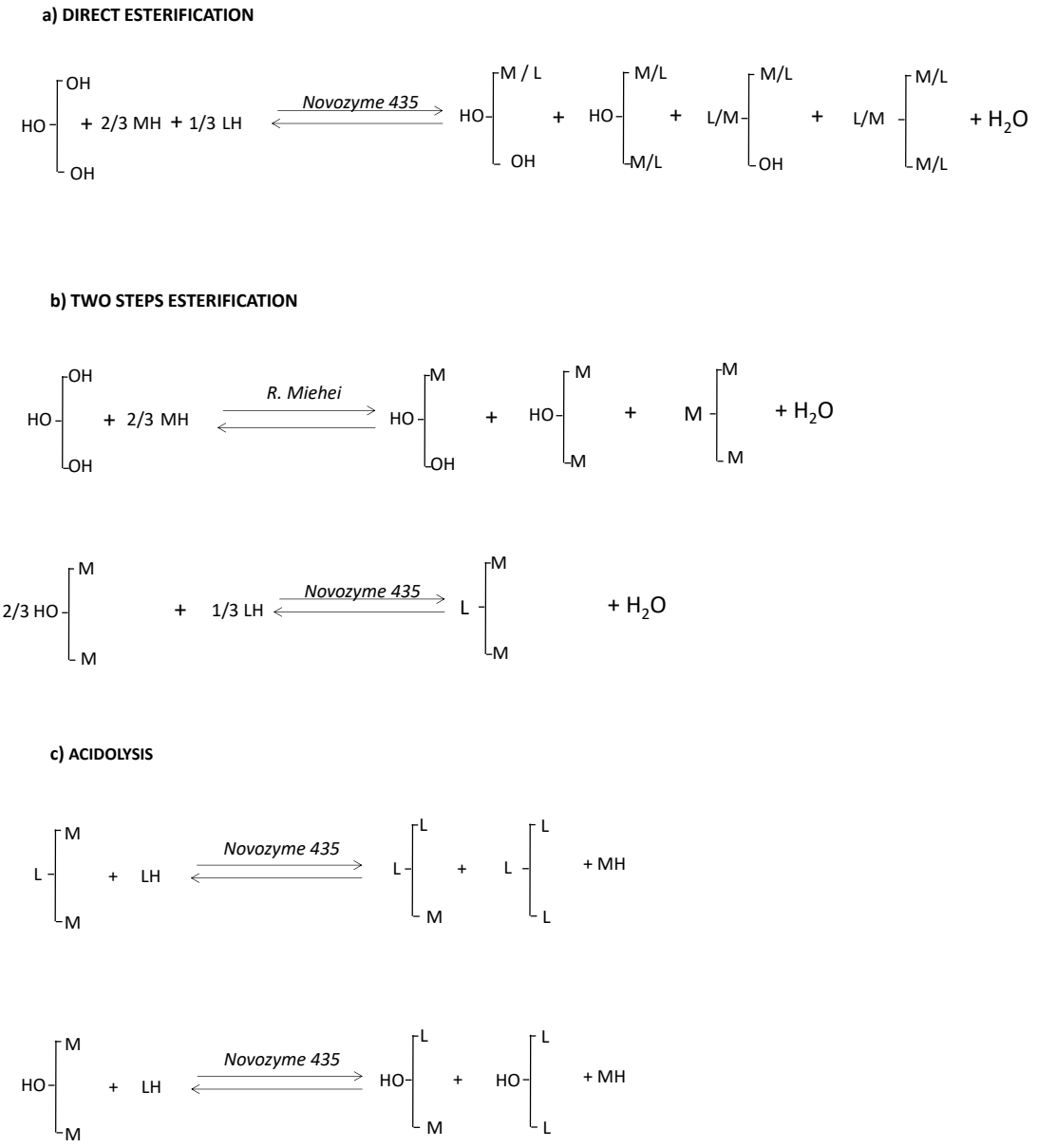


Figure 2. Schematic diagram of the proposed enzymatic treatments: (a) direct esterification and (b) two steps esterification. (c) Acidolysis diagram of TAG and DAG, a feasible parallel and secondary reaction. M and L represent caprylic and polyunsaturated fatty acids, respectively.

Then, after DAG purification, an esterification between the produced diacylglycerols and the concentrate FFA employing Novozyme 435 was conducted (Eq. 4).

2.2.1. Oil Content of sardines and fish oil extraction

Whole sardines were analyzed for determining their oil content following the official method recognized by the A.O.A.C. (2006). Oil was extracted as described elsewhere (Morales-Medina et al., 2015b). Approximately, 2 kg of the whole fish (viscera and gonads included) were preheated at 40ºC for 30 min. Then, they were hydraulically pressed in an electric pressed model ESP-K (Sanahuja, Spain) by employing three steps until achieving a final pressure of 120 bar. Finally, the press liquor was collected and then centrifuged at 20,000×g to recover the oily phase. To prevent the oxidation of oil, it was kept in amber flasks, under nitrogen atmosphere at -85°C until its use.

2.2.2. Omega-3 concentration: free fatty acids (FFA) production and winterization

FFA were released from the TAG by means of basic hydrolysis adapting the method described by Wanasundara and Shahidi, (1999). A detailed description of the whole hydrolysis and purification is described in a previous work of this group (Morales-Medina, De León, Munio, Guadix, & Guadix, 2016).

The Omega-3 content of the FFA was increased *via* low temperature winterization with solvent. To that end, 100 mL of a solution (5 wt%) of FFA in hexane were located in amber flasks (125 mL) and stored in a Panasonic ultra-low temperature freezer MDF-U3386S (Panasonic Healthcare Co, Ltd) at -85ºC for 24 hours. These conditions were selected based on the results of a previous research (Morales-Medina et al., 2016). The crystalized fatty acids were removed by filtering at -85°C, through a 25 µm filter paper. The solvent was removed from the liquid fraction with a Büchi Rotavapor R-210 (Büchi Labortechnik AG) during 15 min at 40°C and 100 mmHg.

2.2.3. Enzymatic esterification

Structured lipids were produced by two different enzymatic procedures (Fig. 2a and 2b). Prior to enzymatic treatment, the molecular weight of the concentrated FFA was calculated (MW 297.12 g/mol). To that end, the fatty acid composition was analyzed by gas chromatography as described below.

Direct esterification

The composition of the reaction mixture was adapted from that described by Munio et al., (2009). Effectively, 288 mg of caprylic acid (MW 144.21 g/mol), 297 mg of concentrated FFA (MW 297.12 g/mol), 300 mg of glycerin (MW 92.09), 14 mL of hexane, 3 g of molecular sieves and 81 mg of the immobilized lipase Novozyme 435 were mixed in 25 mL Erlenmeyer amber flasks with stoppers and under inert atmosphere. The reaction mixture was incubated at 37ºC in an orbital shaker at 200 rpm (Heidolph, Unimax 1010, Germany). After 24h, the reaction was stopped by removing the lipase by filtration. Finally, the solvent was extracted employing a rotary evaporator (40°C and 100 mmHg) and the recovered mass was monitored. The reaction product was diluted in hexane (60 g/L) and kept in amber glasses under nitrogen, at -85ºC until purification.

Two steps esterification

This treatment consisted of the production of dicaprylic glycerols and their consequent reaction with the concentrated FFA (Fig. 2B).

Dicaprylic glycerols were produced by mixing 417.77 mg of caprylic acid, 133 mg of glycerin, 4.8 g of molecular sieves, 144 mg of immobilized lipase Rhizomucor miehei and 48 mL of hexane. The reaction mixture was incubated in 100 mL Erlenmeyer flasks at 37°C and 200 rpm until 10 h. Samples were extracted at several times (1 to 10h) to determine the optimal time for DAG production. To produce the required amount of DAG, this reaction was conducted three times. Finally, dicaprylic glycerols were purified by column chromatography as it is described below.

Then, 346.2 mg of dicaprylic glycerides (MW 345.26 g/mol), 297 mg of concentrated FFA (MW 297.12 g/mol), 48 mL of hexane, 1.54 g of molecular sieves and 143.4 mg of the immobilized lipase Novozyme 435 were mixed in 200 mL amber flask. As described in the direct esterification, samples were incubated at 37ºC in an orbital shaker at 200 rpm for 24 h and the reaction was stopped by filtration. This reaction was conducted three times. The solvent was removed and samples were stored exactly as previously described.

2.2.4. Diacylglycerols and triacylglycerols purification

The reaction mixture consisted of TAG, DAG, FFA and in some cases MAG. To separate them, a combination of organic extractions and liquid chromatography was conducted. Firstly, remaining FFA were removed by neutralization with a hydroethanolic solution of KOH 0.5 N (30 wt% ethanol) at room temperature. Then, MAG, DAG and TAG were

separated by liquid column chromatography adapting the method described by Köse, Tüter, & Aksoy, (2002). Silica gel (50 g) in hexane was transferred to a column of 30 mm diameter. Four grams of the reaction mixture (without FFA) were dissolved in 30 mL of hexane and were added to the column. The elution of the TAG and DAG was accomplished with 1.5 L of hexane:diethylether (85:15 v/v) and 2 L of hexane:diethylether (50:50 v/v), respectively. Flow rate was fixed between 5 and 7 mL/min. Finally, the solvent was removed and samples were stored as described as in the previous section.

2.3. Characterization of the oil

In Fig. 1 it has been numbered (1 to 6) each of the samples which were characterized throughout the whole process: (1) extracted oil, (2) free fatty acids (after chemical hydrolysis), (3) concentrated FFA and (4) saturated FFA (products of the winterization), (5) the reaction mixture and (6) purified TAG (after liquid chromatography).

For each one, the lipid composition (*i.e.* content of MAG, DAG, TAG and FFA) and their fatty acid profile was analyzed. Also, their oxidative state was monitored by measuring peroxide and anisidine value. Finally, the fatty acid regiodistribution of the purified TAG (final product) and the extracted oil was studied.

2.3.1. Lipid Composition and fatty acids profile

The lipid composition of all samples (*i.e.* MAG, DAG; FFA and TAG), but those of the caprylic esterification (Eq. 3), was determined by thin layer chromatography. Also, for each acylglycerol and FFA, the fatty acid composition was determined by means of gas chromatography (Agilent 7890A, Agilent Technologies S.A.). A detailed description of the analysis protocol is found in previous works of this group (Morales-Medina et al., 2015a, 2015b).

In the thin layer chromatography, the separated acylglycerols and FFA are visualized by spraying with iodine vapor. Iodine forms colored complexes with unsaturated carbon bonds and, thus, acyglycerols and FFA can be identified. However, in the caprylic acid esterification all substances are composed of a saturated acid and, consequently they cannot be visually identified. Hence, acylglycerols composed exclusively by caprylic acid were analyzed by UPLC-MS in an Acquity Chromatograph (Waters Corporation, Mildford-MA, USA), using a Waters UPLC BEH C18 column of 100 μm of particle size coupled to a mass spectrometer (Waters Xevo-TG-S) (Moya-Ramírez, García-Román, &

Fernández-Arteaga, 2016). In this case, FFA concentration was determined colorimetrically following the standard ISO 660:2009. All measurements were done in triplicate.

2.3.2. Determination of the positional distribution of fatty acids in the triacylglycerols

The regiodistribution of the fatty acids of the extracted oil and the purified TAG was studied. To that end, an ethanolysis with Novozyme 435 was conducted as described in detail elsewhere (Morales-Medina et al., 2015a, 2015b). Novozyme 435 is a non-specific enzyme that, in the presence of high excess of ethanol, can act as highly 1,3 selective. By this method, all the monoacylglycerols produced are 2-MAG, and they can be easily separated by thin layer chromatography, as previously described. The relative percentage of a given fatty acid at the sn-2 position was calculated as follows:

$$\%\mathrm{FA_i}\ \text{in sn2 position} = \frac{\text{content of } \mathrm{FA_i}\ \text{in sn2 position}}{3 \cdot \text{total content of } \mathrm{FA_i}\ \text{in TAG}} \cdot 100 \quad [5]$$

2.3.3 Oxidative state of the oils: peroxide and anisidine values

The oxidative stability of the oil was studied by monitoring the peroxide value (PV) content and p-anisidine value (AV) as indicators of primary and secondary (volatile) oxidation compounds, respectively. Peroxide value was determined as Drusch et al., (2012) described. P-anisidine value (AV) of the oil samples was determined according to the standard ISO 6885:2006.

3. RESULTS AND DISCUSSION

3.1. Process overview: yield and oxidative stability

As depicted in the Fig. 1. the stages of the process were (i) fish oil extraction by a modification of the wet reduction method, (ii) chemical hydrolysis of FFA (iii) Omega-3 concentration by low temperature winterization (iv) esterification (2 steps process) and (v) extraction of the produced structured lipids (liquid column chromatography).

3.1.1. Individual yields

The individual yields of each step are summarized in Table 1. Whole sardines presented an oil content of 16.0 ± 0,3 wt%. Fish oil extraction (30ºC cooking, hydraulic pressing and centrifugation), presented a yield of 39.5 wt% when referred to the oil content of the sardine. However, this value was dramatically reduced when expressed as a function of the whole fish weight (6.1 wt%). Higher yields can be achieved by increasing the temperature and time of cooking. Nevertheless, the increase of the temperature (>50ºC) implies a more intense oxidation of the extracted oil and a loss of quality (García-Moreno et al., 2014). Effectively, the walls of the cells are broken below 50ºC and fish protein coagulation is completed at 75ºC; both facts increase considerably the yield of extraction but also decrease dramatically the quality of the oil (FAO, 1986). Apart from the traditional wet reduction method, enzymatic hydrolysis or supercritical fluid extraction have been proposed as alternative extractive methods. Enzymatic hydrolysis employs proteases to decrease the size of the fish protein, favoring the release of lipids from the protein matrix (Dumay, Donnay-Moreno, Barnathan, Jaouen, & Bergé, 2006; Laplante, Souchet, & Bryl, 2009). These kind of treatments presented higher yield and recovery rates than the current one, however enzymatic hydrolysis involves heating samples at temperatures around 50ºC for 1-2 hours when proteases as Flavourzyme, Alcalase or Protamex are employed (Dumay et al., 2006). Consequently, oil can be oxidated and its quality can be damaged. In the case of supercritical fluid extraction, the main limitation is economical since it requires expensive equipments and a freeze-drying step as pre-treatment (Rubio-Rodriguez et al., 2010).

Table 1. Oxidative state (peroxide value and anisidine index) and yield of the oil produced in each step. Standard values for crude and refined fish oil are listed. [1]Bimbo (2007).[2]Ackman (2005)

PRODUCT	PV, meq/kg oil	AV	YIELD
Extracted oil	4.60 ± 0.62	<1	39.5 wt %
Free fatty acids (FFA)	4.27 ± 0.98	<1	42.0 wt %
Concentrated FFA	<1	<1	61.6 wt %
Saturated FFA	6.3 ± 0.9	< 1	n.d.
Reaction mixture	6.52 ± 1.82	37.94 ± 1.99	92.1 wt %
Purified TAG	4.01 ± 1.05	18.70 ± 2.83	85.6 wt %
Standard values described in literature			
Crude fish oil [1]	<20	<60	--
Refined fish oil [2]	<5	<20	--

One of the most critical step of the whole process together with the esterification was the concentration step. Winterization at low temperature consists in the selective crystallization of SFA. Consequently, FFA were divided in two phases: a solid one composed mainly of SFA and a liquid one composed of monounsaturated and polyunsaturated fatty acids (MUFA and PUFA). Since the concentration is conducted by a selective crystallization, this step implies a relatively high loss of mass. Effectively, winterization presented a yield of 61.6 wt% when estimated as mass of concentrated fraction per mas of free fatty acids winterized. The main decrease is due to the separation of the SFA present in the oil. When the yield is estimated as a function of the total unsaturated fatty acids (PUFA and MUFA) in the oil, it increases significantly up to 93.5 wt%. In this case, there is a loss of 6.5 wt% of the unsaturated fatty acids. This loss might be due to occlusion of unsaturated fatty acids during the nucleation and growth of the crystals. Nucleation is a heterogeneous mechanism in which external catalytic sites can reduce the energy barrier resulting in the incorporation of PUFA in the crystal (Morales-Medina et al., 2016). Also, inter-solubilization might occur and mixed crystals might be created at higher temperatures than expected (Wanasundara, Wanasundara, & Shahidi, 2005). There are several techniques for PUFA concentration as: enzymatic enrichment, urea precipitation, supercritical fluid extraction, high-performance liquid chromatography (HPLC) or molecular distillation (Lembke, 2013). Lembke (2013) conducted a detailed comparison between the aforementioned methods. Supercritical fluid extraction or HPLC are highly selective and can achieve extremely high concentration levels (99%), however the investment cost is also high. In the case of winterization, Omega-3 concentrates with a purity of 90% can be produced. Although the investment costs are low, the operational costs are relatively high due to the low temperature required and the high volume of solvent (Lembke, 2013). However, this costs can be decreased by recycling the solvent. The selection of the concentration method should be a trade-off decision between the requirement that the oil must accomplish and the economical costs.

The enzymatic treatment and the final product purification presented high yield (92.7 and 85.6 wt%, respectively) (Table 1).

3.1.2. Oxidative evolution of oil through the process

In Fig. 1 it has been numbered (1 to 6) each of the samples which were characterized through the whole process: (1) extracted oil, (2) free fatty acids (after chemical hydrolysis), (3) enriched FFA and (4) saturated FFA (products of the winterization), (5) the reaction

mixture and (6) purified TAG (after liquid chromatography). In Table 1, the peroxide and anisidine values of the aforementioned samples are listed; as well as the maximum PV and AV accepted for crude and refined oil. Crude fish oil must contain a maximum peroxide and anisidine value of 20 meq/kg oil and 60, respectively (Bimbo, 1998). On the other hand, refined fish oil must present better oxidative state (PV < 5 meq/kg oil and AI < 20), (Ackman, 2005) (Table 1).

In the current process, the mild conditions of the extraction led to an extracted sardine oil with a low degree of oxidation (Table 1). The chemical hydrolysis, which was conducted under nitrogen atmosphere (62.5ºC, 1h), did not affect the oxidative stability. Contrary, the concentration step (winterization at -85ºC and solvent evaporation under vacuum) improved the oxidative state of the concentrated fatty acids while it slightly increase the PV of the saturated fraction. Due to the low volatility of hydroperoxides (Jacobsen & Skall Nielsen, 2007), they might have crystallized and remained in the solid phase. Despite having employed mild conditions for esterification (*i.e.* 37ºC, amber flasks, N2 atmosphere, 24 h), the reaction mixture was considerably oxidized during the esterfication (Table 1).

The separation of triglycerides by liquid column chromatography followed by the evaporation of solvent (40ºC, 100 mmHg) improved the oxidative stability of the oil until achieving values of refined oil (Table 1). Silica earth - a compound able to adsorb moisture, trace soaps, phospholipids, trace metals and some oxidation materials (Bimbo, 2007) - was employed as stationary phase. Consequently, in this step a partial adsorption of undesirable compounds might happen, resulting in the observed reduction of the PV. Also, a considerable decrease of the anisidine value (*i.e.* content of volatile compounds) was observed. This could be due to the high volume of organic solvents which were evaporated (around 1.5 L) at 110 mm Hg, 40ºC. Usually, in the refining of crude oils produced by the traditional wet reduction method, deodorization (removal of volatile compounds responsible for odor and flavor) is the last step (Bimbo, 2012). Deodorization is a process in which oils are subjected to high vacuum and high temperature (180 to 200 ºC) (Rubio-Rodriguez et al., 2010). However, depending on the characteristic of the oil, the intensity of the treatment could be adjusted to minimize both the economic costs and the impact on the oil.

In this section, a global description of the oxidative state of the oil throughout the whole process has been conducted. To minimize the extent of the oxidation, further studies

Table 2. Fatty acid composition (molar basis) of the oil produced in each stage of the process: All samples presented a SD< 3 %

	Extracted Oil	FFA	Concentrated FFA	Saturated FFA
C8:0	0.00	0.00	0.00	0.00
C14:0	7.52	7.64	2.29	22.21
C16:0	21.54	21.92	2.96	51.68
C16:1n-7	7.00	7.11	8.86	4.02
C16:2n-4	1.23	1.23	1.86	1.12
C16:3n-4	0.84	0.87	1.28	0.00
C16:4n-1	1.00	1.03	1.77	0.00
C18:0	4.13	4.08	0.71	9.44
C18:1n-7	3.07	3.12	3.75	1.09
C18:1n-9	10.49	10.63	11.89	4.70
C18:2n-6	1.38	1.40	2.04	0.00
C18:3n-3	1.11	1.08	1.56	0.00
C18:4n-3	2.19	2.23	3.65	0.96
C20:1n-9	2.45	2.47	3.14	0.00
C20:3n-6	0.70	0.00	1.37	0.00
C20:4n-3	0.95	0.93	1.11	0.00
C20:4n-6	0.89	0.75	0.60	0.00
C20:5n-3	12.47	12.42	20.02	3.13
C22:1n-9	2.32	2.39	2.27	0.00
C22:5n-3	2.19	2.19	3.40	0.00
C22:6n-3	16.72	16.52	25.19	1.65
SFA	33.20	33.63	5.96	83.34
MUFA	25.32	25.72	29.92	9.80
PUFA	41.67	40.65	63.85	6.86

Table 2 (continuation). Fatty acid composition (molar basis) of the oil produced in each stage of the process: All samples presented a SD< 3 %

	Esterification (one step) Reaction mixture				Esterification (two steps) Reaction mixture		
	MAG	**FFA**	**DAG**	**TAG**	**FFA**	**DAG**	**TAG**
C8:0	76.39	76.25	62.52	72.10	59.04	71.31	58.48
C14:0	1.94	1.41	0.96	1.03	0.00	0.00	1.08
C16:0	6.45	5.14	3.57	2.56	3.71	0.00	1.73
C16:1n-7	0.00	1.85	3.80	2.41	5.73	9.86	4.32
C16:2n-4	0.00	0.00	0.00	0.43	0.00	0.00	0.68
C16:3n-4	0.00	0.00	0.00	0.00	0.00	0.00	0.57
C16:4n-1	0.00	0.00	1.46	0.55	0.00	0.00	0.96
C18:0	2.34	1.74	1.93	0.82	0.00	0.00	0.38
C18:1n-7	0.00	1.13	1.69	1.07	2.58	0.00	1.82
C18:1n-9	9.56	8.12	7.55	4.55	7.47	8.40	5.65
C18:2n-6	3.33	2.27	1.96	1.10	0.00	0.00	0.91
C18:3n-3	0.00	0.00	0.00	0.44	0.00	0.00	0.72
C18:4n-3	0.00	0.00	0.83	0.81	2.00	0.00	1.54
C20:1n-9	0.00	0.00	0.00	0.60	0.00	0.00	1.33
C20:3n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C20:4n-3	0.00	0.00	0.00	0.00	0.00	0.00	0.51
C20:4n-6	0.00	0.00	0.00	0.00	0.00	0.00	0.56
C20:5n-3	0.00	2.08	7.33	4.23	9.87	10.44	7.85
C22:1n-9	0.00	0.00	0.00	0.00	0.00	0.00	0.89
C22:5n-3	0.00	0.00	0.00	0.00	0.00	0.00	1.26
C22:6n-3	0.00	0.00	6.39	7.30	9.59	0.00	8.76
SFA	87.11	84.54	68.98	76.51	62.75	71.31	61.67
MUFA	9.56	11.11	13.04	8.03	15.79	18.26	14.01
PUFA	3.33	4.35	17.98	14.86	21.46	10.44	24.31

focused on the addition of antioxidants (especially for the stabilization of the concentrate FFA and prior to the enzymatic esterification) must be conducted.

3.2. Enzymatic treatment: re-esterification

As previously explained two enzymatic treatments were compared: a direct esterification and a two steps esterification (Eq. 2, 3 and 4). The direct esterification was conducted as a reference to determine the improvement that the two steps process might exert.

Lipases are classified in two groups depending on their regioselectivity: (i) sn-1(3) specific, which preferably react with the external positions of the glycerol and (ii) non-regiospecific or ramdon which shows no regiospecifitiy. Novozyme 435 (*C. antarctica*) is a non-regiospecific enzyme while R. miehei has been described as highly sn-1(3) regiospecific. Contrary, the sn-2 regiospecificity is quite uncommon, fact which presents a challenge in the application of concentrated fatty acids or esters for the production of structured lipids (Hari Krishna & Karanth, 2002). Hence, we propose the esterification of the concentrated FFA with dicaprylic glycerols employing random lipases to favor the esterification in the central position of the glycerol. However, a parallel acidolysis between the DAG and the concentrated fatty acids might happen, decreasing the regioselectivity of the process (Fig. 2.C). In the next section, the influence of this secondary reaction will be described.

The esterification for the production of the DAG was studied to select the time that maximized the DAG content. To that end, the esterification was monitored for 10 hours (Fig. 3.1). After 8 hours the DAG reached a plateau while the production of TAG was increasing. Hence, a time of 8 hours was selected for the production of DAG. It is remarkable that, despite the described regioselectivty of Rhizomucor miehei, (Hari Krishna & Karanth, 2002) there are a considerable percentage of TAG. Dicaprylic glycerols might be produced as 1,2- and 1,3- DAG. Since a 1,3-specific lipase was employed most of produced DAG might be 1,3-DAG. However, 1,3-DAG might be transformed into 1,2-DAG by acyl migration and subsequently esterified to TAG. Acyl migration, involves acyl donors from sn-1(3) to sn-2 and vice versa (Xu, 2000). Also, 1,2- and 2,3-DAG are thermodynamically unstable, being the ratio of equilibrium 66:33 (mol:mol, 1,3-DAG: 1,2-DAG) (Xu, 2000). Factors as temperature, water content, reaction time might favor the acyl migration (Xu, 2000). Consequently the produced DAG might be a mixture of 1,3-DAG: 1,2-DAG with a considerable higher content of 1,3-DAG. In Fig. 3.2. it is depicted

the molar composition of the reaction product for both enzymatic methods. In both cases, the percentage of TAG was similar (~ 70 mol % or ~ 90 wt%). However, when comparing the regiodistribution of the fatty acids there are considerable differences; being the selectivity of the process enhanced when employing a two steps esterification (Table 3). Effectively, in the direct esterification, the PUFA content of the central position of the glycerol backbone contained 10.82 mol%. This value increased up to 41.54 mol % in the case of the two steps esterification. A more detailed discussion about the fatty acid composition and their regiodistribution is conducted in the following section.

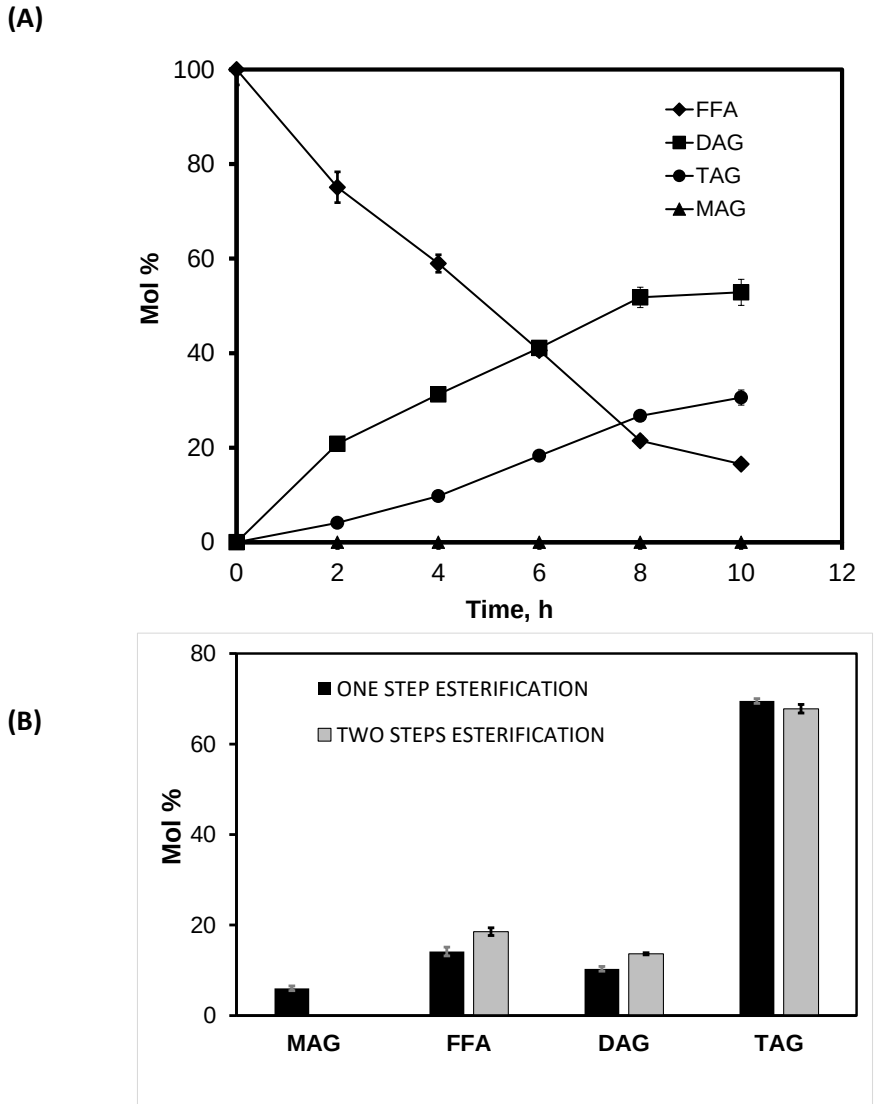


Figure. 3. Molar composition of the acylglycerols (TAG, DAG, MAG, FFA) produced in the esterification of the caprylic acid as a function of time. (b) Comparison of the final molar composition of the reactions mixture (TAG, MAG, DAG, FFA) after each enzymatic treatment.

3.3. Fatty acid composition and regiodistribution

Verification by thin-layer chromatography showed that the extracted oil and the free fatty acids had a purity higher than 99 wt%.

In the Table 2 the molar composition of all the samples studied throughout the process (as well as the reaction mixture of the one step esterification) is listed. The extracted oil presented a high content of PUFA (41.67 mol %) being DHA (16.7 mol %) and EPA (12.47 mol %) the most abundant fatty acids of this fraction. This fatty acid profile agrees with those previously described for sardine (Morales-Medina et al., 2015b). The chemical hydrolysis, a non-selective process, exerted no significant effect on the fatty acids profile. The most relevant changes on the composition were observed after winterization at low temperature, where the PUFA were concentrated 1.6 times. The concentrated free fatty acids contained 20.02 mol% and 25.19 mol% of EPA and DHA (a 1.61 and 1.52-fold increase, respectively). The concentrated FFA contained 610 mg of Omega-3 per g of oil, accomplishing with the minimum concentration required (*i.e.* 600 mg/ g of oil). Contrary, the saturated free fatty acids obtained after winterization contained around 15% of unsaturated fatty acids (Table 2). As previously explained, losses of unsaturated fatty acids in the winterization at low temperature can be due to occlusion during the nucleation or inter-solubilization of the fatty acids (Morales-Medina et al., 2016; Wanasundara et al., 2005).

With regard to the esterification step, the composition of the reaction mixture for the direct and two steps processes is summarized in Table 2. In the case of the direct esterification, a similar composition of all compounds (*i.e.* MAG, FFA, DAG and TAG) was observed for SFA and MUFA (79.3 ± 8.2 mol% and 10.43 ± 2.1 mol% respectively). However, PUFA were more concentrated in DAG and TAG. Due to the number of double C-bonds, PUFA molecules are more rigid and might present some steric hindrance. Consequently, this type of fatty acids might be the last which are esterified and are slightly concentrated in TAG.

A more selective behavior was observed for the products of two steps esterification. In this case, TAG presented a close composition to the theoretical expected (66 mol % SFA, 33 mol% of MUFA and PUFA). However, FFA and DAG composition differed from the expected one. Effectively, FFA had a high content of caprylic acid (59 mol %) in spite of the negligible amount expected. Also, in the case of the DAG, C16:1n-7, C18:1n-9, EPA and DHA were detected in a high proportion (theoretically DAG should be almost pure

Table 3. Fatty acid composition of the sn-2 position of the triacylglycerols of the extracted oil and the purified after the enzymatic treatment. Sn-2 composition referred to the profile of the central position. For a given fatty acid, sn-2% referred to the percentage which was esterified at the central bond compared to the total content that was esterified in the TAG.

	Extracted oil		Two steps esterification		One step esterification	
	sn-2	**sn-2%**	**sn-2**	**sn-2 %**	**sn-2**	**sn-2 %**
C8:0	0.00	0.00	24.13	16.59	73.38	33.31
C14:0	23.08	20.76	4.41	83.55	0.00	0.00
C16:0	12.89	23.66	4.47	83.55	3.84	76.85
C16:1n-7	12.33	21.94	8.10	60.79	2.74	36.37
C16:2n-4	2.67	21.88	0.00	0.00	0.00	0.00
C16:3n-4	1.65	12.73	0.00	0.00	0.00	0.00
C16:4n-1	2.64	14.49	0.00	0.00	0.00	0.00
C18:0	1.86	6.33	1.71	100.00	1.95	86.50
C18:1n-7	2.59	0.00	2.79	50.01	0.00	0.00
C18:1n-9	3.78	11.67	11.61	65.89	7.27	63.23
C18:2n-6	0.53	13.33	1.97	66.97	3.10	100.00
C18:3n-3	0.37	7.93	2.29	0.00	0.00	0.00
C18:4n-3	2.99	21.06	2.67	51.07	0.00	0.00
C20:1n-9	0.79	0.00	1.24	29.92	0.00	0.00
C20:3n-6	0.00	0.00	0.00	0.00	0.00	0.00
C20:4n-3	0.11	3.50	1.48	79.26	0.00	0.00
C20:4n-6	0.00	0.00	0.00	0.00	0.00	0.00
C20:5n-3	8.64	13.33	9.42	34.08	2.91	18.52
C22:1n-9	0.00	0.00	0.00	0.00	0.00	0.00
C22:5n-3	4.46	31.76	0.00	0.00	0.00	0.00
C22:6n-3	18.61	41.19	23.71	76.18	4.81	27.00
SFA	37.83		34.72		79.17	
MUFA	19.49		23.74		10.01	
PUFA	42.68		41.54		10.82	

For a given fatty acid, sn-2 data represents the relative composition of that fatty acid esterified at the central bond of the glycerol backbone.
Sn-2 % represents fatty acid content at sn-2/ all fatty acid content at the three positions as calculated in Eq 5.
All samples presented a SD < 5%.

caprylic acid). This fact suggests that (a) the DAG employed as substrate was a mixture of 1,2-DAG and 1,3-DAG or (b) parallel acidolysis was taken place. Acidolysis is the reaction between FFA and fatty acids bonded to the glycerol backbone (in the form of MAG, DAG or TAG) by which the fatty acids are exchanged (Hita et al., 2007). Hita et al (2007) described acydolysis between TAG (LLL) and FFA (M) as a two steps process, both catalyzed by lipases. Firstly, TAG must be hydrolyzed, resulting in DAG (LL-OH) and a FFA (LH):

$$LLL + H_2O \leftrightarrow LL\text{-}OH + LH \quad [6]$$

In a second step the esterification of the DAG with an odd free fatty acid takes place:

$$LL\text{-}OH + MH \leftrightarrow LLM + H_2O \quad [7]$$

This reaction may also occur by hydrolyzing DAG and re-esterifying them (Fig. 2C). This parallel acidolysis also plays a detrimental role on the regiodistribution of the fatty acids within the glycerol backbone of the produced TAG, as it is described below.

The regiodistribution of the fatty acids in the extracted oil and TAG produced by direct and two steps esterification is listed in Table 3. The column labelled as “sn-2” refers to the composition of the central position, whereas the column “sn-2 %” indicates the percentage of a given fatty acid which is located in the central position referred to the total content of that fatty acids in the TAG. For instance, in the case of DHA, for two steps esterification process, the central position contained an absolute value of 23.71 mol % value which represents the 76.18% of the total DHA that the triglyceride contains.

In the direct esterification, the produced TAG presented a 73.4 mol % of caprylic acid being the content of EPA and DHA 2.91 and 4.81 mol % respectively. TAG produced *via* two steps esterification, showed a much high sn-2 regioselectivity. Indeed, only around 25 mol% of the fatty acids esterified in the central bond were caprylic acid. As previously stated, there might be some 1,2-DAG in the dicaprylic glycerols produced. Also, during esterification a secondary and undesired acidolysis reaction might happen. Since a non-selective enzyme was employed, the acidolysis may affect any bond of the glycerol backbone. A schematic diagram of the feasible parallel reactions is depicted in Fig. 2C. Further research aimed at minimizing the effect of the acidolysis and acyl migration must be conducted.

The EPA and DHA content was higher than the one of the extracted oil. Usually, in the production of MLM structured lipids by acidolysis or by the combination of hydrolysis and esterification the resulting structured lipids show a lower concentration of PUFA than the original oil (Hita et al., 2009; Jennings & Akoh, 2001). In the current work, this loss was overcame by including a concentration step. Additionally, employing a two steps esterification improved considerably the regioselectivity of the process.

4. CONCLUSIONS

In the current work an alternative up-grading process aimed at producing structured lipids (MLM) from sardine discards was proposed. It was composed of (i) fish extraction, (ii) Omega-3 concentration *via* low temperature winterization (iii) enzymatic esterification and (iv) TAG extraction *via* liquid chromatography. Each step presented acceptable yield. Furthermore, the oxidative state of the produced TAG accomplished with the conditions required for refined oils. The esterification was the most oxidative step, while the liquid chromatography purification improved the oxidative state. Further studies focused on minimized the degree of oxidation by adding antioxidants should be conducted.

Additionally, a two steps esterification (i) production and purification of dicaprylic glycerols and (ii) esterification of concentrated fatty acids with the produced dicaprylic glycerols was studied. This enzymatic treatment improved considerably the regiodistribution of the fatty acids of the produced TAG when compared to direct esterification. However, since a considerable percentage of caprylic acid was detected at the sn-2 position, it might possible that (a) 1,2-DAG were produced by acil-migration during the first esterification or (b) acidolysis might be a secondary reaction happening with the second esterification. Further studies, focused exclusively on the enzymatic step, should be conducted aimed at improving the purity of the 1,3-DAG and minimizing the extent of the acidolysis.

5. REFERENCES

A.O.A.C. (Association of Official Analytical Chemist). (2006). Official Methods of Analysis of the AOAC, Association of Official Analytical Chemists. Washington DC.

Ackman, R. G. (2005). Fish oils. In F. Shahidi (Ed.), Bailey's Industrial Oil and Fat Products (6th ed., pp. 279–317). Hoboken, NJ, USA: John Wiley & Sons.

Akoh, C. C., & Kim, B. H. (2008). Structured lipids (pp. 841–872). Conference Proceedings, CRC Press.

Bimbo, A. P. (1998). Guidelines for Characterising Food Grade Fish Oil. Inform.

Bimbo, A. P. (2007). Processing of marine oils. In H. Breivik (Ed.), Long-Chain Omega-3 Specialty Oils (pp. 77–107). Bridgwater, England: The Oily Press.

Bimbo, A. P. (2012). Long-Chain Omega-3 Specialty Oils. Long-Chain Omega-3 Specialty Oils. Elsevier. http://doi.org/10.1533/9780857097897.77

Drusch, S., Serfert, Y., Berger, A., Shaikh, M. Q. Q., Rätzke, K., Zaporojtchenko, V., & Schwarz, K. (2012). New insights into the microencapsulation properties of sodium caseinate and hydrolyzed casein. *Food Hydrocolloids*, 27(2), 332–338. http://doi.org/10.1016/j.foodhyd.2011.10.001

Dumay, J., Donnay-Moreno, C., Barnathan, G., Jaouen, P., & Bergé, J. P. (2006). Improvement of lipid and phospholipid recoveries from sardine (*Sardina pilchardus*) viscera using industrial proteases. *Process Biochemistry*, 41(11), 2327–2332. http://doi.org/10.1016/j.procbio.2006.04.005

EU. (2013). Regulation (EU) no 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Official Journal of the European Union, 22–28.

FAO. (1986). The production of fish meal and oil. Rome: FAO Fisheries and Aquaculture Department, Rome.

García-Moreno, P. J., Morales-Medina, R., Pérez-Gálvez, R., Bandarra, N. M., Guadix, A., & Guadix, E. M. (2014). Optimisation of oil extraction from sardine (*Sardina pilchardus*) by hydraulic pressing. *International Journal of Food Science & Technology*, 49(10), 2167–2175. http://doi.org/10.1111/ijfs.12527

Halim, N. R. A., Yusof, H. M., & Sarbon, N. M. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology*, 51, 24–33. http://doi.org/10.1016/j.tifs.2016.02.007

Hari Krishna, S., & Karanth, N. G. (2002). Lipases and Lipase-Catalyzed Esterification Reactions in Nonaqueous Media. *Catalysis Revie*ws, 44(4), 499–591. http://doi.org/10.1081/CR-120015481

Hita, E., Robles, A., Camacho, B., González, P. A., Esteban, L., Jiménez, M. J., Molina, E. (2009). Production of structured triacylglycerols by acidolysis catalyzed by lipases immobilized in a packed bed reactor. *Biochemical Engineering Journal*, 46(3), 257–264. http://doi.org/10.1016/j.bej.2009.05.015

Hita, E., Robles, A., Camacho, B., Ramírez, A., Esteban, L., Jiménez, M. J. Molina, E. (2007). Production of structured triacylglycerols (STAG) rich in docosahexaenoic acid (DHA) in position 2 by acidolysis of tuna oil catalyzed by lipases. *Process Biochemistry*, 42(3), 415–422. http://doi.org/10.1016/j.procbio.2006.09.023

Jacobsen, C., & Skall Nielsen, N. (2007). Optimization of oxidative stability of Omega-3 enriched food. In H. Breivik (Ed.), Long-Chain Omega-3 Specialty Oils (21st ed., pp. 197–219). Bridgwater, England: The Oily press Lipid Library.

Jennings, B. H., & Akoh, C. C. (2001). Lipase catalyzed modification of fish oil to incorporate capric acid. *Food Chemistry*, 72(3), 273–278.

Johnson, L. A. (2008). Recovery, refining, converting, and stabilizing edible fats and oils (pp. 205–243). Conference Proceedings, CRC Press.

Kelleher, K. (2005). Discards in the world's marine fisheries: An update. FAO. Fisheries Technical Paper.

Kim, B. H., & Akoh, C. C. (2015). Recent Research Trends on the Enzymatic Synthesis of Structured Lipids. *Journal of Food Science*, 80(8), C1713–C1724. http://doi.org/10.1111/1750-3841.12953

Köse, Ö., Tüter, M., & Aksoy, H. A. (2002). Immobilized *Candida antarctica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresource Technology*, 83(2), 125–129. http://doi.org/10.1016/S0960-8524(01)00203-6

Laplante, S., Souchet, N., & Bryl, P. (2009). Comparison of low-temperature processes for oil and coenzyme Q10 extraction from mackerel and herring. *European Journal of Lipid Science and Technology*, 111(2), 135–141. http://doi.org/10.1002/ejlt.200800133

Lawson, L. D., & Hughes, B. G. (1988). Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters. *Biochemical and Biophysical Research Communications*, 152(1), 328–335. http://doi.org/10.1016/S0006-291X(88)80718-6

Lembke, P. (2013). Production Techniques for Omega-3 Concentrates. In F. De Meester, R. R. Watson, & S. Zibadi (Eds.), Omega-6/3 Fatty Acids (pp. 353–364). Humana Press.

Morales-Medina, R., Munio, Maria del Mar, Pérez-Gálvez, R., Guadix, A. and G. E. (2013). Lipids from Marine Sources. In R. Pérez-Gálvez & J.-P. Bergé (Eds.), Utilization of fish waste. Boca Raton: CRC Press.

Morales-Medina, R., De León, G., Munio, M., Guadix, A., & Guadix, E. (2016). Mass transfer modeling of sardine oil polyunsaturated fatty acid (PUFA) concentration by low temperature crystallization. *Journal of Food Engineering*, 183, 16–23. http://doi.org/10.1016/j.jfoodeng.2016.03.009

Morales-Medina, R., García-Moreno, P. J., Pérez-Gálvez, R., Muñío, M., Guadix, A., & Guadix, E. M. (2015a). Seasonal variations in the regiodistribution of oil extracted from small-spotted catshark and bogue. *Food Funct*., 6(8), 2646–2652. http://doi.org/10.1039/C5FO00448A

Morales-Medina, R., García-Moreno, P. J., Pérez-Gálvez, R., Muñío, M. M., Guadix, A., & Guadix, E. M. (2015b). Nutritional indexes, fatty acids profile and regiodistribution of oil extracted from four discarded species of the Alboran Sea: Seasonal effects. *European Journal of Lipid Science and Technology*, In Press. http://doi.org/10.1002/ejlt.201500486

Moya-Ramírez, I., García-Román, M., & Fernández-Arteaga, A. (2016). Waste Frying Oil Hydrolysis in a Reverse Micellar System. *ACS Sustainable Chemistry and Engineering*, 4(3), 1025–1031.

Munio, M. del M., Robles, A., Esteban, L., Gonzalez, P. A., & Molina, E. (2009). Synthesis of structured lipids by two enzymatic steps: Ethanolysis of fish oils and esterification of 2-monoacylglycerols. *Process Biochem*., 44, 723–730. http://doi.org/10.1016/j.procbio.2009.03.002

Pérez-Gálvez, R., Chopin, C., Mastail, M., Ragon, J. Y., Guadix, A., & Bergé, J. P. (2009). Optimisation of liquor yield during the hydraulic pressing of sardine (Sardina pilchardus) discards. *Journal of Food Engineering*, 93(1), 66–71. http://doi.org/10.1016/j.jfoodeng.2008.12.031

Rubio-Rodriguez, N., Beltran, S., Jaime, I., De, D. S. M., Sanz, M. T., & Carballido, J. R. (2010). Production of Omega-3 polyunsaturated fatty acid concentrates: A review. *Innovative Food Sci. Emerging Technol*., 11, 1–12. Journal Article. http://doi.org/10.1016/j.ifset.2009.10.006

Rubio-Rodríguez, N., de Diego, S. M., Beltrán, S., Jaime, I., Sanz, M. T., & Rovira, J. (2012). Supercritical fluid extraction of fish oil from fish by-products: A comparison with other extraction methods. *Journal of Food Engineering*, 109(2), 238–248. http://doi.org/10.1016/j.jfoodeng.2011.10.011

Sathivel, S. (2010). Fish oil extraction, purification and its properties. In C. Alasalvar, F. Shahidi, K. Miyashita, & U. Wanasundara (Eds.), Handbook of Seafood Quality, Safety and Health Applications (1st ed, pp. 423–430). West Sussex: John Wiley & Sons Ltd.

Wanasundara, U. N., & Shahidi, F. (1999). Concentration of Omega 3-polyunsaturated fatty acids of seal blubber oil by urea complexation: Optimization of reaction conditions. *Food Chemistry*, 65(1), 41–49. Journal Article. http://doi.org/10.1016/s0308-8146(98)00153-8

Wanasundara, U. N., Wanasundara, P. K. J. P. D., & Shahidi, F. (2005). Novel Separation Techniques for Isolation and Purification of Fatty Acids and Oil By-Products. In Bailey's Industrial Oil and Fat Products. John Wiley & Sons, Inc.

Xu, X. (2000). Enzymatic production of structured lipids: Process reactions and acyl migration. *INFORM - International News on Fats, Oils and Related Materials*, 11(10), 1121–1130. Journal Article.

VII. Fish Discards as a Source of Health Promoting Biopeptides*

The vast diversity of marine organisms is a source of enormous potential for obtaining bioactive compounds which can be employed in the food industry. In this context, discards (defined as the fraction of the fish catch which is not retained on board but rejected to the sea for reasons such as non-target species, commercial standards or fishing regulations) are regarded as a wastage of resources and an environmental problem. The new EU Common Fisheries Policy introduced a discard ban since 1st January 2015 for pelagic species. Given their proximate composition, 1-18% of lipids and 15-20% of proteins, discarded materials are receiving increasing attention for biotechnological applications. Six of the main discarded species in the Alboran Sea, namely axillary seabream (Pagellus acarne), small-spotted catshark (Scyliorhinus canicula), sardine (Sardina pilchardus), horse mackerel (Trachurus mediterraneus), bogue (Boops boops) and blue whiting (Micromesistius poutassou), represent more than 85% of the total discard (more than 330 ton/year). This chapter mainly focuses on the upgrading of the protein fraction of the aforementioned species into biopeptides. To that end, compacting and separation stages were designed to obtained the lipid and protein fractions. The influence that enzymatic hydrolysis exerted on several bioactivities as antihypertensive, antioxidant, anticholesterolemic or antimicrobial was analyzed. Additionally, the bioavailability of the biopeptides produced was analyzed employing simulated gastrointestinal digestion. Finally, the methods employed for characterizing and identifying the active peptides were described. These biopeptides can be employed in the formulation of functional food.

* BOOK CHAPTER: R Pérez-Gálvez, F. J. Espejo-Carpio, R. Morales-Medina, P. J. García-Moreno, A. Guadix, E. M. Guadix. (2016). In “Handbook of Food Biotechnology: Progress of Biotechnology in Food Industry", Elsevier. In press.

1. INTRODUCTION: DISCARDING PRACTICES IN THE FISHING INDUSTRY

1.1. Definition of discards. Negative impact of discards on the environment

In a context of overexploitation of fish feedstocks, discards in fisheries represent an irresponsible wastage of living resources. According to the FAO, discards are formally defined as "that proportion of the total organic material of animal origin in the catch, which is thrown away, or dumped at sea for whatever reason" (Kelleher, 2005). The discarded fraction not only comprises incidental catches such as non-target fish species, marine mammals or seabirds, but also includes commercial species below minimum landing size or exceeding fishing quotas. Discarded practices are traditionally caused by a number of regulatory, technical and economic reasons:

1. Fisheries regulations, especially those ruling the fishing quotas and minimum landing sizes (MLS), which are responsible for the discarding of fish commercial species.

2. Market or consumer's preferences determine that specimens meeting the MLS are rejected in reason of their low economic value.

3. Technical limitations of the fishing gear. Discard rates are affected by mesh sizes and fishing techniques. FAO estimates an average discard rate of 23% for trawling vessels, while purse seine gears discard on average 10% of the catch.

4. Characteristics of the fisheries such as fishing ground, diversity of fish species, seasonal fluctuations, etc.

Discards causes a negative impact on marine stocks and fishing production. Most of the individuals are dead or dying when dumped back to the sea. In the case of finfish species, the expansion of air in natatory bladders when hailing the nets minimises the survival rate. According to Bozzano and Sardà (2002), 50% of crustaceans and 98% of cephalops do not survive after discarding. Discarded materials represent a superabundant food input which atracts marine scavengers such as seabirds, marine mammals and other depredator species. This has a negative impact on marine trophic chains (Groenewold et al., 2000; Bicknell, Oro, Camphuysen, & Votier, 2013). Furthermore, the dumping of fish waste contribute to

the spread of intestinal parasites (Antelo et al., 2016). Moreover, a large fraction of discarded fish corresponds to juvenile individuals, which has a negative impact on the future yields of fisheries (Jensen, Reider, & Kovalak, 1988; Poos et al., 2013; Antelo et al., 2016).

1.2. Assessment of discard rates: focus on the Alboran Sea

The design and implementation of fisheries regulations on discards requires a periodical estimation of discard rates and catch utilization. Discard rate is defined as the fraction of the fish catch which is rejected. To this regard, last FAO assessment on global discard rates, based on a study during the period from 1992 to 2001, estimated 7.3 million discards per year worldwide. This figure represents an average discard rate of 8%, related to total catches (Kelleher, 2005; FAO, 2014). Recent studies assume that discard rates in a given fishery depend on the main fishing gears employed, the target species and the specifical fishing regulations. The SOFIA report (FAO, 2014) estimates that 40% of the discards observed worldwide correspond to fisheries located in the Atlantic and North Pacific, followed by the West Indian Ocean. Intensive fishing gears such as trawling generate the largest amount of by-catches, which is the case of shrimp trawling (62%) and tuna longliners (29%). Focusing on the Mediterranean Sea, a recent study by Tsagarakis et al., (2012) estimates 230,000 tons per year, which represents 18.6% of total catches. The FAO report underestimates this figure, reporting an average discard rate of 10%. In the same study, Tsagarakis pointed out the lack of studies on by-catches as well as the limited coverage of campaigns. In any case, both studies predict a lower discard rate for the Mediterranean Sea, compared to Atlantic fisheries. Indeed, Mediterranean fisheries are based on traditional fishing gears, such as purse seine, which are more selective and generate lower incidental catches. More specifically, Spanish fisheries represented 14% of total catches of EU in 2008. The major proportion of fish catches are located in Northwest Atlantic coast, employing trawling as fishing gear. According to the FAO report, these fisheries present high discard rates (45 – 65%). In contrast, Spanish fleet based on Mediterranean Sea targets hake and blue whiting as main species, employing traditional fishing gears such as purse seine. These metiers provide a better utilization of fishing catch, with average discard rates of 13 – 15%. The Alboran Sea is a portion of the Mediterranean Sea lying between the southern Spanish coast, from the strait of Gibraltar to

the cape of Gata, and the northern coast of Morocco. This area covers 11,000 km^2 and presents a maximum depth of 800 m.

Spanish fleet in this area employs purse seine (64% of total catches) and trawling (22% of total catches) as fishing gears. Fishing activities in this area discard commercial species such as blue whiting (*Micromesistius poutassou*), sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*) and axillary seabream (*Pagellus acarne*). These discards, which comprise target species, are caused by minimum landing size regulations, fishing quota or high grading practices. Other species, such as bogue (*Boops boops*) or small-spotted catchark (*Scyliorhinus canicula*) are highly discarded due to their low market value. These five species acount for more than 90% of yearly discards in West Mediterranean Sea (García-Moreno et al. 2013). Fish stocks in Alboran Sea have been reduced by 50% in the past two decades (Análisis anual de la producción pesquera andaluza, 2009), which calls for urgent policies providing a better fishing management in the short term (*e.g.* new fishing quotas, discard ban, technical improvements on fishing gears, better utilization of fish by-products). Technical measures in fisheries comprise regulations on minimum landing sizes, fishing quota and time restrictions, as well as those setting the use and design of the fishing gears (specific species which can be caught by a given gear, mesh sizes, catch selectivity), and restricting fishing in some areas or seasons. They basically aim at limiting the catch of juvenile fish and incidental species, promoting a lesser impact of fishing activities on the environment. Mesh size limits should be ideally defined for each target species on the basis of its fish shape, growth rate and size at sexual madurity. Nevertheless, no fishing gear can be completely selective, and the target species are often caught simultaneously to other associated species. Therefore, mesh size requirements should ensure the catch of target species while respecting the maximum levels authorised for incidental species. The recent EU Common Fisheries Policy has implemented some regulations enlarging the mesh size, which has reduced to a large extent incidental catches in some fisheries, such as hake in West Mediterrean Sea (Tsagarakis et al. 2012) or crustacean Nephrops in the Bay of Biscay (Nikolic et al., 2015), among others.

1.3. Fisheries regulations on discards: the EU's Zero discard policy

In past decades, international bodies such as the United Nations, the Kyoto Declaration and the Code of Conduct for Responsible Fisheries have stressed the problems associated to

discards and the need to minimize or eliminate these practices. Earlier countries implementing regulations on discards in Europe were Iceland, Norway and Faeroe Islands. For instance, Norway introduced a discard ban in 2004 for the main target species (*e.g.* cod, haddock, saithe, mackerel, herring, capelin, whiting). This measure was completed with other regulations on mesh size and gear design (Graham, Ferro, Karp, & MacMullen, 2007).

As for the European Union, the EU Commission communications COM 656 (EU Commission, 2002) and COM 136 (EU Commission, 2007) paved the way to the promotion of regulations to avoid incidental catches. Both communications proposed landing obligations on EU fisheries, obliging fishing vessels to land the whole catch, including target and by-catch species. Discard ban was accompanied by technical measures to improve the selectivity of fishing gear and the management of the fish stocks (*e.g.* minimum mesh size requirements, real time closures).

The new Common Fisheries Policy (CFP) introduces the landing obligation of discards for EU fisheries (Article 15). This rule requires fishermen to land and record all the catches subject to catch limits (EU, 2013; Mcilwain, 2015). The implementation of the CFP will mean that discards will be progressively prohibited in European fisheries. More specifically, the article 15 states that all catches from small and large pelagic fisheries, such as anchovy, sardine, mackerel or swordfish should be landed from the 1st January 2015 on. According to the CFP, this measure will be extended to the rest of fisheries in 2017. As an exception, landing obligations will not come into force until the 1st January of 2019 for some specific fisheries such as hake, Norway lobster, common sole and plaice.

2. PRETREATMENTS TO PROCESS FISH DISCARDS AND BY-PRODUCTS

Fish harvest and fish processing industry generate a variable amount of solid wastes and wastewaters whose management, elimination or disposal pose environmental, economic and logistical problems. Arvanitoyannis and Kassaveti (2008) estimated an output of 350-400 kg of solid wastes per ton of fish processed in canning industry. The same authors estimated an output of 400 – 450 kg of solid wastes per ton in the case of oily fish filleting. The costs associated to the processing of organic materials would depend to a large extent on their moisture content. Overall, the moisture content should be limited in order to

reduce transport and handling costs as well as preserving these materials from microbial spoilage (Ghiasinejad & Abduli, 2007; Zafari & Kianmehr, 2013). The daily compaction/dewatering of organic waste is a well-established process in municipal solid waste (MSW) management since it increases the capacity of landfills.

Regarding the dewatering of solid wastes from slaughterhouses, a report from the French Stockbreeding Office evaluated the volume reduction and economic viability of different mechanical dewatering techniques such as filtration devices, centrifuges, decanters and press devices (Pérez-Gálvez, Bergé, & Guadix, 2012). This report concluded that pressing operations were preferred to compact and dewater solid, non-pumpable wastes, due to the range of pressures able to develop by these devices. For instance, the performance of a screw press was tested for several organic wastes such as bovine and ovine manures, pork guts or coagulated bovine blood by a screw press. The yield of water expelled by this device range from 17% (pork guts) to 75% (bovine and ovine manures).

As for the fish processing industry, the bulk of the world's fish meal and oil is today manufactured by the wet method (Bimbo, 2012; Oterhals & Kvamme, 2013). The raw material is cooked (85-95ºC for 15-20 min) in order to coagulate protein and separate this fraction from the fish oil. The cooked mixture is then screened and then pressed to remove most of the water from the mixture. The pressed cake, containing 60-80 of the oil-free dry matter (protein, bones) and oil, is then dried by means of an indirect steam drier or a direct flame dryer. The resulting dried powder is referred as to fish meal, which is used as an ingredient for aquaculture diets. The pressed liquid generated from the pressing operation passes through a decanter to remove most of the sludge, which is fed back to the meal dryer. Oil is separated from the liquid by centrifuges, polished and refined to remove any remaining water and impurities. The separated aqueous phase, referred to as stickwater, contains valuable soluble proteins which are recovered in multi-effect evaporators and reincorporated into the fish meal.

Pressing operations commonly implemented in fish processing industry are based on screw and hydraulic presses. The former operate in a continuous process mode, and are commonly implemented at fish meal and fish oil facilities (Kent, 2010). In contrast, hydraulic-driven presses work batchwise, being preferred to process smaller amounts of raw material or when there is not constant supply of raw material. This is the case of fishing vessels, where the trawling nets are hauled two or three times per day, and the power supply is based on hydraulic devices (*e.g.* to haul the nets). Furthermore, the

maintenance and control of such devices is simple and they require less energy consumption than electric devices. Landing obligations on EU fishing fleet raises economic concerns to fisheries stakeholders, since they are obliged to store and preserve the whole catches on board. In this context, dewatering devices such hydraulic presses are able to reduce the volume of these biomasses and therefore reduce the handling and refrigeration costs. The installation of compaction devices on board fishing vessels was already proposed by the Code of Conduct for Sustainable Fisheries (FAO, 1995):

"Do their utmost to treat waste generated on board as if it were domestic waste, for example, by using a compactor on vessels where that is economically possible to treat refuse and other on-board waste during fishing trips; not dump the waste but retain it for later treatment where suitable structures and equipment exist on land."

The pressing of fish materials generates a partially dewatered press cake and a press liquor, which contains variable amounts of soluble proteins, fish oil and suspended matter (Pérez-Gálvez et al., 2011). The maximization of the economic profit of the pressing operation (*i.e.* maximum volume reduction) may conflict with the discharge of the resulting press wastewaters, which bear high levels of organic matter (*i.e.* high chemical and biological oxygen demand). In this line, Pérez-Gálvez et al. (2009) optimized at laboratory scale the hydraulic pressing of fish discards from sardine (*Sardine pilchardus*) and then scaled up these results to a pilot plant (Pérez-Gálvez et al., 2016). In both cases, the performance of the hydraulic device was optimized to attain a maximum economic revenue (*i.e.* maximum volumen reduction, maximum yield of press liquor) while limiting the organic load (*i.e.* suspended solids, chemical oxygen demand) of the resulting press wastewaters. To this end, the sardine discards were pressed stepwise, employing one to five pressing steps intersected by relaxation periods where the press liquor was allowed to drain out of the press chamber. The pressure supplied by the hydraulic device, the speed of the piston, the number of pressing steps and the duration of the relaxation period were identified as main operation factors affecting both the yield of press liquor and the organic load of the press waters.

Other studies do not regard press liquor as a pollutant discharge, but explore the alternatives to recover the valuable compounds (*e.g.* soluble proteins, fish oil) herein contained. To this regard, Pérez-Gálvez et al. (2011) studied the pressing of sardine discards by a hydraulic- driven press. The press liquor was recovered under different operating conditions (*i.e.* number of pressing steps, target pressure, time of relaxation

between pressing stages, velocity of the piston), and centrifuged at 10,000 g to quantify the amount of fish oil (upper layer), aqueous phase (containing soluble proteins) and sludge. The phase composition of the press liquor was related to the operating conditions of the pressing procedure. This work reviews the current valorization alternatives for each one of these phases, highlighting the recovery of fish oil, soluble proteins from the aqueous phase or the recovery of calcium minerals from the sludges.

García-Moreno et al. (2014) recovered fish oil from sardine discards by means of a mechanical press. To this end, the raw material was preheated in a water bath and then pressed batchwise in a mechanical basket press. The performance of the whole process was optimized to attain a maximum yield of fish oil (*i.e.* mass of oil recovered from mass of raw material) while preserving its oxidative quality (*i.e.* free fatty acids, p-anisidine, peroxide value and Rancimat induction period). The authors concluded that the oxidative stability of the fish oil was affected negatively by the temperature applied in the water bath. Nevertheless, lower temperatures led to a decline in the yield of fish oil recovered from the press liquor. This could alleviated by increasing the pressure in the hydraulic device, as well as applying successive pressing-relaxation cycles.

3. UPGRADING OF DISCARDED MATERIALS: FOCUS ON THE PROTEIN FRACTION

3.1. Introduction: Recovery of the protein fraction from fish materials

The protein content of discards fluctuates depending on species and season, but generally ranges between 15 and 25 % (Morales-Medina et al., In press). After compacting the protein percentage would rise depending on the water content of the original raw material (Pérez-Gálvez et al. 2016). This is a high quality protein source since it has high digestibility and high proportion and availability of essential amino acids (Nunes et al. 2010). The major proteins of fish can be classified in three groups: myofibrillar, sarcoplasmic and stroma proteins. The main myofibrillar proteins (65-75 wt%), myosin and actin are soluble in solution saline solutions. The sarcoplasmic proteins (20-35 wt%) are soluble in water while stroma proteins (2-3 wt%) are water insoluble. Therefore, discards represent a valuable source of high quality protein which can be effectively

recovered by chemical or enzymatic hydrolysis. The enzymatic treatment presents several advantages which made it desirable for the production of discard protein hydrolysates:

a. Because the specificity of the enzymes, side reaction which could produce undesirable products are avoided.

b. The reactions are held at mild conditions of pH and temperature.

c. The nutritional value of the original protein is not altered in the reaction.

In the enzymatic hydrolysis of proteins, proteases catalyze the cleavage of peptide bond between two amino acids consuming a molecule of water per each bond cleaved as shown below:

The products generated in this reaction could be hydrolyzed again. Each consecutive hydrolysis reaction yield products with lower molecular size. Depending on the extent of hydrolysis, a fish byproduct hydrolysate would be composed by a mixture of peptones, peptides or amino acids at different proportions (Adler-Nissen, 1986). The proportion of peptide bonds cleaved is defined as degree of hydrolysis (DH). It should be controlled carefully because of its influence on the hydrolysate properties.

Fish protein hydrolysates show improved characteristics which made them attractive for food industry. They have good solubility over a wide range of pH levels; they also contribute to improve water holding and texture when adding to food (Jemil et al., 2014). Hydrolysates could also be employed as emulsifying and emulsion stabilizing agents in food formulas (Nalinanon et al., 2011). Moreover, hydrolysis reduces the antigenic levels of the native protein (García-Moreno et al., 2016). Nevertheless, in the last years the focus seems to be on the production of bioactive fish hydrolysates which, apart from the nutritional value, have a beneficial health effect (Halim et al., 2016).

In this regard, an increasing number of studies have reported fish protein hydrolysates with a wide range of bioactivities such as antioxidative, antihypertensive, cholesterol lowering, immunomodulatory, antimicrobial, antiviral, antitumor, appetite suppressing and neuroprotective activities (Cheung et al., 2015).

3.2. Identification of bioactive peptides

Protein hydrolysates are composed by a highly complex mixture of peptides with different molecular sizes and sequences. However, only a portion of the peptides released during

hydrolysis reaction would show bioactive activity. The identification of these active sequences present in the hydrolysate is interesting since it allows the characterization of the final bioactive product. Moreover, once the active peptides have been identified, it would be possible to design a filtration process for enriching the hydrolysate in these compounds.

Before carrying out the identification of the amino acid sequence of the active peptide, the peptide should be isolated. Therefore, the first step of the identification process usually involves the fractionation of the hydrolysate in base to the molecular weight of peptides. A large number of studies (Bougatef et al., 2010; Hsu 2010; Nasri et al., 2013; García-Moreno et al., 2015) employed size exclusion chromatography (SEC) for evaluating the bioactive potential of the fractions with different molecular size. SEC is based in the separation of molecules according to their size when passing through a gel filtration medium packed in a column. The molecules do not bind to the chromatography medium, then, buffer does not directly affect resolution. The fractions are detected at 200-300 nm (usually 280 nm) and collected at the end of the column. The peptides would elute the column according their molecular size; smaller molecules flow more slowly through the column because they penetrate deep into the pores, whereas large molecules flow quickly through the column because they do not enter the pores. Therefore, larger molecules elute from the column sooner and smaller molecules later. The columns are commonly packed with a matrix composed by cross-linked dextran (*e.g.* Sephadex) or composite of cross-linked agarose and dextran (*e.g.* Superdex). Distilled water or saline solutions (*e.g.* phosphate buffer) are usually employed as mobile phase.

Another option, for this initial stage, is separating peptides by membrane technology (Zhuang and Sun 2011; Chi et al., 2015). Membranes with molecular weight cut off (MWCO) ranged between 1 and 10 kDa are usually employed for fractionating the hydrolysate. The filtrate obtained from the membrane with the highest cut-off could be consecutively filtrated by lower cut-off membranes in order to have different molecular size fractions. Some authors combine the two methods described before for selecting the most active fractions (Wu et al., 2015; Zou et al., 2014). In this case, the hydrolysate would be initially ultrafiltrated through one or various membranes and the most active permeate obtained would be further fractionated by SEC.

In any case, the most active fractions will be selected for further separation. In the case of antioxidant and antihypertensive bioactivities, the most potent fractions are usually those below 5 kDa (Hsu 2010; Zhuang and Sun 2011; García-Moreno et al., 2015).

Techniques based on differences in charge, hydrophobic interactions, and adsorption affinities among others (Sila & Bougatef 2016) could be employed in combination with the SEC or membrane filtration. However, reverse phase chromatography (RPC) is the most common method for fulfill the purification of the fraction obtained initially by molecular size separation methods. The separation is based on the interaction of the molecules with a hydrophobic stationary phase. The majority of studies dealing with peptide purification employ a high pressure liquid chromatography system mounted with a silica column modified with octadecylsilyl (C18 column) (Zou et al., 2014; Nasri et al., 2013; Bougatef et al., 2010). In RPC, the mobile phase should be polar to favor the interactions between the hydrophobic solutes and the stationary phase. Therefore, hydrophobic compounds will tend to bind the stationary phase while hydrophilic molecules will pass through the column and eluted first. Decreasing the mobile phase polarity reduces the hydrophobic interaction between the molecules and the solid support which allows the elution of hydrophobic compounds. A solution composed of water, acetonitrile and trifluroacetic acid (TFA) is frequently used. The proportions of the mobile phase components can vary between studies, but usually, TFA is fixed at 0.1% and a linear gradient of acetonitrile from 0 to a maximum of 50 % is employed. Nevertheless, some authors employ gradient from 0 to 100% of acetonitrile (Bougatef et al, 2010). As in the case of SEC, UV absorbance is monitored between 200 and 300 nm. Sometimes it is necessary to carry out several purification steps using reverse phase chromatography to get isolated peptides (Hsu, 2010).

Finally the isolated peptide can be analyzed to determine its mass and sequence. Mass spectrometry coupled with electrospray ionization (ESI) source is the most common technique for determining peptide mass (Nasri et al, 2014, Zou et al., 2014). The sequence of the peptide can be determined by analyzing the collision-induced dissociation spectrum of the protonated peptide analyzed $[M + H]^+$ by tandem mass spectrometry. Recently, some authors (Liu et al., 2015) proposed a more rapid method for peptide identification which does not implies the final isolation of the peptides. After some separation stage a complex mixture of peptides is analyzed directly by tandem mass spectrometry obtaining acceptable results. In any case, the MS/MS spectrum is analyzed by software with specific algorithms for deducing the peptide sequence. Most of the authors employ de novo

analysis (Nasri et al., 2014; Guo et al., 2013), which is an analytical process deriving the amino acid sequence from the tandem mass spectrum of a peptide without the assistance of a sequence database. In contrast to identification by database search, de novo sequencing allows to identify novel peptides. The method is based on using of the mass difference between two fragment ions, produced during fragmentation stage of the MS/MS analysis, to calculate the mass of an amino acid residue on the peptide backbone. The calculated mass can usually uniquely determine the residue. The process has some difficulties since some fragment ions could be missing or being incorrectly assigned (Ma & Johnson, 2012), also the existence of noise peak in the spectrum or the similar mass of some residues could generate ambiguity (I = L and K=Q). These factors among others can cause complications in identifying the complete peptide sequence.

Edman degradation method can be also employed for sequencing isolated peptides (Zou et al, 2014; Hsu, 2010). This method will often provide the exact amino acid sequence without ambiguity for a limited run of amino acids, 6-30 amino acids. The process consists in a cyclic degradation of the peptide based on the reaction of phenylisothiocyanate with the free amino group of the N-terminal residue. In each cycle, the first amino acid is cleaved and identified as its phenylthiohydantoin derivative by chromatography or electrophoresis. Once the first amino acid is released, it leaves the new amino terminus for the next degradation cycle. The method can be carried out automatically by a sequencer device. As an example, Garcia-Moreno et al. (2015) carried out an identification process of potential biological active peptides derived from discards. Initially, the bioactivity (ACE-inhibitory activity) of hydrolysates of sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark were evaluated. The combination of subtilisin and trypsin produced the most potent antihypertensive hydrolysate when hydrolyzing horse mackerel and small-spotted catshark. These hydrolysates were selected for analyzing by size exclusion chromatography. A fast liquid chromatography device mounted with a pre-packed column of composite of cross-linked agarose and dextran was employed. Fractions, seven for horse mackerel and 5 for small-spotted catshark were recovered according to the chromatograms obtained. Fig. 1 shows the IC_{50} values of the most potent fractions obtained and its molecular sizes. Since the IC_{50} represents the concentration of hydrolysate which reduces to 50% the activity of angiotensin converting enzyme (ACE), the lower the IC_{50} value is, the more potent the fraction is.

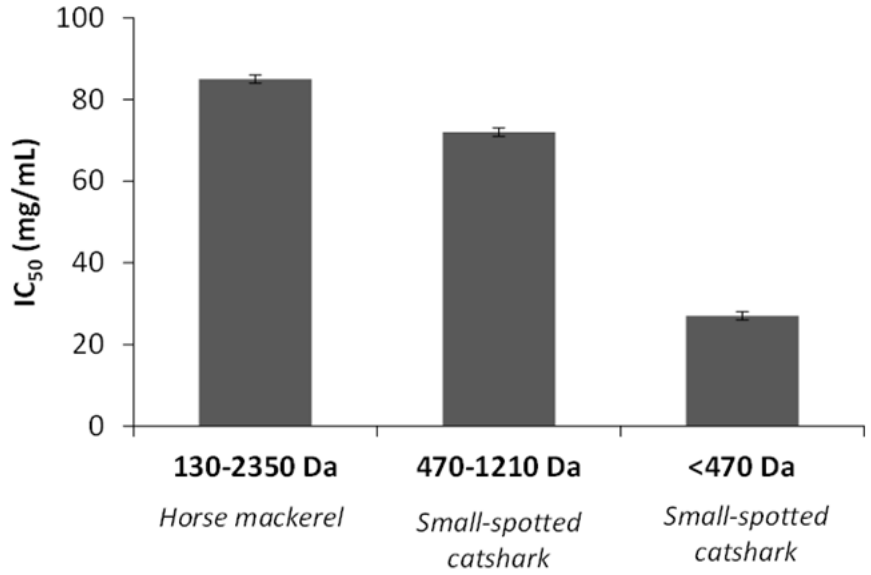


Figure.1. Angiotensin converting enzyme inhibitory activity of most potent fractions obtained for the horse mackerel and small-spotted catshark hydrolysates.

The three fractions were directly analyzed by a HPLC coupled with mass spectrometry as proposed by Liu et al. (2015). The mass spectrum obtained was analyzed by BioLynx software (Micromass UK Ltd., Manchester,United Kingdom) for sequencing peptides. A total of 14 peptides with molecular masses ranged between 400 – 1100 Da were identified. According to quantitative structure-activity relationship models, some of the sequences (HLALT, ELVGV, YLGW and VAMPF) showed a very interesting ACE-inhibitory potential.

Generally, it is not possible to recover the isolated peptide for determining its bioactive capacity as an isolate compound. Therefore, after identifying the sequence, the peptide should be chemically synthesized for verifying its bioactive capacity (Guo et al., 2013). An alternative could be the analysis in silico by quantitative structure activity (QSAR) models which predict the bioactivity of the peptide according to the molecular structure. An increasing number of studies are being published in this area. A recent work (Jahangiri et al., 2014) revises the QSAR models available for evaluating the ACE inhibitory activity of peptides. Apart from ACE inhibitory activity, antioxidant activity has also been studied by QSAR models (Li & Li, 2013).

3.3. Bioavility of active peptides: simulated digestion assays

One of the main applications of bioactive hydrolysates and peptides would be the formulation of functional food. According to the European Commission Concerted Action

on Functional Food Science in Europe (FUFOSE), a food can be regarded as "functional" when apart from its nutritional effects have one or more demonstrated beneficial outcomes for health or well-being. A key point is that, for exerting their biological effect, bioactive peptides should reach their target organs and tissues in an active form. Indeed, several bioactive peptides identified and tested *in vitro* fail when testing *in vivo* (Vermeirssen et al, 2004). This is mainly due to the bioavailability of the bioactive peptides after oral administration. To exert a biological effect after oral ingestion, peptides need to remain active during digestion by human proteases and be transported through the intestinal wall into the blood. Fujita et al. (2000) classified the ACE inhibitory peptides according to their resistance to be degraded by human ACE. Making that classification valid for other bioactivities and other human enzymes, the bioactive peptides could be classified in three groups:

1. Bioactive type: it is not modified by human enzymes.

2. Substrate type: it is degraded by human enzymes decreasing o losing completely their biological activity.

3. Pro-bioactive type: it is hydrolyzed by human enzymes but the resulting peptides increase the bioactive capacity.

Obviously, the bioactive and pro-bioactive types would be the most interesting for producing functional food.

As mentioned before, peptides should resist three stages before reaching the target organ: gastrointestinal digestion, intestinal absorption and blood transport. Gastrointestinal digestion is a key stage for determining the bioavailability of the peptides. During the process enzymes such as pepsin, trypsin, α-chymotrypsin, elastase, carboxypeptidase A or carboxypeptidase B could degrade the active peptide. For evaluating the effect of these digestive enzymes over peptides, a simulated digestion process is usually employed. The method consists in two consecutive digestion stages that reproduce the stomach digestion and the intestinal digestion.

During the stomach digestion the active peptide is subjected to hydrolysis with pepsin at 37 ºC and pH 2. The rest of digestion parameters vary between authors, but reaction time is usually fixed at 1 hour and enzyme substrate ratio at 1-4 (%w/w). Subsequently, the pH is adjusted to 5.3 with a NaHCO3 solution and further to pH 7.5 with NaOH. Then, the intestinal digestion is initiated by adding pancreatine, a mixture of intestinal enzymes. The

enzyme substrate ratio employed is usually around 2-4%. The intestinal digestion is maintained at 37ºC for at least 2 hours. Finally, the reaction is stopped by thermal deactivation of the enzyme and the digested samples are analyzed to check if the original bioactivity has been altered.

Most of the studies dealing with the effect of gastrointestinal activity over bioactive peptides employ fish muscle or fish byproducts hydrolysates. Wiriyaphan et al. (2015) confirmed that the fraction of 1-5 kDa from surimi byproduct hydrolysates retained the antioxidant activity after simulated digestion. Pepsin digestion did not degrade the peptides present in the studied fraction, while pancreatin released a large number of small peptides which increased the bioactive potential 23.7%. Similar results were found by Huang et al. (2012) when evaluating the resistance of three bioactive peptides from tuna cooking juice. They found that, after the simulated digestion process, the peptides maintained or even improve the activity.

However, only a few published works evaluated the resistance to gastrointestinal digestion of bioactive hydrolysates from discard species. Chai et al. (2013) hydrolyzed lanternfish, a small deep-sea fish which is often discarded right after catch due to their low economic value. The hydrolysates produced by Protease-N shown high antioxidant properties which remained constant or improved after *in vitro* digestion. Moreover, the increase in the lower molecular size fraction (>700 Da) originated after digestion did not reduce the bioactive activity of hydrolysate. The authors identified two small active peptides (fraction < 700 Da) which would resist the simulated digestion. Other study (Pérez-Gálvez et al. In Press) employed horse mackerel (*Trachurus mediterraneus*) as source of bioactive peptides. Horse mackerel is a target species which is discarded due to minimum landing size requirements, fishing quota or high grading practices. In this work, the hydrolysis with mixtures of subtilisin and trypsin was optimized for maximizing the ACE inhibitory activity of the hydrolysates. The most potent hydrolysates were subjected to *in vitro* gastrointestinal digestion in order to evaluate changes in bioactivity. As in studies dealing with antioxidant activity, ACE inhibitory activity remained constant after gastrointestinal digestion. However, differently to other studies, the fraction of molecular size ranged between 357 - 172 Da was not modified by digestion. Therefore, it may be concluded that the bioactivity would be mainly determined by this fraction, which contains mostly di- and tripeptides. According to the initial classification these peptides would be peptides of bioactive type.

3.4. ACE Inhibitory activity of FPH from discarded species

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

One of the most common methods to reduce blood pressure is the inhibition of the Angiotensin converting enzyme, ACE, (García-Moreno et al., 2016). ACE (EC 3.4.15.1) is a chloride- dependent zinc metallopeptidase which is present in numerous parts of mammals organisms such as brain, lungs, liver, kidneys, testicles and others, predominantly as a membrane-bound ectoenzyme in vascular endothelial cells (Murray & FitzGerald, 2007). It plays a crucial role on the regulation of blood pressure since, in the renin-angiotensin system, ACE transforms the inactive decapeptide angiotensin I (DRVYIHPFHL) into the potent vasoconstrictor octapeptide, angiotensin II (DRVYIHPF). Furthermore, Angiotensin II favors the secretion of aldosterone which activates the reabsorption of Na+ and the retention of water by the renal tubes, increasing the blood pressure. In addition, in the kallikrein-kinin system, ACE catalyzes the degradation of bradykinin, a vasodilator nonapeptide (Li et al., 2004). Fig. 2 shows in a simplified way the mechanisms of action of ACE.

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

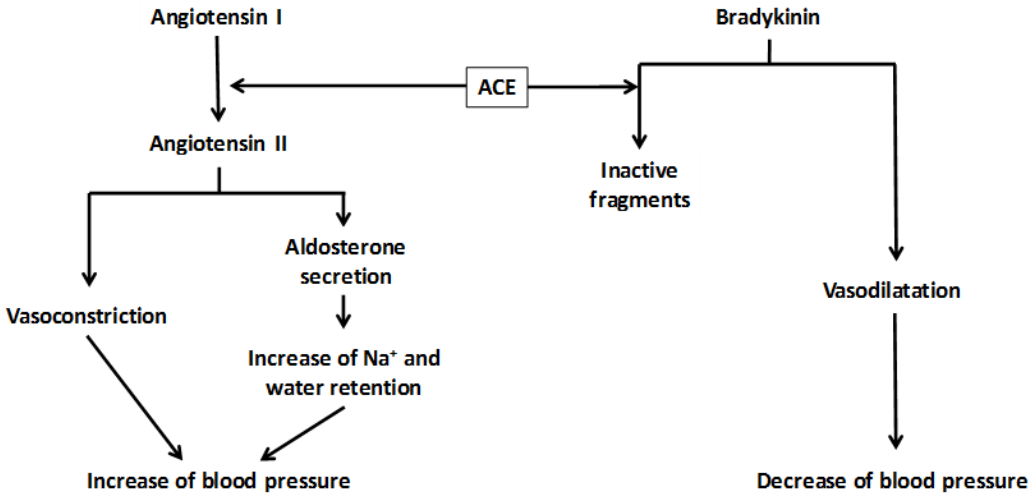


Figure. 2. Mechanisms of action of ACE. Adapted from Li et al. (2004)

In this regard, fish protein hydrolysates have shown strong ACE-Inhibitory activity (Martínez-Maqueda et al., 2012). The ACE-inhibitory peptides are normally constituted by 2-12 amino acids and they normally contain hydrophobic residues (*e.g.* F, W, Y, P) in the C-terminal tripeptide sequence. This enhances the interaction of the peptide with the three hydrophobic zones placed at the active site of the enzyme. In this line, ACE shows weak or no affinity for hydrophilic inhibitors such as those which present dicarboxylic amino acids (*e.g.* E) at the C-terminal. Moreover, the presence of charge amino acids such as K and R at the C-terminal also contribute to the ACE-inhibitory potency. Other structure-activity studies suggest that the inhibitory activity of the peptides with C-terminal proline residue is improved with hydrophobic adjacent amino acids (Li et al., 2004). Therefore, the amino acids composition and sequence of the ACE-inhibitory peptides is highly influenced by: a) properties of the matrix hydrolysed (*e.g.* species, muscle, skin, etc.), b) specificity of the protease employed, c) hydrolysis conditions (temperature, pH and enzyme/substrate ratio), and d) extent of the hydrolysis reaction.

Several studies have been reported on the ACE-inhibitory activity of fish protein hydrolysates produced from fish discards species in the Alboran Sea. For instance, García-Moreno et al. (2015) hydrolyzed defatted and dewatered protein cakes of sardine (Sardina pilchardus), horse mackerel (*Trachurus mediterraneus*), bogue (*Boops boops*), axillary seabream (*Pagellus acarne*) and small-spotted catshark (*Scyliorhinus canicula*) by using combinations of subtilisin and trypsin. The authors reported that the hydrolysates of small-spotted catshark and horse mackerel hydrolysates exhibited the highest ACE-inhibitory activity with IC_{50} values ranging from 279 to 398 µg protein/mL. They were followed by axillary seabream and sardine hydrolysates with IC_{50} values varying from 375 to 489 µg protein/mL, whereas bogue hydrolysates presented the lowest ACE-inhibitory activity with IC_{50} values in the range of 637-768 µg protein/mL. The authors concluded superior ACE-inhibitory activity exhibited by the hydrolysates of small-spotted catshark may be due to its high collagen content, which is rich in proline. Small peptides containing proline at the C-terminal have been reported to play a significant role in the inhibition of ACE (Byun & Kim, 2001). In the case of horse mackerel hydrolysates, their higher values of DH obtained, which imply the presence of a higher number of small peptides, may be the reason for their strong antihypertensive activity. The authors carried out further fractionation of the hydrolysates by using SEC. For horse mackerel hydrolysate, a fraction with peptides between 130 and 2350 Da exhibited the highest ACE-inhibitory activity,

with an IC_{50} value of 85 µg/mL (a 3.3-fold increase of activity compared with that of the hydrolysate). In the case of small-spotted catshark, fractions with peptides 470-1210 Da and 58-470 Da showed the lowest IC_{50} values, 72 and 27 µg/mL respectively. For small-spotted catshark hydrolysate a purified peptide, VAMPF, was identified as one promising ACE-inhibitor due to its tripeptide C-terminal sequence and its low IC_{50} value predicted by the QSAR model (IC_{50} = 0.44 µM).

As another example, García-Moreno et al. (2016) investigated the ACE-inhibitory effect of blue whiting (*Micromesistius poutassou*) hydrolysates. The authors reported that the employment of only subtilisin, which favours the cleavage at the C-terminal of hydrophobic residues, was preferred for the production of FPH with ACE-inhibitory activity when compared to the use of only trypsin or combination of trypsin and subtilisin. Moreover, these authors concluded that blue whiting hydrolysate with high DH (12%), presenting a higher proportion of short peptides than hydrolysates with DH 4 and 8%, was required to better inhibit ACE (IC_{50} =172 µg/mL).

3.5. Antioxidant activity of FPH from discarded species

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

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

Among the existing natural sources, fish proteins have been widely evaluated as raw material for the production of fish protein hydrolysates with antioxidant activity (Samaranayaka & Li-Chan, 2011). The antioxidant activity of a hydrolysate depends on the molecular size of the peptides as well as on their amino acids composition and sequence. In this regard, antioxidant peptides normally contain between 2 and 20 amino acids, with hydrophobic residues such as valine, glycine and proline which enhance the scavenging of lipid soluble radicals (Harnedy & FitzGerald, 2012). Other amino acids such as tyrosine, tryptophan, methionine, lysine, cysteine and hystidine are commonly present in the sequence of antioxidant peptides due to their capacity to donate protons and electrons which stabilize free radicals (He et al., 2013). The release of antioxidant peptides is also highly dependent on the properties of the raw material hydrolyzed, the type of enzyme employed and the reaction conditions, determining the amino acids composition and sequence of the bioactive peptides.

A study from our research group evaluated the antioxidant properties of protein hydrolysates obtained from discarded fish species in the Alboran Sea, namely sardine, horse mackerel, axillary seabream, bogue and small-spotted catshark (García-Moreno et al., 2014b). The authors reported that the highest 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was exhibited by the hydrolysates of sardine (IC_{50} = 0.91 mg protein/mL) and horse mackerel (IC_{50} = 1.47 mg protein/mL), both obtained by the sequential treatment trypsin (2 h) and subtilisin (2 h). The highest chelating activity was found for the hydrolysates of sardine and for the hydrolysates of small spotted catshark obtained by the sequential treatments, exhibiting IC_{50} values of 0.32 mg protein/mL. This binding metal capacity may be related to the presence of peptides containing histidine, which has been reported to exert metal chelating activity through its imidazole ring (Bougatef et al., 2009). The authors also observed that sardine and bogue hydrolysates exhibited the highest reducing power with absorbance higher than 0.7 at a concentration of 20 mg protein/mL, whereas small-spotted catshark hydrolysates presented the lowest. These results obtained in this work were similar to those reported for other fish species such as black scabbardfish (Batista et al., 2010) and hake (Pires et al., 2013).

Another recent study investigated the antioxidant activity of blue whiting hydrolysates (García-Moreno et al., 2016). The authors reported that independently of the enzymatic treatment (only subtilisin, only trypsin or combination of subtilisin and trypsin), FPH with DH 4% presented higher DPPH scavenging activity (IC_{50} = 1-2 mg protein/mL) when

compared to hydrolysates of DH 8 and 12% (IC_{50} = 2-3 mg protein/mL). The authors concluded that blue whiting hydrolysates produced with only subtilisin exhibited the highest antioxidant activity. This is because subtilisin favours the cleavage at the C-terminal of hydrophobic residues (*e.g.* V, G and P) which contribute to the DPPH scavenging activity of the peptides.

3.6. Antiocholesterolemic activity of FPH from discarded species

Cholesterol is a lipid which is essential for cell membrane formation as well as being a precursor in the formation of bile acids, vitamin D and steroid hormones. Despite its physiological function, the accumulation of cholesterol in arteries is strongly associated to the development of several cardiovascular diseases. Cholesterol is segregated as bile acids from the liver, and reaches the intestine at a rate of 800 – 1200 mg per day, where it joins the dietary cholesterol (200 – 500 mg per day). The latter is deesterified by cholesterol esterases, being absorbed through intestinal wall under the form of mixed micelles with plant sterols. As for bile acids, they are reabsorbed by 95% through the ileum section and recycled back to the liver. As a result, it is between 30 – 60% of total cholesterol entering the intestine (*i.e.* dietary cholesterol plus bile acids) is absorbed and incorporated into the bloodstream (Charlton-Menys & Durrington, 2008). The study of the mechanisms of intestinal absorption is of importance in the formulation of drugs to lower the blood cholesterol levels. Indeed, the potency to lower cholesterol of a given compound depends on its ability to inhibit micellar solubilization of dietary cholesterol or to bind to bile acids, impeding their ileum re-absorption.

The sequestration of bile acids reduces their recycle to the liver, which therefore removes the excess of cholesterol in bloodstream to keep bile acid synthesis. Most of the bile acid binding agents identified so far are fiber compounds from vegetal sources (Kahlon & Smith, 2007; Nagarajaiah & Prakash, 2015; Dziedzic et al., 2016; Gannasin, Adzahan, Mustafa, & Muhammad, 2016; Hemati Matin, Shariatmadari, Karimi Torshizi, & Chiba, 2016).

Bile acid sequestrators from animal sources have been investigated to a lesser extent. For instance, Zhou et al. (2006) tested the binding capacity against cholic, deoxycholic and chenodeoxycholic acids of chitosan extracted from shrimp cells. As for the protein compounds, early studies from Lanzini et al. (1987), performed on ileal resection patients, demonstrated the *in vivo* bile acid ability of caseins. Nagaoka et al. (2001) identified

hypocholesterolemic peptides from casein and β-lactoglobulin hydrolysates produced with trypsin. More recently, Nagaoka's collaborators evaluated the cholesterol lowering effect of a protein derived from royal jelly (Kashima et al., 2014).

Few studies have reported to date the hypocholesterolemic potency of proteins of marine origin. Wergedahl et al. (2004) studied the *in vivo* effect of fish protein hydrolates from Atlantic salmon (Salmo salar) on lowering the cholesterol levels of obese Zucher rats. Lin et al. (2010) tested the *in vitro* capacity of freshwater clam (*Curvicual sp.*) to inhibit micellar cholesterol absorption and to sequestrate bile acids. Similarly, Hosomi (2011) studied the hipocholesterolemic efficacy of fish protein hydrolysates from Alaska pollock (*Gadus chalcogrammus*). This author concluded that the hydrolysates studied presented lower micellar solubilization and higher capacity to sequestrate bile acids, compared to the levels commonly reported for casein hydrolysates.

Pérez-Gálvez et al. (2015) studied the binding capacity against cholic and chenodeoxycholic acids of fish protein hydrolysates (FPH) from six Mediterranean species: sardine (*Sardina pilchardus*), horse mackerel (*Trachurus mediterraneus*), axillary seabream (*Pagellus acarne*), bogue (*Boops boops*), small-spotted catshark (*Scyliorhinus canicula*) and blue whiting (*Micromesistius poutassou*). These species are traditionally discarded in the West Mediterranean fisheries due to their low commercial value (*i.e.* bogue, axillary seabream, small-spotted catchark) or former minimum landing size regulations (*i.e.* sardine, horse mackerel and blue whiting). Overall, they account for more than 90% of the finfish discarded in this area (García-Moreno et al., 2013). The protein hydrolysates were produced by three reaction patterns, where subtilisin (purchased as Alcalase 2.4. L, Novozymes) and trypsin (purchased as PTN, Novozymes) were employed sequentially (*i.e.* subtilisin and then trypsin, and conversely) or simultaneously during 4 hours of reaction. The study concluded that the bile acid binding capacity was not affected by the enzymatic treatment, while there were significant differences among fish species. The average levels of bile acids bound by the FPH are shown in Fig. 3. It can be observed that whiting hydrolysates exhibited high ability to bind cholic and chenodeoxycholic acids, regardless the enzymatic treatment (2.85 μmol and 2.64 μmol per 100 mg of protein, respectively). Sardine hydrolysates presented similar levels of binding capacity against cholic acid, while their ability to sequestrate chenodeoxycholic acid was 30% to 50% lower. The other species presented moderate levels of bile acid sequestration, except for horse mackerel hydrolysates produced by the sequential treatment trypsin plus subtilisin,

which bound on average 3.35 μmol of cholic acid per 100 mg of protein. The levels of bile acid capacity reported in this work are similar to those observed in plant proteins such as soy, wheat gluten or beans (Kahlon & Woodruff, 2002).

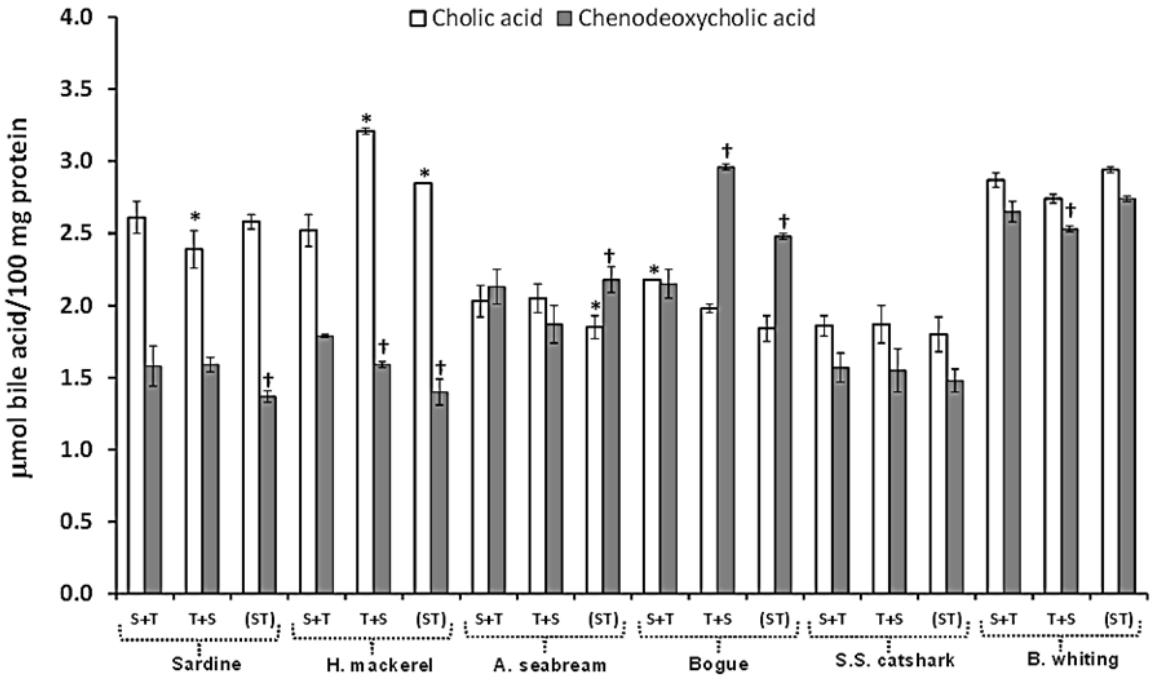


Figure 3. Average levels of bile acids bound by the fish protein hydrolysates studied by Pérez-Gálvez et al. (2015). Enzymatic treatments are noted as: S+T (subtilisin then trypsin); T+S (trypsin then subtilisin) and ST (simultaneous addition of subtilisin and trypsin). Significant differences ($P < 0.05$) within the same species are noted by the symbols * (cholic acid) and † (chenodeoxycholic acid).

4. APPLICATION OF FISH PROTEIN HYDROLYSATES IN THE FOOD INDUSTRY

This section is focused, primary, on the technological properties of FPH (*i.e.* solubility and certain capacities as emulsifying, foaming properties, water holding or oil binding). Also, it reviews the possible application of FPH as stabilizers agents in the production of emulsions and microcapsules by spry-drying. Consequently, a more detailed description of solubility and interfacial properties will be conducted. A detailed revision of technological properties of recent studies has been written by Halim et al., (2016).

Fish hydrolysates (FPH) have proved to present valuable bioactive and technological properties which have been recently described by several authors (Klomklao et al., 2013; Liu et al., 2014; Taheri et al., 2014; Halim et al., 2016). Technological properties are defined as "the physicochemical properties of proteins in the food systems during

processing, storage and consumption" (Halim et al., 2016). FPH have much better solubility than non-hydrolyzed fish protein, indeed FPH are solubilized over a wide range of pH and ionic strengths. Additinally, FPH can be employed for the formulation of foam-based products since the have emulsifying and foaming properties, (Nalinanon et al., 2011). Also, FPH improve the water holding, emulsification properties and texture when incorporated to food (Taheri et al., 2013). Furthermore, due to their oil binding capacity, factor which influence the final product taste, FPH can be employed in the meat and confectionery industries (Pires and Batista, 2013).

4.1. Technological properties of FPH from discarded species

The technological properties of the FPH are highly determined by the enzymatic treatment, as it has been also described for the bioactive properties (Chalamaiah et al., 2012). Effectively, the enzymatic treatment defines the length of the chain and the residues which are in terminal positions; factors which are, to some extent, responsible of the technological properties that FPH present. A limited reduction of the size of the peptides of the protein chain length is critical to improve the technological characteristics of FPH (Chalamaiah et al., 2012). Animal and vegetal protein hydrolysates with lower DH are employed as food texture enhancers whereas hydrolysates with higher DH are used as protein supplements. Indeed, the incorporation of FPH into different systems (*i.e.* cereal products, fish and meat products or desserts) has been successfully tested (Chalamaiah et al., 2012). Finally, one of the main drawbacks for the production of food grade FPH is the formation of bitter and unpalatable tastes (Aspevik et al., 2016). Pires et al., (2013) reviewed several studies which have been conducted in order to prevent and decrease the production of bitter peptides.

4.1.1. Solubility

This property is one of the most important since it affects other technological properties as emulsifying, foaming, thickening and gelling (He et al., 2013). It has been widely reported that the enzymatic hydrolysis considerably improves the solubility of fish proteins within the whole range of pH, being this improvement proportional to the degree of hydrolysis (DH) (Hmidet et al., 2011; García-Moreno et al., 2016a). As an example, raw protein from cuttlefish at pH 7, presented a solubility of 12% while hydrolysates with a DH 18.8 showed a 96% of solubility (Hmidet et al., 2011). Similar results were observed for

defatted skipjack (*Katsuwonous pelamis*) roe hydrolysates produced with Alcalase 2.4 L, in this case hydrolysates whose DH varied from 5 to 50% presented increasing solubility (82 to 99%, respectively) (Intarasirisawat et al., 2012). Hydrolysates with higher DH present a small peptide length chain whose polar and/or hydrophobic residues are better expose to the medium. Also, polar residues are able to create hydrogen bonds with water molecules improving the solubility (Halim et al., 2016).

The influence of the protease selected for hydrolysis has been also studied; subtilisin and flavourzyme were employed for the production of yellow stripe trevally hydrolysates (*Selaroides leptolepis*). Those hydrolysates produced with subtilisin presented slightly lower solubility in the isoelectronic point (Klompong et al., 2007). However, for the blue whiting (*Micromesistius poutassou*) hydrolysates, no significant differences between samples produced with subtilisin and trypsin were described (García-Moreno et al., 2016a). Effectively, the tandem enzyme-substrate has to be studied and optimized as a whole due to the different composition that each fish protein has.

Additionally, pH exerts a strong influence on solubility. Most of FPH present the lowest solubility between pH 4 and 5, corresponding to the isoelectronic point (dos Santos et al., 2011; Hmidet et al., 2011; Sampath Kumar et al., 2011; Halim et al., 2016), where the peptides are neutral and the aggregation of protein is favored by hydrophobic interactions (Hmidet et al., 2011). At pH far from the isoelectric one, there is an increase of the net charge of the peptides and consequently solubility is enhanced (Taheri et al., 2013).

4.1.2. Emulsifying and foaming capacity

FPH can reduce the interfacial and superficial tension (Romero et al., 2011; Taheri et al., 2011; Morales-Medina et al., 2015). The reduction of the interfacial tension proves that protein can act as emulsifiers. On the other hand, dispersed protein can low the surface tension at the water-air interface and, therefore, they can be employed as foaming compounds (Hmidet et al., 2011).

The molecular weight, amino acid sequence and solubility of FPH have been described as the most important intrinsic factors which affect emulsifying or foaming properties (dos Santos et al., 2011; Taheri et al., 2013; Jemil et al., 2014). Indeed, FPH should contain a relatively high amount of peptides with, at least, 20 residues to present good emulsifying properties (Elavarasan et al., 2014) and at least a size of 1 kDa (between 8 and 10 residues) to show appropriate foaming capacity (Nalinanon et al., 2011). The

aforementioned characteristics are mainly determined by the extent of the enzymatic treatment as well as the type of enzyme employed (Elavarasan et al., 2014; García-Moreno et al., 2016a). The influence of the type of protease and the degree of hydrolysis have been studied for a wide variety of species. For instance, in the case of surimi processing by-products, an increase of the DH from 10 to 30 resulted into a considerable decrease of both, foaming and emulsifying properties (Liu et al., 2014), contrary to the solubility behavior. An increase of the DH leads to the presence of smaller peptides which are less effective stabilizing foam or emulsions (Hmidet et al., 2011). Similarly, for hydrolysates of blue whiting (DH 4 to 12%) higher interfacial properties were observed for smaller DH (García-Moreno et al., 2016a). In that work, those hydrolysates produced with trypsin presented slightly higher emulsifying and foaming capacity.

Apart from the own characteristics of the hydrolysate, environmental pH plays a crucial role in the foaming and emulsifying properties (Taheri et al., 2013; Halim et al., 2016). The protein surface hydrophobicity and the protective layer surrounding lipid globules are highly affected by pH (Taheri et al., 2013). As happened to the solubility, interfacial properties also suffer a dramatic decrease at pH values between 4 and 5 close to the isoelectric point (Taheri et al., 2013; Halim et al., 2016).

As previously stated, the emulsifying and foaming properties are governed by the interfacial and superficial tension. However, there are few studies in which both tensions are determined for FPH. Crayfish proteins showed at pH 8 better interfacial activity and solubility than at pH 2. Also larger aggregation was observed at acidic pH. Effectively, the interfacial tension (1 wt% protein solution) at pH 8 was slightly lower than at pH 2 (8,8 and 9,3 mN/m, respectively) (Romero et al. 2011). Contrary, acidic pH favored the interfacial properties in the case of hydrolysates solution of sardine (*S. pilchardus*) and horse mackerel (*T. mediterraneus*) (Morales-Medina et al., 2015). The increase of interfacial activity was related to a higher level of solubility at acidic pH and to an increase of ionic strength with influences the ability of the protein to be adsorbed at the interface. Furthermore, Morales-Medina et al. (2015) compared the tension of hydrolysates (DH 5%) produced with trypsin and subtilisin, being the type of protease only significant in the case of sardine hydrolysates. This influence was explained taking into account the selectivity of the proteases; subtilisin is a less specific one with tendency to cleave hydrophobic residues while trypsin cleaves exclusively Lys and Arg residues.

4.1.3. Fat binding and water holding capacity

Fat biding capacity expresses the amount of oil which can be directly bonded by the protein. Consequently, higher protein bulk density corresponds to greater fat absorption. This capacity influences the final product taste, factor which is especially important in the meat and confectionery industries (Pires & Batista, 2013; Taheri et al., 2013). A negative correlation between fat binding capacity and DH has been described for blue whiting hydrolysates produced with subtilisin (García-Moreno et al. 2016a).

Water holding capacity has importance to the food industry since it affects the sensory attributes of the product (Pires & Batista, 2013). An increase of terminal carboxyl and amino groups can result into a higher amount of absorbed water and strength of the sorption bond which results into an increase of hygroscopicity. Effectively, hydrolysates of rainbow trout presented better water holding capacity as the DH increased (Taheri et al., 2013). Nevertheless, extensive hydrolysis may decrease the FPH water holding capacity because hydrolytic degradation decrease the capacity of protein to bind water (Taheri et al., 2013). However, depending on the substrate and enzymatic treatment different behaviors have been described. For instance, in the case of hydrolysates of blue whiting (DH 8 and 12%) produced with trypsin and subtilisin no significant differences were observed when water holding capacity was compared (García-Moreno et al. 2016a).

4.2. Application of FPH as chemical and physical stabilizers in emulsions and microcapsules

Proteins are widely used in the food industry as a stabilizer against phase separation in several food products (*i.e.* dairy products, baked food or mayonnaise). Egg yolk is the most used emulsifier; however due to consumers' interest in healthier food emulsions new sources of protein are required (Ruiz-Márquez et al., 2013). Hence, several authors have presented alternative proteins as whey protein hydrolysates (Tamm et al., 2015), pea hydrolysates (Tamm et al., 2016) or FPH (Morales-Medina et al., 2015; García-Moreno et al., 2016b).

FPH have been employed as chemical and physical stabilizers for the production of emulsions and microcapsules of fish oil (with high content of Omega-3). This kind of oil presents several benefits to the health but it is easily oxidized (Morales-Medina et al., 2013). Consequently, antioxidants are incorporated to the oil to enlarge their shelf life. In

systems as emulsions or microcapsules the oil oxidation begins at the interface; hence there are an increasing interest in finding new emulsifier which presents also antioxidant activities (Oehlke et al., 2011). In this context, FPH has proved to be a feasible alternative emulsifier for the production of emulsions and microcapsules.

Fish oil emulsions has been physically and chemically stabilized with FPH from sardine (*Sardina pilchardus*) and small-spotted catshark (*Scyliorhinus canicula*) employing DH from 3 to 6 % (García-Moreno et al., 2016b). Sardine hydrolysates with lower DH produced emulsions with lower droplet size, which implies that those hydrolysates had the most effective peptides to physically stabilize emulsions. Also, this emulsion presented higher oxidative stability since more protein was adsorbed at the interface (García-Moreno et al., 2016b). All hydrolysates of sardine were able to produce stables emulsion while in the case of small spotted catshark only hydrolysates with DH 3 yielded to a physical stable emulsion. As explained previously, there is a strong influence of DH and substrate on the emulsifier properties of the FPH. Presumably, the hydrolysates were too small to produce stable emulsions. A similar behavior was observed in the production of emulsions with hydrolysates of horse mackerel and sardine with DH 5 and 10 (Morales-Medina et al., 2015). Only hydrolysates with DH 5 were able to physically stabilize the emulsion. In that work, the influence of the pH was also tested, and emulsions were prepared at pH 2, 3 and 8. FPH were able to physically stabilize emulsions at acidic pH while emulsion at basic conditions were all unstable (Morales-Medina et al., 2015).

With respect to the microencapsulation of fish oil, FPH of sardine and horse mackerel were produced with subtilisin and trypsin. These hydrolysates stabilized physically and chemically the fish oil during the whole process of microencapsulation and storage (>80 days) (Morales-Medina et al., 2015). Minor differences in the physical properties of the microcapsules and on the oxidative state of the fish oil were observed among all the hydrolysates tested.

5. REFERENCES

Adler-Nissen, J., (1986). Enzymic Hydrolysis of Food Proteins. Elsevier Science Ltd, London.

Afonso, M. D., & Bórquez, R. (2002). Review of the treatment of seafood processing wastewaters and recovery of proteins therein by membrane separation processes — prospects of the ultrafiltration of wastewaters from the fish meal industry. *Desalination*, 142(1), 29–45.

Análisis anual de la producción pesquera andaluza. (2009). Sevilla (Spain): Consejería de Agricultura y Pesca.

Antelo, L. T., Ordóñez-del Pazo, T., Lopes, C., Franco-Uría, A., Pérez-Martín, R. I., & Alonso, A. A. (2016). Pollutant levels in discarded fish species by Spanish trawlers operating in the Great Sole Bank and the Atlantic coast of the Iberian Peninsula, 108(1-2), 303–310.

Arvanitoyannis, I. S., & Kassaveti, A. (2008). Fish industry waste: treatments, environmental impacts, current and potential uses. *International Journal of Food Science & Technology*, 43(4), 726–745.

Aspevik, T.; Totland, C.; Lea, P.; Oterhals, Å.(2016) Sensory and Surface-Active Properties of Protein Hydrolysates Based on Atlantic Salmon (*Salmo Salar*) by-Products. *Process Biochemistry*, 51 8, 1006–1014.

Batista, I., Ramos, C., Coutinho, J., Bandarra, N. M., & Nunes, M. L. (2010). Characterization of protein hydrolysates and lipids obtained from black scabbardfish (aphanopus carbo) by-products and antioxidative activity of the hydrolysates produced. *Process Biochemistry*, 45(1), 18-24.

Bicknell, A. W. J., Oro, D., Camphuysen, K. C. J., & Votier, S. C. (2013). Potential consequences of discard reform for seabird communities. *Journal of Applied Ecology*, 50(3), 649-658.

Bimbo, A. P. (2012). The Seafood Industry. (L. A. Granata, G. J. Flick, & R. E. Martin, Eds.) The Seafood Industry: Species, Products, Processing, and Safety: Second Edition. Oxford, UK: Wiley-Blackwell.

Bougatef, A. Nedjar-Arroume, N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D., Nasri, M. (2010) Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. *Food Chemistry*, 118 , pp. 559–565

Bozzano, A., & Sardà, F. (2002). Fishery discard consumption rate and scavenging activity in the northwestern Mediterranean Sea. *ICES Journal of Marine Science: Journal Du Conseil*, 59(1), 15–28.

Butterfield, D. A., Castegna, A., Pocernich, C. B., Drake, J., Scapagnini, G., & Calabrese, V. (2002). Nutritional approaches to combat oxidative stress in alzheimer's disease. *Journal of Nutritional Biochemistry*, 13(8), 444-461.

Byun, H., & Kim, S. (2001). Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from alaska pollack (*Theragra chalcogramma*) skin. *Process Biochemistry*, 36(12), 1155-1162.

Chai, H.-J., Chan, Y.-L., Li, T.-L., Shiau, C.-Y., Wu, C.-J. (2013). Evaluation of lanternfish (Benthosema pterotum) hydrolysates as antioxidants against hydrogen peroxide induced oxidative injury. *Food Research International*, 54, 1409-1418

Chalamaiah, M.; Dinesh Kumar, B.; Hemalatha, R.; Jyothirmayi, T. (2012) Fish Protein Hydrolysates: Proximate Composition, Amino Acid Composition, Antioxidant Activities and Applications: A Review. *Food chemistry*, 135 4, 3020–3038.

Charlton-Menys, V., & Durrington, P. N. (2008). Human cholesterol metabolism and therapeutic molecules. *Experimental Physiology*, 93(1), 27-42.

Cheung, R. C. F., Ng, T. B., & Wong, J. H. (2015). Marine Peptides: Bioactivities and Applications. *Marine Drugs*, 13(7), 4006–4043.

Chi, C.F., Wang, B., Hu, F.Y., Wang, Y.M., Zhang, B., Deng, S.G., Wu, C.W. (2015) Purification and identification of three novel antioxidant peptides from protein hydrolysate of bluefin leatherjacket (Navodon septentrionalis) skin. *Food Research International*, 73 , pp. 124–139

dos Santos, S. D. A.; Martins, V. G.; Salas-Mellado, M.; Prentice, C.(2011) Evaluation of Functional Properties in Protein Hydrolysates from Bluewing Searobin (*Prionotus Punctatus*)

Obtained with Different Microbial Enzymes. *Food and Bioprocess Technology*, 4 8, 1399–1406.

Elavarasan, K.; Naveen Kumar, V.; Shamasundar, B. A.(2014) Antioxidant and Functional Properties of Fish Protein Hydrolysates from Fresh Water Carp (*Catla Catla*) as Influenced by the Nature of Enzyme. *Journal of Food Processing and Preservation*, 38 3, 1207–1214.

EU Commission. EU communication COM (2002) 656. Community Action Plan to reduce discards of fish.

EU Commission. EU communication COM (2007) 136. A policy to reduce unwanted by-catches and eliminate discards in European fisheries.

EU. Regulation (EU) no 1380/(2013) of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy.

FAO. (1995). Code of conduct for responsible fisheries. FAO Fisheries and Aquaculture Department, Rome.

FAO. (2014). The state of world fisheries and aquaculture. Food and Agriculture Oraganization of the United Nations (Vol. 2014).

Fujita, H., Eiichiyokoyama, K., & Yoshikawa, M. (2000). Classification and Antihypertensive Activity of Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Food Proteins. *Journal of Food Science*, 65(4), 564–569.

García-Moreno, P.J., Pérez-Gálvez, R., Morales-Medina, R., Guadix, A., Guadix, E. M. (2013). Discarded species in the west Mediterranean Sea as sources of Omega-3 PUFA. *European Journal of Lipid Science and Technology*, 115(9), 982–989.

García-Moreno, P. J., Morales-Medina, R., Pérez-Gálvez, R., Bandarra, N. M., Guadix, A., & Guadix, E. M. (2014a). Optimisation of oil extraction from sardine (*Sardina pilchardus*) by hydraulic pressing. International *Journal of Food Science & Technology* 49(10), 2167–2175.

García-Moreno, P. J., Batista, I., Pires, C., Bandarra, N. M., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2014b). Antioxidant activity of protein hydrolysates obtained from discarded mediterranean fish species. *Food Research International*, 65(PC), 469-476.

García-Moreno, P. J.; Guadix, A.; Guadix, E. M.; Jacobsen, C. (2016b) Physical and Oxidative Stability of Fish Oil-in-Water Emulsions Stabilized with Fish Protein Hydrolysates. *Food chemistry*, 203, 124–135.

García-Moreno, P.J., Espejo-Carpio, F.J., Guadix, A., Guadix, E.M., (2015). Production and identification of Angiotensin I-Converting Enzyme (ACE) inhibitory peptides from Mediterranean fish discards. *Journal of Functional Foods* 18, Part A, 95–105.

García-Moreno, P.J., Pérez-Gálvez, R., Espejo-Carpio, F.J., Ruiz-Quesada, C., Pérez-Morilla, A.I., Martínez-Agustín, O., Guadix, A., Guadix, E.M. (2016) Functional, bioactive and antigenicity properties of blue whiting protein hydrolysates: effect of enzymatic treatment and degree of hydrolysis. *Journal of the Science of Food and Agriculture* (In press)

Ghiasinejad, H., & Abduli, S. (2007). Technical and economical selection of optimum transfer-transport method in solid waste management in metropolitan cities. *International Journal of Environmental Research*, 1(2), 179–187.

Graham, N., Ferro, R. S. T., Karp, W. A., & MacMullen, P. (2007). Fishing practice, gear design, and the ecosystem approach - Three case studies demonstrating the effect of management strategy on gear selectivity and discards (Vol. 64, pp. 744–750).

Groenewold, S., Damalas, D., Maravelias, C. D., Osio, G. C., Maynou, F., Sbrana, M., Casey, J. (2000). Effects on benthic scavengers of discards and damaged benthos produced by the beam-trawl fishery in the southern North Sea. *ICES Journal of Marine Science*, 57(5), 1395–1406.

Guo, L., Hou, H., Li, B., Zhang, Z., Wang, S. & Zhao, X. (2013) Preparation, isolation and identification of iron-chelating peptides derived from Alaska pollock skin. *Process Biochemistry* 48, 988–993.

Halim, N. R. A.; Yusof, H. M.; Sarbon, N. M. Functional and Bioactive Properties of Fish Protein Hydolysates and Peptides: A Comprehensive Review. *Trends in Food Science & Technology* 2016, 51, 24–33.

Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods*, 4(1), 6-24.

He, S., Franco, C., & Zhang, W. (2013). Functions, applications and production of protein hydrolysates from fish processing co-products (FPCP). *Food Research International*, 50(1), 289-297.

Hmidet, N.; Balti, R.; Nasri, R.; Sila, A.; Bougatef, A.; Nasri, M. (2011) Improvement of Functional Properties and Antioxidant Activities of Cuttlefish (*Sepia Officinalis*) Muscle Proteins Hydrolyzed by Bacillus Mojavensis A21 Proteases. *Food Research International*, 44 9, 2703–2711.

Hosomi, R., Fukunaga, K., Arai, H., Kanda, S., Nishiyama, T., & Yoshida, M. (2011). Fish protein decreases serum cholesterol in rats by inhibition of cholesterol and bile acid absorption. *Journal of Food Science* 76(4), H116-H121.

Hsu, K.C. (2010) Purification of antioxidative peptides prepared from enzymatic hydrolysates of tuna dark muscle by-product. *Food Chemistry* 122, 42–48.

Huang, S.-L., Jao, C.-L., Ho, K.-P., Hsu, K.-C. (2012) Dipeptidyl-peptidase IV inhibitory activity of peptides derived from tuna cooking juice hydrolysates. *Peptides* 35, 114-121.

Intarasirisawat, R.; Benjakul, S.; Visessanguan, W.; Wu, J. (2012) Antioxidative and Functional Properties of Protein Hydrolysate from Defatted Skipjack (*Katsuwonous Pelamis*) Roe. *Food chemistry*, 135 4, 3039–3048.

Ito, N., Fukushima, S., & Tsuda, H. (1985). Carcinogenicity and modification of the carcinogenic response by BHA, BHT, and other antioxidants. *Critical Reviews in Toxicology*, 15(2), 109-150.

Jahangiri, R., Soltani, S., & Barzegar, A. (2014). A Review of QSAR Studies to Predict Activity of ACE Peptide Inhibitors. *Pharmaceutical Sciences* 20(3), 122-129.

Jemil, I.; Jridi, M.; Nasri, R.; Ktari, N.; Ben Slama-Ben Salem, R.; Mehiri, M.; Hajji, M.; Nasri, M.(2014) Functional, Antioxidant and Antibacterial Properties of Protein Hydrolysates Prepared from Fish Meat Fermented by Bacillus Subtilis A26. *Process Biochemistry*, 49 6, 963–972.

Jensen, A. L., Reider, R. H., & Kovalak, W. P. (1988). Estimation of Production Forgone. North American Journal of Fisheries Management 8(2), 191–198.

Kahlon, T. S., & Smith, G. E. (2007). *In vitro* binding of bile acids by blueberries (*Vaccinium spp*.), plums (*Prunus spp.*), prunes (*Prunus spp.*), strawberries (*Fragaria X ananassa*), cherries (*Malpighia punicifolia*), cranberries (*Vaccinium macrocarpon*) and apples (*Malus sylvestris*). *Food Chemistry* 100(3), 1182–1187.

Kahlon, T. S., & Woodruff, C. L. (2002). *In vitro* binding of bile acids by soy protein, pinto beans, black beans and wheat gluten. *Food Chemistry* 79(4), 425–429.

Kashima, Y., Kanematsu, S., Asai, S., Kusada, M., Watanabe, S., Kawashima, T., Nakamura, T., Goto, T., Nagaoka, S. (2014). Identification of a novel hypocholesterolemic protein, major royal jelly protein 1, derived from royal jelly. *PLoS ONE* 9(8), e105073.

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K., & He, J. (2005). Global burden of hypertension: Analysis of worldwide data. *Lancet*, 365(9455), 217-223.

Kelleher, K. (2005). Discards in the world's marine fisheries. An update. Rome: FAO Fisheries and Aquaculture Department, Rome.

Kent, J. A. (Ed.). (2010). Kent and Riegel's Handbook of Industrial Chemistry and Biotechnology (11th ed.). New York: Springer Science & Business Media.

Klomklao, S.; Benjakul, S.; Kishimura, H.(2013) Functional Properties and Antioxidative Activity of Protein Hydrolysates from Toothed Ponyfish Muscle Treated with Viscera Extract from Hybrid Catfish. *International Journal of Food Science & Technology* , 48 7, 1483–1489.

Klompong, V.; Benjakul, S.; Kantachote, D.; Shahidi, F. (2007) Antioxidative Activity and Functional Properties of Protein Hydrolysate of Yellow Stripe Trevally (*Selaroides Leptolepis*) as Influenced by the Degree of Hydrolysis and Enzyme Type. *Food Chemistry*, 102 4, 1317–1327.

Lanzini, A., Fitzpatrick, W. J. F., Pigozzi, M. G., & Northfield, T. C. (1987). Bile acid binding to dietary casein: A study *in vitro* and *in vivo*. *Clinical Science*, 73(4), 343–350.

Laroque, D., Chabeaud, A., and Guérard, F., (2008). Antioxidant capacity of marine protein hydrolysates, in: Bergé, J.P. (Ed.), Added Value to Fisheries Waste. Transworld Research Network, Kerala, pp. 147-161.

Li, G., Le, G., Shi, Y., & Shrestha, S. (2004). Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research*, 24(7), 469-486.

Li, Y.-W., Li, B. (2013). Characterization of structure-antioxidant activity relationship of peptides in free radical systems using QSAR models: Key sequence positions and their amino acid properties. *Journal of Theoretical Biology*, 318, 29-43.

Lin, Y.-H., Tsai, J.-S., Hung, L.-B., & Pan, B. S. (2010). Hypocholesterolemic effect of compounded freshwater clam protein hydrolysate and Gracilaria. *Food Chemistry*, 123(2), 395–399.

Liu, R., Zheng, W., Li, J., Wang, L., Wu, H., Wang, X., & Shi, L. (2015). Rapid identification of bioactive peptides with antioxidant activity from the enzymatic hydrolysate of *Mactra veneriformis* by UHPLC–Q-TOF mass spectrometry. *Food Chemistry*, 167, 484–489.

Liu, Y.; Li, X.; Chen, Z.; Yu, J.; Wang, F.; Wang, J. (2014) Characterization of Structural and Functional Properties of Fish Protein Hydrolysates from Surimi Processing by-Products. *Food chemistry*, 151, 459–465.

Ma, B. & Johnson, R. (2012) De novo sequencing and homology Searching. *Molecular & Cellular Proteomics* 11, O111.014902.

Martínez-Maqueda, D., Miralles, B., Recio, I., & Hernández-Ledesma, B. (2012). Antihypertensive peptides from food proteins: A review. *Food and Function*, 3(4), 350-361.

Mcilwain, K. (2015). The EU Discard Reduction Manual. Environmental Defense Fund.

Morales-Medina, R., García-Moreno, P.J., Pérez-Gálvez, R., Muñío, M.M., Guadix, A., Guadix, E.M. (2016). Nutritional indexes, fatty acids profile, and regiodistribution of oil extracted from four discarded species of the Alboran Sea: Seasonal effects. *European Journal of Lipid Science and Technology*, .In Press.

Morales-Medina, R., Munio, M, Pérez-Gálvez, R., Guadix, A. and Guadix. E. (2013) Lipids from Marine Sources. In Utilization of fish waste; Pérez-Gálvez, R., Bergé, J.P., Eds.; CRC Press: Boca Raton.

Morales-Medina, R.; Tamm, F.; Guadix, A. M.; Guadix, E. M.; Drusch, S. (2015) Functional and Antioxidant Properties of Hydrolysates of Sardine (*S. Pilchardus*) and Horse Mackerel (*T. Mediterraneus*) for the Microencapsulation of Fish Oil by Spray-Drying. *Food Chemistry*, 194, 1208–1216.

Murray, B. A., & FitzGerald, R. J. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production. *Current Pharmaceutical Design*, 13(8), 773-791.

Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., Tadashi, K., Kuwata, T. (2001). Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin. *Biochemical and Biophysical Research Communications* 281(1), 11–17.

Nalinanon, S. Benjakul, S., Kishimura, H., Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry* 124, 1354–1362.

Nasri, R., Chataigné, G., Bougatef, A., Chaâbouni, M.K., Dhulster, P. Nasri, M., Nedjar-Arroume, N. (2013) Novel angiotensin I-converting enzyme inhibitory peptides from enzymatic hydrolysates of goby (*Zosterisessor ophiocephalus*) muscle proteins. *Journal of Proteomics* 91, 444–452.

Nikolic, N., Diméet, J., Fifas, S., Salaün, M., Ravard, D., Fauconnet, L., & Rochet, M. J. (2015). Efficacy of selective devices in reducing discards in the Nephrops trawl fishery in the Bay of Biscay. *ICES Journal of Marine Science* 72(6), 1869–1881.

Nunes M.L., Bandarra N.M. and I. Batista (2010). Health benefits associated with seafood consumption. In Alasalvar C., Miyashita K., Shahidi F., Wanasundara U. (Eds.) Handbook of seafood quality, safety and health applications. Chapter 42 pp 518.

Oehlke, K.; Heins, A.; Stöckmann, H.; Sönnichsen, F.; Schwarz, K.(2011) New Insights into the Antioxidant Activity of Trolox in O/W Emulsions. *Food Chemistry*, 124 3, 781–787.

Oterhals, Å., & Kvamme, B. (2013). Optimization of an oil leaching process to reduce the level of dioxins and dioxin-like PCBs in fishmeal. *Journal of the Science of Food and Agriculture* 93(7), 1649–1659.

Pérez-Gálvez, R., Chopin, C., Mastail, M., Ragon, J. Y., Guadix, A., & Bergé, J. P. (2009). Optimisation of liquor yield during the hydraulic pressing of sardine (*Sardina pilchardus*) discards. *Journal of Food Engineering*, 93(1), 66–71.

Pérez-Gálvez, R., García-Moreno, P. J., Morales-Medina, R., Guadix, A., & Guadix, E. M. (2015). Bile acid binding capacity of fish protein hydrolysates from discard species of the West Mediterranean Sea, *Food and Function*, 6(4), 1261–1267.

Pérez-Gálvez, R., García-Moreno, P.J., Huong, N.T.-M., Guadix, E.M., Guadix, A. & Bergé, J.-P. (2016). Multiobjective optimization of a pilot plant to process fish discards and by-products on board. *Clean Technologies and Environmental Policy*, 18 (3), 935-948.

Pérez-Gálvez, R., Guadix, A., Almécija, M. C., Guadix, E. M., & Bergé, J.-P. (2011). Response Surface Modeling of the Multiphase Juice Composition from the Compaction of Sardine Discards. *Food and Bioprocess Technology*, 5(6), 2172–2182.

Pérez-Gálvez, R., Morales-Medina R., Espejo-Carpio, F., Guadix A. & Guadix E.M. (2016). Modeling of the production of ACE inhibitory hydrolysates of horse mackerel (*Trachurus mediterraneus*) using protease mixtures. Food and Function, In press.

Pires, C., Clemente, T., & Batista, I. (2013). Functional and antioxidative properties of protein hydrolysates from cape hake by-products prepared by three different methodologies. *Journal of the Science of Food and Agriculture*, 93(4), 771-780.

Pires, C.; Batista, I. (2013) Functional Properties of Fish Protein Hydrolysates. In Utilization of fish waste; Pérez-Gálvez, R., Bergé, J. P., Eds.; CRC Press - Taylor & Francis Group: Boca Raton, Florida.

Pires, C.; Clemente, T.; Batista, I. (2013) Functional and Antioxidative Properties of Protein Hydrolysates from Cape Hake by-Products Prepared by Three Different Methodologies. *Journal of the science of food and agriculture*, 93 4, 771–780.

Poos, J. J., Aarts, G., Vandemaele, S., Willems, W., Bolle, L. J., & van Helmond, A. T. M. (2013). Estimating spatial and temporal variability of juvenile North Sea plaice from opportunistic data. *Journal of Sea Research*, 75, 118–128.

Romero, A.; Beaumal, V.; David-Briand, E.; Cordobés, F.; Anton, M.; Guerrero, A. (2011) Interfacial and Emulsifying Behaviour of Crayfish Protein Isolate. *LWT - Food Science and Technology*, 44 7, 1603–1610.

Ruiz-Márquez, D.; Partal, P.; Franco, J.; Gallegos, C. (2013) Linear Viscoelastic Behaviour of Oil-in-Water Food Emulsions Stabilised by Tuna-Protein Isolates. *Food science and technology international*, 19 1, 3–10.

Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of Functional Foods*, 3(4), 229-254.

Sampath Kumar, N. S.; Nazeer, R. A.; Jai Ganesh, R.(2011) Functional Properties of Protein Hydrolysates from Different Body Parts of Horse Mackerel (*Magalaspis Cordyla*) and Croaker (*Otolithes Ruber*). *Mediterranean Journal of Nutrition and Metabolism*, 5 2, 105–110.

Sila, A., & Bougatef, A. (2016). Antioxidant peptides from marine by-products: Isolation, identification and application in food systems. A review. *Journal of Functional Foods*, 21, 10–26.

Taheri, A.; Abedian Kenari, A.; Motamedzadegan, A.; Habibi Rezaie, M. (2011) Optimization of Goldstripe Sardine (Sardinella Gibbosa) Protein Hydrolysate Using Alcalase® 2.4L by Response Surface Methodology *Journal of Food*, 9 2, 114–120.

Taheri, A.; Anvar, S. A. A.; Ahari, H.; Fogliano, V.(2013)Comparison the Functional Properties of Protein Hydrolysates from Poultry Byproducts and Rainbow Trout (*Onchorhynchus Mykiss*) *Viscera*., 12 1, 154–169.

Taheri, A.; Sabeena Farvin, K. H.; Jacobsen, C.; Baron, C. P.(2014) Antioxidant Activities and Functional Properties of Protein and Peptide Fractions Isolated from Salted Herring Brine. *Food chemistry*, 142, 318–326.

Tamm, F.; Gies, K.; Diekmann, S.; Serfert, Y.; Strunskus, T.; Brodkorb, A.; Drusch, S.(2015) Whey Protein Hydrolysates Reduce Autoxidation in Microencapsulated Long Chain Polyunsaturated Fatty Acids. *European Journal of Lipid Science and Technology*, In press,

Tamm, F.; Herbst, S.; Brodkorb, A.; Drusch, S. (2016) Functional Properties of Pea Protein Hydrolysates in Emulsions and Spray-Dried Microcapsules. *Food Hydrocolloids*, 58, 204–214.

Torres, J.A., Chen, Y.C., Rodrigo-García, J. & Jaczynski, J. (2006). Recovery of by-products from seafood processing streams. In F. Shahidi (Ed.), Maximising the value of marine by-products, (1st ed., pp. 65-90). Woodhead Publishing Limited, Cambridge.

Tsagarakis, K., Vassilopoulou, V., Kallianiotis, A., & Machias, A. (2012). Discards of the purse seine fishery targeting small pelagic fish in the eastern Mediterranean Sea. *Scientia Marina*, 76(3), 561–572.

Vermeirssen, V., Camp, J. V., & Verstraete, W. (2004). Bioavailability of Angiotensin I Converting Enzyme Inhibitory Peptides. *British Journal of Nutrition*, 92(3), 357–366.

Wergedahl, H., Liaset, B., Gudbrandsen, O. A., Lied, E., Espe, M., Muna, Z., Mørk, S., Berge, R. K. (2004). Fish protein hydrolysate reduces plasma total cholesterol, increases the proportion of HDL cholesterol, and lowers acyl-CoA:cholesterol acyltransferase activity in liver of Zucker rats. *The Journal of Nutrition*, 134(6), 1320–1327.

Wijesekara, I., & Kim, S. (2010). Angiotensin-I-converting enzyme (ACE) inhibitors from marine resources: Prospects in the pharmaceutical industry. *Marine Drugs*, 8(4), 1080-1093.

Wiriyaphan, C., Xiao, H., Decker, E.A., Yongsawatdigul, J. (2015) Chemical and cellular antioxidative properties of threadfin bream (*Nemipterus spp.*) surimi byproduct hydrolysates fractionated by ultrafiltration. *Food Chemistry*, 167, 7-15.

Wu, S., Feng, X., Lan, X., Xu, Y. & Liao, D. (2015) Purification and identification of angiotensin-I converting enzyme (ACE) inhibitory peptide from lizard fish (*Saurida elongata*) hydrolysate. *Journal of Functional Foods*, 13, 295–299

Zafari, A., & Kianmehr, M. H. (2013). Application of densification process in organic waste management. Waste Management & Research : *The Journal of the International Solid Wastes and Public Cleansing Association*, ISWA, 31(7), 684–91.

Zhou, K., Xia, W., Zhang, C., & (Lucy) Yu, L. (2006). *In vitro* binding of bile acids and triglycerides by selected chitosan preparations and their physico-chemical properties. *LWT - Food Science and Technology*, 39(10), 1087–1092.

Zhuang, Y.L. & Sun L.P. (2011) Preparation of reactive oxygen scavenging peptides from tilapia (*Oreochromis niloticus*) skin gelatin: Optimization using response surface methodology. *Journal of Food Science*, 76 C483–C489.

Zou, P., Wang, J.-L., He, G.-Q. & Wu, J. (2014). Purification, identification, and *in vivo* activity of angiotensin I-converting enzyme inhibitory peptide, from ribbonfish (*Trichiurus haumela*) backbone. *Journal of Food Science*, 79, C1-C7

VIII. Artificial Neuronal Networks Modelling of the Enzymatic Hydrolysis of Horse Mackerel Protein Using Protease Mixtures [*]

In enzymatic reactions, the modeling of the degree of hydrolysis (DH) is desirable given its influence on many functional and biological activities. Empirical approaches are preferred to phenomenological ones to model the complexity of enzymatic reactions. Artificial Neuronal Networks (ANN) are able to process large sets of data where no linear relationships between them are expected. In this work, a feedforward ANN, comprising 10 neurons in the hidden layer, was successfully employed to model the DH as a function of the initial concentration of horse mackerel protein (tested at 2.5 g/L, 5 g/L and 7.5 g/L), the reaction temperature (40ºC, 47.5ºC and 55ºC), the time of reaction (up to 4 h) and the percentage of subtilisin in the enzyme mixture (0%, 25%, 50%, 75% and 100%). The resulting ANN model was optimized by an evolutionary algorithm, obtaining a maximum (DH 17.1%) at 2.54 g/L, 40ºC, 4 h and an enzyme mixture comprising 38.3% of subtilisin and the rest of trypsin. The combination of trypsin and subtilisin led to higher DH than the sole use of subtilisin (DH 15.5% at 52ºC). Furthermore, the former optimum was attained at lower reaction temperature, which reduces both the operational costs and the nutritional losses.

[*] JOURNAL PAPER: R. Morales-Medina, R. Pérez-Gálvez, A. Guadix and E. M. Guadix. Artificial Neuronal Networks Modelling of the Enzymatic Hydrolysis of Horse Mackerel Protein Using Protease Mixtures. *Biochemical Engineering Journal* 105:364-370. http://dx.doi.org/10.1016/j.bej.2015.10.009

1. INTRODUCTION

Extractive fishing generates an important amount of discards (*i.e.* fraction of the catch returned to the sea), which comprise small-sized individuals and non-target species with low commercial value. New EU Fisheries Common Policy avoids discarding for most of the pelagic fisheries (*e.g.* herring, sardine, horse mackerel) since the 1st of January of 2015. This ban will be extended to the rest of species in the following two years (EU, 2013). In application of new EU regulations, all the finfish and crustaceans caught must be landed, generating a supplementary amount of biomass. This calls for the search of new solutions to handle these underutilized materials, involving the commercial promotion of non-target species, improvements in the fishing gears or up-grading strategies to convert these biomaterials into valuable products (FAO, 2010).

In this context, the processing of the fish protein fraction from fish discards arises as a promising alternative. Effectively, previous studies have demonstrated that fish protein hydrolysates (FPH) do not only present improved technological properties but they also exhibit a number of biological activities (Harnedy & FitzGerald, 2012; He, Franco, & Zhang, 2013; Kristinsson & Rasco, 2000). A key parameter explaining most of these properties is the degree of hydrolysis (*i.e.* the percentage of peptide bonds cleaved during hydrolysis). Extensive hydrolysis results in a product rich in free amino acids and low-molecular-weight peptides (*i.e.* below < 4kDa). Kristinsson & Rasco, (2000) and Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, (2012) pointed out that, as a general trend, low molecular weight hydrolysates present enhanced properties such as solubility, antioxidant or antihypertensive activities.

The hydrolysis of fish substrates is a heterogeneous reaction involving a number of simultaneous physical (*e.g.* substrate solubility, enzyme deactivation) and chemical phenomena (*i.e.* cleavage of peptide bonds). The solubility of the substrate increases the availability of peptide bonds for enzyme attack. However, it may slow down the reaction kinetics due to substrate inhibition (Qian, Zhang, & Liao, 2011). As for the reaction temperature, it increases the substrate solubility while its effect on the enzyme activity may be detrimental due to thermal deactivation (Li, Jiang, Zhang, Mu, & Liu, 2008; Valencia, Pinto, & Almonacid, 2014). Deterministic models, based on Michaelis-Menten theory, present a wide scope of applicability (Cavaille & Combes, 1995; Qian et al., 2011;

Valencia et al., 2014; Zhou, Chen, & Li, 2003). However, the complexity of the model increased with the number of phenomena considered. Therefore, empirical approaches such as response surface methodology (RSM) or artificial neural networks (ANN) are preferred for these purposes (Abakarov, Teixeira, Simpson, Pinto, & Almonacid, 2011; Baş & Boyacı, n.d., 2007). RSM are commonly used for the modelling and optimization of protein hydrolysis (Valencia, Espinoza, Ceballos, Pinto, & Almonacid, 2015). The advantage of this technique is the small number of experimental data required (Keskin Gündoğdu et al., 2014). As main drawback, not all the biochemical processes are adequately modeled by the polynomial functions (*e.g.* quadratic or cubic) usually employed in RSM (Baş & Boyacı, 2007).

ANN arise as an alternative with high predicting capabilities, especially when involving a large volume of data (Baş & Boyacı, 2007.; G. Zhang, Eddy Patuwo, & Y. Hu, 1998; Y. Zhang, Xu, & Yuan, 2009). These are empirical models inspired in human brain, where the neurons are arranged in layers and interconnected by mathematical functions. Each neuron receives a weighted signal from the previous layer, which is processed by mathematical functions (*e.g.* hyperbolic, sigmoidal). The strength of this approach lies on its ability to learn from provided examples by training algorithms. These algorithms iteratively update the model parameters (*i.e.* weights and bias) until minimizing the error between the actual value of the output variable and the experimental one. In the field of enzymatic hydrolysis, this technique has been successfully employed to model reactions catalyzed by single enzymes (Pérez-Gálvez, Espejo-Carpio, Guadix, & Guadix, 2015; Rivera, Rabelo, dos Reis Garcia, Filho, & da Costa, 2010). So far, ANN modeling has not been considered in literature to predict optimal composition of an enzyme mixture.

The aim of this study was to model and optimize an artificial neural network to predict the time evolution of DH as a function of the initial concentration of substrate, the temperature and the composition of a binary mixture of proteases (trypsin and subtilisin).

2. MATERIALS AND METHODS

2.1. Characterization and pretreatment of the substrate

Horse mackerel (*Trachurus mediterraneus*), was chosen for this study since it presents a high discard rate in the west Mediterranean Sea. The raw material was provided by the fishing harbor of Motril (Spain).

The substrate of the enzymatic hydrolysis was a partially dewatered cake of horse mackerel, obtained by hydraulic pressing. To this end, 3 kg of fresh fish were preheated at 40ºC for 30 min in a water bath and then were fed into a hydraulic press (model ESP-K, Sanahuja, Spain). The pressing procedure comprised three cycles where the fish was pressed at 120 bar, intersected by relaxation periods where the press liquor was allowed to release from the press chamber. After completing three cycles (pressing plus relaxation), the press cake was recovered, partially deprived of water and oil. It was conserved under refrigeration before being fed to the hydrolysis reactor.

The proximate composition of the press cake was determined according to the official methods recognized by the Association of the Official Analytical Chemists (AOAC). The moisture and ash content were estimated gravimetrically by heating the samples until attaining constant weight at 103ºC and 550ºC, respectively (A.O.A.C., 2006). Total Nitrogen content was determined by the Kjeldahl method and reported to crude protein employing a conversion factor of 6.25 (Adler Nissen, 1986).

2.2. Enzymes and hydrolysis procedure

The press cakes were hydrolyzed employing two serine endoproteases, one of bacterial origin (subtilisin, EC 3.4.21.62) and other from an animal source (trypsin, EC 3.4.21.4), both purchased from Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S, respectively. Each sample of press cake was grinded and then homogenized with demineralized water until completing 200 mL. This suspension was then fed into a jacketed reactor of volume capacity 250 mL attached to a water bath. An enzyme mixture comprising subtilisin and trypsin was employed as catalyst. The enzyme-substrate ratio was kept at 1 wt%, larger enzyme/protein ratios will shorten the time required to reach this steady value. Nevertheless, from an industrial point of view, the main operation cost is related to the amount of enzyme, which is normally set below 5% referred to the substrate. Additionaly,

the enzyme composition was varied according to the experimental design explained below. After completing 4 hours, the hydrolysis was stopped by heating the reaction solution at 100ºC for 15 min.

The degree of hydrolysis was monitored throughout the reaction by means of an automatic titrator (718 Stat Titrino, Metrohm, Switzerland) which employed NaOH 0.5 N as titration agent. According to the pH-stat method, the volume of base consumed to keep the pH constant in the course of the reaction can be related to the degree of hydrolysis by Eq. 1:

$$DH = \frac{V_b \cdot N_b}{m_P} \cdot \frac{1}{\alpha} \cdot \frac{1}{h_{tot}} \cdot 100 \quad [1]$$

where DH stands for the degree of hydrolysis, defined as the percentage of peptide bonds cleaved; V_b (mL) is the amount of base consumed; N_b (eq/L) is the normality of the base; m_P (g) is the mass of protein in the substrate and h_{tot} (meq /g) is the number of milliequivalents of peptide bonds per gram of protein. The value of htot was assumed to be 8.6 milliequivalents of peptide bonds per gram of protein, as commonly accepted for fish materials (Adler Nissen, 1986). The average degree of dissociation of the α-NH2 amino groups (α) was calculated employing Eq. 2

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \quad [2]$$

where pH was fixed at 8 and pK is expressed as a function of the temperature as follows (Adler Nissen, 1986).

$$pK = 7.8 + \frac{298 - T}{298 \cdot T} \cdot 2400 \quad [3]$$

2.3. Experimental design

A crossed mixture-process design was proposed in this work, involving the percentage of subtilisin in the enzyme mixture, the concentration of protein in the reaction volume, the temperature and the reaction time as input variables. The percentage of subtilisin (X_1) in the enzyme preparation (comprising subtilisin and trypsin) is a mixture variable which was varied from 0% (*i.e.* pure trypsin) to 100% (*i.e.* pure subtilisin) at five experimental levels: 0%, 25%, 50%, 75% and 100%. The initial substrate concentration, the temperature and

the reaction time were chosen as process variables. The initial substrate concentration in the reactor was assayed at three levels: 2.5 g/L, 5 g/L and 7.5 g/L. Higher values could prevent an adequate homogenization and stirring of the reaction mixture. In contrast, substrate concentrations below 2.5 g/L entail higher energy consumption for the stabilization of the final hydrolysate (*e.g.* by spray or freeze drying) prior to their incorporation into a food matrix. The thermal stability of the two enzymes was considered when selecting the levels of reaction temperature: 40ºC, 47.5ºC and 55ºC. Indeed, trypsin presents maximum activity at 40ºC, while subtilisin does at 55ºC.

By combination of these levels, a total number of 5 x 3 x 3 = 45 experimental runs were conducted. For each hydrolysis reaction, the pH-stat allowed the determination of DH every minute, for a total reaction time of 4 h.

2.4. ANN model of the degree of hydrolysis

A feedforward ANN was built by means of Matlab 7.0 Neural Network Toolbox, comprising a single hidden layer with a variable number of neurons (1 – 10). The neuronal network was fed with four input variables (X_i): concentration of protein (X_1), temperature (X_2), percentage of subtilisin in the protease mixture (X_3) and time of hydrolysis (X_4). Therefore, the input layer comprised 4 neurons, where each of them generated a single weighted function (s_k) by linear combination of weight factors (w_{ki}) and a bias (b_k):

$$s_k = \sum_{i=1}^{4} w_{ki} \cdot X_i + b_k \qquad [4]$$

These signals were processed by each neuron in the hidden layer by means of the logsig function Φ(s) as follows:

$$\Phi(s_k) = \frac{1}{1+e^{-s_k}} \qquad [5]$$

This function processes a response within the interval [0,1]. The output layer, containing one neuron, receives a combined response from the hidden layer. This response is finally processed by means of a new transfer function. To this regard, three transfer functions were assayed: purelin, poslin, tansig. The output of this layer is the calculated value of the degree of hydrolysis for the vector of input variables assayed.

The input data were divided into three subsets where the first one, containing 70% of the experimental data, was employed to train the neural network. Training consists in finding the vector of weights and bias which fit the output responses to the input variables. The Levenberg-Marquardt algorithm (lm), implemented in the software Matlab 7.0, was chosen to update iteratively the values of weights and bias so the mean squared error between actual and predicted output variables is minimum. For a given number of neurons in the hidden layer (1-10), 30 trainings were performed to ensure an appropriate data population. For each single training, the number of iterations (*i.e.* epoch) was limited to 10000.

The validation subset comprised 15% of the experimental data. Along the iteration process, the errors from this subset were monitored and employed as criterion for early stopping. By this approach, the training process stopped after recording an increase in the error of validation subset for 10 iterations. This avoids overfitting, which is the decrease in predictive performance caused by excessive training of the neural network. The remaining 15% of experimental data were processed independently to compute the test error which is commonly employed to estimate the predictability of the ANN model, since it is independent of the training process.

2.5. Evolutionary algorithm

An evolutionary algorithm is a stochastic search method that mimics the natural biological evolution (Elbeltagi, Hegazy, & Grierson, 2005). In this case, it was employed to obtain the experimental conditions leading to the maximum DH. To this purpose, an initial population of solutions including 100 individuals was randomly generated. At each iteration, the algorithm created the next generation by the procedures of elite (1%), crossover (91%) and mutation (8%). The algorithm was set to stop after 60 s of computing time. This algorithm was employed as implemented in the Solver tool of the Microsoft Excel software.

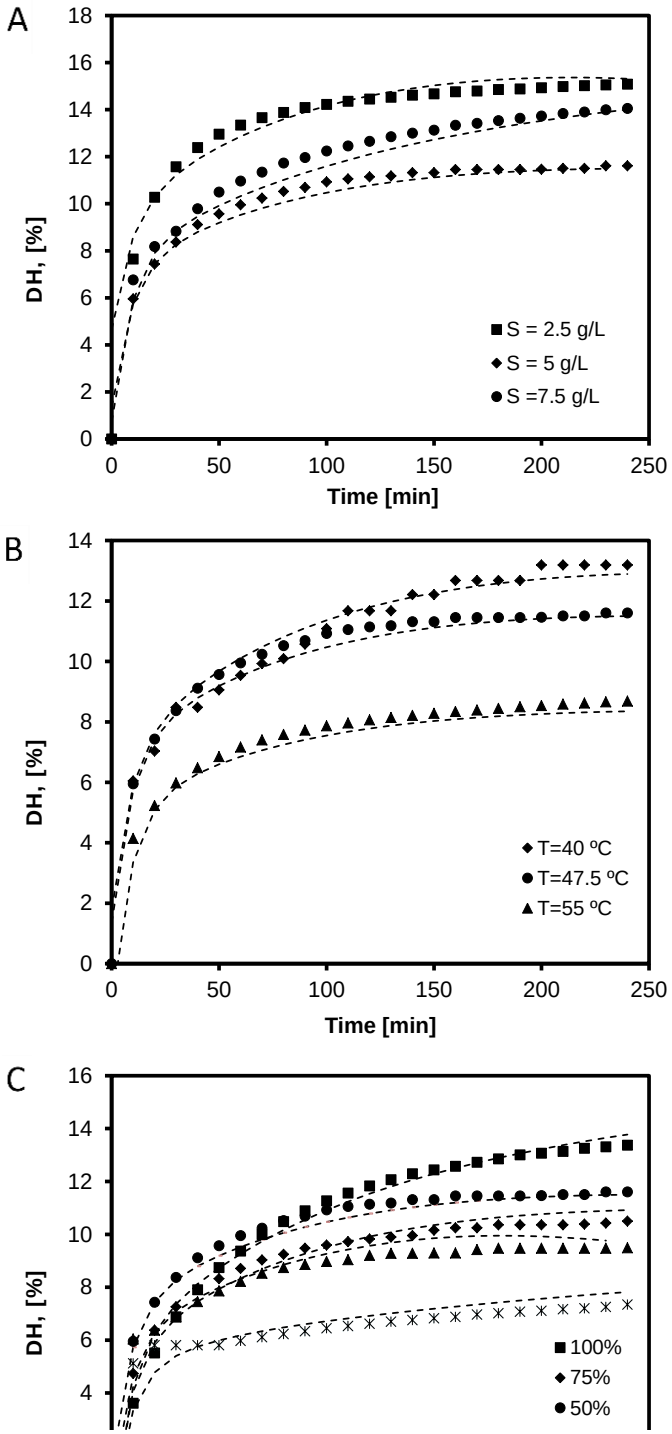


Figure 1. Empirical (symbols) and predicted (dash lines) hydrolysis curves for horse mackerel protein with mixtures of subtilisin and trypsin. (a) Influence of the initial substrate concentration (g/L) at 47.5ºC and an enzyme mixture ratio 1:1. (b) Influence of the temperature at an initial substrate concentration of 5g/L and an enzyme mixture ratio 1:1. (c) Influence of the composition of the enzyme mixture at 5g/L and 47.5ºC.

Table 1. Experimental design and measured values for the experimental final DH.

Nº Exp	S_0, g/L	T, ºC	% Alcalase	DH_f
1	2.5	40	100	13.24
2	2.5	40	75	13.40
3	2.5	40	50	16.48
4	2.5	40	25	15.91
5	2.5	40	0	13.40
6	5	40	100	12.74
7	5	40	75	14.12
8	5	40	50	13.19
9	5	40	25	12.97
10	5	40	0	10.70
11	7.5	40	100	13.04
12	7.5	40	75	12.17
13	7.5	40	50	13.71
14	7.5	40	25	13.15
15	7.5	40	0	8.97
16	2.5	47.5	100	14.15
17	2.5	47.5	75	14.81
18	2.5	47.5	50	15.09
19	2.5	47.5	25	14.32
20	2.5	47.5	0	11.16
21	5	47.5	100	13.37
22	5	47.5	75	10.50
23	5	47.5	50	11.61

Table 1. (Continuation) Experimental design and measured values for the experimental final DH.

Nº Exp	S_0, g/L	T, ºC	% Alcalase	DH_f
24	5	47.5	25	9.49
25	5	47.5	0	7.34
26	7.5	47.5	100	13.01
27	7.5	47.5	75	10.85
28	7.5	47.5	50	14.05
29	7.5	47.5	25	12.74
30	7.5	47.5	0	9.17
31	2.5	55	100	12.24
32	2.5	55	75	11.59
33	2.5	55	50	12.12
34	2.5	55	25	12.24
35	2.5	55	0	8.90
36	5	55	100	14.73
37	5	55	75	9.70
38	5	55	50	8.69
39	5	55	25	10.96
40	5	55	0	7.39
41	7.5	55	100	13.89
42	7.5	55	75	13.37
43	7.5	55	50	12.29
44	7.5	55	25	13.98
45	7.5	55	0	10.14

3. RESULTS AND DISCUSSION

3.1. Experimental data

In Fig. 1, some example plots are presented showing the influence of the experimental factors on the hydrolysis curves. It can be observed that DH varied with the operation conditions of substrate initial concentration (X_1, g/L), temperature (X_2, ºC) and percentage of subtilisin in the enzyme mixture (X_3, %). The DH against the substrate concentration at 47.5ºC and a mixture 1:1 of enzymes is plotted in Fig. 1a. It can be observed that the minimum values of DH were attained at the central level of concentration (5 g/L), while the highest results were obtained at 2.5 g/L, followed by 7.5 g/L. This trend differs from that described for the hydrolysis of fish protein with 1398 neutrase (Qian et al., 2011), where the substrate inhibition was the controlling pathway. Nevertheless, in the same work, a similar behavior was observed for salmon muscle hydrolyzed by Alcalase. This trend was justified by a protective effect of the substrate against the thermal inactivation of the enzymes, overcoming the influence of substrate inhibition (Valencia et al., 2014).

The influence of the reaction temperature on the hydrolysis curves is depicted in Fig. 1b, where the initial substrate concentration and the percentage of subtilisin in the enzyme mixture were fixed at 5 g/L and 50%, respectively. It can be seen that the increase of reaction temperature plays a detrimental effect on the final DH. This behavior is a trade-off between the solubility of the substrate protein and the intervals of stability of the enzymes employed. Effectively, higher temperatures favor the solubility of the substrate, and hence, the availability of peptide bonds. By contrast, subtilisin is stable at high temperatures (maximum activity at 50ºC) while trypsin presents an optimal activity at 37ºC (Najafian & Babji, 2012).

As shown in Fig. 1c, the influence of the composition of the mixture of enzymes does not follow a clear tendency. Indeed, for concentrations of 5 g/L and 47.5ºC, the maximum value of final DH was obtained when employing pure subtilisin, followed by mixtures employing 50%, 75%, 25% and 0%. In Table 1 the final experimental DH are listed for each initial substrate concentration, temperature and composition of the enzyme mixture. Globally, the final values of experimental DH ranged from 7.3% (5g/L; 47.5ºC and 0 %) to 16.5% (2.5g/L; 40ºC and 50%). Among them, experiments 3, 4 and 18 (Table 1),

employing mixtures of enzymes, presented the highest DH. These data suggest a synergic effect between both proteases. In fact, trypsin cleaves exclusively lysine and arginine residues (Olsen, Ong, & Mann, 2004) while subtilisin cleaves a wide range of peptides, preferably aromatic acid and methionine residues (Adamson & Reynolds, 1996). Hence, their combination might increase the availability of potentially cleavage peptide bonds. Additionally, trypsin presents a chymotrypsin-like behavior for temperatures ranging 40 to 45ºC. This means that it can cleave new peptides bonds presenting tyrosine, tryptophan and phenylalanine residues at the carboxyl side (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010).

3.2. Artificial neural network model of the degree of hydrolysis

The selection of the transfer function (purelin, tansig or poslin) for the output layer was conducted by minimizing the mean square error between the actual and the predicted DH. The highest absolute errors were found at the beginning of the reaction (±2%), as described in the porcine blood hydrolysis (Pérez-Gálvez et al., 2015), being some of the predicted DH negative. To overcome this limitation, tansig (a tangent sigmoidal transfer function) and poslin (a positive linear transfer function) were assayed. However, these functions were not able to improve the correlation achieved by purelin. The asymptotic behavior shown by tansig and poslin led to initial DH greater than 7%. Therefore, it can be concluded that purelin transfer function was the most appropriate for modeling the kinetic hydrolysis.

An increase of the number of neurons in the hidden layer decreased the test MSE while improved the coefficient of determination r^2 (Fig. 2). The rate of improvement (*i.e.* slope of MSE against number of neurons) was high up to 2 neurons and then continued at a lower rate until 10 neurons where the MSE was minimum (0.005±0.002) and r^2 maximum (0.978±0.007). Due to this asymptotic behavior, employing a higher number of neurons may imply slight improvement at the expense of increasing significantly the processing time.

The frequency distribution of absolute errors (difference between experimental and theoretical DH) for 1, 5 and 10 neurons was depicted in Fig. 3. It can be observed that an increase in the number of neurons resulted in a narrower distribution, centered at zero. For 1 neuron, (Fig. 3a) the absolute error distribution was mainly flat, with average frequencies around 1000. A noticeable improvement was achieved for 5 neurons (Fig. 3b), where most

of the errors fell within the interval [-1,1]. In the case of 10 neurons, the absolute errors followed a Normal distribution, with 9,167 data out of 10845 (85%) presenting an error between -0.5 and 0.5. The proposed model was able to predict the time evolution of the DH, with more than 10,000 data, with high coefficient of determination ($r^2 = 0.987$).

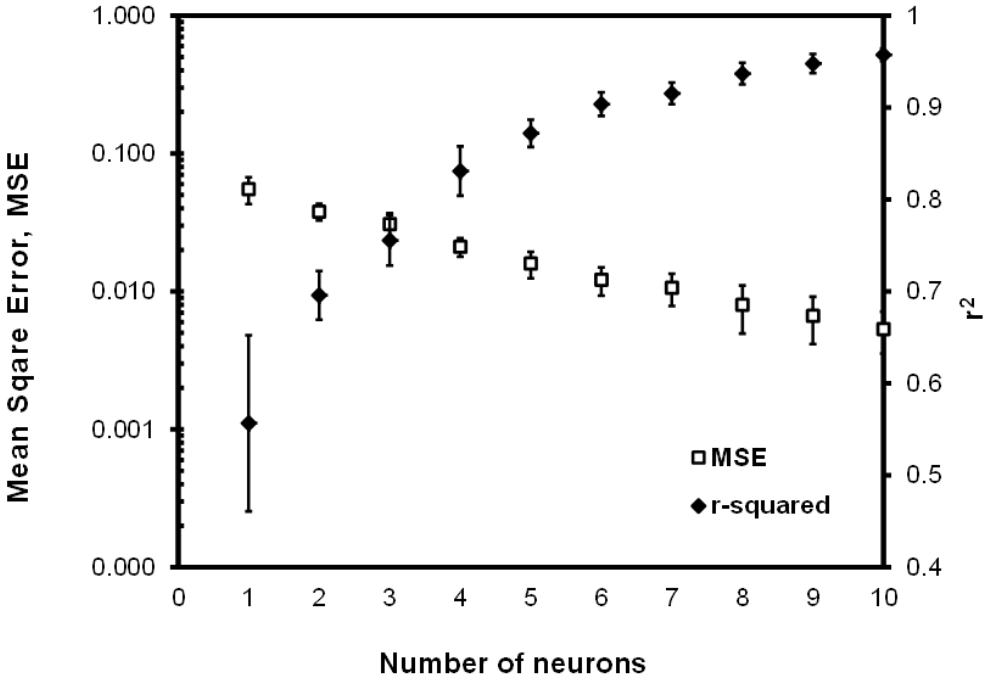


Figure 2. Mean square errors and coefficients of determination (r^2) of the test subsets as a function of the number of neurons in the hidden layer.

Previous works have employed feedforward ANN to model the time evolution of enzymatic hydrolysis of different protein substrates. The novelty of this work lies in the fact that a mixture of enzymes was employed for the hydrolysis. Time evolution of the DH has been studied and modeled in other systems such as the hydrolysis of squid or blood protein (Abakarov et al., 2011; Pérez-Gálvez et al., 2015). Abakarov et al. (Abakarov et al., 2011) modeled the time evolution of the DH in the hydrolysis of squid waste protein with subtilisin employing an ANN comprising a double hidden layer and gradient descend as training algorithm. The correlation coefficients were similar to that obtained in the current work. Pérez-Gálvez et al.,(2015) modeled the time evolution of DH of porcine blood hydrolysis with subtilisine employing a feedforward ANN with 10 neurons in the hidden layer trained by the Levenberg–Marquardt algorithm.

3.3. Optimization of the degree of hydrolysis by evolutionary algorithm

Some technological properties are closely related to the molecular weight distribution of the hydrolysate, such as the solubility, which increases with the degree of hydrolysis (and hence lower average peptide chain length) (Kristinsson & Rasco, 2000). Furthermore, some biological activities such as chelating or ACE inhibition are favored with higher values of DH (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Vercruysse, Van Camp & Smagghe, 2005). Therefore, the goal of the optimization problem proposed in this work was to maximize the DH.

To this end, the evolutionary optimization algorithm was set to employ the ANN model presented above as an objective function. As a result, an optimal DH of 17.1% was reached at an initial protein concentration of 2.54 g/L, 40ºC, 4h of hydrolysis and an enzyme mixture comprising 38.3% of subtilisin and 61.7% trypsin. This optimal value was confirmed by conducting duplicate hydrolysis under those operation conditions achieving an average final DH of 16.9%. Consequently, the absolute error between the experimental and theoretical values (-0.2%) falls into the interval [-0.5, 0.5] where 85% of the errors are comprised. Due to the selectivity of proteases, not all peptide bonds are susceptible to be cleaved. Hence, the extent of the enzymatic hydrolysis is always far from 100%. Effectively, Valencia et al. (2015) attained a maximum steady DH of 26.2% in the hydrolysis of salmon protein employing subtilisin at high enzyme-substrate ratio and long reaction time. Given the limited conditions employed in the current work (4h and 1% of enzyme-substrate ratio), the optimal DH attained (17.1%) is far below this theoretical value (26.2%). Furthermore, each fish species presents a specific degradability and amino acids composition.

As stated before, the evolutionary algorithm generates a set of new solutions based on an initial population. Table 2 summarizes the parameters of the final population leading to this optimal solution.

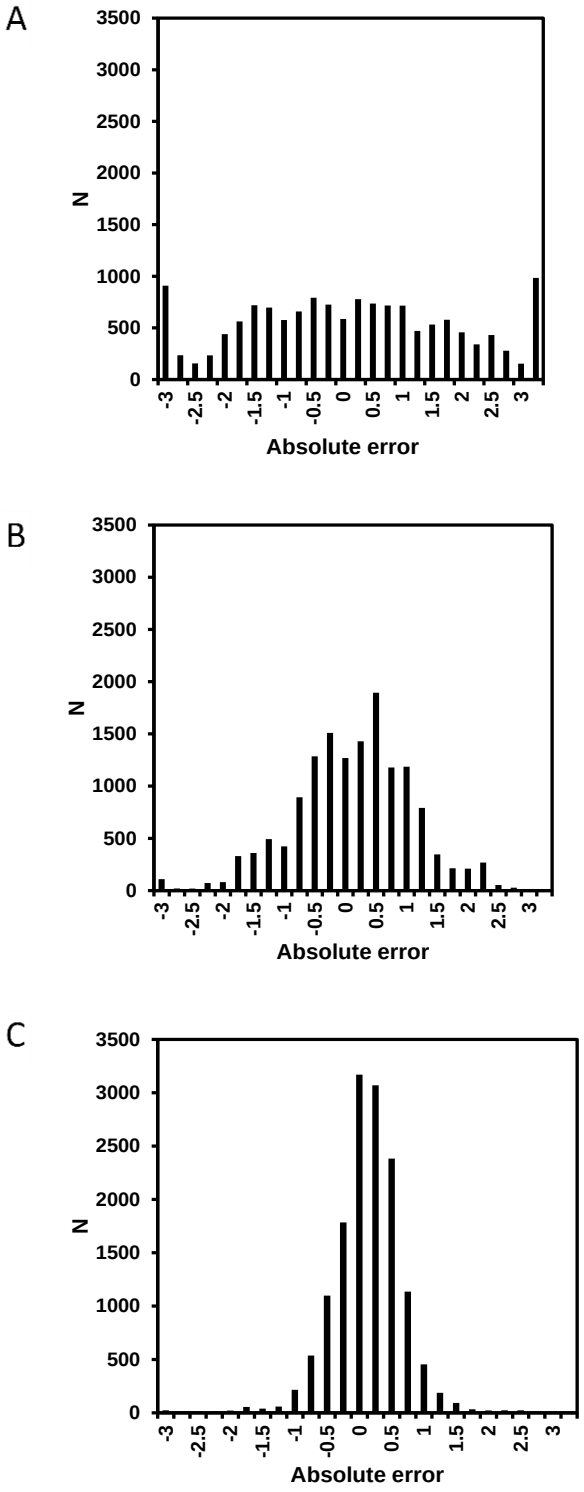


Figure 3. Absolute error histograms of the neural network model when employing (a) 1, (b) 5 and (c) 10 neurons in the hidden layer.

Table 2. Characterization of the population where the maximum DH (17.1%) was obtained.

Variable	Optimal	Mean	SD	Min	Max
Substrate concentration, g/L	2.536	2.525	0.011	2.5	2.538
T, ºC	40.000	40.000	0.000	40	40
Percentage subtilisin, %	38.280	37.491	6.247	0	38.630
Time, min	240.000	233.684	38.933	0	240

Fig. 4 shows the contour plot of the degree of hydrolysis as a function of the reaction temperature and the enzyme composition. Both the substrate concentration and the reaction time were fixed at their optimal values: 2.54 g/L and 240 min, respectively. The maximum DH (17.1%) was placed at the lowest reaction temperature assayed (40ºC). This temperature is within the interval of maximum activity for trypsin, but below the optimal conditions for subtilisin (50 – 55ºC). Additionally, the contour presents a local maximum of DH (~16.5%), for a mixture 1:1 of both enzymes at 50ºC. Under these conditions, the proteolytic activity of subtilisin is maximum, while that of trypsin should be weak.

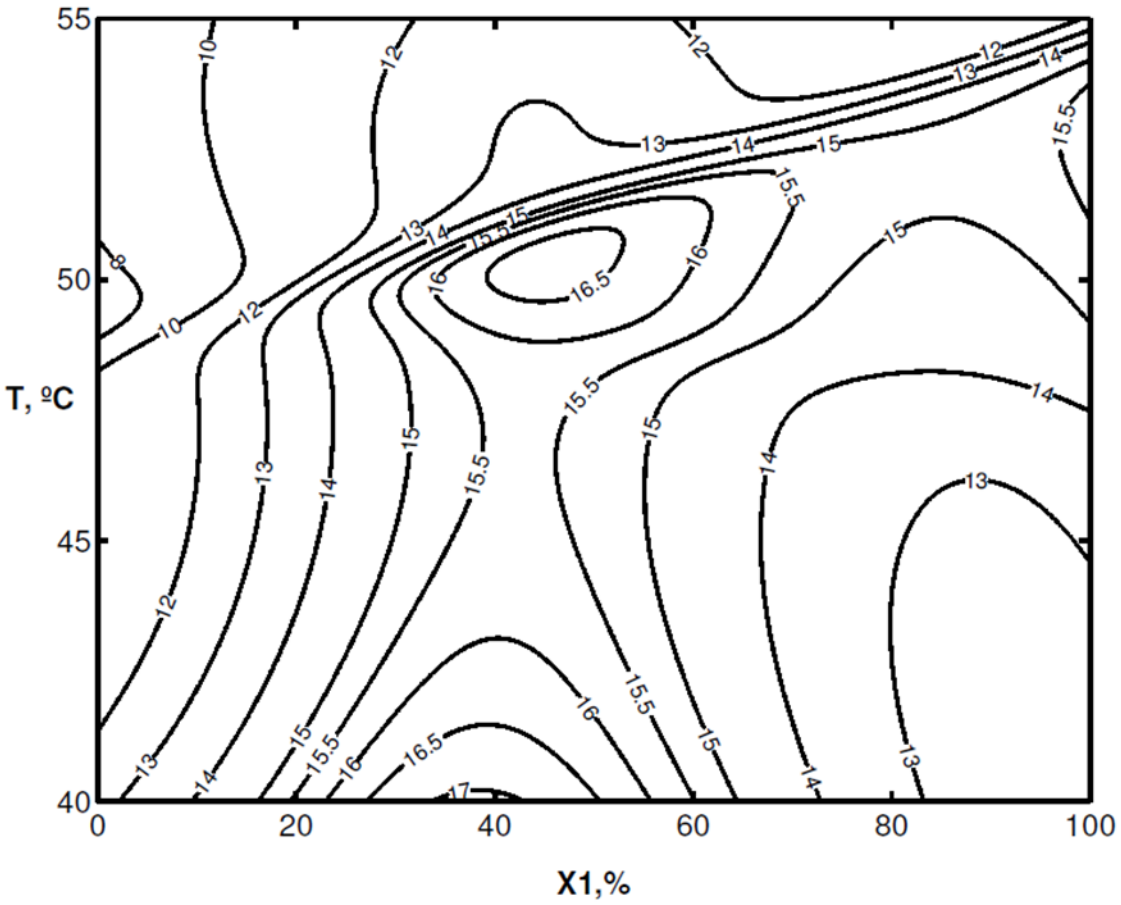


Figure 4. Final DH contour plot against percentage of subtilisin in the enzyme mixture and the reaction temperature. Initial substrate concentration and time were fixed at 2.54g/L and 240 min, respectively.

It can be concluded that the fact of adding trypsin to the enzyme solution led to a higher final DH (17.1%), compared to the single use of subtilisin (maximum DH of 15.5%). Moreover, the former optimum was attained at lower temperatures (40ºC instead of 52ºC). Enzyme processing at the lower temperature (40ºC) presents several economical and nutritional advantages. Firstly, the thermal deactivation of the enzyme is prevented. Given the thermal stability ranges of subtilisin and trypsin, at 40ºC both enzymes keep their proteolytic activity (*i.e*. the effect of enzyme deactivation can be neglected). Additionally, from an industrial point of view, it involves a decrease in the operational costs. Furthermore, lower temperatures minimize nutritional losses of thermolabile compounds.

4. CONCLUSIONS

The degree of hydrolysis is a key variable in the production of hydrolysates, since many functional and biological properties depend on the peptide chain length. However, the complexity of the enzymatic processing of fish protein hinders to mechanistically model this process. In this study, the hydrolysis of horse mackerel protein with a mixture of enzymes (trypsin and subtilisin) was successfully modeled ($r^2 > 0.98$) by an ANN. The experimental data were fitted to a feed-forward ANN comprising 10 neurons in the hidden layer, trained by the Levenberg-Marquardt algorithm. This model is valid for a range of temperatures between 40 – 55ºC, substrate concentration 2.5 to 7.5g/L and any composition of the enzyme mixture keeping the enzyme-substrate ratio at 1w:w%. The optimization of the ANN was conducted employing an evolutionary algorithm, which determined a maximum value of DH (17.1%) at 240 min, 2.54 g/L of substrate concentration, 40ºC and 38.3% of subtilisin in the enzyme preparation. It was concluded that the combination of enzymes improved the final DH expected by the use of a single protease (*i.e.* trypsin or subtilisin). In addition, the optimal DH was reached at low temperature (40ºC), which is desirable from an economical and nutritional point of view.

APPENDIX 1. PARAMETERS OF THE ANN WITH 10 NEURONS IN THE HIDDEN LAYER

k	INPUT LAYER					HIDDEN LAYER	
	w_{k1}	w_{k2}	w_{k3}	w_{k4}	b_k	ω_k	β
1	2.2865	1.3632	-14.7004	35.6435	20.3191	0.096	17.0105
2	-8.2347	3.8218	18.1066	-0.8204	16.2424	0.057	-
3	4.0544	0.6122	-5.4517	-0.0433	1.55105	-0.598	-
4	-0.0567	-0.6082	-0.5771	0.0265	0.14785	-13.394	-
5	-0.0405	-0.0586	0.3317	-7.4888	-10.082	-12.217	-
6	-12.0042	-5.9804	0.0960	-0.0985	-5.8777	0.436	-
7	0.0170	0.5857	0.0294	-0.1099	0.77699	-11.403	-
8	-0.7799	19.6414	3.1823	-11.0203	6.62399	0.113	-
9	-0.1795	0.3261	1.8460	-0.0432	-0.4524	-5.230	-
10	5.2449	-29.9378	12.0887	1.1513	20.7445	0.477	-

5. REFERENCES

A.O.A.C. (Association of Official Analytical Chemist). (2006). Official Methods of Analysis of the AOAC, Association of Official Analytical Chemists. Washington DC.

Abakarov, A., Teixeira, A., Simpson, R., Pinto, M., & Almonacid, S. (2011). Modeling of squid protein hydrolysis: artificial neural network approach. *Journal of Food Process Engineering*, 34(6), 2026–2046. http://doi.org/10.1111/j.1745-4530.2009.00567.x

Adamson, N. J., & Reynolds, E. C. (1996). Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. *Enzyme and Microbial Technology*, 19(3), 202–207. http://doi.org/10.1016/0141-0229(95)00232-4

Adler Nissen. (1986). Enzymic hydrolysis of food proteins. London: Elsevier Applied Science Publishers LTD.

Baş, D., & Boyacı, İ. H. (n.d.). Modeling and optimization II: Comparison of estimation capabilities of response surface methodology with artificial neural networks in a biochemical reaction. *Journal of Food Engineerin*g, 78(3), 846–854. http://doi.org/10.1016/j.jfoodeng.2005.11.025

Baş, D., & Boyacı, İ. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*, 78(3), 836–845. http://doi.org/10.1016/j.jfoodeng.2005.11.024

Cavaille, D., & Combes, D. (1995). Characterization of β-galactosidase from *Kluyveromyces lactis*. *Biotechnology and Applied Biochemistry*, 22(1), 55–64.

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food Chemistry*, 135(4), 3020–38. http://doi.org/10.1016/j.foodchem.2012.06.100

Cheison, S. C., Schmitt, M., Leeb, E., Letzel, T., & Kulozik, U. (2010). Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β-lactoglobulin: Analysis by LC–ESI-TOF/MS. *Food Chemistry*, 121(2), 457–467. http://doi.org/10.1016/j.foodchem.2009.12.065

Elbeltagi, E., Hegazy, T., & Grierson, D. (2005). Comparison among five evolutionary-based optimization algorithms. *Advanced Engineering Informatics*, 19(1), 43–53. http://doi.org/ 10.1016/ j.aei.2005.01.004

EU. (2013). Regulation (EU) no 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Official Journal of the European Union, 22–28.

FAO. (2010). Report of the Technical Consultation to Develop International Guidelines on By-catch Management and Reduction of Discards. Report 957, Rome.

Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods*, 4(1), 6–24. http://doi.org/10.1016/ j.jff.2011.09.001

He, S., Franco, C., & Zhang, W. (2013). Functions, applications and production of protein hydrolysates from fish processing co-products (FPCP). *Food Research International*. http://doi.org/10.1016/j.foodres.2012.10.031

Keskin Gündoğdu, T., Deniz, I. İ., Calışkan, G., Sahin, E. S., Azbar, N., Çalışkan,.Azbar, N. (2014). Experimental design methods for bioengineering applications. *Critical Reviews in Biotechnology*, 8551(31), 1–21. http://doi.org/10.3109/07388551.2014.973014

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, 102(4), 1317–1327. http://doi.org/10.1016/j.foodchem.2006.07.016

Kristinsson, H. G., & Rasco, B. A. (2000). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition*, 40(1), 43–81. http://doi.org/10.1080/10408690091189266

Li, Y., Jiang, B., Zhang, T., Mu, W., & Liu, J. (2008). Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food Chemistry*, 106(2), 444–450. http://doi.org/10.1016/j.foodchem.2007.04.067

Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. *Peptides*. http://doi.org/10.1016/ j.peptides.2011.11.013

Olsen, J. V, Ong, S. E., & Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular & Cellular Proteomics : MCP*, 3(6), 608–14. http://doi.org/10.1074/mcp.T400003-MCP200

Pérez-Gálvez, R., Espejo-Carpio, F. J., Guadix, E. M., & Guadix, A. (2015). Artificial neural networks to model the production of blood protein hydrolysates for plant fertilisation. *Journal of the Science of Food and Agriculture*. http://doi.org/10.1002/jsfa.7083

Qian, J., Zhang, H., & Liao, Q. (2011). The properties and kinetics of enzymatic reaction in the process of the enzymatic extraction of fish oil. *Journal of Food Science and Technology*, 48(3), 280–284. http://doi.org/10.1007/s13197-010-0128-8

Rivera, E. C., Rabelo, S. C., dos Reis Garcia, D., Filho, R. M., & da Costa, A. C. (2010). Enzymatic hydrolysis of sugarcane bagasse for bioethanol production: determining optimal enzyme loading using neural networks. *Journal of Chemical Technology & Biotechnology*, 85(7), 983–992. http://doi.org/10.1002/jctb.2391

Valencia, P., Espinoza, K., Ceballos, A., Pinto, M., & Almonacid, S. (2015). Novel modeling methodology for the characterization of enzymatic hydrolysis of proteins. *Process Biochemistry*, 50(4), 589–597. http://doi.org/10.1016/j.procbio.2014.12.028

Valencia, P., Pinto, M., & Almonacid, S. (2014). Identification of the key mechanisms involved in the hydrolysis of fish protein by Alcalase. *Process Biochemistry*, 49(2), 258–264. http://doi.org/10.1016/j.procbio.2013.11.012

Vercruysse, L., Van Camp, J., & Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A review. *Journal of Agricultural and Food Chemistry*. http://doi.org/10.1021/jf0508908

Zhang, G., Eddy Patuwo, B., & Y. Hu, M. (1998). Forecasting with artificial neural networks: *International Journal of Forecasting*. http://doi.org/10.1016/S0169-2070(97)00044-7

Zhang, Y., Xu, J.-L., & Yuan, Z.-H. (2009). Modeling and Prediction in the Enzymatic Hydrolysis of Cellulose Using Artificial Neural Networks. In Fifth International Conference on Natural Computation, 2009. ICNC '09 (Vol. 2, pp. 158–162). http://doi.org/10.1109/ICNC.2009.334

Zhou, Q. Z., Chen, X. D., & Li, X. (2003). Kinetics of lactose hydrolysis by beta-galactosidase of Kluyveromyces lactis immobilized on cotton fabric. *Biotechnology and Bioengineering*, 81(2), 127–33. http://doi.org/10.1002/bit.10414

IX. Modeling of the Production of ACE Inhibitory Hydrolysates of Mediterranean Horse Mackerel (*T.mediterraneus*) Using Protease Mixtures [*]

Fish protein hydrolysates from Mediterranean horse mackerel were produced by a mixture of two commercial endoproteases (*i.e.* subtilisin and trypsin) under different levels of substrate concentration (2.5 g/L, 5 g/L, 7.5 g/L of protein), temperature (40ºC, 47.5ºC, 55ºC) and percentage of subtilisin in the enzyme mixture (0%, 25%, 50%, 75% and 100%). A crossed mixture process model was employed to predict the degree of hydrolysis (DH) and the ACE inhibitory activity of the final hydrolysates as a function of the experimental factors. Both models were optimized for a maximum DH and ACE inhibition. A maximum DH (17.1%) was predicted at 2.54 g/L of substrate concentration, 40ºC and an enzyme mixture comprising 38.3% of subtilisin and 61.7% of trypsin. Although its proteolytic activity is limited, the presence of trypsin in the enzyme mixture allowed obtaining higher degrees of hydrolysis at low temperatures, which is desirable to minimize thermal deactivation of the proteins. Similarly, a percentage of ACE inhibition above 48% was attained at 2.5 g/L of protein, 40ºC and a mixture 1:1 of both proteases. Higher values of ACE inhibition could be attained by increasing both the temperature and the amount of trypsin in the enzyme mixture (*e.g.* 50% ACE inhibition at 55ºC and 81.5 % of trypsin). Finally, those hydrolysates exhibiting the highest levels of ACE inhibition were subjected to simulated gastrointestinal digestion. These assays confirmed the resistance of active fractions against their degradation by digestive enzymes.

[*] JOURNAL PAPER: R. Pérez-Gálvez, R. Morales-Medina, F. Espejo-Carpio, A. Guadix and E. M. Guadix. (2016). Modeling of the Production of ACE Inhibitory Hydrolysates of Mediterranean Horse Mackerel (*Trachurus mediterraneus*) Using Protease Mixtures. *Food and Function* 7:3890-3901.

http://dx.doi.org/10.1039/c6fo00716c

1. INTRODUCTION

According to the Magnuson-Stevens Fishery Conservation and Management Act (MSA), by-catch is defined as "fish which are harvested in a fishery, but which are not sold or kept for personal use, and includes economic and regulatory by-catch."(Karp, Desfosse, 2011) Bycatch comprises target species which are discarded due to legal, economic or personal considerations (*i.e.* minimum landing size, prohibitions on the retention of particular species, sexes or size ranges, fishing quota, low commercial value, highgrading practices). This definition also includes incidental catches (*i.e.* retained catches of non-targeted species) as well as "unobserved mortalities resulting from a direct encounter with fishing gear" (Bane et al., 1998). International instruments, such as the FAO have highlighted the impact of bycatch on the sustainability of fishing. (Kelleher, 2005)

In compliance with the recent EU Fisheries Common Policy, by-catch is banned in European fisheries for most of pelagic fisheries (*e.g.* herring, sardine or Mediterranean horse mackerel) since the 1st of January of 2015. This measure will be extended to the rest of species in the following two years (EU, 2013). As a consequence, a supplementary amount of unwanted biomass will be no longer returned to the sea but brought ashore. This calls for the search of new solutions to handle and valorize these materials, involving the commercial promotion of non-target species, improvements in the fishing gears or up-grading strategies to obtain valuable products (FAO, 2010).

In this context, enzymatic processing arises as a promising alternative to recover protein and lipid fractions from fish biomasses (*e.g.* by-catches, non edible fractions and other wastes from fish processing). The use of proteases ensures high protein recovery rates and allows converting the native proteins into fish protein hydrolysates (FPH). These do not only present improved technological properties (Pires & Batista, 2013), but they also exhibit a number of biological activities such as antioxidant, antihypertensive or antimicrobial (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; G.-H. Li, Le, Shi, & Shrestha, 2004; Najafian & Babji, 2012; Vercruysse, Van Camp, & Smagghe, 2005). The main group of peptides displaying antihypertensive activity corresponds to the inhibitors of Angiotensin Converting Enzyme (ACE). Fish protein hydrolysates from marine origin have been widely reported to exhibit ACE-inhibitory activity, some of them belonging to target species in Mediterranean fisheries such as sardinelle (*Sardinella aurita*)

(Bougatef et al., 2008), cuttlefish (*Sepia officinalis*) (Balti et al., 2010), sardine (*Sardina pilchardus*) or Mediterranean horse mackerel (*Trachurus mediterraneus*) (García-Moreno et al., 2013), among others. Apart from their intrinsic activity, bioactive peptides should resist gastrointestinal digestion before reaching the target organs and exerting their physiological effect. To this regard, *in vitro* digestion processes, which simulate the digestion process by employing gastrointestinal proteases in a reaction vessel, are effective to evaluate the bioavailabity of active peptides (Borawska, Darewicz, Vegarud, Iwaniak, & Minkiewicz, 2015; Pérez-Vega, Olivera-Castillo, Gómez-Ruiz, & Hernández-Ledesma, 2013).

Enzymatic reactions entail a number of simultaneous phenomena (*e.g.* substrate solubilization, product or substrate inhibition, thermal enzyme deactivation) which cannot be accurately predicted by classical approaches (*i.e.* Michaelis-Menten mechanisms). Empirical models overcome these limitations since they are based on direct observation of experimental data, without considering the underlying mechanism (Baş & Boyacı, 2007). These techniques require a small number of experimental data, arranged according to an experimental design, and have been successfully employed to model or optimize the yield of enzymatic reactions (Nikolaev et al., 2016; Pérez-Gálvez, Almécija, Espejo, Guadix, & Guadix, 2011; Vázquez, Blanco, Fraguas, Pastrana, & Pérez-Martín, 2015). For instance, previous works on fish protein hydrolysis have predicted the optimum conditions for maximum degree of hydrolysis (Morales-Medina, Pérez-Gálvez, Guadix, & Guadix, 2016; Vázquez et al., 2015) which has a positive impact on many technological properties (*e.g.* protein solubility, water or lipid binding capacities). Other range of optimization problems target the maximization of the levels of some biological activities (*e.g.* ACE inhibition, DPPH scavenging, antimicrobial activity) in the final hydrolysate (Abedin et al., 2015; L. Li, Wang, Zhao, Cui, & Jiang, 2006; Ren et al., 2012).

This work studied the enzymatic hydrolysis of Mediterranean horse mackerel (Trachurus mediterraneus) by a variable mixture of two commercial endoproteases (subtilisin and trypsin). A design of experiments was performed to investigate the influence of the enzyme mixture and operating conditions (*i.e.* reaction temperature and enzyme-substrate ratio) on both the final degree of hydrolysis (DH) and the *in vitro* ACE inhibitory activity of the final hydrolysates (ACEI). Two predictive models were constructed for the final DH and ACEI, by means of a crossed mixture-process approach. These equations allowed the optimization for maximum DH and ACEI. Subsequently, those hydrolysates presenting the

highest levels of ACEI were subjected to simulated gastrointestinal digestion to evaluate the loss of bioactivity of the hydrolysate after digestion.

2. MATERIALS AND METHODS

2.1. Proximate composition of the raw material

Mediterranean horse mackerel (*Trachurus mediterraneus*), was chosen as model species for this study. According to previous studies, this species is highly discarded in the Alboran Sea (*i.e.* portion of the Mediterranean Sea lying between northern Morocco and southern Spain)(Bellido Millán, Carbonell Quetglas, Garcia Rodriguez, Garcia Jimenez, & González Aguilar, 2014; Damalas et al., 2015). The raw material was provided by the fishing harbor of Motril (Spain) and kept in ice during transportation.

The raw material was partially dewatered prior to hydrolysis. To this end, 3 kg of fresh fish were preheated at 40ºC for 30 min in a water bath and then pressed stepwise at 120 bar by means of a hydraulic press (model ESP-K, Sanahuja, Spain). After completion of three pressing cycles the dewatered press cakes were recovered and analyzed for their proximate composition.

The moisture and ash content of press cakes was determined according to the official methods recognized by the A.O.A.C., (2012). Total Nitrogen, determined by the Kjeldahl method, was reported to the content of crude protein by a conversion factor of 6.25 (Adler Nissen, 1986).

2.2. Production of the fish protein hydrolysates (FPH)

The hydrolysis experiments were conducted with two serine endoproteases: subtilisin (EC 3.4.21.62) and trypsin (EC 3.4.21.4), purchased from Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S, respectively. A sample of grinded press cake, containing the desired amount of crude protein, was suspended in 200 mL of demineralized water. This suspension was transferred to a jacketed reactor of capacity 250 mL, where it was adjusted at pH 8 and the desired temperature. An enzyme mixture of subtilisin and trypsin was employed as catalyst, whose composition was varied according to the experiment design. The amount of enzyme mixture was adjusted at 1% w/w of the protein content in the reactor. After addition of the enzymes, the hydrolysis reaction was allowed for 4 h. The

degree of hydrolysis (DH) was monitored in the course of the reaction by the pH-stat method (Adler Nissen, 1986), employing an automatic titrator (718 Stat Titrino, Metrohm, Switzerland) and NaOH 0.5 N as titration agent. The degree of hydrolysis can be related to the amount of base consumed throughout the reaction to maintain the pH at 8, according to the Eq. 1:

$$DH = \frac{V_b \cdot N_b}{m_P} \cdot \frac{1}{\alpha} \cdot \frac{1}{h_{tot}} \cdot 100 \quad [1]$$

where DH stands for the degree of hydrolysis; V_b (mL) is the amount of base consumed; N_b (eq/L) is the normality of the base; m_P (g) is the mass of protein in the substrate; α is the average degree of dissociation of the α-NH_2 amino groups and htot (meq /g) was assumed to be 8.6 milliequivalents of peptide bonds per gram of protein, as commonly accepted for fish materials (Adler Nissen, 1986; Kristinsson & Rasco, 2000).

After completing 4 h, the hydrolysis was stopped by heating the reaction mixture at 100ºC for 15 min. These conditions ensure complete enzyme deactivation. Samples were then stored at -20ºC until freeze drying in a Labconco freeze drying system (Kansas City, MO, USA)

2.3. Experimental design

A crossed mixture-process design was proposed in this work, comprising two mixture variables: the percentage of subtilisin and trypsin in the enzyme preparation (X_1, X_2, %), and two process variables: the concentration of substrate (*i.e.* protein) in the reaction vessel (S, g/L) and the reaction temperature (T, ºC). The percentage of subtilisin was tested at five experimental levels: 0% (pure trypsin), 25%, 50%, 75% and 100 % (pure subtilisin). The substrate concentration was varied at three levels: 2.5 g/L, 5 g/L and 7.5 g/L. Protein concentrations above 7.5 g/L could hinder the correct stirring of the reactor vessel. On the contrary, producing hydrolysates with protein concentration below 2.5 g/L requires a high energy consumption for purification and stabilization. Three levels of reaction temperature were assayed: 40ºC, 47.5ºC and 55ºC. The lower bound corresponds to the optimal temperature of trypsin (around 37ºC), while the maximum activity of subtilisin is reported to be within the interval 50ºC - 60ºC (Adler Nissen, 1986). The combination of these levels led to 45 experimental runs. As response variables, the final DH (at 4 h of reaction) and the

ACE inhibition of the powdered hydrolysate were determined for each experiment. These results are summarized in Table 1.

2.4. Crossed mixture-process models for DH and ACEI

The designed experiment described above allowed obtaining mathematical models relating two response variables, the degree of hydrolysis (DH) and the ACE inhibitory activity (ACEI), to the three experimental factors assayed: enzyme composition (X_1, X_2, %), substrate concentration (S, g/L) and temperature (T, ºC). The mathematical models proposed were constructed by combination of a binary mixture model, which relates the responses with the composition of the enzyme preparation (*i.e.* percentage of subtilisin and trypsin) and a factorial design involving two process variables (*i.e.* substrate concentration and reaction temperature). Each of the responses could be related to the enzyme components by means of mixture model of second order (Cornell, 2002), as shown in Eq. 2:

$$Y(X_1, X_2) = \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_{12} \cdot X_1 X_2 \quad [2]$$

where Y denotes any of the response variables DH or ACEI, X_1 is the percentage of subtilisin in the enzyme mixture and X_2 is the percentage of trypsin in the enzyme mixture.

As for the process variables, a second order factorial model was proposed as follows:

$$Y(S, T) = \alpha_0 + \alpha_1 \cdot S + \alpha_2 \cdot T + \alpha_{12} \cdot S \cdot T + \alpha_{11} \cdot S^2 + \alpha_{22} \cdot T^2 \quad [3]$$

Finally, a crossed mixture process model, containing 18 terms, was obtained by multiplication of both sub-models, as expressed by Eq. 4:

$$Y(X_1, X_2, S, T) = (\alpha_0 + \alpha_1 \cdot S + \alpha_2 \cdot T + \alpha_{12} \cdot S \cdot T + \alpha_{11} \cdot S^2 + \alpha_{22} \cdot T^2) \cdot (\beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_{12} \cdot X_1 X_2) \quad [4]$$

Every term of the complete model was estimated by non linear regression of the experimental data. The significance of each term was then judged statistically by computing the associated probability (p-value) at a confidence level of 95%. This means that those terms whose p-value was below 0.05 were statistically significant on the output variable. The non significant terms can be sequentially removed from the regression model by backward selection. This approach starts with the complete model and eliminates the

term with the highest p-value. This process is repeated with the new model until obtaining a reduced model where all the terms are statistically significant.(Kroese & Chan, 2014) The goodness of the reduced model was assessed by the coefficient of determination r^2, as well as the mean absolute error (*i.e.* average value of residuals) and the standard error of estimate (standard deviation of the residuals).

2.5. Determination of the ACE inhibitory activity of the FPH

The ACE inhibitory activity of the freeze dried hydrolysates was determined *in vitro* by the assay proposed by Shalaby, Zakora, & Otte (2006) This method is based on the hydrolysis of the synthetic tripeptide N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG, Sigma-Aldrich, St. Louis, USA) by the enzyme ACE (EC 3.4.15.1, Sigma-Aldrich, St. Louis, USA), which can be followed spectrophotometrically. The assays were conducted in a 96-well microplate at 37ºC, where the absorbance at 340 nm was monitored during 30 min by means of a Multiskan FC microplate photometer (Thermo Scientific, Finland). The hydrolysis of the substrate FAPGG causes a linear decrease of absorbance with time, whose slope is commonly related to the enzyme activity. Therefore, the percentage inhibition of the ACE activity by the hydrolysate can be expressed as:

$$\%\ \text{ACE Inhibition} = \left(1 - \frac{\rho_i}{\rho_0}\right) \cdot 100 \qquad [5]$$

where ρ_i and ρ_0 are the slopes of the absorbance curves in the presence and in the absence of inhibitor (hydrolysate), respectively. The slopes were calculated in the interval between 10 to 25 min, where the best linearity was observed.

2.6. *In vitro* digestion of the FPH

A modification of the method proposed by Garrett, Failla, & Sarama, (1999) was employed to evaluate the effect of digestive enzymes. The reaction was carried out at 37 ºC with a continuous shaking (300 rpm) in a temperature-controlled shaker (Heidolph, Germany). Firstly, the lyophilized samples were dissolved in distilled water 5% (w/w) and the pH was set to 2 with 1M HCl. Subsequently, pepsin (EC 3.4.23.1, Merck, Darmstadt, Germany) was added at enzyme-substrate ratio of 4% (w/w). After 1h of reaction, a solution of 0.9 M NaHCO3 was added to raise the pH until 5.3. Then, a mixture of porcine pancreas enzymes (Pancreatin from Sigma-Aldrich, USA) was added and the pH was set to 7.5 with 1M NaOH. The digestion was maintained for 2 more hours and finally, the enzymes were

thermally deactivated (100 ºC for 15 min). The samples were freeze-dried and stored until analysis.

2.7. Size exclusion Chromatography (SEC)

The hydrolysates with higher *in vitro* ACE inhibition and their digest were analyzed by fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) employing a Superdex Peptide 10/300GL column (GE Healthcare, Uppsala, Sweden). Aliquots of 100 μL (5 mg protein/mL) were eluted at 0.5 mL/min with mobile phase composed of 70:30 water/acetonitrile and 0.1% TFA. The absorbance was measured at 280 nm. The column was calibrated with the following standards: glycine (75 Da), alanine (89 Da), Phe-Gly-Gly (279 Da), $(Gly)_6$ (360 Da), vitamin B12 (1355 Da), insulin (5733 Da), aprotinin (6511 Da) and ribonuclease (13700 Da).

2.8. Optimization of DH and ACEI by evolutionary algorithm

The empiric models presented above allowed the optimization of the operating conditions (X_1, X_2, S and T) for a maximum degree of hydrolysis and ACE inhibitory activity. Among the optimization strategies currently available, an evolutionary algorithm was chosen for this work (Ashlock, 2006). This algorithm is implemented in the Solver tool of the Microsoft Excel software. The optimization procedure starts with the generation of an initial random population of 100 individuals (*i.e.* combinations of S, T and t within their range of application) whose quality is evaluated by means of a fitness function (*i.e.* maximization of DH or ACEI). The best candidates are then combined to create a new population, employing a range of procedures inspired in biological evolution (*i.e.* elite, crossover and mutation). This procedure was repeated iteratively until completing 60 s of computation time.

3. RESULTS AND DISCUSSION

3.1. Curves of hydrolysis

The time evolution of the degree of hydrolysis followed the general pattern described for enzymatic reactions (Adler Nissen, 1986; Valencia, Espinoza, Ceballos, Pinto, & Almonacid, 2015).

Table 1. Experimental design and measured values for the final DH and ACE inhibition

Nº	S, (g/L)	T, (ºC)	X_1, (%)	X_2, (%)	Final DH (%)	ACE Inhibition (%)
1	2.5	40	100	0	13.24	40.40
2	2.5	40	75	25	13.4	45.33
3	2.5	40	50	50	16.48	47.33
4	2.5	40	25	75	15.91	43.45
5	2.5	40	0	100	13.4	40.27
6	5	40	100	0	12.74	37.50
7	5	40	75	25	14.12	39.93
8	5	40	50	50	13.19	37.87
9	5	40	25	75	12.97	31.29
10	5	40	0	100	10.7	36.78
11	7.5	40	100	0	13.04	37.40
12	7.5	40	75	25	12.17	39.06
13	7.5	40	50	50	13.71	34.33
14	7.5	40	25	75	13.15	38.94
15	7.5	40	0	100	8.97	30.09
16	2.5	47.5	100	0	14.15	43.84
17	2.5	47.5	75	25	14.81	48.33
18	2.5	47.5	50	50	15.09	43.14
19	2.5	47.5	25	75	14.32	47.26
20	2.5	47.5	0	100	11.16	48.66
21	5	47.5	100	0	13.37	38.74
22	5	47.5	75	25	10.5	32.65
23	5	47.5	50	50	11.61	30.71

Table 1. (Continuation) Experimental design and measured values for the final DH and ACE inhibition

Nº	S, (g/L)	T, (ºC)	X_1, (%)	X_2, (%)	Final DH (%)	ACE Inhibition (%)
24	5	47.5	25	75	9.49	31.02
25	5	47.5	0	100	7.34	38.43
26	7.5	47.5	100	0	13.01	37.59
27	7.5	47.5	75	25	10.85	34.29
28	7.5	47.5	50	50	14.05	37.28
29	7.5	47.5	25	75	12.74	36.48
30	7.5	47.5	0	100	9.17	32.87
31	2.5	55	100	0	12.24	45.93
32	2.5	55	75	25	11.59	44.04
33	2.5	55	50	50	12.12	42.18
34	2.5	55	25	75	12.24	50.23
35	2.5	55	0	100	8.9	56.12
36	5	55	100	0	14.73	44.32
37	5	55	75	25	9.7	33.70
38	5	55	50	50	8.69	35.32
39	5	55	25	75	10.96	33.27
40	5	55	0	100	7.39	38.04
41	7.5	55	100	0	13.89	39.18
42	7.5	55	75	25	13.37	36.57
43	7.5	55	50	50	12.29	36.71
44	7.5	55	25	75	13.98	31.22
45	7.5	55	0	100	10.14	35.76

In general, hydrolysis curves presented a high reaction rate at the beginning and then decreased progressively until attaining a steady state. This trend is explained by the exhaustion of peptide bonds available in the protein, combined to other phenomena such as thermal enzyme inactivation or product inhibition, among others.(Valencia, Pinto, & Almonacid, 2014) As for the interactions between enzyme and temperature, the Fig. 1 presents two examples of hydrolysis curves, illustrating the single use of subtilisin (Fig. 1a) or trypsin (Fig. 1b) at protein concentration of 5 g/L and increasing reaction temperature (experiments # 6, 10, 21, 25, 36 and 40 in Table 1). Subtilisin cleaves a wide range of peptide bonds, preferably those involving aromatic and methionine residues.(Adamson & Reynolds, 1996) This enzyme exhibits high resistance against thermal degradation, presenting maximum proteolitic activity in the interval 50-60ºC (Adler Nissen, 1986; Valencia et al., 2014). Indeed, increasing temperatures favored the reaction rate, so the highest final value of DH was attained at 55ºC (DH = 14.73%). The hydrolysis curve at 55ºC was the only one attaining a steady state from 160 min on, suggesting possible loss of enzyme activity. In contrast, as shown in Fig. 1b, trypsin presented its highest proteolytic activity at 40ºC (final DH = 10.7%) while the curves at 47.5ºC and 55ºC flattened after 30 min, attaining a steady value of DH = 7.4 %. This enzyme presents a narrow selectivity, since it cleaves exclusively peptide bonds with participation of lysine or arginine residues (Olsen, Ong, & Mann, 2004). Furthermore, trypsin is highly susceptible to thermal deactivation, combined to loss of activity by autolysis. Zhang, He, & Guan, (1999) reported maximum proteolytic activity of trypsin at 37-40ºC, which decreased in 50% at 47ºC. This is reflected by the curves at 47.5ºC and 55ºC, which present rapid loss of enzyme activity by thermal inactivation.

3.2. Modelization of the degree of hydrolysis

The experimental values of DH at the end of the hydrolysis (4 h) are summarized in Table 1. It can be observed that the final DH varied from 7.34% (single trypsin at 47.5ºC and substrate concentration 5 g/L) to 16.48% (mixture subtilisin-trypsin 1:1 at 40ºC and substrate concentration 2.5 g/L). These experimental data were fitted to the crossed mixture-process model proposed in Eq. 4, obtaining the set of 18 regression coefficients by non linear regression. The statistical significance of each term on the calculated DH was evaluated by the probability value (p-value) at a level of confidence of 95%.

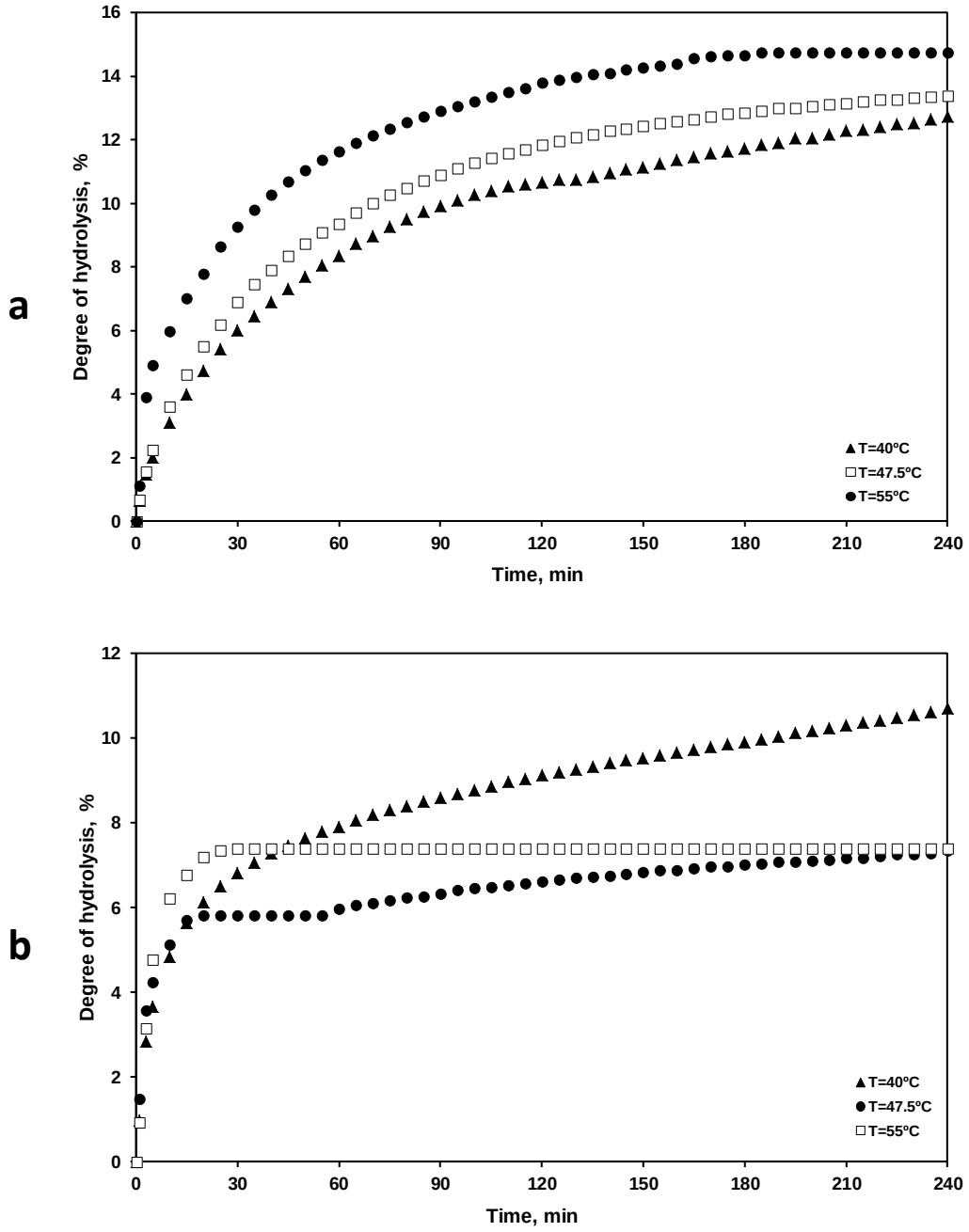


Figure 1. Effect of individual enzymes and reaction temperature on the hydrolysis curves: (a) 5 g/L of protein with subtilisin at enzyme-substrate ratio of 1% w/w; (b) 5 g/L of protein with trypsin at enzyme-substrate ratio of 1% w/w.

Those terms with non-significant impact on the full model (*i.e.* those with p-value > 0.05) were removed by backward elimination, obtaining a reduced model with 8 terms, (Eq. 6):

$$+ 4.66 \cdot 10^{-3} \cdot X_2 \cdot S^2 + 7.02 \cdot 10^{-5} \cdot X_1 \cdot X_2 \cdot T - 1.20 \cdot 10^{-6} \cdot X_1 \cdot X_2 \cdot T^2 \quad [6]$$

The goodness of fit of the reduced model was confirmed by the value of the coefficient of determination (r^2 =0.9913, r^2 adjusted to the degrees of freedom = 0.9813) and the

distribution of the residuals (*i.e.* difference between observed and calculated DH), which presented an average value of 1.2803 ± 0.9590 %.

The predicted model allowed the generation of the contour plots presented in Fig. 2, where the final degree of hydrolysis calculated by Eq. 6 was plotted against the temperature and the percentage of subtilisin in the enzyme mixture at the three levels of substrate concentration assayed (*i.e.* 2.5, 5 and 7.5 g/L). The highest values of final DH were attained at 2.5 g/L of substrate in the reaction mixture (Fig. 2a). The optimization procedure calculated a maximum value of DH (15.94%), attained at 2.5 g/L, 40ºC and 41.2% of subtilisin in the enzyme mixture. This value is the absolute maximum for DH inside the experimental range of temperature, enzyme composition and substrate assayed. The current results are in line with a previous work by Morales-Medina et al., (2016), where DH was modeled by an artificial neuronal network comprising 10 neurons in the hidden layer. In that case, the model which also included the time as a variable, predicted an absolute optimum for DH (17.1%) at 2.54 g/L of substrate concentration, 40ºC and 38.3% of subtilisin in the enzyme mixture

Overall, the final values of DH for 5 g/L (Fig. 2b) were significantly inferior, ranging between 8% and 13.5%. Similarly, the final values of DH were slightly improved in the case of 7.5 g/L (10-13.7%, Fig. 2c). These results are in agreement with the mathematical model, where several interactions between trypsin and substrate concentration are significant on the response variable. Indeed, the interaction $X_2 \cdot S$ affected negatively the final DH, while $X_2 \cdot S \cdot T$ and $X_2 \cdot S_2$ did positively. No interaction between subtilisin and substrate concentration was significant on the response variable. The inhibitory effect of substrate on the proteolysis has been reported for fish protein hydrolysates,(Qian, Zhang, & Liao, 2011; Valencia et al., 2014) and could explain the optimum DH at substrate concentration 2.5 g/L. Effectively, increasing levels of substrate slowed the reaction rate, leading to lower values of final DH. However, this negative modulation vanished at

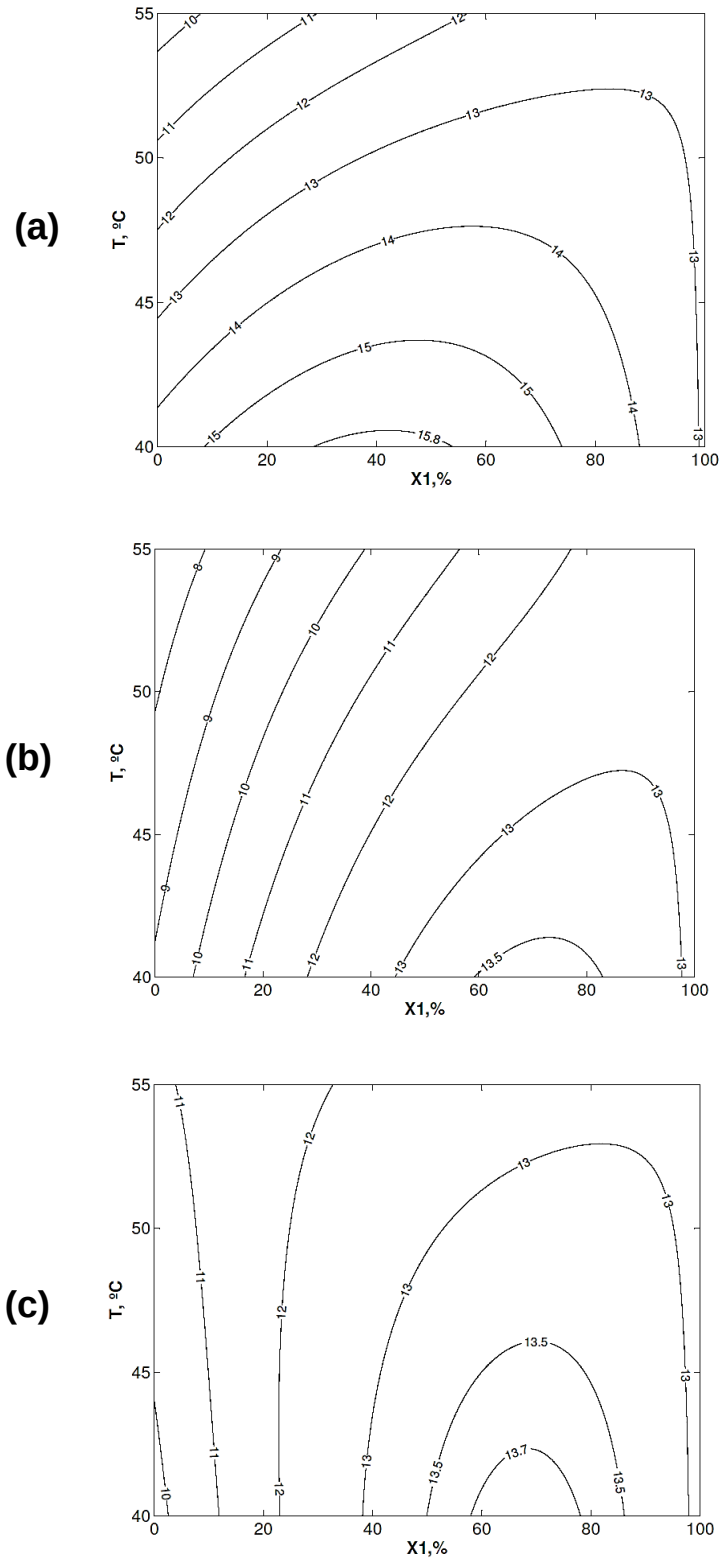


Figure 2. Contour plots for the final degree of hydrolysis (4 h) as a function of the reaction temperature and the percentage of subtilisin in the enzyme mixture at three levels of substrate concentration: (a) 2.5g/L (b) 5 g/L and (c) 7.5 g/L.

substrate concentrations above 5 g/L. Furthermore, high levels of substrate above 6.5 - 7 g/L exerted a positive effect on the final DH, especially in combination with low temperatures (40ºC – 47.5ºC). To this regard, Valencia et al. (2014) reported that increasing levels of substrate (salmon muscle) protected Alcalase against thermal denaturation. In this case, this effect was more remarkable for trypsin and vanished when reaction temperatures above 50ºC were employed.

The mathematical model predicts a synergic effect between subtilisin and trypsin on the DH, modulated by the reaction temperature, as confirmed by the significant interactions $X_1 \cdot X_2 \cdot T$ and $X_1 \cdot X_2 \cdot T^2$. Overall, the combination of subtilin and trypsin improved the final DH, in comparison with their single use. This synergy has been reported in previous studies on other fish species such as sardine, mackerel or Mediterranean horse mackerel (García-Moreno et al., 2013). As shown in the contour plots, the optimal values of DH correspond to combinations of both enzymes. As the reaction temperature increased, so did the percentage of subtilisin required for maximum DH. For instance, as shown in Fig. 2a, the maximum DH reachable at 40ºC required 41.2% of subtilisin in the enzyme preparation, while this percentage rose to 66.7% (50ºC) and 100% at 55ºC. As mentioned above, this is attributed to the different thermal stability of subtilisin and trypsin.

3.3. Modelization of the ACE inhibitory activity

The observed values of ACE inhibition for the final hydrolysates (Table 1) ranged from 30.1% (single trypsin at 40ºC and substrate concentration 7.5 g/L) to 56.12% (single trypsin at 55ºC and substrate concentration 2.5 g/L). The ACE inhibitory activity of the final hydrolysate was fitted to the operation conditions by means of the crossed model expressed by Eq. 4. A set of 18 coefficients was estimated, whose statistical significance was evaluated by the associated probabilities. A backward elimination procedure was employed to remove those terms with associated probability higher than 5%. In contrast with the DH model, the reduced model for ACE inhibition was more complex, comprising 11 terms and significant interactions of third ($X_1 \cdot X_2 \cdot S$, $X_1 \cdot X_2 \cdot T$) and fourth order ($X_1 \cdot X_2 \cdot T^2$).

$$\begin{aligned} ACE = {} & 0.3708 \cdot X_1 - 2.61 \cdot 10^{-4} \cdot X_1 \cdot S \cdot T + 4.28 \cdot 10^{-5} \cdot X_1 \cdot T^2 + 0.0139 \cdot X_2 \cdot T \\ & - 1.77 \cdot 10^{-3} \cdot X_2 \cdot S \cdot T + 5.38 \cdot 10^{-3} \cdot X_2 \cdot S_2^2 + 0.0266 \cdot X_1 \cdot X_2 \\ & - 5.49 \cdot 10^{-3} \cdot X_1 \cdot X_2 \cdot S - 4.19 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot T + 3.71 \cdot 10^{-5} \cdot X_1 \cdot X_2 \cdot S \cdot T \\ & + 3.84 \cdot 10^{-4} \cdot X_1 \cdot X_2 \cdot T^2 \end{aligned} \quad [7]$$

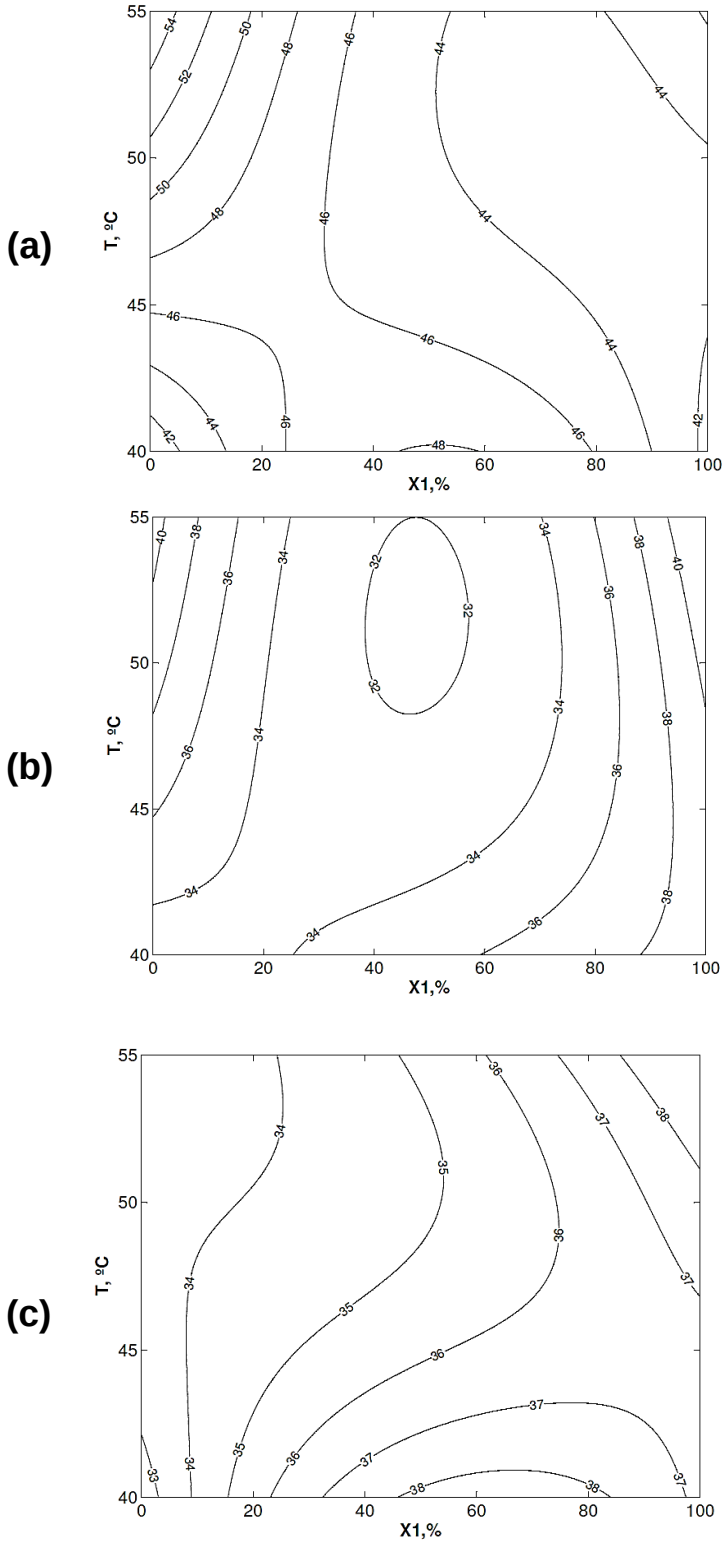


Figure 3. Contour plots for the ACE inhibition as a function of the reaction temperature and the percentage of subtilisin in the enzyme mixture at three levels of substrate concentration: (a) 2.5g/L (b) 5 g/L and (c) 7.5 g/L.

As expected, the goodness of fit of this model was higher than that of the DH. Indeed, the coefficient of determination was r^2=0.9972 (r^2 adjusted to the degrees of freedom = 0.9963). As for the residuals, they presented an average value of 2.4333 ± 1.7581 %. The contour plots shown in Fig. 3a-3c illustrate the influence of operation conditions on the ACE inhibitory activity of the final hydrolysate. The highest levels of ACE inhibition were observed at 2.5 g/L of substrate, 55ºC and only trypsin in the enzyme preparation (56.12%, experiment 35 in Table 1). This value was confirmed by the regression model. Indeed, the contour plot at 2.5 g/L (Fig. 3a) shows that the ACE inhibition increased with both the percentage of trypsin in the enzyme mixture and the temperature, reaching an absolute maximum of ACE inhibition (55.3%) at 2.5 g/L, 55ºC and 100% of trypsin. Under these conditions (*i.e.* trypsin at 55ºC) the extent of the proteolysis was limited, with final DH around 9%. As shown in the contour 3a, levels of ACE inhibition above 50% were detected for tryptic hydrolysates processed above 48ºC. Increasing amounts of subtilisin in the enzyme mixture allowed obtaining similar inhibitory levels, at the expense of higher processing temperatures (*e.g.* 50% ACE inhibition at 55ºC and 18.5% of subtilisin).

The ACE inhibitory effect of a given hydrolysate is favored by the presence of peptides containing hydrophobic residues (*e.g.* Pro, Phe, Tyr) in the tripeptide sequence at the C-terminal end, since it facilitates the interaction with the active site of the Angiotensin I Converting Enzyme (Li et al., 2004). To this regard, it is reported that subtilisin cleaves preferably peptide bonds with participation of hydrophobic residues (Je, Lee, Lee, & Ahn, 2009), while trypsin does specifically with those containing arginine and lysine residues.(Olsen et al., 2004) Although both amino acids are charged positively, they are reported to favor ACE inhibition when placed at the C- terminus (Li et al., 2004). Furthermore, Cheison, Schmitt, Leeb, Letzel, & Kulozik, (2010) affirmed that trypsin behaves like chymotrypsin at high temperatures k above 50ºC). As a result, new peptides with hydrophobic residues (Tyr, Trp, Phe) at the carboxyl side are released to the medium. In our case, these latter may be responsible for the enhanced inhibitory potency observed for the tryptic hydrolysates above 45ºC.

At low temperatures, where trypsin displays the highest specificity, the inhibitory activity of the hydrolysate was favored by increasing levels of subtilisin in the enzyme preparation (*e.g.* 46% ACE inhibition at 40ºC and 21.6% of subtilisin). According to the predictive model, a local maximum (48.2% of ACE inhibition) was detected at 40ºC by employing a mixture 1:1 of both enzymes. As observed in the contour in Fig. 2a, these conditions

favored the extensive hydrolysis of the substrate, attaining values of final DH above 15.8%. Although the potential ACE inhibitory effect of a given peptide depends on a range of factors (*e.g.* residue composition, hydrophobicity), most of the active peptides identified to date are di- and tripeptides (Y. Li, Zhou, Huang, Sun, & Zeng, 2012; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). It is expectable that increasing DH would decrease the average size of the resulting peptides and thus contribute to the presence of potentially ACE inhibitors.

The levels of ACE inhibitory activity of the final hydrolysates obtained at substrate concentration 5 g/L and 7.5 g/L (contours in Fig. 3b and 3c, respectively) were significantly lower, ranging from 32% to 40%. Under these conditions, the extent of the proteolysis was limited (contours in Fig. 2b and 2c), which could be related to the lower inhibitory activity of the resulting hydrolysates.

3.4. *In vitro* digestion of the hydrolysates

The hydrolysates with the highest ACE inhibitory activity for each reaction temperature assayed (*i.e.* those produced by experiments no. 3, 20 and 35 in Table 1) were selected for assessing the effect of gastrointestinal enzymes on the ACE inhibitory activity. The size distribution profiles of both the crude hydrolysates (solid line) and their digests (dotted line) are shown in the Fig. 4a to 4c. The SEC profiles identified four main fractions, whose percentages areas are listed in Table 2.

Table 2. ACE inhibitory activity and area percentage of the SEC-fractions of the raw hydrolysates number 3 (2.5 g/L, 40ºC, 50% subtilisin), 20 (2.5 g/L, 47.5ºC, 100% trypsin) and 35 (2.5 g/L, 55ºC, 100% trypsin) and their respective digests. Mean values in the same column with different superscript letters are significantly different ($p < 0.05$) (d* refers samples after digestion)

Sample	ACEI activity	Area percentage of fractions			
	IC_{50} (µg/mL)	Fraction A (>734 Da)	Fraction B (734-337 Da)	Fraction C (337-172 Da)	Fraction D (<172 Da)
3	330.2 ± 10.2^{a}	20.1 ± 1.7^{a}	21.8 ± 1.4^{a}	18.2 ± 0.7^{a}	39.8 ± 0.3^{a}
3d*	314.6 ± 14.2^{a}	22.5 ± 1.1^{ab}	18.9 ± 1.1^{b}	15.7 ± 1.4^{b}	42.9 ± 0.7^{b}
20	272.1 ± 11.1^{b}	27. ± 0.5^{c}	19. ± 0.5^{b}	15. ± 1.5^{bc}	39. ± 1.1^{a}
20d	261.3 ± 30.3^{b}	23.6 ± 0.6^{bd}	18.4 ± 0.5^{b}	13.4 ± 1.5^{c}	44.6 ± 0.2^{c}
35	253.0 ± 29.1^{b}	32.8 ± 1.0^{e}	16.4 ± 0.8^{c}	15.9 ± 0.5^{b}	34.9 ± 0.4^{d}
35d	265.6 ± 13.7^{b}	25.4 ± 1.8^{cd}	17.7 ± 0.1^{bc}	15.2 ± 0.3^{bc}	41.6 ± 0.8^{e}

The effect of digestive enzymes on the ACE inhibitory activity was assessed by determining the IC_{50} value of both the crude hydrolysates and their digests (Table 2). The IC_{50} values of the selected crude hydrolysates ranged between 253 and 330 µg/mL (experiments 35 and 3, respectively). These values were better than those obtained for Mediterranean horse mackerel using combination 1:1 of trypsin and subtilisin (García-

Moreno, Espejo-Carpio, Guadix, & Guadix, 2015). In contrast, higher ACE inhibitory potential was found in thermolysin hydrolysates of salmon (Ono, Hosokawa, Miyashita, & Takahashi, 2006).

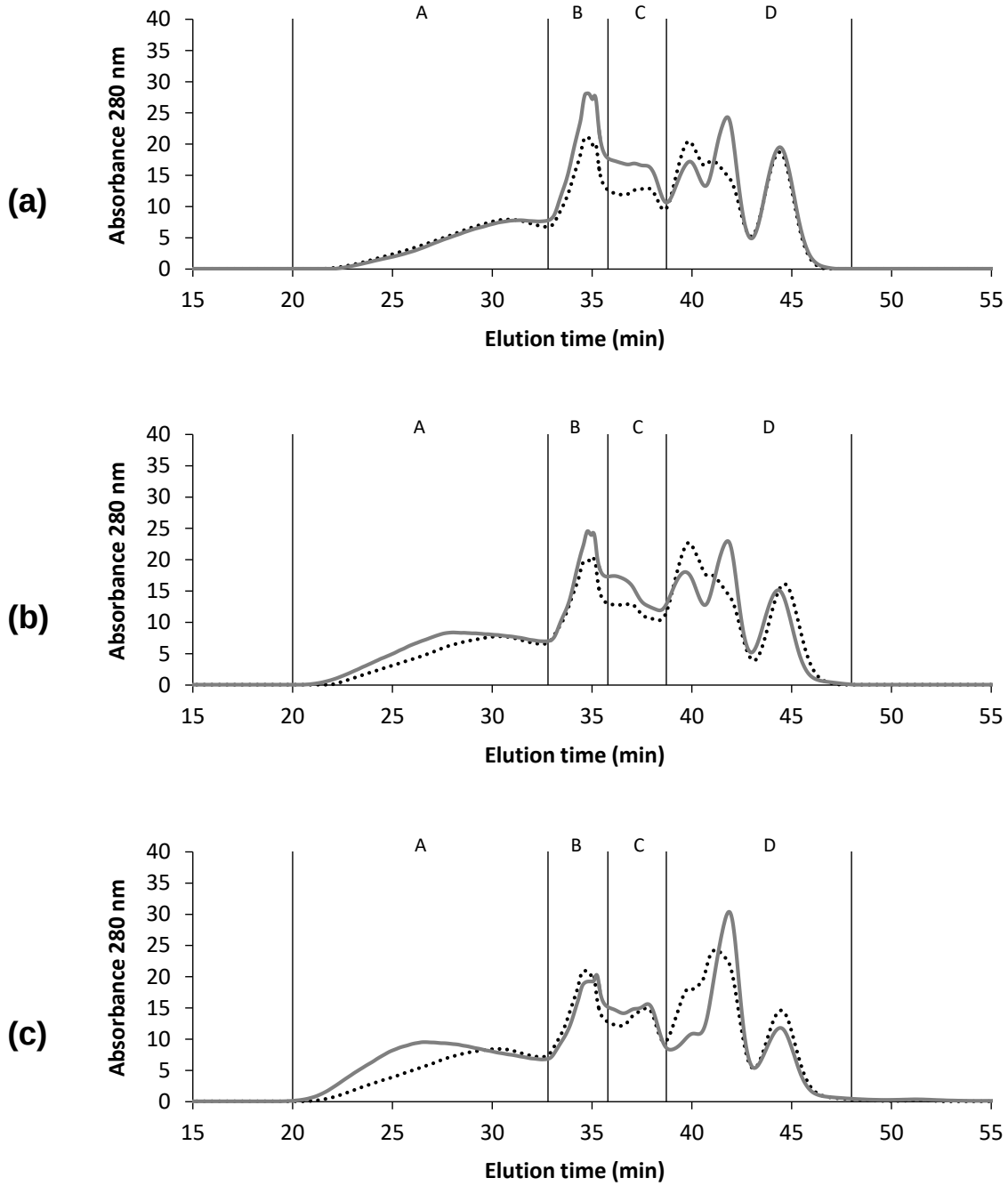


Figure 4. Molecular size distribution of the selected hydrolysates and their respective digests: (a) experiment no. 3 (2.5 g/L, 40ºC, 50% subtilisin), (b) experiment no. 20 (2.5 g/L, 47.5ºC, 100% trypsin) and (c) experiment no. 35 (2.5 g/L, 55ºC, 100% trypsin). Dotted lines represent the digested samples.

Since the variations between the crude and the digested hydrolysates were not significant (Table 2), it was concluded that the ACE inhibitory activity of the selected hydrolysates was not altered by digestive enzymes. This is a very interesting feature, since gastrointestinal digestion is one of the main processes reducing the bioavailability of

bioactive peptides. Indeed, many peptides showing high levels of *in vitro* ACE inhibitory activity failed to show *in vivo* effect due to their degradation by digestive enzymes (Fujita, Yokoyama, & Yoshikawa, 2000). Only a few studies have dealt with the effect of digestive enzymes on the ACE inhibitory activity of fish hydrolysates. In line with our results, Hwang, (2010) reported that ACE inhibitory activity of tuna cooking juice did not change after gastrointestinal digestion. Similar trend was described by Samaranayaka, Kitts, & Li-Chan, (2010) for Pacific hake autolisates, while Cinq-Mars, Hu, Kitts, & Li-Chan, (2008) found that Pacific hake hydrolysates increased its ACE inhibitory activity after simulated digestion.

Regarding the variation in molecular size distribution, all the fractions in the hydrolysate no. 3 (50% subtilisin-50% trypsin) except fraction A were altered during the digestion process. However, the final IC_{50} value remained constant. This can be explained because the degradation of active peptides in fraction B and C would be balanced with the generation of new active peptides. This behavior has been also suggested by Samaranayaka et al.,(2010) for pacific hake hydrolysates.

The crude hydrolysates no. 20 and 35 presented different size exclusion profiles, except for the fraction C (337 – 172 Da) which accounted for 15% of the total area. However, both samples presented similar values of IC_{50}. It may be concluded that the ACE inhibitory activity of these hydrolysates would be mainly determined by the fraction C (337-172 Da), which might contain di- and tripeptides as major species. Indeed, small peptides of 2 or 3 amino acids are usually identified as potent ACE inhibitory peptides (Ono et al., 2006; Wu et al., 2008). The IC_{50} values for both tryptic hydrolysates remained unaltered after the simulated digestion. Furthermore, the profiles in Fig. 4b and 4c showed no significant differences in the percentage area of fractions B and C after digestion. These results agree with previous studies where Mediterranean horse mackerel was hydrolyzed employing subtilisin and trypsin. In this work, some low molecular active peptides, such as HLALT, RQLAGP and ELSAP, were identified (García-Moreno et al., 2015) as potential ACE inhibitors. In addition, the low ACEI variation between crude and digested hydrolysates has been previously explained by Salampessy, Reddy, Kailasapathy, & Phillips, (2015), who reported that the fractions of trevally hydrolysates containing di- and tripeptides maintained its inhibitory potency after gastrointestinal digestion. More generally, Seki, Osajima, Matsufuji, Matsui, & Osajima, (1996) concluded that short chain peptides derived from sardine resisted the digestion process without modification.

4. CONCLUSIONS

The crossed mixture process model chosen in this work fitted adequately the observed data of final DH and ACE inhibition, with determination coefficients r^2=0.9913 and r^2=0.9972, respectively. This model predicted a maximum DH (15,94%) at 2.5 g/L of substrate concentration, 40ºC and an enzyme mixture comprising 41.2% of subtilisin and 59.8% of trypsin. The hydrolysis curves confirmed that the reaction rate was inhibited by increasing levels of substrate concentration, as well as the higher proteolytic activity of subtilisin. Nevertheless, the addition of trypsin to the enzyme mixture allowed obtaining higher degrees of hydrolysis at lower temperatures than those achieved when only subtilisin was employed. Levels of ACE inhibition above 50% were obtained for trypsin hydrolysates at the lowest substrate concentration and temperatures over 48ºC, which was attributed to the increasing affinity of trypsin towards peptide bonds containing hydrophobic residues such as Tyr, Trp or Phe. At lower temperatures the selectivity of trypsin was restrained to Arg and Lys residues, and a maximum of 48.2% ACE inhibition was predicted at 40ºC employing a mixture 1:1 of both endoproteases. Size exclusion chromatograms confirmed that the fractions containing small-sized peptides (*e.g.* di- and tripeptides) were mainly responsible for the ACE inhibitory activity. Furthermore, the experiments of simulated digestion confirmed that these fractions were resistant to digestive enzymes. Indeed, no significant differences in the levels of ACE inhibition were detected between the raw hydrolysates and their digests.

5. REFERENCES

A.O.A.C. (2012). Official Methods of Analysis of the AOAC (19th ed.). Washington DC: Association of Official Analytical Chemists.

Abedin, M. Z., Karim, A. A., Gan, C. Y., Ghazali, F. C., Barzideh, Z., Zzaman, W., & Zaidul, I. S. M. (2015). Identification of angiotensin I converting enzyme inhibitory and radical scavenging bioactive peptides from sea cucumber (*Stichopus vastus*) collagen hydrolysates through optimization. International *Food Research Journal*, 22(3), 1074–1082.

Adamson, N. J., & Reynolds, E. C. (1996). Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. *Enzyme and Microbial Technology*, 19(3), 202–207. http://doi.org/10.1016/0141-0229(95)00232-4

Adler Nissen. (1986). Enzymic hydrolysis of food proteins. London: Elsevier Applied Science Publishers LTD.

Ashlock, D. (2006). Evolutionary Computation for Modeling and Optimization. New York: Springer-Verlag New York.

Balti, R., Bougatef, A., El-Hadj Ali, N., Zekri, D., Barkia, A., & Nasri, M. (2010). Influence of degree of hydrolysis on functional properties and angiotensin I-converting enzyme-inhibitory activity of protein hydrolysates from cuttlefish (*Sepia officinalis*) by-products. *Journal of the Science of Food and Agriculture*, 90(12), 2006–14. http://doi.org/10.1002/jsfa.4045

Bane, A. W., Boggs, C. H., Conser, R., Lauck, E. W., McCallum, J. K., McInnis, R. R., Witzig, J. F. (1998). Managing the nations bycatch: priorities, programs and actions for the national marine fisheries service. *Managing*, 197.

Baş, D., & Boyacı, İ. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*, 78(3), 836–845. http://doi.org/10.1016/j.jfoodeng.2005.11.024

Bellido Millán, J. M., Carbonell Quetglas, A., Garcia Rodriguez, M., Garcia Jimenez, T., & González Aguilar, M. (2014). The obligation to land all catches. Consequences for the mediterranean. Policy Department B. Structural and Cohesion Policies. European Parliament. http://doi.org/10.2861/59268

Borawska, J., Darewicz, M., Vegarud, G. E., Iwaniak, A., & Minkiewicz, P. (2015). Ex vivo digestion of carp muscle tissue--ACE inhibitory and antioxidant activities of the obtained hydrolysates. *Food & Function*, 6(1), 211–8. http://doi.org/10.1039/c4fo00621f

Bougatef, A., Nedjar-Arroume, N., Ravallec-Plé, R., Leroy, Y., Guillochon, D., Barkia, A., & Nasri, M. (2008). Angiotensin I-converting enzyme (ACE) inhibitory activities of sardinelle (*Sardinella aurita*) by-products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases. *Food Chemistry*, 111(2), 350–356.

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food Chemistry*, 135(4), 3020–38. http://doi.org/10.1016/j.foodchem.2012.06.100

Cheison, S. C., Schmitt, M., Leeb, E., Letzel, T., & Kulozik, U. (2010). Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β-lactoglobulin: Analysis by LC–ESI-TOF/MS. *Food Chemistry*, 121(2), 457–467. http://doi.org/10.1016/j.foodchem.2009.12.065

Cinq-Mars, C. D., Hu, C., Kitts, D. D., & Li-Chan, E. C. Y. (2008). Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-converting enzyme-inhibitory peptides in Pacific hake (*Merluccius productus*) fillet hydrolysate. *Journal of Agricultural and Food Chemistry*, 56(2), 410–9. http://doi.org/10.1021/jf072277p

Cornell, J. A. (2002). Experiments with mixtures : designs, models, and the analysis of mixture data. New York: John Wiley & Sons, Inc.

Damalas, D., Maravelias, C. D., Osio, G. C., Maynou, F., Sbrana, M., Sartor, P., & Casey, J. (2015). Historical discarding in Mediterranean fisheries: a fishers' perception. *ICES Journal of Marine Science: Journal Du Conseil*, 72(9), 2600–2608. http://doi.org/10.1093/icesjms/fsv141

EU. (2013). Regulation (EU) no 1380/2013 of the European Parliament and of the Council of 11 December 2013 on the Common Fisheries Policy. Official Journal of the European Union, 22–28.

FAO. (2010). Report of the Technical Consultation to Develop International Guidelines on By-catch Management and Reduction of Discards. Report 957, Rome.

Fujita, H., Yokoyama, K., & Yoshikawa, M. (2000). Classification and antihypertensive activity of angiostensin I-converting enzyme inhibitory peptides derived from food proteins. *Journal of Food Science*, 65(4), 564–569.

García-Moreno, P. J., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2015). Production and identification of angiotensin I-converting enzyme (ACE) inhibitory peptides from Mediterranean fish discards. *Journal of Functional Foods*, 18, 95–105. http://doi.org/10.1016/j.jff.2015.06.062

García-Moreno, P. J., Pérez-Gálvez, R., Espejo-Carpio, F. J., Muñío, M. M., Guadix, A., & Guadix, E. M. (2013). Lipid characterization and properties of protein hydrolysates obtained from discarded Mediterranean fish species. *Journal of the Science of Food and Agriculture*, 93(15), 3777–3784. http://doi.org/10.1002/jsfa.6266

Garrett, D. A., Failla, M. L., & Sarama, R. J. (1999). Development of an *in Vitro* Digestion Method To Assess Carotenoid Bioavailability from Meals. *Journal of Agricultural and Food Chemistry*, 47(10), 4301–4309. http://doi.org/10.1021/jf9903298

Hwang, J.-S. (2010). Impact of processing on stability of angiotensin I-converting enzyme (ACE) inhibitory peptides obtained from tuna cooking juice. *Food Research International*, 43(3), 902–906. http://doi.org/10.1016/j.foodres.2009.12.012

Je, J.-Y., Lee, K.-H., Lee, M. H., & Ahn, C.-B. (2009). Antioxidant and antihypertensive protein hydrolysates produced from tuna liver by enzymatic hydrolysis. *Food Research International*, 42(9), 1266–1272. http://doi.org/10.1016/j.foodres.2009.06.013

Kelleher, K. (2005). Discards in the world's marine fisheries: An update. FAO. Fisheries Technical Paper.

Kristinsson, H. G., & Rasco, B. A. (2000). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition*, 40(1), 43–81. http://doi.org/10.1080/10408690091189266

Kroese, D. P., & Chan, J. C. C. (2014). Statistical Modeling and Computation. New York: Springer-Verlag New York.

Li, G.-H., Le, G.-W., Shi, Y.-H., & Shrestha, S. (2004). Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research*, 24(7), 469–486. http://doi.org/10.1016/j.nutres.2003.10.014

Li, L., Wang, J., Zhao, M., Cui, C., & Jiang, Y. (2006). Artificial neural network for production of antioxidant peptides derived from bighead carp muscles with alcalase. *Food Technology and Biotechnology*, 44(3), 441–448.

Li, Y., Zhou, J., Huang, K., Sun, Y., & Zeng, X. (2012). Purification of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide with an antihypertensive effect from loach (Misgurnus anguillicaudatus). Journal of Agricultural and Food Chemistry, 60(5), 1320–5. http://doi.org/10.1021/jf204118n

Morales-Medina, R., Pérez-Gálvez, R., Guadix, A., & Guadix, E. M. (2016). Artificial neuronal network modeling of the enzymatic hydrolysis of horse mackerel protein using protease mixtures. *Biochemical Engineering Journal*, 105, 364–370. http://doi.org/10.1016/j.bej.2015.10.009

Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. *Peptides*. Journal Article. http://doi.org/10.1016/j.peptides.2011.11.013

Nikolaev, I. V, Sforza, S., Lambertini, F., Ismailova, D. Y., Khotchenkov, V. P., Volik, V. G., Koroleva, O. V. (2016). Biocatalytic conversion of poultry processing leftovers: Optimization of hydrolytic conditions and peptide hydrolysate characterization. *Food Chemistry*, 197(Pt A), 611–21. http://doi.org/10.1016/j.foodchem.2015.10.114

Olsen, J. V, Ong, S. E., & Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular & Cellular Proteomics : MCP*, 3(6), 608–14. http://doi.org/10.1074/mcp.T400003-MCP200

Ono, S., Hosokawa, M., Miyashita, K., & Takahashi, K. (2006). Inhibition properties of dipeptides from salmon muscle hydrolysate on angiotensin I-converting enzyme. International *Journal of Food Science and Technology*, 41(4), 383–386.

Pérez-Gálvez, R., Almécija, M. C., Espejo, F. J., Guadix, E. M., & Guadix, A. (2011). Bi-objective optimisation of the enzymatic hydrolysis of porcine blood protein. *Biochemical Engineering Journal*, 53(3), 305–310. http://doi.org/10.1016/j.bej.2010.12.004

Pérez-Vega, J. A., Olivera-Castillo, L., Gómez-Ruiz, J. Á., & Hernández-Ledesma, B. (2013). Release of multifunctional peptides by gastrointestinal digestion of sea cucumber (*Isostichopus badionotus*). *Journal of Functional Foods*, 5(2), 869–877. http://doi.org/10.1016/j.jff.2013.01.036

Pires, C., & Batista, I. (2013). Functional Properties of Fish Protein Hydrolysates. In R. Pérez-Gálvez & J. P. Bergé (Eds.), Utilization of fish waste (pp. 59–75). Boca Raton, Florida: CRC Press - Taylor & Francis Group.

Qian, J., Zhang, H., & Liao, Q. (2011). The properties and kinetics of enzymatic reaction in the process of the enzymatic extraction of fish oil. *Journal of Food Science and Technology*, 48(3), 280–284. http://doi.org/10.1007/s13197-010-0128-8

Ren, X., Ma, L., Chu, J., Wang, Y., Zhuang, Y., Zhang, (2012). Optimization of Enzymatic Hydrolysis of Channel Catfish Bones for Preparing Antimicrobial Agents. *Journal of Aquatic Food Product Technology*, 21(2), 99–110. http://doi.org/10.1080/10498850.2011.586136

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: Meat and fish. *Nutrients*, 3(9), 765–791.

Salampessy, J., Reddy, N., Kailasapathy, K., & Phillips, M. (2015). Functional and potential therapeutic ACE-inhibitory peptides derived from bromelain hydrolysis of trevally proteins. *Journal of Functional Foods*, 14, 716–725. http://doi.org/10.1016/j.jff.2015.02.037

Samaranayaka, A. G. P., Kitts, D. D., & Li-Chan, E. C. Y. (2010). Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a Pacific Hake (*Merluccius productus*) fish protein hydrolysate subjected to simulated gastrointestinal digestion and Caco-2 cell permeation. *Journal of Agricultural and Food Chemistry*, 58(3), 1535–42. http://doi.org/10.1021/jf9033199

Seki, E., Osajima, K., Matsufuji, H., Matsui, T., & Osajima, Y. (1996). Resistance to gastrointestinal proteases of the short chain peptides having reductive effect in blood pressure. *Nippon Shokuhin Kagaku Kogaku Kaishi*, 43(5), 520–525.

Shalaby, S. M., Zakora, M., & Otte, J. (2006). Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. *The Journal of Dairy Research*, 73(2), 178–86. http://doi.org/10.1017/S0022029905001639

W. A. Karp, L. L. Desfosse, S. G. B., (2011). US National Bycatch Report. NOAA Tech, 1–508.

Valencia, P., Espinoza, K., Ceballos, A., Pinto, M., & Almonacid, S. (2015). Novel modeling methodology for the characterization of enzymatic hydrolysis of proteins. *Process Biochemistry*, 50(4), 589–597. http://doi.org/10.1016/j.procbio.2014.12.028

Valencia, P., Pinto, M., & Almonacid, S. (2014). Identification of the key mechanisms involved in the hydrolysis of fish protein by Alcalase. *Process Biochemistry*, 49(2), 258–264. http://doi.org/10.1016/j.procbio.2013.11.012

Vázquez, J. A., Blanco, M., Fraguas, J., Pastrana, L., & Pérez-Martín, R. (2015). Optimisation of the extraction and purification of chondroitin sulphate from head by-products of Prionace glauca by environmental friendly processes. *Food Chemistry*, 198, 28–35. http://doi.org/10.1016/j.foodchem.2015.10.087

Vercruysse, L., Van Camp, J., & Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A review. *Journal of Agricultural and Food Chemistry*. Journal Article. http://doi.org/10.1021/jf0508908

Wu, H., He, H.-L. L., Chen, X.-L. L., Sun, C.-Y. Y., Zhang, Y.-Z. Z., & Zhou, B.-C. C. (2008). Purification and identification of novel angiotensin-I-converting enzyme inhibitory peptides from shark meat hydrolysate. *Process Biochemistry*, 43(4), 457–461. Journal Article. http://doi.org/10.1016/j.procbio.2008.01.018

Zhang, Z., He, Z., & Guan, G. (1999). Thermal stability and thermodynamic analysis of native and methoxypolyethylene glycol modified trypsin. *Biotechnology Techniques*, 13, 781–786. http://doi.org/10.3109/10242429909003203

X. Multiobjective Optimization of the Antioxidant Activities of Horse Mackerel Hydrolysates Produced with Protease Mixtures [*]

Fish protein hydrolysates (FPH) from horse mackerel were produced by employing an enzyme mixture of subtilisin and trypsin. The antioxidant activity of fish hydrolysates (DPPH scavenging activity, Fe^{2+} chelating activity and Fe^{3+} reducing power) was modelled as a function of the operating conditions for the hydrolysis (*i.e.* protein concentration, temperature and composition of the enzyme mixture). The antioxidant activities showed different behavior depending on whether their controlling pathway was the transference of electrons/protons (*i.e.* DPPH scavenging activity and Fe^{3+} reducing power) or metal chelation. In the first case, the antioxidant activities increased with the decrease of substrate concentration and temperature when pure trypsin (DPPH scavenging activity) or a mixture of enzymes (Fe^{3+} reducing capacity) was employed. Contrary, hydrolysates showed higher Fe^{2+} chelating activities at moderate concentration and high temperature (*i.e.* 5g/L and 55ºC) employing solely subtilisin. The conflictive behavior among the antioxidant properties suggested using a multiobjective optimization technique. The Ɛ-constraint method was chosen for this purpose. This approach allows determining the most adequate operational conditions for producing hydrolysate with a specific antioxidant profile which is the first approximation to the production of taylor-made antioxidant hydrolysates.

---

[*] JOURNAL PAPER: R. Morales-Medina, R. Pérez-Gálvez, A. Guadix and E. M. Guadix. Multiobjective Optimization of the Antioxidant Activities of Horse Mackerel Hydrolysates Produced with Protease Mixtures *Process Biochemistry*. Accepted for publication.

1. INTRODUCTION

Lipid oxidation is the main cause of food quality deterioration. In the human body, it is responsible for oxidative stress, which might cause a number of diseases associated to cellular oxidative damage (Shahidi & Zhong, 2015). Antioxidant compounds are generally employed to prevent lipid oxidation in food systems. Antioxidants enlarge lipids' shelf life and prevent rancidity, which is associated to the loss of nutritional value and the development of off-flavors (Shahidi & Zhong, 2010). In food systems, lipid oxidation is catalyzed by heat, metals and light among others. Consequently, oxidation processes can follow several pathways such as autoxidation, photooxidation, thermal or enzymatic oxidation; being autoxidation the most common process in oxidative deterioration of foods (Shahidi, 2000). There are several compounds able to partially inhibit oxidative reactions, as radical scavengers, inactivators of peroxides (and other reactive oxygen species), singlet oxygen quenchers, reducing agents or metal ion chelators (Shahidi & Zhong, 2006). Hence, the measurement of antioxidant activity must be conducted employing a range of analysis which measures the aforementioned oxidation mechanisms as DPPH scavenging activity, ABTS, Fe^{3+} reducing power or Fe^{2+} chelating capacity (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002).

In the last decades, there has been an increasing interest in finding effective natural antioxidants able to replace the synthetic ones. In this context, fish hydrolysates have been widely described as a potential natural antioxidant (García-Moreno et al., 2014; Klompong, Benjakul, Kantachote, Hayes, & Shahidi, 2008; Kristinsson & Rasco, 2000; Zhou et al., 2012). Hence, the antioxidant potency of some available marine sources such as by-catches (the portion of the fishing catch which is not retained for sale but returned to the sea for any reason (Kelleher, 2005)) or fish by-products has been evaluated (Abdelhedi et al., 2016; Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; García-Moreno et al., 2014; Klompong, Benjakul, Kantachote, & Shahidi, 2007). Focusing on fish by-catch, since 1st of January of 2015, the EU Fisheries Common Policy has banned the discard of most pelagic fisheries such as horse mackerel or sardine. Consequently, a supplementary amount of by-catches with no commercial value must be retained on board and brought ashore. These materials represent a cheap and renewable raw material for the production of hydrolysates. In this sense, discarded species of the Alboran Sea (*i.e.* horse mackerel, sardine, bogue, small-spotted catshark among others) have been described as good raw

material for hydrolysates production since they present a high and constant protein content (16 to 22 wt%) throughout the year (Morales-Medina et al., 2015a, 2015b).

The antioxidant capacity of protein hydrolysates is mainly determined by the raw material and the extent of the enzymatic treatment. The raw protein determines both the amino acid composition and their sequence in the active peptides. Both properties strongly determine the antioxidant capacity of the peptides. Furthermore, the antioxidant activity of a hydrolysate is influenced by its molecular weight distribution, which is determined by the enzymatic treatment (*i.e.* type of protease and extent of the hydrolysis). In this case, high degrees of hydrolysis (DH) have been described to benefit the antioxidant activity. Several fish hydrolysates from yellow stripe trevally (*Selaroides leptolepis*), tuna backbone or cod produced with subtilisin, trypsin or flavourzyme have been described to present scavenging, reducing and chelating capacity (Farvin et al., 2014; Je, Qian, Byun, & Kim, 2007; Klompong et al., 2008, 2007). Also, the employment of mixture of proteases have shown a synergic effect in the case of horse mackerel (*Trachurus mediterraneus*), sardine (*Sardine pilchardus*) or bogue (*Boops boops*) treated with equimassic mixtures of subtilisin and trypsin (García-Moreno et al., 2014). Furthermore, fish hydrolysates have demonstrated their antioxidant capacity in the chemical stabilization oil in water emulsions where cod hydrolysates prevented oxidation (Farvin et al., 2014; Petursson, Decker, & McClements, 2004). Recently, oil in water emulsions were both physically and oxidatively stabilized employing solely fish hydrolysates (García-Moreno, Guadix, Guadix, & Jacobsen, 2016; Morales-Medina, Tamm, Guadix, Guadix, & Drusch, 2015).

Previous works have studied the effect of hydrolysis parameters on the antioxidant activities of protein hydrolysates (Klompong et al., 2007). However, mathematical models linking the main hydrolysis parameters (*e.g.* substrate concentration, temperature or enzyme substrate ratio) with the potential antioxidant properties of the resulting hydrolysates are scarce (Vaštag, Popović, Popović, Krimer, & Peričin, 2010). Furthermore, to the authors' knowledge, no studies have so far employed a mixture of proteases to produce antioxidant hydrolysates.

In this work horse mackerel (*Trachurus mediterraneus*) hydrolysates were produced employing a variable mixture of two commercial endoproteases (subtilisin and trypsin) and different operating conditions (*e.g.* temperature or substrate concentration). Three antioxidant properties, namely DPPH scavenging activity, Fe^{3+} reducing power and Fe^{2+} chelating activity were analyzed. Each antioxidant activity was related to the operating

conditions (*i.e.* protein concentration, temperature and composition of the enzyme binary mixture) by means of crossed mixture-process models. These equations allowed the optimization of every single antioxidant property. Finally, a multiobjective optimization was proposed, in order to obtain a set of intermediary solutions allowing the simultaneous maximization of the DPPH scavenging activity or Fe^{3+} reducing capacity for a given level of Fe^{2+} chelating power.

2. MATERIALS AND METHODS

2.1. Raw material and reagents

Raw horse mackerel (*Trachurus mediterraneus*) was provided by the fishing harbor of Motril (Spain). The raw material was kept in ice during the transportation and stored at -20ºC until protein separation.

The reagents 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (Ferrozine), butyl hydroxytoluene (BHT), α-tocopherol and ascorbic acid were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All other chemicals, namely ammonium thiocyanate, ferric chloride, EDTA and sodium hydroxide were of analytical grade (Sigma-Aldrich, Inc., St. Louis, MO, USA).

2.2. Substrate pretreatment

Prior to hydrolysis, the whole fish (including heads and viscera) was partially dewatered and defatted by hydraulic pressing. To that purpose, 3 kg of fishes were preheated at 40ºC for 30 min in a water bath and then they were fed in a hydraulic press (model ESP-K, Sanahuja, Spain). The pressing process consisted in three cycles at 120 bar interrupted by relaxation periods where the press liquor was released from the press chamber. The partially dewatered and defatted cake was homogenized in a cutter (SK-3, Sammic, Sevilla, Spain) and then stored at -20ºC until their use.

The proximate composition of the press cake was analyzed following the official methods described by the Association of the Official Analytical Chemists (AOAC). Total Nitrogen was measured according to the Kjeldahl method and expressed as crude protein using a conversion factor of 6.25 (Adler Nissen, 1986). The lipid content was analyzed following the Soxhlet semi-continuous extraction method. Finally, the moisture and ash content was

determined gravimetrically by heating the samples until achieving a constant weight at 103 and 550ºC, respectively (A.O.A.C., 2006).

2.3. Enzymes and hydrolysis procedure

Two serine endoproteases were employed for the enzymatic hydrolysis: subtilisin (EC 3.4.21.62) of bacterial origin secreted by Bacillus subtilis and trypsin (EC 3.4.21.4) from an animal source. Both enzymes were purchased from Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S, respectively.

The substrate of the hydrolysis was the dewatered cake remaining from the hydraulic pressing. The cake presented a composition of 18.54% of protein, 3.93% of lipid, 2.06% of ashes and 70.77% of water. A certain mass of press cake was homogenized with water until a final volume of 200 mL. The mixture was then fed into a jacketed reactor attached to a water bath. As catalyst, a mixture of the aforementioned proteases was added to the reaction vessel, adjusting the enzyme-substrate ratio at 1 wt% (related to the mass of protein in the reactor vessel) for all the hydrolysis. A designed experiment was built by varying the concentrations of protein in the reaction vessel (S, g/L), as well as the weight percentage of subtilisin (denoted as X_1, %) and trypsin (X_2, %) in the enzyme mixture. The mass of cake suspended in the vessel was calculated so the concentration of protein was 2.5, 5 or 7.5 grams of protein per liter of reaction mixture. After 4 hours, the hydrolysis was stopped by thermal deactivation of the proteases. To that end, the reaction mixture was heated at 100 ºC for 15 min in a water bath. This mixture was subsequently filtered through 8 µm filter paper (Whatman Grade 40, Sigma Aldrich Inc., St. Louis, MO, USA) to remove insoluble matter (*e.g.* scales, bones). The filtered hydrolysate was then freeze dried in a Labconco freeze drying system (Kansas City, MO, USA) at 5 mmHg and 15ºC. The resulting powders were stored at -20ºC until further analyses.

2.4. Experimental design and mathematical modeling

In this work, a crossed mixture-process was employed to relate the antioxidant properties to the reaction conditions. The percentage of subtilisin (X_1, %) and trypsin (X_2, %) in the enzyme mixture, the concentration of protein (S, g/L) and the temperature (T, ºC) were chosen as input variables. The composition of the enzyme mixture was varied from pure trypsin (*i.e.* 0% subtilisin) to pure subtilisin (*i.e.* 100% subtilisin) with three intermediate experimental levels containing 25%, 50% and 75% of subtilisin. As explained above, the

protein concentration in the reactor was tested at three levels: 2.5 g/L, 5 g/L and 7.5 g/L. This range was chosen taking into consideration that higher concentrations might hinder the homogenization and stirring of the reaction mixture. On the contrary, lower substrate concentration implies high energy cost in the stabilization process of the hydrolysate (*e.g.* by spray or freeze drying). Finally, the levels of reaction temperature were: 40ºC, 47.5ºC and 55ºC. In this range, both enzymes are thermally stable: subtilisin presents its maximum activity at 55ºC whereas trypsin at 40ºC. Consequently, 45 experimental runs ($5 \cdot 3 \cdot 3$) were conducted.

The mathematical models proposed in this work were constructed by combination of a binary mixture model and a factorial one. Each output variable (Y: DPPH scavenging activity, Fe^{3+} reducing power or Fe^{2+} chelating activity) were predicted as a function of the aforementioned input variables.

The relationship between the output variables and the enzyme composition can be expressed by a binary mixture model as shown in Eq. 1:

$$Y(X_1, X_2) = \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_{12} \cdot X_1 X_2 \quad [1]$$

where X_1 and X_2 correspond to the percentage of subtilisin and trypsin in the enzyme mixture, respectively. Due to the linear dependence between mixture variables (*i.e.* X_1 and X_2 add 100% for all the experimental runs), mixture models do not contain intercept, since any constant in Eq. 1 would be linear combination of X_1 and X_2.

As for the process variables (*i.e.* S and T), they were processed by employing a factorial model of second order:

$$Y(S, T) = \alpha_0 + \alpha_1 \cdot S + \alpha_2 \cdot T + \alpha_{12} \cdot S \cdot T + \alpha_{11} \cdot S^2 + \alpha_{22} \cdot T^2 \quad [2]$$

where S refers to the substrate concentration (g/L) and T to the reactor temperature (ºC).

A crossed mixture-process model was constructed relating the antioxidant properties to both mixture and process variables (Anderson & Whitcomb, 2000; Cornell, 2002). This new model was obtained by combination of Eq. 1 and Eq. 2 as follows:

$$Y(X_1, X_2, S, T) = (\alpha_0 + \alpha_1 \cdot S + \alpha_2 \cdot T + \alpha_{12} \cdot S \cdot T + \alpha_{11} \cdot S^2 + \alpha_{22} \cdot T^2) \cdot (\beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_{12} \cdot X_1 X_2) \quad [3]$$

The combined model expressed by Eq. 3 contains 18 coefficients which were estimated by multiple regression, employing a multivariate analysis software (StatGraphics 5.1, Statpoint Technologies, Inc., Warrenton, VA, USA). The statistical significance of each term in the crossed mixture process model was assessed by means of an ANOVA analysis. To this end, every term in the polynomial was associated to a p-value obtained by the Fisher's statistical test at a confidence level of 95%. Those terms judged as non-significant were removed from the model by stepwise backward selection, obtaining a simplified model where all the factors were significant. Finally the reduced models for the DPPH scavenging activity, the reducing power and the chelating activity were optimized to determine the conditions which maximize each single variable.

The fitness of the experimental data to the empirical models was assessed by the determination coefficient r^2, as well as their values adjusted to the degrees of freedom, which are preferred when comparing different models. Furthermore, the absolute optimums for DPPH, reducing and chelating activity were validated experimentally. To this end, the operating conditions (*i.e.* substrate concentration, temperature, enzyme composition) for the maximization of each single antioxidant activity were reproduced in the reactor vessel. Each optimum was validated by triplicate, producing nine protein hydrolysates whose antioxidant activities were determined. These experiments allowed evaluating the experimental error among triplicates as well as the deviation between the predicted optimums and their actual values.

2.5. Determination of the degree of hydrolysis

The degree of hydrolysis (DH) was monitored employing an automatic titrator (718 Stat Titrino, Metrohm, Switzerland) which used a 0.5 N solution of NaOH as titration agent.

As proposed in the pH-stat method, the DH can be calculated as a function (Eq. 4) of the base consumed to maintain the pH constant in the course of the reaction.

$$DH = \frac{V_b \cdot N_b}{m_P} \cdot \frac{1}{\alpha} \cdot \frac{1}{h_{tot}} \cdot 100 \qquad [4]$$

In Eq. 4, Vb (mL) is the amount of base consumed; Nb (meq/mL) is the normality of the base; mP (g) is the mass of protein in the substrate, htot (meq /g) is the number of milliequivalents of peptide bonds per gram of protein and (α) is the average degree of

dissociation of the α -NH2 amino groups. As widely accepted for fish proteins (Adler Nissen, 1986), the value of htot was fixed to 8.6 milliequivalents of peptide bonds per gram of protein. The average degree of dissociation, α, was calculated using Eq. 5:

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{pK}}}{1+10^{\mathrm{pH}-\mathrm{pK}}} \quad [5]$$

The pH was fixed at 8 and pK estimated as a function of temperature as following as described elsewhere (Steinhardt & Beychok, 1964)

$$\mathrm{pK} = 7.8 + \frac{298-\mathrm{T}}{298\cdot\mathrm{T}}\cdot\ 2400 \quad [6]$$

2.6. Determination of the antioxidant activity

Since antioxidant activity can be exerted by several pathways, three methods were employed to characterize the hydrolysates: DPPH scavenging activity, Fe^{3+} reducing power and iron (Fe^{2+}) chelating activity.

Each antioxidant property was determined at a fixed concentration, *i.e.* 3 mg/mL for DPPH scavenging activity, 10 mg/mL for Fe^{3+} reducing power and 0,6 mg/mL for Fe^{2+} chelating activity. The relation between the concentration and the antioxidant activity have been described for other substrates and DH (Klompong et al., 2008; Morales-Medina et al., 2015). In all cases, these properties show a linear dependence with the concentration until achieving a plateau at relatively high concentrations. These concentrations were selected taking into account a preliminary test (data not published) in which the linearity between the concentration and the antioxidant properties were studied.

2.6.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay

The DPPH scavenging activity of the hydrolysates was measured as described by Picot et al., (2010). Briefly, 1 mL of hydrolysate solution (3 mg/mL) was mixed with 1 mL of 0.1 mM DPPH in methanol. Then, samples were stirred and incubated for 30 min in the dark. The absorbance was measured at 517 nm. Additionally, a blank and a sample control were analyzed. The blank was prepared as described above but using distilled water instead of hydrolysate solution. In the sample control, methanol was employed instead of the DPPH solution. All measurements were done in duplicate. As positive control, a solution of BHT (0.02% w/w) was employed. DPPH scavenging activity was calculated by Eq. 7:

$$\text{DPPH scavenging activity, \%} = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \cdot 100 \quad [7]$$

2.6.2. Fe^{3+} reducing power assay

The reducing power was analyzed following the method described by Oyaizu, (1986). An aliquot of 2 mL of hydrolysate solution (10 mg/mL) was mixed with 2 mL of 0.2 mM phosphate buffer (pH 6.6) and 2 mL of 1% (w/v) potassium ferricyanide. The mixture was then stirred and incubated (50 °C, 20 min). After incubation, 2 mL of 10% (w/v) trichloroacetic acid was added and samples were centrifuged at 1500×g for 10 min. Then, 2 mL of supernatant were extracted and mixed with 2 mL of distilled water and 0.4 mL of 0.1 % (w/v) ferric chloride solution. This mixture was stirred and incubated at room temperature for 10 minutes. Finally, the absorbance was measured at 700 nm. Analyses were carried out in duplicate. An ascorbic acid solution of 0.009% w/w was employed as positive control.

2.6.3. Iron (Fe^{2+}) chelating activity assay

The iron chelating activity was determined as Decker & Welch, (1990) proposed. A mixture of 3.7 mL of distilled water, 0.1 mL of ferrous chloride solution (2 mM) and 1 mL of hydrolysate solution (0.6 mg/mL) was stirred and incubated at room temperature for 3 min. Then, 0.2 mL of ferrozine 5 mM was added and samples were then incubated at room temperature for 10 min. Finally absorbance was measured at 562 nm. In the case of the blank, water was employed instead of hydrolysate solution. Sample control was prepared without ferrozine and analysis were done in duplicate. A solution of EDTA (0.006% w/w) was employed as positive control. The chelating activity was calculated with Eq. 8:

$$Fe^{2+}\text{Chelating activity, \%} = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \cdot 100 \quad [8]$$

2.7. Size exclusion chromatography (SEC)

The operating conditions for the maximization of each single antioxidant property were reproduced in the reactor vessel. The resulting hydrolysates were freeze dried before analysis. Their molecular weight distribution was determined by means of a fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden)

employing a Superdex Peptide 10/300GL column (GE Healthcare, Uppsala, Sweden). To this end, aliquots of 100 μL of hydrolysate, containing 5 mg protein/mL, were eluted at 0.5 mL/min employing a mobile phase of 70:30 water/acetonitrile and 0.1% trifluoroacetic acid. The absorbance was measured at 280 nm. The following standards were employed for calibration: glycine (75 Da), alanine (89 Da), Phe-Gly-Gly (279 Da), $(Gly)_6$ (360 Da), vitamin B12 (1355 Da), insulin (5733 Da), aprotinin (6511 Da) and ribonuclease (13700 Da).

2.8. Multiobjective optimization

Multiple optimization problems arise when a set of objectives, possibly conflicting, need to be satisfied. Two or more objectives are conflicting when the improvement of one of them is detrimental for the others. Contrary to single optimization, the result of a multiple optimization problem is a set of intermediary solutions (*i.e.* non inferior solutions), so called the Pareto Front, which satisfies to a certain degree the different objectives (Collette & Siarry, 2013). There are several techniques employed to create Pareto Front, as weighed sum functions or ε-constraint. The former consists in optimizing an objective function which is formulated as combination of the single objectives (Kim & de Weck, 2004). The ε-constraint method was chosen in this work to obtain the Pareto Front (Halsall-Whitney & Thibault, 2006). This method consists in maximizing/minimizing one single objective while the others are formulated as constraint equations of the optimization problem. In our case, for a fixed value of chelating activity (noted as ε) both the DPPH activity and the reducing power were individually maximized. Both optimization problems can be formulated as follows:

$$
\begin{aligned}
&\textit{Maximize } DPPH(S,T,X_1,X_2)\\
&\qquad Reducing(S,T,X_1,X_2)\\
&\textit{Subjected to:}\\
&\qquad 2.5 \le S \le 7.5\\
&\qquad 40 \le T \le 55\\
&\qquad 0 \le X_1 \le 100\\
&\qquad X_2 = 100 - X_1\\
&\qquad Chelating(S,T,X_1,X_2) = \varepsilon
\end{aligned}
\qquad [9]
$$

Iterating this calculation within the experimental range of chelating activity (15-50% in this work), two Pareto Fronts were obtained for DPPH and Fe^{3+} reducing power, respectively. The GRG non-linear algorithm, implemented in the Solver Tool of the MS Excel software, was chosen for this purpose.

3. RESULTS AND DISCUSSION

3.1. Modeling and optimization of the antioxidant properties

DPPH scavenging assay, a reactive oxygen scavenging test, is commonly employed to determine the ability of a certain substrate to stabilize free radicals (*e.g.* by electron or hydrogen transfer). Reducing power assay, on the contrary, belongs to the redox potential tests. It measures the capacity of a compound to donate exclusively electrons to free radicals. However, reducing power might also exert a pro-oxidant activity when the reduced agents are metals considering that reduced metals are more effective oxidative catalyzers than the oxidized metals (Sathivel, 2010; Shahidi & Zhong, 2010). Electron and hydrogen atom transfer may also occur in parallel and the controlling mechanism varies depending on the properties and structure of the antioxidant (Prior, Wu, & Schaich, 2005). Additionally, metals catalyze lipid oxidation, they decompose hydroperoxides, produce alkyl radicals and reactive oxygen species (Shahidi & Zhong, 2010). Indeed, by means of Fenton reaction, reduced metal ion (cuprous and ferrous) may produce hydroxyl radicals which are one of the most harmful reactive oxygen in the case of lipid oxidation. Hence, metal chelators can act as antioxidant by metal coordination preventing catalytic effect of metals.

Antioxidant activity of peptides is closely related to their amino acids constituents, their sequences and their molecular weight (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Sabeena Farvin et al., 2016). The molecular weight distribution of the hydrolysates could be indirectly expressed as degree of hydrolysis (DH), the higher the DH the smaller the average size of hydrolysate. Peptides with molecular weight between 500 and 1500 Da have been described to present high antioxidant properties (Nalinanon, Benjakul, Kishimura, & Shahidi, 2011). In Table 1, the final DH of each run is listed. In most cases, high antioxidant activities corresponded to high DH (values larger than 12%), which implies low molecular weight distribution. Additionally, the composition and

distribution of the constituent amino acids within the peptide chain strongly determine the antioxidant activities. In this case, those amino acids located in terminal positions are of special interest (Chalamaiah et al., 2013). Consequently, main variations of antioxidant activities with similar DH may be mainly due to both the composition and distribution of amino acids in the peptide chain.

Since DPPH scavenging activity and reducing power are governed by the transfer of electrons or hydrogen, both models, optimization and validation are discussed together. In contrast, chelating activity is described individually since it follows a different antioxidant pathway.

3.1.1. DPPH scavenging activity and Fe^{3+} reducing power

Table 1 presents the observed values of DPPH scavenging activity and reducing power of the final hydrolysates under the experimental conditions assayed. The experimental DPPH scavenging activity ranged from 34.78 to 78.78 % (Table 1, # 37 and 4, respectively) whereas the experimental reducing power varied from 0.57 to 1.12 (Table 1, # 13 and 2). The current results are slightly higher than those reported for horse mackerel (T. mediterraneus) treated with an equimassic mixture of subtilisin and trypsin (García-Moreno et al., 2014) or with pure subtilisin or trypsin (Morales-Medina et al., 2015).

Both DPPH scavenging activity and reducing power were fitted to the composed model expressed by Eq. 3, yielding full polynomials of 18 coefficients (Table 2). After backward selection, two reduced models were obtained, containing 8 and 10 significant terms for the reducing power and DPPH, respectively. In both cases, coefficients of determination were greater than 0.99 (r^2 adjusted to the degree of freedom > 0.99).

Table 1.(A) Experimental design and measured values for the final DH, DPPH, Fe^{3+} reducing power and Fe^{2+} chelating activity. Experimental variables are substrate concentration (S, g/L), temperature (T, °C), percentage of subtilisin (X_1,%) and trypsin (X_2, %) in the enzyme mixture.

Nº	S, g/L	T, ºC	X_1, %	X_2,%	DH, %	DPPH	Reducing power	Chelating activity
1	2.5	40	100	0	13.24	77.78	1.074	29.23
2	2.5	40	75	25	13.40	78.40	1.124	28.33
3	2.5	40	50	50	16.48	77.75	1.052	30.55
4	2.5	40	25	75	15.91	78.78	1.023	35.13
5	2.5	40	0	100	13.40	74.56	0.839	24.63
6	5	40	100	0	12.74	52.88	0.673	31.91
7	5	40	75	25	14.12	48.01	0.770	25.88
8	5	40	50	50	13.19	45.72	0.765	22.28
9	5	40	25	75	12.97	48.36	0.697	18.52
10	5	40	0	100	10.70	52.61	0.602	18.87
11	7.5	40	100	0	13.04	50.40	0.750	28.38
12	7.5	40	75	25	12.17	43.11	0.596	19.34
13	7.5	40	50	50	13.71	47.83	0.570	9.95
14	7.5	40	25	75	13.15	40.01	0.601	29.53
15	7.5	40	0	100	8.97	51.22	0.656	15.86
16	2.5	47.5	100	0	14.15	72.28	0.890	13.93
17	2.5	47.5	75	25	14.81	66.63	0.839	22.48
18	2.5	47.5	50	50	15.09	72.27	0.761	30.13
19	2.5	47.5	25	75	14.32	74.39	0.970	19.71
20	2.5	47.5	0	100	11.16	74.84	0.801	21.96
21	5	47.5	100	0	13.37	42.18	0.650	28.55
22	5	47.5	75	25	10.50	36.33	0.614	31.10
23	5	47.5	50	50	11.61	36.02	0.647	18.35

Table 1. (B). Experimental design and measured values for the final DH, DPPH, Fe^{3+} reducing power and Fe^{2+} chelating activity. Experimental variables are substrate concentration (S, g/L), temperature (T, °C), percentage of subtilisin (X_1,%) and trypsin (X_2, %) in the enzyme mixture.

Nº	S, g/L	T, ºC	X_1, %	X_2,%	DH, %	DPPH	Reducing power	Chelating activity
24	5	47.5	25	75	9.49	37.13	0.635	13.44
25	5	47.5	0	100	7.34	47.68	0.666	20.98
26	7.5	47.5	100	0	13.01	43.51	0.775	22.48
27	7.5	47.5	75	25	10.85	43.37	0.761	10.12
28	7.5	47.5	50	50	14.05	46.70	0.792	39.34
29	7.5	47.5	25	75	12.74	41.30	0.708	42.56
30	7.5	47.5	0	100	9.17	39.67	0.578	30.09
31	2.5	55	100	0	12.24	65.44	0.867	35.24
32	2.5	55	75	25	11.59	70.71	0.935	37.36
33	2.5	55	50	50	12.12	68.93	0.792	38.87
34	2.5	55	25	75	12.24	66.73	0.753	28.55
35	2.5	55	0	100	8.90	67.17	0.624	25.07
36	5	55	100	0	14.73	44.85	0.945	53.02
37	5	55	75	25	9.70	34.78	0.617	11.06
38	5	55	50	50	8.69	36.41	0.650	20.12
39	5	55	25	75	10.96	41.92	0.724	19.24
40	5	55	0	100	7.39	42.09	0.677	19.48
41	7.5	55	100	0	13.89	51.09	0.812	37.16
42	7.5	55	75	25	13.37	41.60	0.904	34.12
43	7.5	55	50	50	12.29	47.85	0.833	32.65
44	7.5	55	25	75	13.98	49.21	0.710	20.49
45	7.5	55	0	100	10.14	53.57	0.771	35.87

The contour plots depicted in Fig. 1 and 2 present, for a fixed substrate concentration, the variation of the antioxidant properties as a function of the temperature and the subtilisin content in the enzyme mixture. Despite that DPPH scavenging activity and the Fe^{3+} reducing power act by different pathways, they both are related to electronic transfer and, generally, were influenced in a similar trend by the operational variables. Effectively, both properties presented their absolute maximum when substrate concentration was 2.5 g/L and at 40ºC (Fig. 1a and 2a). Contrary, the effect of the enzyme composition on both properties was different. Indeed, the theoretical maximum of DPPH scavenging activity (80.09 %, Table 3) was produced when pure trypsin was employed. In the case of reducing power, its theoretical maximum (1.25, Table 3) was achieved with an enzyme mixture containing 58% subtilisin and 42% trypsin.

Table 2. Regression coefficients for the crossed mixture process models. The variables are noted as: S (substrate concentration, g/L), T (temperature, ºC) and percentage composition of the enzyme mixture (subtilisin X_1, trypsin X_2). Associated probability values were computed for each term at 95% level of confidence.

Coefficient	DPPH, %		Fe^{3+} Reducing power		Fe^{2+} Chelating activity	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
X_1	3.56	p< 0.001	1.4E-02	p< 0.001	3.70471	p< 0.001
$X_1 \cdot S$	-3.2E-01	p< 0.001	-2.2E-03	0.014	1.4E-01	0.005
$X_1 \cdot S^2$	2.7E-02	p< 0.001	1.9E-04	0.033	-1.4E-02	0.006
$X_1 \cdot T$	-9.0E-02	0.012	0	ns	-1.7E-01	p< 0.001
$X_1 \cdot T^2$	8.9E-04	ns	0	ns	1.9E-03	p< 0.001
$X_1 \cdot S \cdot T$	0	ns	0	ns	0	ns
X_2	1.996	p< 0.001	0	ns	1.01805	p< 0.001
$X_2 \cdot S$	-4.0E-01	p< 0.001	0	ns	-3.0E-01	p< 0.001
$X_2 \cdot S^2$	2.5E-02	p< 0.001	0	ns	1.4E-02	0.005
$X_2 \cdot T$	-1.4E-02	0.002	3.0E-04	p< 0.001	-1.1E-02	0.005
$X_2 \cdot T^2$	0	ns	-3.1E-06	0.001	0	ns
$X_2 \cdot S \cdot T$	2.3E-03	0.005	0	ns	3.4E-03	0.003
$X_1 \cdot X_2$	0	ns	1.2E-03	p< 0.001	0	ns
$X_1 \cdot X_2 \cdot S$	-2.8E-04	0.009	-2.7E-04	p< 0.001	0	ns
$X_1 \cdot X_2 \cdot S^2$	0	ns	8.8E-06	0.012	7.2E-04	p< 0.001
$X_1 \cdot X_2 \cdot T$	0	ns	-1.9E-05	p< 0.001	3.1E-04	p< 0.001
$X_1 \cdot X_2 \cdot T^2$	0	ns	0	ns	0	ns
$X_1 \cdot X_2 \cdot S \cdot T$	0	ns	3.5E-06	p< 0.001	-1.4E-04	p< 0.001
Model		p< 0.001		p< 0.001		p< 0.001
R^2	0.9966		0.9922		0.9815	
R^2 (df)*	0.9953		0.9900		0.9732	

ns Non significant at 95% confidence level ($p > 0.05$)
*r^2 (df): r^2 adjusted to the degrees of freedom

This value is higher than the empirical ones determined for enzyme mixture containing 50 or 75% of subtilisin (Table 1, #2 and #3, 1.12 and 1.05, respectively). For intermediate substrate concentration (5 mg/mL), both antioxidant properties were maximized at 40ºC and employing only trypsin in the enzymatic mixture (Fig. 1b and 2b). Otherwise, at 7.5 g/L the local maximum of reducing power was produced at 55 ºC and employing 60% of subtilisin, whereas the local maximum for DPPH scavenging activity was obtained at 40ºC with pure trypsin (Fig. 1c and 2c). It can be concluded that those hydrolysates produced at lower temperatures presented higher antioxidant properties, except for the reducing power of the hydrolysates at 7.5 g/L.

The ratio between both proteases strongly determines the composition of the hydrolysates at the N and C-terminal. Effectively, subtilisin cleaves aromatic acid and methionine residues (Adamson & Reynolds, 1996) while trypsin is more selective, cleaving exclusively arginine and lysine residues (Olsen, Ong, & Mann, 2004). Additionally, at temperatures comprised between 40 and 45ºC trypsin also cleaves peptides bonds with Tyr, Trp and Phe at the carboxyl side (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010). High DPPH scavenging activity is related to the presence of hydrophobic amino acids and to the presence of Val, Leu, Ile, Ala, Phe or Lys at the N-terminal (Suetsuna, Ukeda, & Ochi, 2000). In the case of horse mackerel (*Trachurus mediterraneus*) muscle, these residues represent 30 wt% of the amino acid composition (Morales-Medina et al., 2015). By means of hydrolysis with subtilisin Met, Phe, Tyr and Trp residues might be released. Furthermore, at low temperature (<45ºC) trypsin presents enhanced proteolytic activity (*i.e.* it releases Tyr, Trp or Phe, apart from Lys and Arg) which leads to hydrolysates with improved scavenging properties. Indeed, Tyr residues located at the C-terminal have exhibited high scavenging properties (Chen et al., 1998).

The operating conditions (temperature, protein concentration and subtilisin content) for the maximization of the DPPH scavenging activity and reducing power were reproduced to validate the results predicted by the proposed model (Table 3). DPPH scavenging activity presented an optimal value of 80.09 % (T = 40ºC, S_0 = 2.5 mg/mL, X_1 =0), while the experimental value of hydrolysates produced under those conditions was 75.6 ± 4.5 % (relative error of 5.6%). In the case of reducing power, both theoretical and experimental values (produced at T 40ºC, S0 = 2.5 mg/mL, X_1 =58%) were similar 1.25 and 1.04 ± 0.14, (relative error: 10 %). Hence, the proposed models are able to satisfactorily predict the optimal values with acceptable deviation.

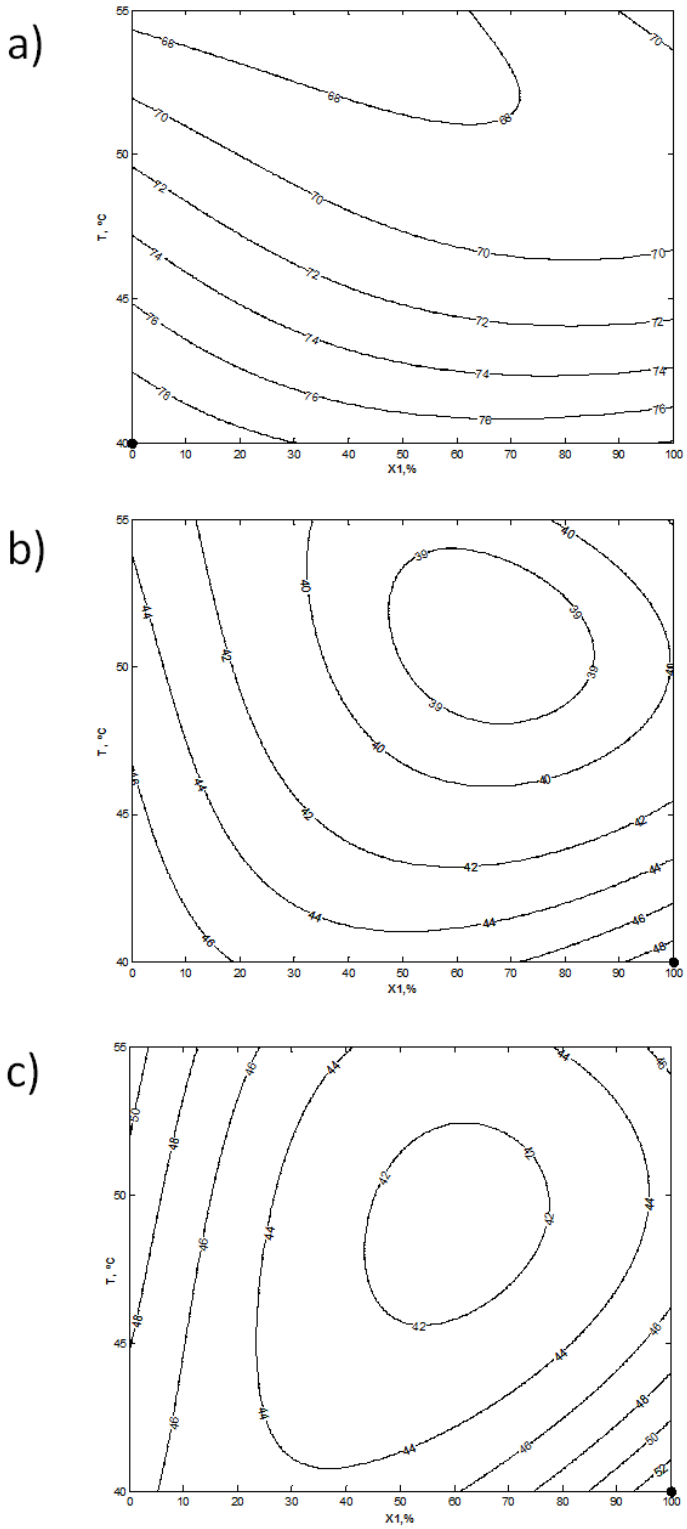


Figure 1. Contour plot of the DPPH scavenging activity as a function of the reaction temperature (T,ºC) and the percentage of subtilisin in the enzyme mixture (X1, %) at three levels of substrate concentration assayed: (a) 2.5 g/L, (b) 5 g/L and (c) 7.5 g/L. Relative maximums are marked with black dots.

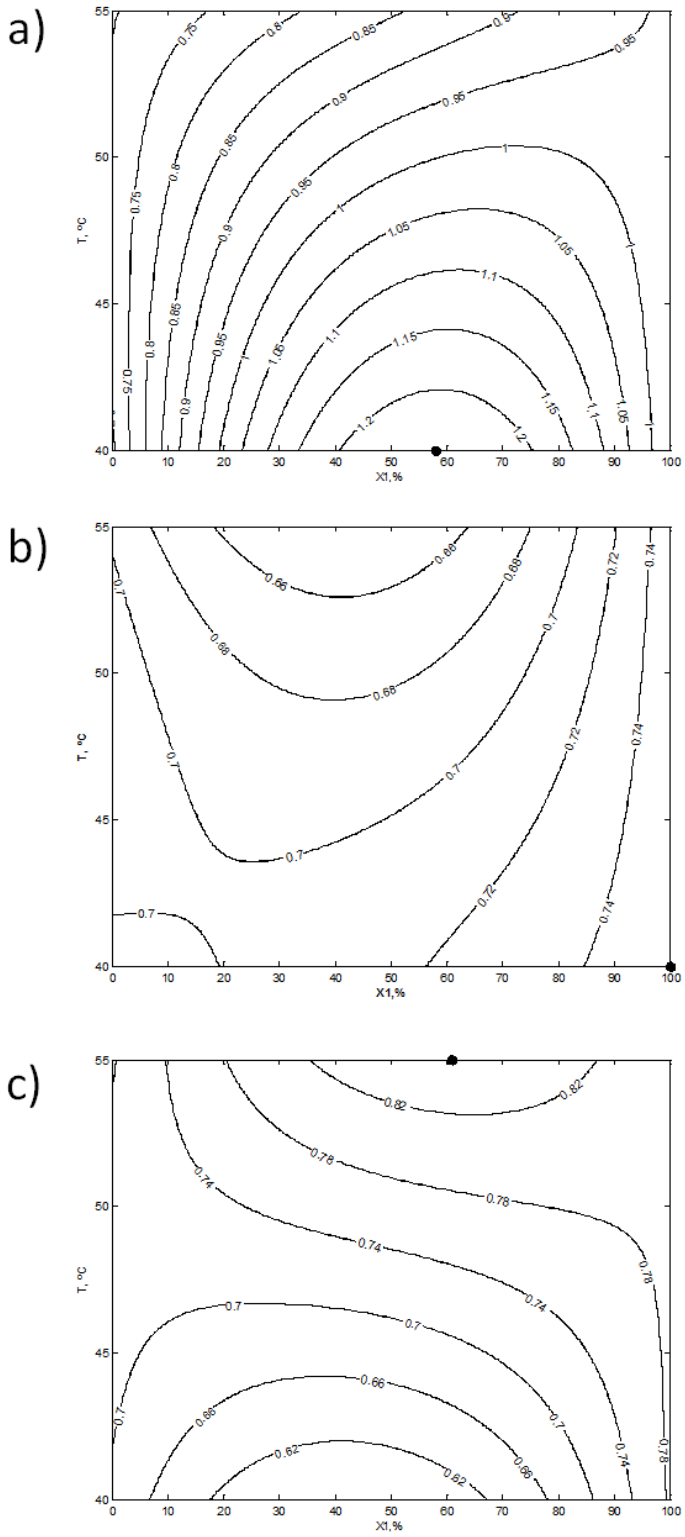


Figure 2. Contour plot of the reducing power as a function of the reaction temperature (T, ºC) and the percentage of subtilisin in the enzyme mixture (X1, %) at three levels of substrate concentration assayed: (a) 2.5 g/L, (b) 5 g/L and (c) 7.5 g/L. Relative maximums are marked with black dots.

Additionally, the molecular weight distribution of the hydrolysates produced under the optimal conditions was analyzed (Table 3). It has been described that, usually, antioxidant peptides are short (between 5 and 16 amino acids) (Halim, Yusof, & Sarbon, 2016) with molecular weight between 500 and 1500 Da (Nalinanon et al., 2011). In the case of hydrolysates with maximum DPPH scavenging activity and reducing power, they contained around 15% of hydrolysates with size between 500 and 1500 Da. The most significant fraction corresponded to low weight oligopeptides (100 to 500 Da) accounting around 40 % of the total. Hexapeptide Asn-His-Arg-Tyr-Asp-Arg (856 Da) and Ala–Cys–Phe–Leu (518.5 Da) have been identified as high scavengers present in hydrolysates of horse mackerel (Sampath Kumar, Nazeer, & Jaiganesh, 2011, 2012).Also, in the case of hydrolysates from Cobia skin (Rachycentron canadum), peptides with size below 700 Da were those with more intense scavenging activity (Yang, Ho, Chu, & Chow, 2008). Additionally, a relative high content of free amino acids has been described to favor the scavenging activity (Yang et al., 2008). Similar influence of the molecular weight distribution of peptides has been described for reducing power. Effectively, a mixture of peptides composed mainly by low molecular weight components and free amino acids, showed significant higher reducing properties than larger peptides (Farvin et al., 2014).

3.1.2. Fe^{2+} Chelating activity

Experimental results of chelating activity varied between 9.95% and 53.02% (Table 1. #13 and 36). Although these values are lower than those obtained for DPPH, it must be taken into account that chelating activities were analyzed in a much lower hydrolysate concentration (0.6 mg/mL) than the DPPH scavenging activity or reducing power. Consequently, among the three antioxidant activities studied, chelating was the most powerful. As happened to the previous antioxidant activities, chelating activity of hydrolysates is mainly determined by the amino acids composition and distribution. Glu, Asp, Lys or Arg at side chains can act as chelators (Liu, Kong, Xiong, & Xia, 2010), also His residues show higher affinity to metal coordination when located at the N-terminus rather than at the C-terminus (Chen et al., 1998). Fish proteins present a high content of glutamic and aspartic acid (Limin, Feng, & Jing, 2006), in the case of horse mackerel these amino acids represented a ~ 45 wt% of the total percentage that increased up to ~54 wt% when including Lys and Arg (Morales-Medina et al., 2015). Hence, the high chelating activity is caused by the high content of amino acids able to present chelating activity.

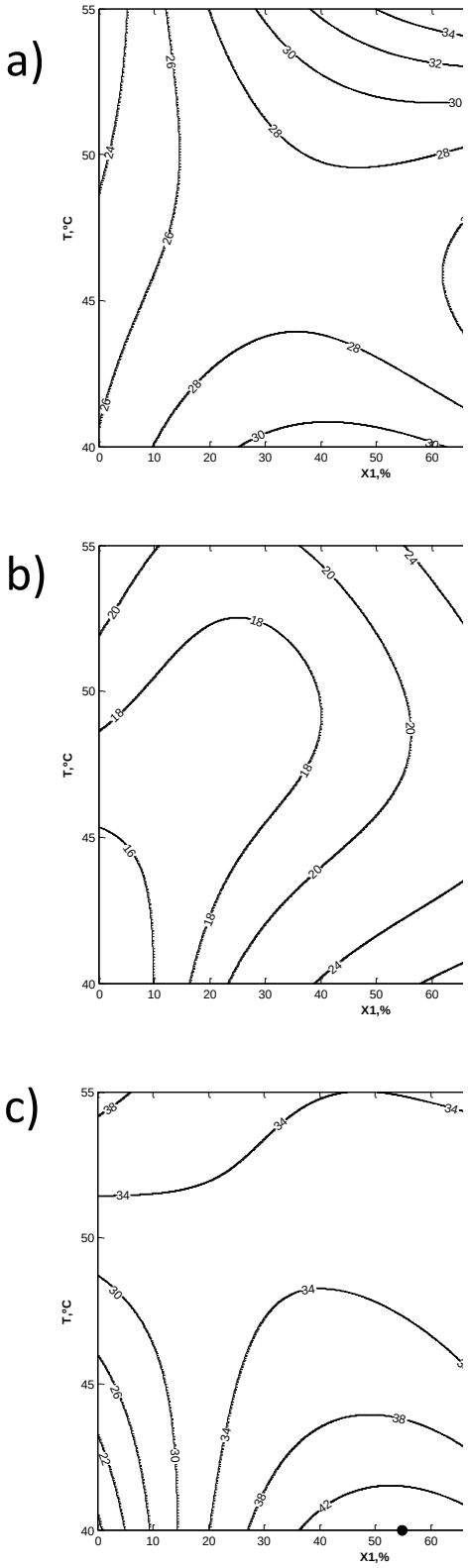


Figure 3. Contour plot of the chelating activity as a function of the reaction temperature and the percentage of subtilisin in the enzyme mixture (X1) at three levels of substrate concentration assayed: (a) 2.5 g/L, (b) 5 g/L and (c) 7.5 g/L. Relative maximums are marked with black dots

As done in the previous section, the experimental data were fitted to the complete model expressed by Eq. 3 and a backward selection reduction was employed to simplify the model until the remaining terms were significant. Chelating activity was finally fitted to a polynomial containing 13 coefficients (Table 2). The predicted model was employed to generate three c plots (Fig. 3) where the substrate concentration was fixed.

Chelating activity presents a theoretical absolute maximum (47.44%) for the hydrolysate produced at 5g/L, 55ºC and employing pure subtilisin (Fig. 3b). This predicted value is slightly lower than the experimental one (53%, Table 1, #36). A similar behavior was observed at the lowest substrate concentration (Fig. 3a) where the local maximum was obtained in comparable temperature and enzyme mixture composition. Contrary, at increasing levels of substrate concentration, the local maximum was found at 40ºC and employing ~55% of subtilisin.

Subtilisin exerts its higher enzymatic activity at 55ºC and, additionally, it shows preference for sites containing hydrophobic residues, particularly when Glu is adjacent to the N-terminal position (Adamson & Reynolds, 1996). Hence, the use of pure subtilisin at its optimal temperature may lead to a maximum production of hydrolysates with Glu in the terminal position and, consequently, to higher chelating activity. Effectively, these conditions led to one of the highest values of DH (14.73%, Table 1 #36). Also, trypsin may produce peptides containing Lys and Arg residues at terminal position, which may be responsible for the chelating activity (Liu et al., 2010). Hence, in these complex system, the resulting antioxidant properties are a trade-off between enzyme selectivity and activity and the effect of temperature and substrate concentration. Effectively, higher temperature favors the solubility of the substrate but might cause enzyme denaturation. Also, high concentrations of protein may result in substrate inhibition and low solubility of the substrate. In a previous work, the influence of the operational variable on the extent of the hydrolysis was described in detail (Morales-Medina, Pérez-Gálvez, Guadix, & Guadix, 2016).

As done for the other two antioxidant properties, the optimal conditions that led to the maximum chelating activity were replicate (three times) achieving values closed to the predicted by the model (Table 3). Effectively, the variation between the theoretical maximum (47.44 %) and the validation (50.61 ± 5.8%) represented a variation smaller than 7%. Additionally, the molecular weight distribution of the hydrolysates produced for the validation was analyzed (Table 3). Due to the intense conditions of the hydrolysis (DH

~15%) ~90 % of peptides were smaller than 1500 Da. Some studies have described high metal chelating activities for low molecular weight protein fractions and an increase of this property with increasing degree of hydrolysis (Klompong et al., 2007). Small peptides as Ser-Cys-His (MW: 345 Da) produced from Alaska pollock skin has been identified as good chelators (Guo et al., 2013). However, also large molecular weight peptides are able to show high chelating activities as happened to Ser-$(Gly)_7$-Leu-Gly-Ser-$(Gly)_2$-Ser-Ile-Arg and Ile-$(Glu)_2$-Leu-$(Glu)_3$-Ile-Glu-Ala-Glu-Arg produced from anchovy muscle with average size of 8000 Da (Wu, Liu, Zhao, & Zeng, 2012).

Table 3. Summary of the absolute optimums of DPPH scavenging activity, Fe^{3+} reducing power and Fe^{2+} chelating activity: operating conditions, validation and molecular weight distribution.

Antioxidant property	Operating conditions				Optimal activity		Molecular weight distribution (percentage area under the curve)			
	T, ºC	S, g/L	X_1, %	X_2, %	Predicted	Experimental*	>1500 Da	1500 to 500 Da	500 to 100 Da	< 100 Da
DPPH	40	2.5	0	100	80.1%	75.6 ± 4.5 %	10.4	13.8	42.0	33.7
Fe^{3+} Reducing	40	2.5	58	42	1.25	1.04 ± 0.14	12.2	14.5	42.5	30.9
Fe^{2+} Chelating	55	5	100	0	47.4%	50.6 ± 5.8 %	11.6	13.9	44.5	30.0

3.2. Multi-objective optimization

The mechanisms by which reducing power and DPPH scavenging activity act are related to electronic transference, contrary, chelating activity follows a different pathway. Hence, the bi-objective optimization problem was stated as the maximization of DPPH or reducing power while the chelating activity was restrained at a fixed value. In the Fig. 4, for a given chelating activity value it is shown the maximum value of DPPH scavenging activity (Fig. 4a) and reducing capacity (Fig. 4b), respectively.

Both graphs present a quite similar shape with three different sections: (i) increase of DPPH scavenging or reducing power with the chelating activity, (ii) a plateau where DPPH scavenging and reducing power remained almost unaltered (iii) decrease of the DPPH scavenging or reducing power with the increase of the chelating capacity. Consequently, the production of hydrolysates with high chelating activity can be accomplished at the expense of lower DPPH scavenging activity and reducing power. Additionally, most of the optimal values of DPPH scavenging activity or reducing power were attained under the same operating conditions (*i.e.* temperature, substrate concentration and enzyme composition). Considering the first region, where chelating activities ranged between 15

and 30 %, the scavenging activity increased until achieving an absolute maximum (80.09 % Table 3) and then slightly decreased. Contrary, Fe^{3+} reducing power continuously increased until achieving an absolute maximum (1.25, Table 3). In this first section, all the individual optimal solutions were obtained at low substrate concentration (2.5 g/L), while the temperature decreased from 45 to 40ºC and the subtilisin content was decreasing from 100 to 10 %.

In the second section of the graphs (Fig. 4), both scavenging activity and reducing power remained almost constant with values closed to 70% and 0.9, respectively. These antioxidant properties were produced with pure subtilisin, 2.5 g/L of substrate and at high temperature (53 to 55ºC). Finally, in the third section (chelating activity values from 35 to 50%) pure subtilisin and high temperature (55ºC) were required while the substrate concentration increased from 2.6 to 4.7 g/L. In this section, both the DPPH scavenging activity and reducing capacity decreased continuously until achieving minimum values of 44 % and 0.766, respectively.

In the food industry antioxidants are widely employed for stabilizing oils with high content of polyunsaturated fatty acids. These antioxidants can be incorporated directly to the oil bulk, although to enhance the shelf life of oils they are usually stabilized as oil in water emulsions or microcapsules in the presence of antioxidant (Farvin et al., 2014; García-Moreno et al., 2016; Morales-Medina et al., 2015; Petursson et al., 2004). In this context fish hydrolysates are gaining interest since they present both interfacial and antioxidant activities. Hence, hydrolysates with hydrophobic residues are desired since they can enhance the solubility of the hydrolysates in a lipid matrix and, subsequently, improving the contact to hydrophobic radicals (Farvin et al., 2014). Also, hydrolysates with hydrophobic residues can act in water-lipid interfaces allowing close contact with lipids (Mendis, Rajapakse, & Kim, 2005) and preventing oxidation. Furthermore, hydrophobic residues are the main responsible of scavenging and reducing capacity. Horse mackerel hydrolysates have high *in vitro* antioxidant activities and these properties can be modulated by varying the operational variables, *i.e.* hydrolysates with high chelating activity might be produced for stabilizing oils with a high metal content. However, further studies should be conducted to study the efficiency of these antioxidants in *in vivo* systems and to elucidate the relation between *in vitro* and *in vivo* antioxidant activities.

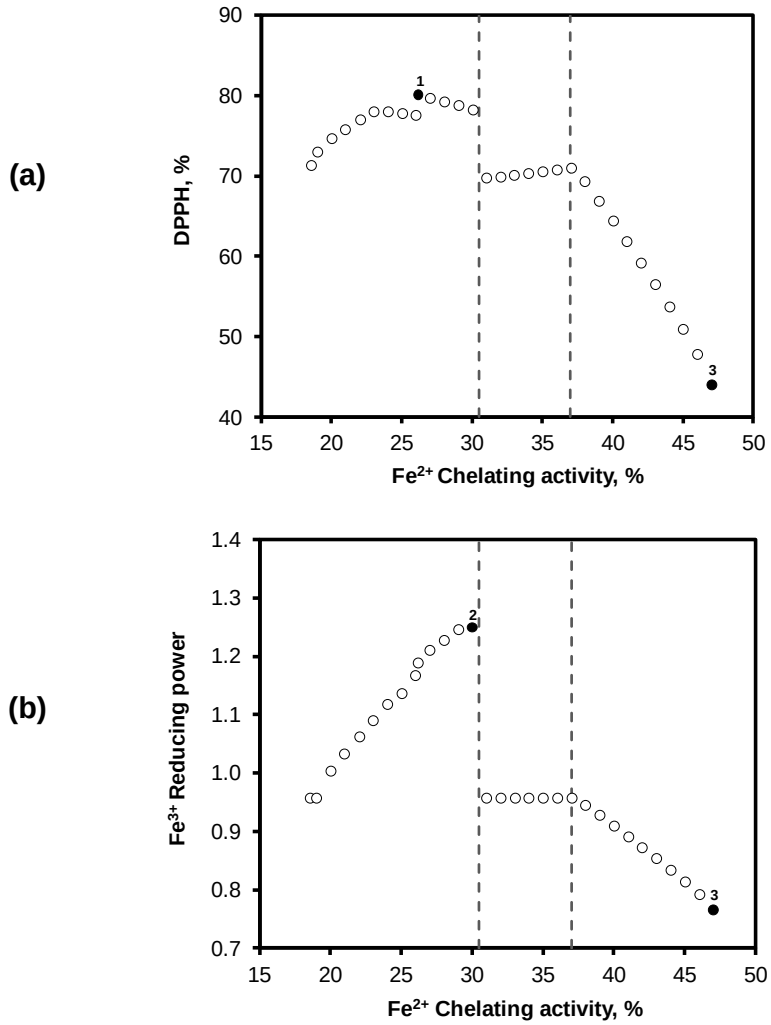


Figure 4. Solutions of the multiobjective optimization (Pareto Front): (a) maximum DPPH scavenging activity against Fe^{2+} chelating activity or (b) maximum Fe^{3+} reducing power against Fe^{2+} chelating activity . The absolute optimums of DPPH scavenging activity, Fe^{3+} reducing power and Fe^{2+} chelating activity (summarized in Table 3) are marked as 1, 2 and 3, respectively

4. CONCLUSIONS

Horse mackerel hydrolysates produced with a mixture of subtilisin and trypsin presented high antioxidant activities (DPPH scavenging activity, Fe^{3+} reducing power and Fe^{2+} chelating activity). The proposed crossed process- mixture model fitted satisfactorily the observed antioxidant activities and the predicted ones ($r^2 > 0.9815$). Furthermore, these mathematical models were optimized to determine the operating conditions maximizing each antioxidant activity. Optimal DPPH scavenging activity (80.09%) and reducing power (1.25) were attained under the same levels of substrate (2.5 g/L of protein in the reactor vessel) and temperature (40ºC). However, the composition of the enzyme mixture was different for each optimum. Indeed, the maximum scavenging activity was reached by employing pure trypsin as catalyst, while that of reducing power corresponded to an

enzyme mixture comprising 58% of subtilisin and 42% trypsin. In the case of chelating activity, the optimal conditions were 5 g/L of substrate, 55ºC and 100% of subtilisin. It was observed that the experimental conditions leading to an optimal activity (*e.g*. low temperature for maximum DPPH) could affect negatively the others (*e.g.* chelating activity). This conflictive behavior suggested employing a multi-objective optimization technique. To this regard, the ε-constraint method allowed obtaining a set of intermediary solutions (the so-called Pareto Front) which maximized to a certain degree the three activities.

Further studies are required to study the efficiency of these antioxidants *in vivo* systems and elucidate the relation between *in vitro* and *in vivo* antioxidant activities.

5. REFERENCES

A.O.A.C. (Association of Official Analytical Chemist). (2006). Official Methods of Analysis of the AOAC, Association of Official Analytical Chemists,. Washington DC.

Abdelhedi, O., Jridi, M., Jemil, I., Mora, L., Toldrá, F., Aristoy, M.-C., Nasri, R. (2016). Combined biocatalytic conversion of smooth hound viscera: Protein hydrolysates elaboration and assessment of their antioxidant, anti-ACE and antibacterial activities. *Food Research International*, 86, 9–23. http://doi.org/10.1016/j.foodres.2016.05.013

Adamson, N. J., & Reynolds, E. C. (1996). Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. *Enzyme and Microbial Technology*, 19(3), 202–207. http://doi.org/10.1016/0141-0229(95)00232-4

Adler Nissen. (1986). Enzymic hydrolysis of food proteins. London: Elsevier Applied Science Publishers LTD.

Anderson, M. J., & Whitcomb, P. J. (2000). Design experiments that combine mixture components with process factors. *Chemical Engineering Progress*, 96(12), 27–32.

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. (2002). Methods for testing antioxidant activity. *The Analyst*, 127(1), 183–198. http://doi.org/10.1039/b009171p

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food Chemistry*, 135(4), 3020–38. http://doi.org/10.1016/j.foodchem.2012.06.100

Chalamaiah, M., Jyothirmayi, T., Bhaskarachary, K., Vajreswari,, Hemalatha, R., & Dinesh Kumar, B. (2013). Chemical composition, molecular mass distribution and antioxidant capacity of rohu (*Labeo rohita*) roe (egg) protein hydrolysates prepared by gastrointestinal proteases. *Food Research International*, 52(1), 221–229. http://doi.org/10.1016/j.foodres.2013.03.020

Cheison, S. C., Schmitt, M., Leeb, E., Letzel, T., & Kulozik, U. (2010). Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β-lactoglobulin: Analysis by LC–ESI-TOF/MS. *Food Chemistry*, 121(2), 457–467. http://doi.org/10.1016/j.foodchem.2009.12.065

Chen, H., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative Properties of Histidine-Containing Peptides Designed from Peptide Fragments Found in the

Digests of a Soybean Protein. *Journal of Agricultural and Food Chemistry*, 46(1), 49–53. http://doi.org/Doi 10.1021/Jf970649w

Collette, Y., & Siarry, P. (2013). Multiobjective Optimization: Principles and Case Studies. Springer Science & Business Media.

Cornell, J. A. (2002). Experiments with mixtures : designs, models, and the analysis of mixture data. New York: John Wiley & Sons, Inc.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. Journal of Agricultural and *Food Chemistry*, 38(3), 674–677. http://doi.org/10.1021 /jf00093a019

Farvin, K. H. S., Lystbæk Andersen, L., Hauch Nielsen, H., Jacobsen, C., Jakobsen, G., Johansson, I., & Jessen, F. (2014). Antioxidant activity of Cod (*Gadus morhua*) protein hydrolysates: *in vitro* assays and evaluation in 5% fish oil-in-water emulsion. *Food Chemistry*, 149, 326–34. http://doi.org/10.1016/j.foodchem.2013.03.075

García-Moreno, P. J., Batista, I., Pires, C., Bandarra, N. M., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2014). Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species. *Food Research International*, 65, Part C, 469–476. http://doi.org/10.1016/j.foodres.2014.03.061

García-Moreno, P. J., Guadix, A., Guadix, E. M., & Jacobsen, C. (2016). Physical and oxidative stability of fish oil-in-water emulsions stabilized with fish protein hydrolysates. *Food Chemistry*, 203, 124–35. http://doi.org/10.1016/j.foodchem.2016.02.073

Guo, L., Hou, H., Li, B., Zhang, Z., Wang, S., & Zhao, X. (2013). Preparation, isolation and identification of iron-chelating peptides derived from Alaska pollock skin. *Process Biochemistry*, 48(5–6), 988–993. http://doi.org/10.1016/j.procbio.2013.04.013

Halim, N. R. A., Yusof, H. M., & Sarbon, N. M. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology*, 51, 24–33. http://doi.org/10.1016/j.tifs.2016.02.007

Halsall-Whitney, H., & Thibault, J. (2006). Multi-objective optimization for chemical processes and controller design: Approximating and classifying the Pareto domain. *Computers and Chemical Engineering*, 30(6–7), 1155–1168.

Je, J. Y., Qian, Z. J., Byun, H. G., & Kim, S. K. (2007). Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry*, 42(5), 840–846. http://doi.org/10.1016/j.procbio.2007.02.006

Kelleher, K. (2005). Discards in the world's marine fisheries: An update. FAO. Fisheries Technical Paper.

Kim, I. Y., & de Weck, O. L. (2004). Adaptive weighted-sum method for bi-objective optimization: Pareto front generation. *Structural and Multidisciplinary Optimization*, 29(2), 149–158. http://doi.org/10.1007/s00158-004-0465-1

Klompong, V., Benjakul, S., Kantachote, D., Hayes, K. D., & Shahidi, F. (2008). Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. International *Journal of Food Science & Technology*, 43(6), 1019–1026. http://doi.org/10.1111/j.1365-2621.2007.01555.x

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, 102(4), 1317–1327. http://doi.org/10.1016/j.foodchem.2006.07.016

Kristinsson, H. G., & Rasco, B. A. (2000). Biochemical and functional properties of Atlantic salmon (Salmo salar) muscle proteins hydrolyzed with various alkaline proteases. *Journal of Agricultural and Food Chemistry*, 48(3), 657–666.

Limin, L., Feng, X., & Jing, H. (2006). Amino acids composition difference and nutritive evaluation of the muscle of five species of marine fish, *Pseudosciaena crocea* (large yellow croaker), *Lateolabrax japonicus* (common sea perch), *Pagrosomus major* (red seabream), *Seriola dumerili* (Dumeril's) *Aquaculture Nutrition*, 12(1), 53–59. http://doi.org/10.1111/j.1365-2095.2006.00381.x

Liu, Q., Kong, B., Xiong, Y. L., & Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry*, 118(2), 403–410. http://doi.org/10.1016/j.foodchem.2009.05.013

Mendis, E., Rajapakse, N., & Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of Agricultural and Food Chemistry*, 53(3), 581–587.

Morales-Medina, R., García-Moreno, P. J., Pérez-Gálvez, R., Muñío, M., Guadix, A., & Guadix, E. M. (2015). Seasonal variations in the regiodistribution of oil extracted from small-spotted catshark and bogue. *Food Funct*., 6(8), 2646–2652. http://doi.org/10.1039/C5FO00448A

Morales-Medina, R., García-Moreno, P. J., Pérez-Gálvez, R., Muñío, M. M., Guadix, A., & Guadix, E. M. (2015). Nutritional indexes, fatty acids profile and regiodistribution of oil extracted from four discarded species of the Alboran Sea: *Seasonal effects. European Journal of Lipid Science and Technology*, In Press. http://doi.org/10.1002/ejlt.201500486

Morales-Medina, R., Pérez-Gálvez, R., Guadix, A., & Guadix, E. M. (2016). Artificial neuronal network modeling of the enzymatic hydrolysis of horse mackerel protein using protease mixtures. *Biochemical Engineering Journal*, 105, 364–370. http://doi.org/10.1016/j.bej.2015.10.009

Morales-Medina, R., Tamm, F., Guadix, A. M., Guadix, E. M., & Drusch, S. (2015). Functional and antioxidant properties of hydrolysates of sardine (*S. pilchardus*) and horse mackerel (*T. mediterraneus*) for the microencapsulation of fish oil by spray-drying. *Food Chemistry*, 194, 1208–1216. http://doi.org/10.1016/j.foodchem.2015.08.122

Nalinanon, S., Benjakul, S., Kishimura, H., & Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry*, 124(4), 1354–1362. http://doi.org/10.1016/j.foodchem.2010.07.089

Olsen, J. V, Ong, S. E., & Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular & Cellular Proteomics : MCP*, 3(6), 608–14. http://doi.org/10.1074/mcp.T400003-MCP200

Oyaizu, M. (1986). Studies on products of browning reactions: Antioxidative activities of browning products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, 44, 307–315. http://doi.org/http://dx.doi.org/10.5264/eiyogakuzashi.44.307

Petursson, S., Decker, E. a, & McClements, D. J. (2004). Stabilization of oil-in-water emulsions by cod protein extracts. Journal of Agricultural and *Food Chemistry*, 52(12), 3996–4001. http://doi.org/10.1021/jf035251g

Picot, L., Ravallec, R., Fouchereau-Péron, M., Vandanjon, L., Jaouen, P., Chaplain-Derouiniot, M., Bourseau, P. (2010). Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. *Journal of the Science of Food and Agriculture*, 90(11), 1819–26. Journal Article. http://doi.org/10.1002/jsfa.4020

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53(10), 4290–302. http://doi.org/10.1021/jf0502698

Sabeena Farvin, K. H., Andersen, L. L., Otte, J., Nielsen, H. H., Jessen, F., & Jacobsen, C. (2016). Antioxidant activity of cod (*Gadus morhua*) protein hydrolysates: Fractionation and

characterisation of peptide fractions. *Food Chemistry*, 204, 409–419. http://doi.org/10.1016/j.foodchem.2016.02.145

Sampath Kumar, N. S., Nazeer, R. A., & Jaiganesh, R. (2011). Purification and biochemical characterization of antioxidant peptide from horse mackerel (*Magalaspis cordyla*) viscera protein. *Peptides*, 32(7), 1496–501. http://doi.org/10.1016/j.peptides.2011.05.020

Sampath Kumar, N. S., Nazeer, R. A., & Jaiganesh, R. (2012). Purification and identification of antioxidant peptides from the skin protein hydrolysate of two marine fishes, horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*). *Amino Acids*, 42(5), 1641–9. http://doi.org/10.1007/s00726-011-0858-6

Sathivel, S. (2010). Fish oil extraction, purification and its properties. In C. Alasalvar, F. Shahidi, K. Miyashita, & U. Wanasundara (Eds.), Handbook of Seafood Quality, Safety and Health Applications (1st ed, pp. 423–430). West Sussex: John Wiley & Sons Ltd.

Shahidi, F. (2000). Antioxidants in food and food antioxidants. *Die Nahrung*.

Shahidi, F., & Zhong, Y. (2006). Antioxidants from marine by-products. In F. Shahidi (Ed.), Maximising the value of marine by-products (pp. 397–412). Cambridge: Woodhead Publishing. http://doi.org/10.1533/9781845692087.2.397

Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews*, 39(11), 4067–79. http://doi.org/10.1039/b922183m

Shahidi, F., & Zhong, Y. (2015). Measurement of antioxidant activity. *Journal of Functional Foods*, 18, 757–781. http://doi.org/10.1016/j.jff.2015.01.047

Steinhardt, H., & Beychok, S. (1964). Interaction of Proteins with Hydrogen Ions and Other Small Ions and Molecules. In H. Neurath (Ed.), The Proteins (pp. 139–304). New York: Academic Press. http://doi.org/10.1016/B978-0-12-395724-5.50012-0

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry*, 11(3), 128–131. http://doi.org/10.1016/S0955-2863(99)00083-2

Vaštag, Ž., Popović, L., Popović, S., Krimer, V., & Peričin, D. (2010). Hydrolysis of pumpkin oil cake protein isolate and free radical scavenging activity of hydrolysates: Influence of temperature, enzyme/substrate ratio and time. *Food and Bioproducts Processing*, 88(2–3), 277–282. http://doi.org/10.1016/j.fbp.2009.12.003

Wu, H., Liu, Z., Zhao, Y., & Zeng, M. (2012). Enzymatic preparation and characterization of iron-chelating peptides from anchovy (*Engraulis japonicus*) muscle protein. *Food Research Internationa*l, 48(2), 435–441. http://doi.org/10.1016/j.foodres.2012.04.013

Yang, J. I., Ho, H. Y., Chu, Y. J., & Chow, C. J. (2008). Characteristic and antioxidant activity of retorted gelatin hydrolysates from cobia (*Rachycentron canadum*) skin. *Food Chemistry*, 110(1), 128–136. http://doi.org/10.1016/j.foodchem.2008.01.072

Zhou, D. Y., Zhu, B. W., Qiao, L., Wu, H. T., Li, D. M., Yang, J. F., & Murata, Y. (2012). *In vitro* antioxidant activity of enzymatic hydrolysates prepared from abalone (*Haliotis discus hannai Ino*) viscera. *Food and Bioproducts Processing*, 90(2), 148–154. http://doi.org/10.1016/j.fbp.2011.02.002

XI. Functional and Antioxidant Properties of Hydrolysates of Sardine (*S. pilchardus*) and Horse Mackerel (*T. mediterraneus*) for the Microencapsulation of Fish Oil by Spray-drying [*]

The functionality of fish protein hydrolysates (FPH) for the microencapsulation of fish oil was investigated. Muscle protein from sardine (*S.pilchardus*) and horse mackerel (*T. mediterraneus*) was hydrolyzed using Alcalase or trypsin. Physically stable emulsions suitable for spray-drying were obtained when using FPH with a degree of hydrolysis of 5%. Microencapsulation efficiency amounted to 98±0.1% and oxidative stability of the encapsulated oil over a period of twelve weeks was in a similar range as it is reported for other matrix systems. Therefore, the suitability of FPH for use in spray-dried emulsions has been shown for the first time. Since no clear correlation between the antioxidative activity of the FPH and the course of lipid oxidation could be established future research is required to more specifically characterize the molecular structure of the peptides and its impact on protein alteration and role in lipid oxidation.

[*] JOURNAL PAPER. R. Morales-Medina, F. Tamm, A.M. Guadix, E.M. Guadix and S. Drusch. Functional and Antioxidant Properties of Hydrolysates of Sardine (*S. pilchardus*) and Horse Mackerel (*T. mediterraneus*) for the Microencapsulation of Fish Oil by Spray-drying. *Food Chemistry* 194:1208-1216. http://dx.doi.org/10.1016/j.foodchem.2015.08.122

1. INTRODUCTION

Fish industry produces a large amount of by-products among whom discards are considered the most significant waste of fish resources (Davies, Cripps, Nickson, & Porter, 2009). The term discard refers to "the portion of the total organic material of animal origin in the catch, which is thrown away, or dumped at sea for whatever reason" and in 2004 the average amount of discards was estimated around 7.3 million of tonnes/year (Kelleher, 2005). Discards do not only have a great ecological impact due to the alteration of trophic channels but also considerably affect the economic viability of the fishing sector. Due to this critical situation, the European Commission is undertaking an extensive reform in the common fisheries policy, adopting a set of measures towards the complete elimination of discards. However, the adaptation to the new policy requires technical solutions allowing the use of discards as raw materials for the production of added-value compounds.

In this context, functional and bioactive properties (antihypertensive, antioxidant or antimicrobial) of fish protein hydrolysates gain importance. Non-hydrolysed fish protein does not possess these properties because bioactive and functional peptide sequences are poorly accessible (Kim & Wijesekara, 2010). Another functional ingredient for promoting health is fish oil with its unique composition, namely its high content of polyunsaturated fatty acids (PUFA) of the Omega-3 family. More specifically, eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA) have been described as substances with anti-thrombotic, anti-arrhythmic and anti-inflammatory effects (Ruxton, 2011). However, due to the high level of unsaturation of Omega-3 PUFA they get easily oxidized resulting in the loss of their good organoleptic and nutritional properties. Hence, the stabilization of the oil against autoxidation is essential for its industrial use.

The addition of a combination of antioxidants with different mode of action to bulk fish oils (Drusch, Groß, & Schwarz, 2008) or emulsions (Serfert, Drusch, & Schwarz, 2009) achieved efficient stabilization. In addition, microencapsulation of fish oil by spray-drying in presence of antioxidants can further extend the shelf life of fish oil (Serfert et al., 2009). The enhancement of the stability is due to the amorphous structure of the carbohydrate-based glassy-state matrix of the microcapsules reducing the molecular mobility of oxygen and thus slowing down the rate of lipid oxidation. Regarding the use of antioxidants, it has been shown that antioxidants efficient in stabilizing liquid systems do not necessarily

increase the stability of an oil encapsulated by spray-drying (Serfert et al., 2009). Furthermore, the location of the antioxidants in the disperse system has impact on its antioxidant activity, ideally being positioned at the oil/water-interface with its specific microenvironment where autoxidation begins (Oehlke, Heins, Stöckmann, Sönnichsen, & Schwarz, 2011). Hence, there is increasing interest in the utilization of emulsifying compounds with antioxidant properties like proteins or protein hydrolysates. Recently it was shown that whey protein hydrolysates can increase the stability of microencapsulated fish oil compared to microcapsules stabilized with non-hydrolyzed whey protein (Tamm et al., 2015).

The antioxidant properties of FPH have been studied for a wide range of species and enzymes (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; García-Moreno et al., 2014; Klompong, Benjakul, Kantachote, & Shahidi, 2007; Wu, Chen, & Shiau, 2003). The antioxidant activity of the hydrolysates is affected by the protein source and the conditions of hydrolysis (*i.e.* degree of hydrolysis; enzyme, pH, substrate to enzyme ratio, etc.), since these factors determine the peptide profile being essentially responsible for the antioxidant and functional activities (Chalamaiah et al., 2012). Some of the discard species of Alboran Sea, such as sardine (*Sardina pilchardus*) or horse mackerel (*Trachurus mediterraneus*) have been described as an adequate substrate for the production of hydrolysates with antioxidative properties when treated with Alcalase, trypsin or their mixture even when achieving a relative high DH (>14%). These species exhibited DPPH radical scavenging activity, reducing power and iron chelating activity, however a high DH may involve too small peptides with no suitable technological properties for the stabilisation of emulsions (García-Moreno et al., 2014). The antioxidant and technological properties of FPH have been studied for hydrolysates of yellow stripe trevally (*S. leptolepis*) at different degree of hydrolysis (DH) and employing different enzymes (Klompong et al., 2007). However, the technological properties were described in terms of emulsifying or foaming activity, but did not include experiments on the stability of FPH emulsions or the behaviour during spray-drying. With respect to the use of FPH for the physical and oxidative stabilisation of emulsions, different molecular weight fractions of cod hydrolysates have been employed; nonetheless, the physical stabilisation of emulsions was conducted by adding emulsifiers such as Citrem instead of taking advantage of the interfacial properties of the FPH (Farvin et al., 2014). Physical stability of emulsions prepared using cod hydrolysates has been described, but no research on the oxidative

stability of the emulsified oil was conducted (Petursson, Decker, & McClements, 2004). To the best of the authors' knowledge, there are no previous studies related to the microencapsulation of fish oil stabilised with FPH.

In this work, two discarded species of the Alboran Sea, namely sardine (*Sardine pilchardus*) and horse mackerel (*Trachurus mediterraneu*s) were hydrolysed to a degree of hydrolysis (DH) 5 and 10 % (FPH(5) and FPH(10)) with two different enzymes: Alcalase and trypsin. FPH were used as emulsifiers and antioxidants in the production of emulsions (oil load 5%) and subsequent spray-drying to yield microcapsules with an oil load of 14.33%. Finally the course of lipid oxidation in these microcapsules (stored at 20ºC and 33% relative humidity) was monitored for a period of 12 weeks. The aim of this work was to study both functional and antioxidative properties of FPH to evaluate the potential of the use of FPH for the microencapsulation of sensitive lipophilic substances.

2. MATERIALS AND METHODS

Raw sardines (*Sardine pilchardus*) and horse mackerels (*Trachurus mediterraneus*) were purchased from the fishing harbour of Motril (Spain) in March 2014. They were kept in ice during the transportation and storage. Muscle meat was manually prepared by retiring bones, skin and viscera, grinded in a cutter SK-3 (Sammic, Guipúzcoa, Spain) and kept at -80°C until use. Refined fish oil (Omevital 18/12 TG Gold; 21% of EPA and 14% of DHA) was acquired from BASF Personal Care and Nutrition GmbH (Illertissen, Germany). For the enzymatic hydrolysis, two serine endoproteases were employed; subtilisine EC 3.4.21.62 of bacterial origin, and pancreatic trypsin, EC 3.4.21.4 from animal sources. Both enzymes were provided by Novozymes (Bagsvaerd, Denmark) namely Alcalase 2.4 L and PTN 6.0 S, respectively. Glucose syrup (DE38, C*Dry 1934) and MCT oil (CremerCOOR MCT 60/40) were purchased from Cargill Germany GmbH (Krefeld, Germany) and Cremer Oleo GmbH & Co. KG (Hamburg, Germany), respectively. All other chemicals and solvents used were of analytical grade.

2.1. Characterisation of the composition of the fish muscle meat and the fish protein hydrolysate

The proximate composition of the muscle meat and lyophilized hydrolysates was determined following the official methods of the AOAC (2006). Briefly, ash and moisture

content were gravimetrically measured by heating the samples until constant weight at 550ºC and 103ºC, respectively. Protein content was analysed following the Kjeldahl method and employing a nitrogen-to-protein conversion factor of 6.25. Total lipid fraction was evaluated according to the Soxhlet extraction method.

Amino acid composition of the protein hydrolysates was determined by complete acid digestion of the samples coupled with separation by reversed phase chromatography (H. Liu, Chang, Yan, Yu, & Liu, 1995). To this end, 100 mg of powdered sample were dissolved in 5 mL of 6 M HCl for 22 h at 112ºC. This reaction was conducted under nitrogen atmosphere to avoid amino acid oxidation. After cooling down, the hydrolysate was diluted 50-fold with MilliQ water and filtered through a 0.45 μm syringe filter (Cronun 25 mm PES FFPS2545, EMD Millipore, Billerica, MA, USA) to remove suspended particles. An aliquot of 10 μL of this filtrate were mixed with 70 μL of borate buffer and 20 μL of the derivatisation reagent (AccQ•Fluor Reagent WAT052880, Waters Corporation), consisting of a solution of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in acetonitrile. The reaction took place at 55ºC for 10 min, forming asymmetric urea derivatives which present high fluorescence emission.

The derivatives were subsequently separated by reversed-phase chromatography, employing a column of 2.1 mm (inner diameter) × 100 mm (length) packed with silica-based particles of 1.7 μm (AccQ•Tag Ultra RP Column 186003837, Waters Corporation, Milford, Massachusetts, USA).

The sample was eluted in a mobile phase composed of AccQ Tag Eluent A, Acetonitrile and water at 37ºC and the derivatives were detected after separation by UV detection at 248 nm.

To analyse the molecular weight distribution of the peptides produced, a tris-tricine SDS-PAGE under reducing conditions was conducted as described elsewhere (Tamm et al., 2015). Samples were diluted with tris-tricine buffer (Bio-Rad Laboratories GmbH, München, Germany). In order to detect the presence of disulphide bonds, these analyses were conducted in the presence and absence of 1 M DL-dithiothreitol (DTT) solution (Sigma Aldrich, Taufkierchen Germany) as reducing agent.

Samples were then heated to 90ºC for 5 min and loaded on a 16.5% tris-tricine gel (Bio-Rad cat# 345-0065). The separation was conducted employing tris-tricine running buffer (Bio-Rad, cat#161-0744). The gels fixation was conducted using an aqueous

methanol/acetic acid (40/10%) solution with 0.025% Coomassie brilliant blue G250 (Serva Electrophoresis GmbH, Heidelberg, Germany) were used to fisex gels. Finally, as marker a commercial mixture of seven peptides, which covered the range between 2.5 and 17 kDa (cat#MWSDS17S, Sigma Aldrich) was employed.

2.2. Enzymatic hydrolysis of fish muscle meat

An aliquot of muscle meat, containing 50g of protein, was homogenised with demineralised water until reaching a final volume of 1 L. For both degrees of hydrolysis (DH5 and DH10) the enzyme-substrate ratio was set to 0.5 for Alcalase and 0.125 for trypsin, whereas the temperature was fixed to 50°C and the pH to 8. Hydrolysis was conducted in an automatic titrator 718 Stat Titrino (Metrohm AG, Herisau, Switzerland) and the DH was estimated with the pH-stat-method, as a function of the base consumption (Eq. 1):

$$DH = \frac{V_b \cdot N_b}{m_P} \cdot \frac{1}{\alpha} \cdot \frac{1}{h_{tot}} \cdot 100 \quad [1]$$

In the equation 1, B refers to the amount of base consumed, Nb to the normality of the base, α to the average degree of dissociation of the α-NH_2 amino groups released during the hydrolysis, mp to the mass of protein in the substrate and h_{TOT} to the number of equivalents of peptide bonds per gram of protein. At pH 8 and temperature of 50°C, the 88.5% of the amino groups are dissociated, while h_{TOT} was assumed to be 8.6 meq/g of protein, as reported in literature (Adler Nissen, 1986). A solution of 1 M sodium hydroxide was employed to automatically adjust the pH. Samples were heated to 100°C for 15 min to deactivate the enzyme and then centrifuged (20.000g, 15 min) to extract manually the residual oil and remove the remaining solids. Finally the sample was filtered under vacuum (pore size: 10 µm). The purification step was carried out twice and samples were stored at -20°C until they were lyophilized in a Labconco freeze drying system (Kansas City, MO, USA).

2.3.Determination of the antioxidant activity and the interfacial tension of the fish protein hydrolysates

Antioxidant activity was measured employing three methods: DPPH scavenging activity, Fe^{3+} reducing power and iron (Fe^{2+}) chelating activity.

In order to evaluate the antioxidant activity, the evolution of the DPPH scavenging activity of the hydrolysates the method described by Picot et al. (2010) was used. Briefly, 1 mL of each protein hydrolysates solutions (ranging from 1 to 30 mg/mL) was mixed with 1 mL of 0.1 mM DPPH in methanol. The mixture was stirred and incubated for 30 min in the dark. Finally, the absorbance was measured at 517 nm. Furthermore, a blank was run in the same way by using distilled water instead of sample, and a sample control, using methanol instead of DPPH solution, was made for each sample. All measurements were done in duplicate. DPPH scavenging activity was calculated by Eq. 2:

$$\text{DPPH scavenging activity}, \% = \left(1 - \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{blank}}}\right) \cdot 100 \quad [2]$$

The reducing power was determined according to the method of Oyaizu (1986). An aliquot of 2 mL of each hydrolysate (1 to 30 mg/mL) were mixed with 2 mL of 0.2 mM phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. Then, samples were incubated (50°C, 20 min). After that, 2 mL of 10% TCA was added and they were centrifuged at 1500×g for 10 min. Finally, 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride were added to 2 mL of supernatant. After 10 minutes, the absorbance was measured at 700 nm. As a control, instead of sample distilled water was used. Analyses were carried out in triplicate.

The iron chelating activity of the fish protein hydrolysates was estimated by the method described by Decker and Welch, (1990). In short, 1 mL of hydrolysate solution (0.2 - 1.2mg/mL) was mixed with 3.7 mL of distilled water and 0.1 mL of ferrous chloride 2 mM. After 3 min, by adding 0.2 mL of ferrozine 5 mM the reaction was inhibited. Then, sample were stirred and incubated at room temperature during 10 min. Finally absorbance was measured at 562 nm. As blank water was used instead of sample and as for the sample control ferrozine was not added. Duplicate measurements were done. The chelating activity was calculated with Eq 3:

$$Fe^{2+}\text{Chelating activity}, \% = \left(1 - \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{blank}}}\right) \cdot 100 \quad [3]$$

The interfacial tension at the oil/water-interface was measured using a drop tensiometer OCA20 (Dataphysics GmbH, Filderstadt, Germany). The oil phase employed was MCT oil treated with magnesium silicate (Florisil ® 60-100 mesh) to remove surface-active compounds. A drop of the dissolved protein hydrolysate (0.01 to 2% by weight (wt%); pH:

2, 3 and 8 adjusted with a solution of 0.1 M HCl) was created at the tip of a needle using a computer-controlled dosing unit. The interfacial tension was automatically calculated from the drop shape and was monitored for 30 min. All experiments were conducted at 20°C in duplicate.

2.4. Preparation of FPH-stabilised emulsions and evaluation of the stability of the liquid emulsions

For optimising the composition of emulsions with fish protein hydrolysates (FPH) at DH 5 and DH 10 the influence of the protein content (0.5; 1 and 2wt %) and pH (2, 3 and 8) was varied, whilst the load of MCT oil was fixed at 5wt%. In all cases the pH was adjusted by addition of 0.5 M HCl and the solution was stirred over night to allow complete rehydration of the protein. Then, a pre-emulsion was prepared by shear homogenisation (22,000 rpm, 90 sec, Ystral GmbH, Ballrechten-Dottingen, Germany). Finally, the emulsion was homogenised in a high-pressure homogeniser (Panda 2K; Niro Soavi Deutschland, Lübeck, Germany) at a pressure range of 300/50 bar and applying 2 passes.

The oil droplet size distribution (ODSD) was determined by laser diffraction using a LA-950 (Horiba Jobin Yvon GmbH, Unterhaching, Germany). Results of the volume distribution are presented as the 10th, 50th and 90th percentiles of the oil droplets. The zeta potential was determined employing electrophoretic light scattering (Zetasizer Nano-ZS, Malvern Instruments GmbH, Herrenberg, Germany) once emulsions were diluted 20-folds in water. Both measurements were conducted in triplicate.

Stability against creaming was evaluated as described by Petursson et al (2004). Emulsions were store in glass tubes at room temperature for 7 days and the creaming index was calculated as the percentage of phase separation. This parameter is used as an indirect measurement of the droplet aggregation. Emulsions were qualitatively characterised using light microscopy with 40x increment using a Motic B3 series microscope (Motic Deutschland GmbH, Wetzlar, Germany) to visually check whether there was coalescence or aggregation in the emulsions. Finally, conductivity of emulsions was measured with a WTW conductivity-meter LF96 (WTW GmbH, Weilheim, Germany).

2.5. Emulsion preparation and spray-drying of emulsions for microencapsulation of fish oil

Emulsions for subsequent spray-drying were prepared with 2wt% of FPH(5), 5% of fish oil and 28% of glucose syrup as described in 2.4, glucose syrup was added prior to emulsification. The pH was adjusted to 2.0 by adding 0.5N HCl. Spray-drying was conducted on a Mobile Minor (Niro A/S, Copenhagen, Denmark) at 180/70°C inlet/outlet temperature and 4 bar with rotary atomization resulting in 22,000 rpm.

ODSD of fresh and reconstituted emulsions; and zeta-potential (ζ-potential) and conductivity of reconstituted emulsions were measured as explained in section 2.4. The efficiency of the encapsulation can be measured by extracting the non-encapsulated oil, which is mainly located at the particle surface or in interstitial voids located close to the surface (Drusch & Berg, 2008). The extractable oil content was determined gravimetrically after extraction of the fat with petrol ether (Westergaard, 2004). Results are expressed in wt% of the extracted mass against the total fat content of the powder.

2.6. Storage and determination of the hydroperoxide content of microencapsulated fish oil over time

To examine the stability of the encapsulated oil the microcapsules were stored in the dark in desiccators over a saturated solution of magnesium chloride (resulting in 33% of relative humidity in the headspace) at 20°C. The hydroperoxide content was analysed once per week during 12 weeks. Extraction of the oil was conducted by blending the rediluted powder with a mixture of 2-propanol/isooctane (1:1, v/v). The hydroperoxide content was measured using the thiocyanate assay as described by Drusch et al. (2012). Briefly, a mixture of the extracted oil was diluted with 2-propanol and incubated at 60°C for 30 min after the addition of iron-II-chloride and ammonium thiocyanate solution. After cooling down the sample to room temperature the absorbance was measured at 485 nm (Novaspec II Spektralphotometer, Uppsala, Sweden). Extractions were done in duplicate and hydroperoxide determination of each extract at least with two replicates.

2.7. Statistical analysis

The Statgraphics software (version 5.1) was used to conduct a one way analysis of variance (ANOVA) on the data. Mean values were compared by employing Tukey's test. Differences between means were considered significant at $p \leq 0.05$. Furthermore, Grubb's test was employed for identifying outlier values of the ODSD.

3. RESULTS AND DISCUSSION

3.1. Proximate composition and amino acid composition of fresh muscle meat and lyophilized FPH

The fresh muscle meat of both species showed similar proximate composition with low percentages of oil and ash (Table 1). These data slightly differ from those of the whole fish (including bones, skin and viscera) published by García-Moreno, Pérez-Gálvez, Morales-Medina, Guadix, & Guadix, (2013). The content of ash found in the literature was higher, which is due to the presence of bones and skin in the whole fish sample but not in purified muscle meat. With respect to the FPH, all samples exhibited a similar content of protein, whilst the amount of oil is almost double in horse mackerel samples. The ash content was, in all cases, higher compared to the fresh muscle, this may be due to the increase of salts in the media caused by the addition of 1 M sodium hydroxide solution during hydrolysis.

The amino acid composition of the raw protein and hydrolysates is shown in Table 1. All samples contained aspartic and glutamic acids as major amino acids, representing almost 45wt% of the total amino acid content. Lysine, arginine and leucine were also present in relatively high amounts. These data are in accordance with those reviewed by (Chalamaiah et al., 2012) where species like capelin (*M. villosus*), pacific whiting (*M. productus*) or red salmon (*S. elongate*) also contained aspartic and glutamic acid in a high proportion. Nevertheless, in the case of herring (*C. harengus*) the content of lysine is higher than glutamic acid. On the contrary, it is also common in fish protein and FPH to show low levels of cysteine and tryptophan (Chalamaiah et al., 2012). There was a considerable difference between the average recovered mass of hydrolysates with DH 5 (22.6±1.5g) and with DH 10 (31.9±4.1g). This variation may be related to the increase of the solubility of hydrolysates with the DH (Klompong et al., 2007). However, the amino

acid composition remained almost constant for the non hydrolysed protein and hydrolysates with both DHs (data not shown).

Table. 1. Proximate composition and amino acid composition of muscle of horse mackerel and sardine as well as lyophilized hydrolysates thereof hydrolysed with alcalase or trypsin.

	Sardine muscle	H. mackerel muscle	SAH(5)	STH(5)	HAH(5)	HTH(5)
			Proximate composition, wt%			
PROTEIN	18.83	21.35	85.82	86.61	86.34	87.63
OIL	1.15	1.04	0.3	0.49	0.82	0.84
WATER	78.12	77.47	3.04	5.31	4.23	6.68
ASHES	1.52	1.5	12.72	10.91	12.35	11.16
			Amino acids composition, wt%			
ASP	27.64	29.35	23.99	29.99	28.56	28.79
SER	2.94	2.49	3.76	2.45	2.66	2.58
GLU	15.00	15.65	16.40	17.28	16.35	16.27
GLY	5.24	3.49	4.44	3.62	3.84	3.71
HIS	1.90	2.30	3.79	2.66	3.31	2.82
ARG	6.08	5.52	5.60	5.71	5.85	6.24
THR	3.36	3.34	3.37	2.93	3.25	3.29
ALA	4.42	4.37	5.04	4.59	4.43	4.38
PRO	2.90	2.32	2.54	2.21	2.44	2.44
CYS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TYR	3.89	2.80	2.41	2.00	2.08	2.32
VAL	4.04	4.26	4.26	3.69	3.91	3.96
MET	2.07	2.16	1.94	1.69	1.81	1.83
LYS	7.72	8.80	9.59	9.73	9.74	9.39
ILE	3.58	3.87	3.49	3.19	3.22	3.35
LEU	6.00	6.19	6.48	5.82	6.02	6.01
PHE	3.21	3.09	2.88	2.44	2.53	2.62

3.2. Molecular weight distribution of muscle and FPH by SDS-PAGE

As depicted in Fig. 1 hydrolysis led to significant changes in the molecular weight distribution of both proteins for all FPH. Non-hydrolysed sardine and horse mackerel muscle contained a high amount of peptides larger than 17 kDa with a slight band at around 10.6 kDa. FPH(5) showed a continuous band from 2.5 to 10.6 kDa. Among FPH(10) exclusively SAH (10) presented a slight band between 3.5 and 8.2 kDa, in the other samples the absence of band may indicate that all peptides were smaller than 2.5

kDa and diffused through the gel. No differences between patterns prepared in the presence and absence of a reducing agent (DL-dithiothreitol, DTT) were found (data not shown). These findings are supported by the results of the amino acid composition (Table 1), as no cysteine was detected in any of the samples. Accordingly no disulphide bonds can be formed in the muscle protein and in the FPH.

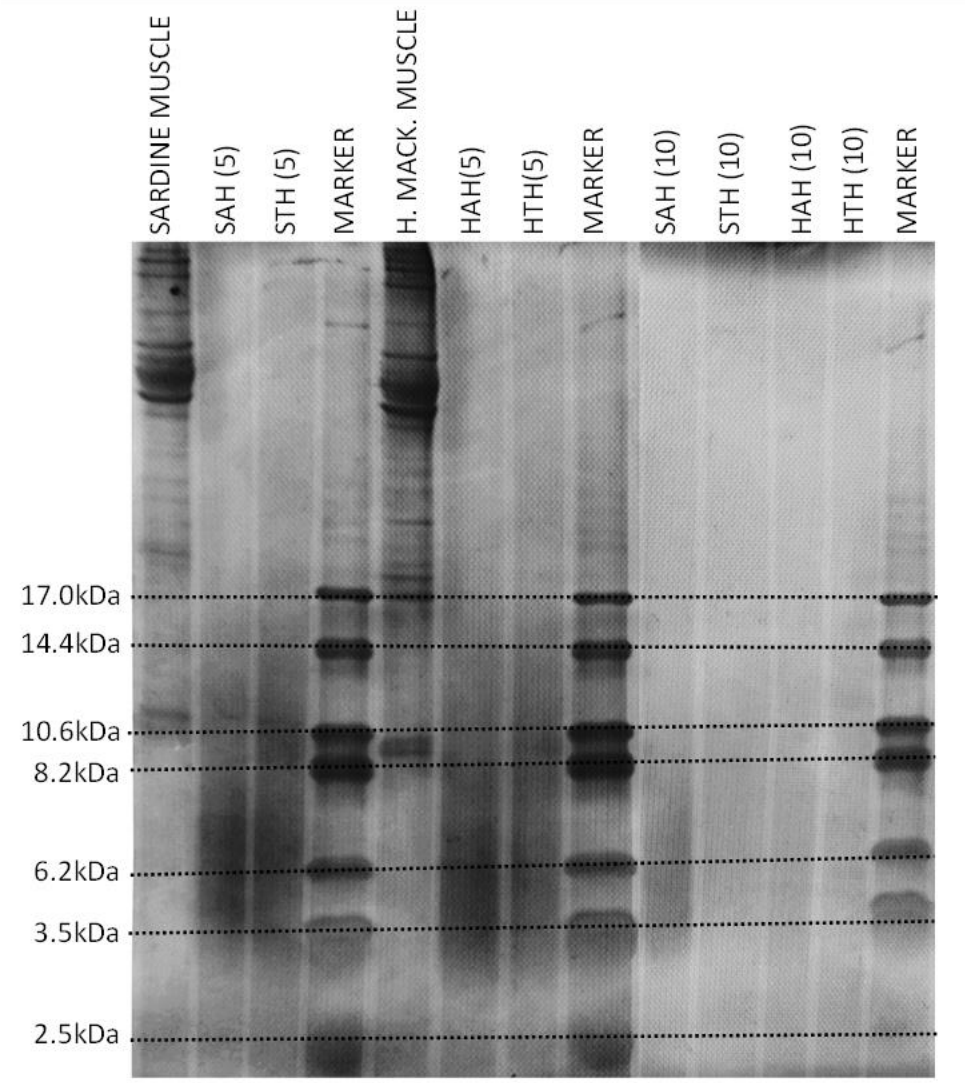


Figure. 1 SDS Page of sardine and horse mackerel raw protein and hydrolysates produced with alcalase and trypsin (DH 5 and DH 10).

The molecular weight distribution of the hydrolysates depends not only on the conditions of hydrolysis but also on the intensity of the purification step. For instance, in the case of the hydrolysis of yellow stripe trevally using Alcalase (50ºC, pH 8.5) with subsequent purification at 2,000g, the molecular weight profile contained a high proportion of large

peptides compared to the hydrolysates of the present study at DH 5, which were purified at 20.000g (Klompong, Benjakul, Kantachote, Hayes, & Shahidi, 2008).

3.3. *In vitro* antioxidant properties of FPH

Since antioxidants can act by several mechanisms, three different *in vitro* assays were conducted: DPPH radical scavenging activity, Fe^{2+} chelating activity and Fe^{3+} reducing power (Fig. 2). DPPH determines the ability of a substrate to transfer electrons or hydrogen atoms which can react with free radicals to form more stable compounds. The chelation of metal ions may be an effective tool to decrease the amount of metals available in the media and to avoid their contact to reactive oxygen species. It is well known that, metals catalyse lipid oxidation due to their ability to produce alkyl radicals and reactive oxygen species. Furthermore, they decompose hydroperoxides by accelerating the autoxidation process (Shahidi & Zhong, 2010). Reducing power is related to the ability of compound to donate an electron to free radicals converting them into more stable compounds. By this electron donation, an oxidised antioxidant molecule can be regenerated (Shahidi & Zhong, 2010). However, they could also act as pro-oxidants by reducing metals present in the medium. Ferrous iron, considered the most powerful pro-oxidant, is much stronger (100-fold) than ferric iron due to its higher solubility and reactivity (Shahidi & Zhong, 2010). Additionally, by means of the Fenton reaction, reduced metal ions (mainly cuprous and ferrous) can produce hydroxyl radicals, which are considered the most harmful reactive oxygen species with respect to lipid oxidation (Shahidi & Zhong, 2010).

Among the range of concentrations studied, all antioxidant activities showed a dose-dependent behaviour. In the case of DPPH, the activity increased linearly ($r^2 > 0.99$) and with a sharp slope in case the concentrations ranged between 1 and 10 mg/mL, whereas for higher concentrations the slope was much lower. On the contrary, for reducting and chelating capacities, the slope remained constant ($r^2 > 0.95$). As a general trend, DPPH activity and reducing power decreased significantly ($p < 0.05$) with the increase of DH (data not shown) whereas the opposite behaviour was found for the chelating activity. The effect of the enzyme and the fish species on the antioxidant properties were exclusively significant at DH5.

These current results are in line with those reported for horse mackerel *(T. mediterraneus*) and sardine (*S. pilchardus*) treated with mixtures of Alcalase and trypsin (García-Moreno

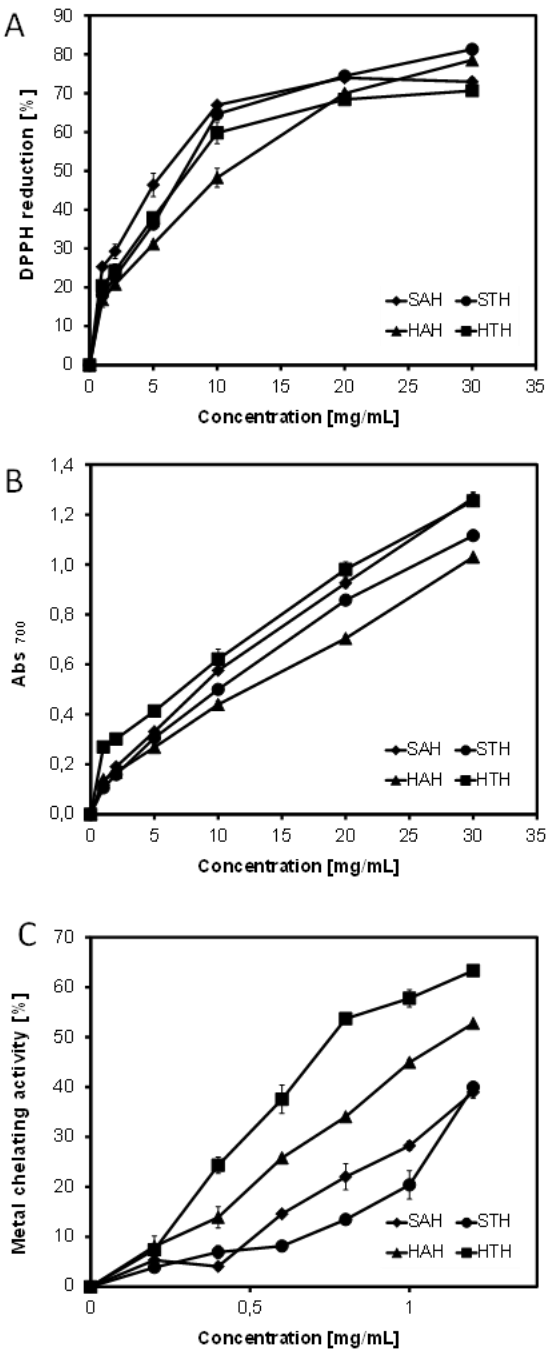


Figure. 2 *In vitro* antioxidant activity of FPH(5) produced with alcalase or trypsin (a) DPPH radical scavenging activity, (b) chelating activity and (c) reducing power.

et al., 2014). On the contrary, yellow stripe trevally hydrolysed with Alcalase or Flavourzyme (Klompong et al., 2007) exhibited lower activities.

Since all FPH tested showed scavenging and reducing properties, they may contain peptides capable of transferring electrons or hydrogen atoms and may be able to react with free radicals to form more stable compounds. The difference of the activities that samples presented may be related to the amino acid composition and the peptides size. High DPPH radical scavenging ability is associated to the presence of hydrophobic amino acids (Suetsuna, Ukeda, & Ochi, 2000). Peptides containing amino acid residues as Val, Leu, Ile, Ala, Phe, Cys or Lys at the N-terminal position have been reported to act as good scavengers (Suetsuna et al., 2000). These amino acids are present in a similar proportion (Table 1) being their average content 29.8±0.6wt%. Moreover, Tyr residues, especially when located at the C-terminal, present strong scavenging and histidine residues have also shown antioxidant properties which may be related to their indolic and imidazole groups (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). As it is shown in Table 1, the average percentage of Tyr and His is 5.1±0.4wt%. On the other hand, some carboxyl (Glu, Asp) and amino groups (Lys, Arg) at side chains can also act as chelators (Q. Liu, Kong, Xiong, & Xia, 2010). Glutamic and aspartamic acids are the predominant amino acids, their average sum being 45.6±1.5wt%, increasing up to 53.8±1.0wt% when taking into account lysine and arginine (Table 1). This percentage is considerably higher than that of possible scavenger amino acids and may explain the increased chelating activity compared to the radical scavenging activity. Additionally, the position of the amino acids plays an important role in the chelating activity. His residues, whose imidazole ring may coordinate with iron, showed higher affinity when located at the N-terminus rather than at C-terminus (Chen et al., 1998). Hence, the differences observed in the chelating activity of the FPH may be due to the size and position of amino acids.

3.4. Interfacial activity of FPH

The impact of protein content and pH on the interfacial tension of SAH(5) solutions is illustrated in Fig. 3a. At acidic pH (2, 3) and for concentrations above 0.1wt% the interfacial tension values were much lower than at pH 8. These variations may be mainly related to the influence which pH exerts on solubility: in the case of sardine non-hydrolysed protein at pH 8 only 20% is soluble whereas at pH 2 this value increases to 80% (Batista, Pires, & Nelhas, 2007). Despite the increased solubility by intensifying the

enzymatic treatment, non-hydrolysed and hydrolysed proteins follow the same trend with pH, as indicated by the isoelectric pH remaining constant (Klompong et al., 2007). Moreover, these differences could be related to the solvent composition (*i.e.* pH and ionic strength), which influences the ability of the protein to adsorb to interfaces.

Among all FPH at pH 2, SAH with a concentration of 2wt% exhibited the lowest interfacial tension (5.3±0.3 mN/m) followed by HTH (6.6±0.1 mN/m and STH (6.8±0.1 mN/m) Fig. 3b). The protease employed in the enzymatic treatment had a significant effect on the interfacial tension in the case of sardine but not for horse mackerel. On the other hand, comparing data with a concentration of 0.1wt%, the STH (10.8±0.1 mN/m) followed by SAH (11.5±0.5 mN/m) showed the lowest interfacial activity. The influence of the enzyme may be explained by the selectivity of the proteases employed during hydrolysis: trypsin cleaves at a few specific sites of the amino acid chain while Alcalase cleaves less specific. A more detailed description of the specific cleaves sites of each enzyme has been summarized by Tamm et al. (2015).

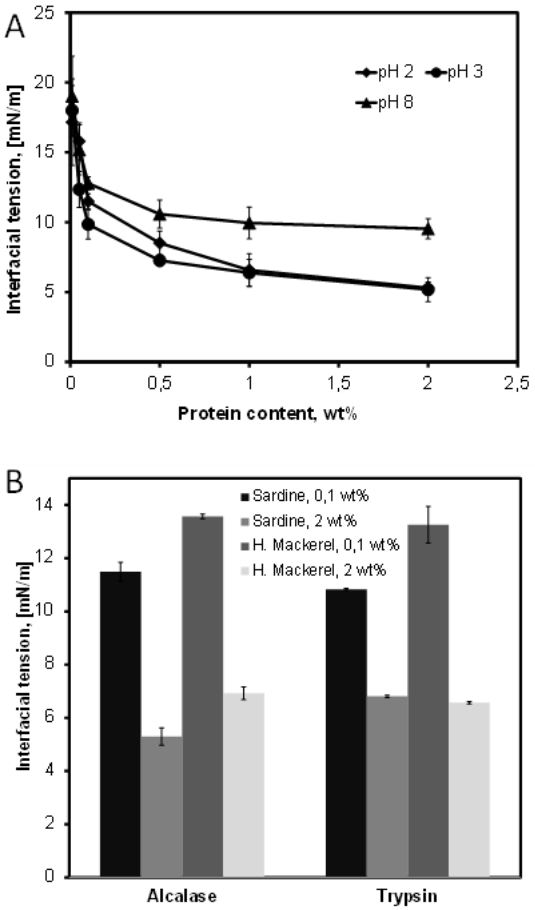


Figure. 3 Interfacial activity of fish protein hydrolysates with a degree of hydrolysis of 5 % at the oil/water-interface. (A) Impact of protein content (SAH) and pH. (B) Impact of of enzyme type at pH 2 at different concentrations.

3.5. Selection of pH and composition of the emulsion for microencapsulation by spray-drying

Among all samples of protein, only when using FPH(5) stable emulsions for spray-drying could be produced. The solubility of the lyophilised unmodified substrate was found poor and the peptides in FPH (10), most of them less than 2.5 kDa as shown in Fig. 1, are too small for adapting themselves to the interface with good emulsifying properties. Apparently, a relatively high amount of peptides larger than 2 kDa is required for avoiding emulsion instability, *e.g.* hydrolysates possessing only peptides smaller than 500 Da did not give stable emulsions (Caessens, Daamen, Gruppen, Visser, & Voragen, 1999).

Different emulsion formulations were tested in the present study with respect to the physical stability, varying the pH (2, 3 and 8) and the protein content of FPH(5) (0.5, 1 and 2wt%). FPH(5) stabilized emulsions at pH 2 and 3 but not at 8 (data not shown). The stability of the emulsions increased as the percentage of protein was increased until a maximum of 2wt%. At acidic pH, the emulsions with the lower percentage of protein (0.5%) presented creaming (6.2%) at the first day, which was mainly caused by coalescence and minor aggregation. The ODSD of fresh emulsions showed a median of 0.60 ±0.10μm and 0.48±0.07 μm at pH 2 and pH3, respectively. As a consequence of coalescence and aggregation, the median increased to 0.94±0.01 μm and 2.00±0.09 μm after one day of storage. In the case of emulsions with 1wt% of protein less creaming (3%) and coalescence occurred. The ODSD increased from 0.58±0.08 μm and 0.40±0.02 μm to 1.22±0.06 μm and 1.50±0.2μm at pH 2 and pH 3, respectively. In the higher protein concentration tested (2wt%), emulsions at pH 2 were the most stable with no creaming during 7 days. Furthermore, the median increased from 0.36±0.00 to 0.44±0.05 μm during storage.

At acidic pH, the ζ-potential of all emulsions ranged from 40 and 55 mV. These results are to some extent higher than those obtained with cod protein hydrolysate at pH 2 (30 mV) (Petursson et al., 2004). High absolute values of the ζ-potential are important for stabilisation against aggregation by means of electrostatic repulsion. Independent from the protein content, the ζ-potential slightly increased with pH, *e.g.*, emulsions with 1wt% of FPH at pH 3 presented: 53.0±0.9 mV and at pH 2: 45.0±0.1 mV. In addition, all emulsions produced at pH 2 showed higher conductivity and hence, higher ionic strength. Although, it has been described that an increase in the ionic strength may result in electrostatic

screening effects with a consequent reduction in the ζ-potential, in the present study the opposite was observed. This can be explained when taking into account that the ζ-potential increases with the concentration of protein as well as the ionic strength of media (due to the addition of 0.5N HCl). It seems that the ζ-potential of the emulsions in the present study is more affected by the increase of the protein content rather than electrostatic screening effects. For emulsions stabilised with FPH, pH plays an important role on their stability because it may govern not only solubility but interfacial properties and, consequently, the steric conformation at the interface. In summary, emulsions stabilized with 2% of hydrolysates at pH 2 were the most stable, they were selected for the process of microencapsulation.

3.6. Characterisation of fresh emulsions, microcapsules and reconstituted emulsions after spray-drying

Focusing on ODSD of emulsions before spray-drying (Table 2), SAH(5) presented the smallest droplets in all the percentiles. With regard to sardine hydrolysates, the effect of enzyme was only significant in the 90th percentile, whereas for horse mackerel it was significant in all percentiles. Since all samples had the same protein:oil ratio, the ODSD is mainly affected by the technological properties of the peptides, *i.e.* by their molecular weight distribution and by their amino acids composition. As all emulsions presented median values smaller than 1.5μm, they were stable enough for spray-drying purposes (Drusch, Serfert, Scampicchio, Schmidt-Hansberg, & Schwarz, 2007).

When comparing the ODSD of fresh and reconstituted spray-dried emulsions, no significant changes were observed for hydrolysates treated with Alcalase, whereas a significant increase was found for samples hydrolysed using trypsin. The extractable oil content for all microcapsules was 2.0±0.1% resulting in a high microencapsulation efficiency of 98.0±0.1% (data not shown); hence despite the changes of ODSD in samples treated with trypsin the integrity of the interface was maintained during spray-drying and all hydrolysates effectively stabilised the oil droplets during atomisation and drying.

Table. 1. Zeta potential, conductivity and oil droplet size distribution of oil in water emulsions (fresh and reconstituted after spray-drying) stabilised with hydrolysates with a degree of hydrolysis of 5 % of sardine and horse mackerel produced with alcalase or trypsin. Values within a column with different superscript letters indicate significant differences ($p<0.05$)

Sample	Percentile of oil droplet size, µm			Span	Conductivity mS/cm	Zeta potential, mV
	10th	50th	90th			
Liquid emulsions before spray-drying						
SAH	0.18 ± 0.04^{a}	0.35 ± 0.06^{a}	1.13 ± 0.15^{a}	2.43	nd	nd
STH	0.19 ± 0.01^{ab}	0.44 ± 0.01^{bc}	1.54 ± 0.10^{bc}	3.1	nd	nd
HAH	0.21 ± 0.01^{bc}	0.48 ± 0.04^{c}	1.45 ± 0.01^{b}	2.6	nd	nd
HTH	0.26 ± 0.01^{d}	0.73 ± 0.03^{d}	1.58 ± 0.07^{bc}	1.82	nd	nd
Reconstituted emulsion after spray- drying						
SAH	0.17 ± 0.03^{a}	0.37 ± 0.08^{ab}	1.12 ± 0.12^{a}	2.57	1.8	46.90 ± 0.85^{a}
STH	0.24 ± 0.01^{cd}	0.67 ± 0.04^{d}	1.76 ± 0.03^{c}	2.29	1.53	45.13 ± 0.31^{b}
HAH	0.21 ± 0.05^{ab}	0.45 ± 0.08^{bc}	1.44 ± 0.03^{b}	2.71	1.71	48.87 ± 0.38^{c}
HTH	0.27 ± 0.01^{d}	0.82 ± 0.03^{e}	1.69 ± 0.13^{c}	1.72	1.53	44.13 ± 0.15^{d}

3.7. Lipid oxidation of fish oil in spray-dried emulsions stabilised by FPH

The degree of oxidation of the fresh oil employed for the production of emulsions and microcapsules was below the detection limit, whereas fresh microcapsules contained up to 5.5±0.9 mmol/kg oil in the case of STH. This increment is in accordance with previous works describing how lipid oxidation can take place during the microencapsulation process due to homogenisation and drying (Serfert et al., 2009). The homogenisation of the emulsion involves intense mechanical stress and turbulences, which can lead to oxygen inclusion and to a better distribution of pro-oxidant species. Additionally, as previously stated, the presence of shear forces during atomisation (Vega & Roos, 2006) as well as the heat of the drying process can to some extent oxidise the lipids (Serfert et al., 2009). The course of the lipid oxidation in the microcapsules stabilised with FPH is illustrated in Fig. 4. After 12 weeks of storage, the hydroperoxide content ranged from 114±18 to 136±17mmol/kg oil for HAH(5) and HTH(5) respectively. The slope of the oxidation curves provide a rough value of the oxidation rate, SAH(5) with 1.42mmol/kg oil per day being the fewer value followed by HAH(5) with 1.48mmol/kg oil per day. Microcapsules stabilised with FPH produced with trypsin, regardless the substrate, showed similar slope values: ~1.61mmol/kg oil per day. All curves presented a highly linear behaviour (r^2> 0.97) for the entire time of study, with no lag or exponential phase.

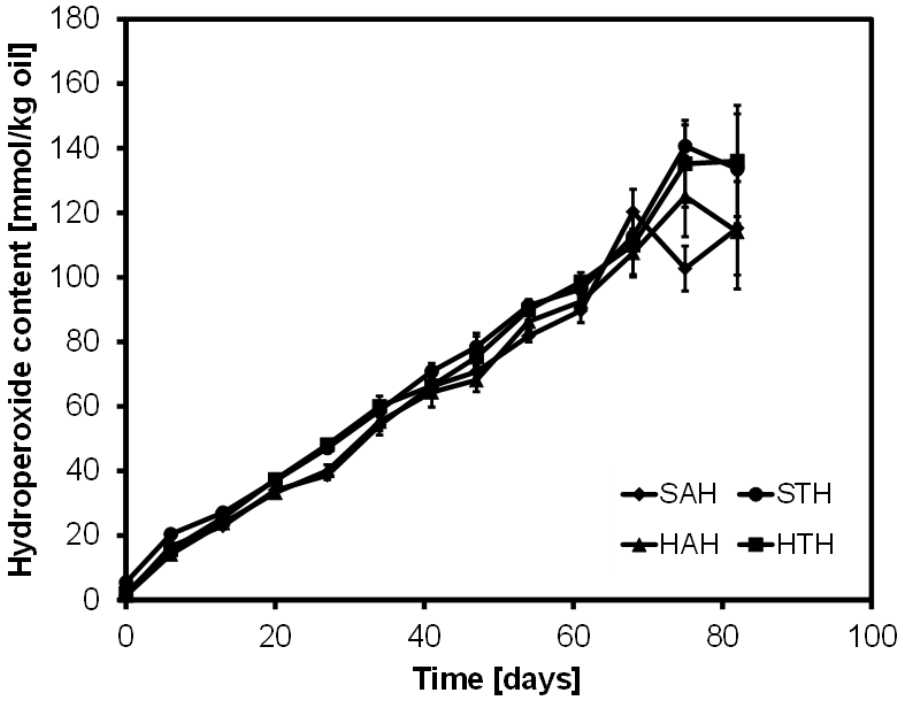


Figure. 4 Development of hydroperoxide content in microcapsules stabilised with FPH produced with alcalase or trypsin in a DH of 5 %.

Since no stable emulsions could be prepared using the non-hydrolysed protein, a direct evaluation of the release of antioxidative peptides through hydrolysis is not possible. Concerning the general protective effect of the microencapsulation, a comparison with the available literature shows that the lipid oxidation is a similar magnitude as it occurs in other sytems. A similar behaviour was found in the case of microcapsules stabilised with nOSA starch, in which the oxidation presented a linear drift (r^2=0.96) with a similar slope as FPH produced with Alcalase (1.4 mmol/kg oil per day) (Drusch et al., 2009). Additionally, fish oil has been microencapsulated by using milk proteins (sodium caseinate and hydrolysed casein, DH 7.6) at different protein concentrations (0.25 to 5wt%) with glucose syrup and 18wt% (wet basis) of oil (Drusch et al., 2012). The encapsulation efficiency for microcapsules produced with sodium caseinate was similar to those obtained in this work, whereas casein hydrolysates presented lower values.

More specifically, the current results can be compared to those obtained for the microencapsulation by spray-drying of emulsions stabilised using unhydrolysed and hydrolysed whey proteins (Tamm et al., 2015). Microcapsules in the cited study were produced under the same conditions using the same source of fish oil. In the case of non-hydrolysed whey proteins the hydroperoxide content after 11 weeks amounted to 113 mmol/kg oil showing a lag phase of 20 days and was in a similar range like the data for the

spray-dried FPH-stabilised emulsions in the present study. Emulsions prepared with whey protein hydrolysates produced with trypsin showed much lower values of 55 and 40 mmol/kg for DH 3 and 6, respectively and thus more effectively protected against oxidation than those stabilised with FPH. In general this difference might be related to several factors: the ODSD of parent emulsions, the encapsulation efficiency, antioxidative properties of the hydrolysates, degradation of microcapsules or to physical protection of oil due to the protecting barrier formed around the oil (Elias, Kellerby, & Decker, 2008). The molecular weight profile of the hydrolysates, the amino acids composition and their distribution within the backbone are the parameters which govern the aforementioned properties. Although differences in the molecular weight profile occur, the ODSD and the encapsulation efficiency are similar in both studies. Hence, the differences between oxidation rates might be related to the antioxidative properties of the hydrolysates, a consumption of radicals through radical attack of proteins or to the efficiency of the physical barrier at the interface as discussed in the following section.

Apart from the positive overall performance of the FPH-stabilised emulsions, no correlation between rate of oxidation and antioxidative properties of the FPH was found: SAH(5) and HAH(5) presented similar values of scavenging activity whereas SAH(5) presented better reducing capacity, however the degree of oxidation of both microcapsule was similar. The absence of a clear correlation might be related to several factors, as for instance the narrow range of variation of the antioxidant properties. Furthermore, antioxidant activity in food systems depends on several factors as physical location of the antioxidant, environmental conditions (*e.g.* pH), or interactions with other food components. However *in vitro* test often correlate to a specific antioxidant activity against a unique oxidant agent, hence a considerable simplification of the real system is conducted (Decker, Warner, Richards, & Shahidi, 2005). In this study, the emulsions were produced at pH 2 whereas the antioxidant tests were evaluated around neutral pH (as specified in the analysis protocol), hence the structure of the protein can vary and consequently the antioxidant activity. Chelating activity tested *in vitro* has been found to decrease at acidic pH whereas DPPH radical scavenging activity of ferric reducing power was reported to remain constant (Klompong et al., 2008). In addition, *in vitro* tests are usually performed in the absence of lipids; the conformation of the protein may vary when located at the interface (Decker et al., 2005) and, hence, the antioxidant properties. So as to determine whether the *in vitro* antioxidant tests can be a useful tool for the prediction of oxidation of

microencapsulated oil, further comparisons between in vivo and *in vitro* test must be done. Finally apart from an antioxidative effect, consumption of radicals through reaction with proteins may retard lipid oxidation. Oxidation pathways of proteins have been reviewed in the literature, *i.e.* electrophilic radicals can produce the cleavage of the polypeptide backbone as well as aldehydes can react with histidine, lysine or cysteine by Michael addition (Elias et al., 2008). By means of the hydrolysates oxidation, which can happen if available amino acids are more oxidatively labile than the unsaturated fatty acids, the oil is protected. Further characterization of hydrolysates should be conducted so as to determinate the degree of degradation of the proteins, *i.e.* analyzing the protein thiol and protein carbonyl groups as indicators of protein oxidation (Eymard, Baron, & Jacobsen, 2009).

4. CONCLUSIONS

Hydrolysis of fish protein is a possibility to improve its technological properties. However, type of enzyme and degree of hydrolysis heavily affect the performance of the FPH. FPHs with a degree of hydrolysis of 5 % were capable to physically stabilise emulsions, in contrast to the non-hydrolysed protein and FPH(10). Emulsions stabilised with FPH(5) were also physically and chemically stable after the homogenisation and subsequent microencapsulation by spray-drying. During storage the encapsulated oil exhibited a similar course of hydroperoxide content as it was reported for microencapsulated fish oil prepared from other matrix systems showing that FPH may serve as an alternative matrix constituent. No differences among substrates (species of fish) and type of enzyme were found. Future research should focus on a more specific characterization of hydrolysates with a DH lower than 5% and possibilities to produce and purify hydrolysates with a well-defined, tailor-made peptide profile to define a compromise between solubility, emulsifying activity and functionality in spray-dried systems. Hydrolysates may provide additional stability through their localization at the interface and their antioxidative activity. More basic research on the complex interplay between protein impact, its alteration and lipid oxidation is required to fully understand the chemical background and its impact on functionality of proteins in these amorphous systems.

5. REFERENCES

A.O.A.C. (2006). Official Methods of Analysis of the A.O.A.C. Washington DC: Association of Official Analytical Chemists.

Adler Nissen. (1986). Enzymic hydrolysis of food proteins. London: Elsevier Applied Science Publishers LTD.

Batista, I., Pires, C., & Nelhas, R. (2007). Extraction of Sardine Proteins by Acidic and Alkaline Solubilisation. *Food Science and Technology International*, 13(3), 189–194. doi:10.1177/1082013207079619

Caessens, P. W. J. R., Daamen, W. F., Gruppen, H., Visser, S., & Voragen, A. G. J. (1999). β-Lactoglobulin Hydrolysis. 2. Peptide Identification, SH/SS Exchange, and Functional Properties of Hydrolysate Fractions Formed by the Action of Plasmin. *Journal of Agricultural and Food Chemistry*, 47(8), 2980–2990. doi:10.1021/jf981230o

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food Chemistry*, 135(4), 3020–38. doi:10.1016/j.foodchem.2012.06.100

Chen, H., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative Properties of Histidine-Containing Peptides Designed from Peptide Fragments Found in the Digests of a Soybean Protein. *Journal of Agricultural and Food Chemistry*, 46(1), 49–53. doi: 10.1021/Jf970649w

Davies, R. W. D., Cripps, S. J., Nickson, A., & Porter, G. (2009). Defining and estimating global marine fisheries bycatch. Marine Policy, 33(4), 661–672. doi:10.1016/j.marpol.2009.01.003

Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring antioxidant effectiveness in food. Journal of Agricultural and Food Chemistry, 53(10), 4303–10. doi:10.1021/jf058012x

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, 38(3), 674–677. doi:10.1021/jf00093a019

Drusch, S., & Berg, S. (2008). Extractable oil in microcapsules prepared by spray-drying: Localisation, determination and impact on oxidative stability. *Food Chemistry*, 109(1), 17–24. doi:10.1016/j.foodchem.2007.12.016

Drusch, S., Groß, N., & Schwarz, K. (2008). Efficient stabilization of bulk fish oil rich in long-chain polyunsaturated fatty acids. European *Journal of Lipid Science and Technology*, 110(4), 351–359. doi:10.1002/ejlt.200700195

Drusch, S., Rätzke, K., Shaikh, M. Q., Serfert, Y., Steckel, H., Scampicchio, M., … Mannino, S. (2009). Differences in Free Volume Elements of the Carrier Matrix Affect the Stability of Microencapsulated Lipophilic Food Ingredients. *Food Biophysics*, 4(1), 42–48. doi:10.1007/s11483-008-9100-9

Drusch, S., Serfert, Y., Berger, A., Shaikh, M. Q. Q., Rätzke, K., Zaporojtchenko, V., & Schwarz, K. (2012). New insights into the microencapsulation properties of sodium caseinate and hydrolyzed casein. *Food Hydrocolloids*, 27(2), 332–338. doi:10.1016/j.foodhyd.2011.10.001

Drusch, S., Serfert, Y., Scampicchio, M., Schmidt-Hansberg, B., & Schwarz, K. (2007). Impact of physicochemical characteristics on the oxidative stability of fish oil microencapsulated by spray-drying. *Journal of Agricultural and Food Chemistry*, 55(26), 11044–51. doi:10.1021/jf072536a

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition*, 48(5), 430–41. doi:10.1080/ 10408390701425615

Eymard, S., Baron, C. P., & Jacobsen, C. (2009). Oxidation of lipid and protein in horse mackerel (Trachurus trachurus) mince and washed minces during processing and storage. *Food Chemistry*, 114(1), 57–65. doi:10.1016/j.foodchem.2008.09.030

Farvin, K. H. S., Lystbæk Andersen, L., Hauch Nielsen, H., Jacobsen, C., Jakobsen, G., Johansson, I., & Jessen, F. (2014). Antioxidant activity of Cod (*Gadus morhua*) protein hydrolysates: *in vitro* assays and evaluation in 5% fish oil-in-water emulsion. *Food Chemistry*, 149, 326–34. doi:10.1016/j.foodchem.2013.03.075

García Moreno, P. J., Pérez-Gálvez, R., Morales-Medina, R., Guadix, A., Guadix, E. M., (2013). Discarded species in the west Mediterranean Sea as sources of Omega-3 PUFA. *European Journal of Lipid Science and Technology*, 115(9), 982–989. doi:10.1002/ejlt.201300021

García-Moreno, P. J., Batista, I., Pires, C., Bandarra, N. M., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2014). Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species. *Food Research International*, 65, Part C, 469–476. doi:10.1016/j.foodres.2014.03.061

Kelleher, K. (2005). Discards in the world's marine fisheries: An update. FAO. Fisheries Technical Paper.

Kim, S.-K., & Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: A review. *Journal of Functional Foods*, 2(1), 1–9. doi:10.1016/j.jff.2010.01.003

Klompong, V., Benjakul, S., Kantachote, D., Hayes, K. D., & Shahidi, F. (2008). Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. *International Journal of Food Science & Technology*, 43(6), 1019–1026. doi:10.1111/j.1365-2621.2007.01555.x

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, 102(4), 1317–1327. doi:10.1016/j.foodchem.2006.07.016

Liu, H., Chang, B., Yan, H., Yu, F., & Liu, X. (1995). Determination of amino acids in food and feed by derivatization with 6-aminoquionolyl-N-hydroxysuccinimidyl carbamate and reversed-phase liquid chromatographic separation. *Journal of AOAC*, 78, 736–744. doi:10.1007/s13197-010-0130-1

Liu, Q., Kong, B., Xiong, Y. L., & Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry*, 118(2), 403–410. doi:10.1016/j.foodchem.2009.05.013

Oehlke, K., Heins, A., Stöckmann, H., Sönnichsen, F., & Schwarz, K. (2011). New insights into the antioxidant activity of Trolox in o/w emulsions. *Food Chemistry*, 124(3), 781–787. doi:10.1016/j.foodchem.2010.06.095

Oyaizu, M. (1986). Studies on products of browning reactions: Antioxidative activities of browning products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, 44, 307–315. doi: 10.5264/eiyogakuzashi.44.307

Petursson, S., Decker, E. a, & McClements, D. J. (2004). Stabilization of oil-in-water emulsions by cod protein extracts. *Journal of Agricultural and Food Chemistry*, 52(12), 3996–4001. doi:10.1021/jf035251g

Picot, L., Ravallec, R., Fouchereau-Péron, M., Vandanjon, L., Jaouen, P., Chaplain-Derouiniot, M., … Bourseau, P. (2010). Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. *Journal of the Science of Food and Agriculture*, 90(11), 1819–26. doi:10.1002/jsfa.4020

Ruxton, C. H. S. (2011). The benefits of fish consumption. *Nutrition Bulletin*, 36(1), 6–19. doi:10.1111/j.1467-3010.2010.01869.x

Serfert, Y., Drusch, S., & Schwarz, K. (2009). Chemical stabilisation of oils rich in long-chain polyunsaturated fatty acids during homogenisation, microencapsulation and storage. *Food Chemistry*, 113(4), 1106–1112. doi:10.1016/j.foodchem.2008.08.079

Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews*, 39(11), 4067–79. doi:10.1039/b922183m

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry*, 11(3), 128–131. doi:10.1016/S0955-2863(99)00083-2

Tamm, F., Gies, K., Diekmann, S., Serfert, Y., Strunskus, T., Brodkorb, A., & Drusch, S. (2015). Whey protein hydrolysates reduce autoxidation in microencapsulated long chain polyunsaturated fatty acids. *European Journal of Lipid Science and Technology*, In press, n/a–n/a. doi:10.1002/ejlt.201400574

Van der Ven, C., Gruppen, H., de Bont, D. B. A., & Voragen, A. G. J. (2001). Emulsion Properties of Casein and Whey Protein Hydrolysates and the Relation with Other Hydrolysate Characteristics. *Journal of Agricultural and Food Chemistry*, 49(10), 5005–5012. doi:10.1021/jf010144c

Vega, C., & Roos, Y. H. (2006). Invited review: spray-dried dairy and dairy-like emulsions--compositional considerations. *Journal of Dairy Science*, 89(2), 383–401. doi:10.3168/jds.S0022-0302(06)72103-8

Westergaard, V. (2004). Milk Powder technology: Evaportion and Spray Drying. Copenhagen: Niro A/S.

Wu, H.-C., Chen, H.-M., & Shiau, C.-Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, 36(9-10), 949–957. doi:10.1016/S0963-9969(03)00104-2

Conclusiones Finales

De la investigación desarrollada se extraen las siguientes conclusiones:

1. Los aceites extraídos de seis especies de descarte (sardina, jurel, aligote, boga, pintarroja y bacaladilla) presentaron un alto contenido en ácidos grasos poliinsaturados (PUFA) (>25% en peso) y valores de índices nutricionales adecuados para su aplicación como compuestos nutricionales (TI<0.2; AI<0.7; HH<3.4). Los aceites extraídos de la pintarroja y de la boga contenían PUFA con alta regioselectividad hacia la posición sn-2 y se han propuesto como sustrato para la producción de 2-monoglicéridos (2-MAG). Los aceites extraídos de sardina, aligote, jurel y bacaladilla tenían un alto contenido global de PUFA distribuidos de forma más aleatoria. Por ello, estos aceites han sido propuestos como sustrato para la producción de concentrados de PUFA por métodos físicos.

2. Por medio de la winterización a baja temperatura se han obtenido concentrados en PUFA con un factor de concentración de 1.7 a -85ºC y tras 24 h. El proceso de concentración se favoreció a bajas temperaturas y largos tiempos. La evolución temporal de la masa de la fracción líquida se modeló satisfactoriamente siguiendo la teoría de transferencia de materia ($r^2 > 0.94$) y empleando dos constantes (KG·A y meq). La fracción másica de PUFA, EPA y DHA en la fase líquida se modeló empleando el modelo anterior y coeficientes de pérdida ($r^2 > 0.83$).

3. La hidrólisis de aceite de sardina catalizada con la lipasa inmovilizada R.miehei en sistemas microacuosos incrementó hasta 2 veces el contenido de DHA en los diglicéridos (DAG) y triglicéridos (TAG). Sin embargo, independientemente de la actividad del agua, el contenido de PUFA permaneció virtualmente constante en los acilgliceroles y ácidos grasos libres (FFA). La hidrólisis se modeló siguiendo un mecanismo de dos reacciones reversibles de segundo orden. Las constantes cinéticas mostraron que la hidrólisis del primer ácido graso del TAG era independiente del grado de insaturación (k1=0.031 min-1), mientras que la liberación del segundo ácido graso mostraba una dependencia inversa al grado de insaturación. Así, en este último caso, los PUFA fueron los ácidos grasos que presentaron la menor tendencia a hidrólisis ($k_{2global}$=0.42 min^{-1}, k_{2SFA}=0.82 min^{-1} and k_{2MUFA}=0.82 min^{-1})

4. La producción de lípidos estructurados (MLM) a partir de descartes de sardinas se ha presentado como proceso de revalorización de los descartes. Este proceso consta

de: (i) extracción del aceite, (ii) concentración de ácidos grasos Omega-3 mediante winterización a baja temperatura, (iii) esterificación enzimática y (iv) purificación de los TAG mediante cromatografía en columna. Cada etapa presentó un rendimiento aceptable y los lípidos estructurados producidos, a nivel de estabilidad oxidativa, cumplían con los estándares exigidos para un aceite refinado. La esterificación propuesta constaba de dos etapas y supuso una mejora del 72% de la selectividad del proceso (respecto al ácido caprílico). cuando se comparaba con una esterificación realizada en una única etapa

5. En la hidrólisis de la proteína de jurel, la influencia de la concentración del sustrato, la temperatura, la proporción de subtilisina en la mezcla enzimática y el tiempo se modeló empleando redes neuronales artificiales ($r^2 > 0.98$). Este modelo predijo un valor máximo de DH (17.1%) obtenido a 2.54 g/L de proteína en el reactor mezcla, a 40ºC, tras 4 horas de reacción y añadiendo un 38.3% de subtilisina en la mezcla de proteasas. La combinación de enzimas presentó un efecto sinérgico, obteniéndose grados de hidrólisis mayores al usar mezclas de proteasas.

6. Los hidrolizados de proteína de jurel mostraron una alta capacidad inhibidora de la enzima convertidora de angiotensina (ACEI). Un modelo mixto de proceso y mezcla se eligió para modelizar la ACEI (r^2=0.9972). Este modelo predijo la máxima ACEI (55.3%) a 2.5 g/L, 55ºC y empleando exclusivamente tripsina en la mezcla de proteasas. La actividad de estos hidrolizados no se alteró tras ser sometidos a disgestión *in vitro*.

7. Los hidrolizados de proteína de jurel mostraron alta actividad antioxidante, en concreto, capacidad inhibitoria del radical DPPH, poder reductor y actividad quelante; siendo la actividad quelante la más intensa de las tres estudiadas. Un modelo mixto de proceso y mezcla se empleó para modelizar las actividades en función de la concentración de sustrato, temperatura y contenido de subtilisina en la mezcla enzimática ($r^2 > 0.9815$). El óptimo valor de capacidad inhibitoria de DPPH (80.09% medido a 3 mg/mL) y de capacidad reductora (1.25 medido a 10 mg/mL) se obtuvo a 2.5 g/l y 40 ºC con tripsina pura y con una mezcla que contenía subtilisina en un 58%, respectivamente. La actividad quelante máxima (47.4 % determinada a 0.6 mg/mL) se produjo a 5g/L, 55ºC y empleando subtilisina pura. Los máximos valores de actividad inhibidora de DPPH y capacidad reductora, coincidían con las actividades más bajas de actividad quelante y viceversa.

8. Se han producido emulsiones y microcápsulas estables empleando hidrolizados de sardina y jurel con grado de hidrólisis 5% como agentes emulsificantes y antioxidantes. El proceso de emulsionado y de secado por atomización no afectó a la estabilidad oxidativa del aceite encapsulado y presentó altos rendimientos (98%). Tras 80 días de almacenamiento a temperatura ambiente y 30% de humedad, se observó que las microcápsulas presentaban contenidos de peróxidos que variaban entre 114 y 136 mmol/ kg de aceite. La velocidad de oxidación de estas microcápsulas es comparable a otras observadas empleando otras matrices alimentarias. Por ello, los hidrolizados de proteína de pescado pueden utilizarse como una posible alternativa para la estabilización de aceite de pescado por medio del secado por atomización.

Final Conclusions

Based on the research carried out for the upgrading of lipids and protein compounds, the following conclusions have been drawn:

1. Oil extracted from discard species (sardine, horse mackerel, axillary seabream, bogue, small-spotted catshark and blue whiting) presented high PUFA content (>25%) and appropriate TI, AI and HH indexes for nutraceutical applications (TI<0.2; AI<0.7; HH<3.4). Oils extracted from small-spotted catshark and bogue contained PUFA with high sn-2 regioselectivity and have been proposed as substrates for the production of 2-MAG. In the case of oils extracted from sardine, axillary seabream, blue whiting and horse mackerel PUFA were more randomly distributed. Consequently, these oils have been recommended as substrates for the production of PUFA concentrates by physical methods.

2. Employing low temperature winterization, a maximum increase (1.7-fold) of the PUFA content was obtained at -85 ºC and 24 h. The concentration process was favored by lower temperatures and long times. The time evolution of the liquid mass was modeled (r^2 > 0.94) following the theory of mass transference employing two coefficients ($K_{G \cdot A}$ and m_{eq}). The mass fraction of PUFA, EPA and DHA was modeled by employing the aforementioned model and solid mass loss coefficients (r^2 > 0.83).

3. By the *R.miehei* lipase-mediated hydrolysis of sardine oil the DHA content increased up to 2-fold in the DAG and TAG fraction. However, the PUFA content remained virtually constant in acylglycerols and free fatty acids, regardless the conditions. The hydrolysis was modelled following a two reversible second-order mechanism. The kinetic constant showed that the release of the first fatty acid from the triacylglycerol was independent on the unsaturation degree (k_1=0.031 min^{-1}), while the hydrolysis of the second one was highly affected by the degree of unsaturaturation, PUFA being the fatty acids that presented the lowest rate ($k_{2global}$=0.42 min^{-1}, k_{2SFA}=0.82 min^{-1} and k_{2MUFA}=0.82 min^{-1}).

4. An upgrading process aimed at producing MLM structured lipids from sardine was developed. It was composed of (i) fish oil extraction, (ii) Omega-3 concentration *via* low temperature winterization, (iii) enzymatic esterification and (iv) TAG extraction *via* liquid chromatography. Each step presented acceptable yield and the produced MLM structured lipid met the oxidative conditions required for refined

oils. The proposed two steps esterification process improved in a 72 mol% the selectivity of the caprylic acid esterification when compared to direct esterification.

5. In the hydrolysis of horse mackerel protein, the influence of the substrate concentration, temperature, proportion of subtilisin in the mixture of enzymes and time was successfully modeled) by an artificial neuronal network ($r^2 > 0.98$. Its optimization determined a maximum value of DH (17.1%) 2.54 g/L of substrate concentration, 40 ºC and 38.3% of subtilisin in the enzyme preparation and at 240 min. The combination of enzymes showed a synergic effect, since hydrolysates with higher DH were produced when employing proteases mixtures.

6. Horse mackerel hydrolysates presented high angiotensine converting enzyme inhibitory activity (ACEI). The maximum ACEI (55.3 %) was predicted at 2.5 g/L, 55ºC and employing pure trypsin in the enzyme preparation. The crossed-mixture process model chosen in this work fitted adequately the observed data (r^2=0.9972). The ACEI of the hydrolysate remained constant after simulated digestion.

7. Horse mackerel hydrolysates presented high antioxidant activities (DPPH scavenging activity, Fe^{3+} reducing power and Fe^{2+} chelating activity), chelating activity being the most intense one. The proposed crossed process-mixture model fitted satisfactorily the observed antioxidant activities and the predicted ones (r^2> 0.9815). Optimum DPPH scavenging activity (80.09%, measured at 3 mg/mL) and reducing power (1.25, measured at 10 mg/mL) were attained under at 2.5 g/L, 40 ºC with pure trypsin and 58% of subtilisin, respectively. In the case of the maximum chelating activity (47.4% measured at 0.6 mg/mL), the optimal conditions were 5 g/L of substrate, 55 ºC and 100% of subtilisin. Experimental conditions that led to optimal activity of DPPH scavenging activity and Fe^{3+} reducing power resulted in low Fe^{2+} chelating activity and *vice versa*.

8. Stable emulsions and microcapsules were produced with hydrolysates (DH 5%) of sardine and horse mackerel. The process of homogenization and spray-drying did not affect the stability of the oil and presented high yield (98%). After 80 days of storage, the encapsulated oil exhibited a similar course of hydroperoxide content as reported for microencapsulated fish oil prepared from other matrix systems, showing that FPH may serve as an alternative matrix constituent (PV ranged from 114 to 136 mmol/kg of oil).