Technological University Dublin
ARROW@TU Dublin

Doctoral Science

2010-11-01

Functional and Bioactive Components from Mackerel (Scomber scombrus) and Blue Whiting (Micromesistius poutassou) Processing Waste

Zied Khiari
Technological University Dublin

Follow this and additional works at: https://arrow.tudublin.ie/sciendoc

Part of the Food Science Commons

Recommended Citation

Khiari, Z. (2010). *Functional and Bioactive Components from Mackerel (Scomber scombrus) and Blue Whiting (Micromesistius poutassou) Processing Waste*. Doctoral Thesis. Technological University Dublin. doi:10.21427/D7FK50

This Theses, Ph.D is brought to you for free and open access by the Science at ARROW@TU Dublin. It has been accepted for inclusion in Doctoral by an authorized administrator of ARROW@TU Dublin. For more information, please contact yvonne.desmond@tudublin.ie, arrow.admin@tudublin.ie, brian.widdis@tudublin.ie.

This work is licensed under a Creative Commons Attribution-Noncommercial-Share Alike 3.0 License

Functional and bioactive components from mackerel (*Scomber scombrus*) and blue whiting (*Micromesistius poutassou*) processing waste

Zied Khiari

PhD **2010**

Functional and bioactive components from mackerel (*Scomber scombrus*) and blue whiting (*Micromesistius poutassou*) processing waste

A thesis submitted to the Dublin Institute of Technology in fulfillment of requirements for the degree of

DOCTOR OF PHILOSOPHY

Zied Khiari, Dipl.Eng, PGDip, M.Sc.

School of Food Science and Environmental Health
Dublin Institute of Technology

Supervisors:
Dr. Catherine Barry-Ryan
Dr. Ana Belen Martin-Diana

November 2010

ABSTRACT

The use of fish processing waste from an oily fish model (mackerel, *Scomber scombrus*) and a white fish model (blue whiting, *Micromesistius poutassou*) as a source of functional and bioactive compounds was investigated.

Gelatines were extracted from fish heads and skins using pre-treatment with different organic acids (acetic, citric, lactic, tartaric and malic acids). Gelatines from fish bones were extracted after pre-treating the bones either chemically or enzymatically. The type of pre-treatment affected the yield, colour, turbidity, amino acid profiles as well as functional properties of fish gelatines. Mackerel gelatines formed stronger and more stable gels than blue whiting. Acetic acid pre-treatment of mackerel and blue whiting heads and skins produced stronger gelatine gels. The enzymatic pre-treatment of fish bones resulted in gelatines with poor rheological properties.

Hydrolysates obtained from the hydrolysis of mackerel head and skin gelatines with pepsin exhibited the highest antioxidant activity (DPPH ~ 80 %). These hydrolysates had high anti-inflammatory activity (inhibition of SSAO activity by 50 %) and antihypertensive activity (inhibition of ACE activity by 75 %). Skin gelatine hydrolysates, were also able to inhibit platelet aggregation.

The hydrolysis of mackerel viscera with Flavourzyme produced hydrolysates with high antioxidant activity (73 %) and inhibited the SSAO activity by 45.8 % and ACE by 60 %.

Different procedures (chemical, physical or enzymatic) were developed to extract oil rich in ω-3 fatty acids from mackerel heads and skins. The yield varied significantly ($p < 0.05$) depending on the extraction method. The physicochemical properties of the majority of oils extracted were acceptable according to the *Codex Alimentarius* criteria. Polar oil fractions extracted from mackerel heads after enzymatic hydrolysis with Alcalase had high DPPH radical scavenging activity which was due to the presence of carotenoids.

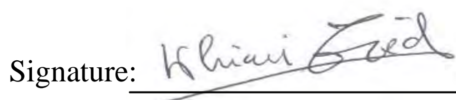
DECLARATION

I certify that this thesis which I now submit for examination for the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported in this thesis conforms to the principles and requirements of the Institute guidelines for ethics in research.

The institute has permission to keep, to lend or to copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged.

Signature:  Date: 05 November 2010

Zied Khiari

To my mother Hajar

To the memory of my father

To my family

To those who love me.

ACKNOWLEDGEMENTS

I owe a debt of gratitude to my supervisors, Dr. Catherine Barry-Ryan and Dr. Ana Belen Martin Diana, from whom I have learned much about research. I wish to acknowledge their support and guidance throughout the project. Their encouragements were well appreciated.

I am greatly thankful to Dr. Daniel Rico for his assistance, encouragement, useful comments and suggestions and his insight throughout the development of this project.

This work was conducted with the financial support from the Irish government under the FIRM 2006. I would like to acknowledge them for giving me the opportunity to carry out my research.

I would also like to convey thanks to DIT administrative staff for providing the financial means and laboratory facilities.

I am grateful to the DIT technical staff: Noel, Jyothi, Plunkett, Tony and Denis for all their help and assistance.

Many thanks go to Dr. Javier Fontecha for his assistance and help, Mr. John Fagan for providing the fish, Dr. Padraig McLoughlin for running the NMR experiments, Dr. Nigel Bruntton for his help in Teagasc and Prof Niamh Moran for her help in the RCSI.

I am extremely grateful to my friends and colleagues who provided an inspiring and friendly atmosphere and made my work easier.

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

List of Abbreviations

a*	CIELAB redness/greenness value
AA	Acetic acid
Abs	Absorbance
ACD	Acid citrate dextrose
ACE	Angiotensin I-converting enzyme
ADP	Adenosine diphosphate
Ala	Alanine
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
Arg	Arginine
ASA	Aspirin
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
A_x	Absorbance values at x nm
b*	CIELAB yellowness/blueness value
BIM	Bord Iascaigh Mhara
BSE	Bovine spongiform encephalopathy
BSI	British standard institution
C	Concentration
°C	Degree Celsius
CA	Citric acid
Chem (NaOH)	Alkaline pre-treatment
Cryo-SEM	Cryogenic scanning electron microscopy
Ctr	Control
Cys	Cysteine
D_2O	Deuterium oxide
DAG	Diacylgylcerols
Da	Daltons

DH	Degree of hydrolysis
DHA	Docosahexaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
DVB	Dynamic viscoelastic behaviour
E/S	Enzyme to substrate ratio
EAI	Emulsifying activity index
EFSA	European Food Safety Authority
ELSD	Evaporative light scattering detector
Enz (A)	Flavourzyme
Enz (B)	Alcalase
EPA	Eicosapentaenoic acid
ESI	Emulsion stability index
EU	European Union
eV	Electron volts
FA	Fatty acids
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organisation
FA-P	Furylacryloylphenylalanine
FA-PGG	N-[3-(2-furyl) acryloyl]-L-phenylalanyl- glycylglycine
FC	Foaming capacity
FFA	Free fatty acids
FID	Flame ionisation detector
FPH	Fish protein hydrolysates
FRAP	Ferric reducing antioxidant power
FS	Foam stability
FTIR	Fourier transform infra-red
FTU	Formazin turbidity units
G'	Elastic modulus
G''	Viscous modulus
G*	Complex shear
GC	Gas Ghromatography

GC-FID	Gas ghromatography-flame ionisation detector
GC-MS	Gas ghromatography-mass spectroscopy
GG	Glycylglycine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GME	Gelatine Manufacturers of Europe
h	Hour
H	Number of peptide bonds cleaved
HA	Hippuric acid
HATR	Horizontal attenuated total reflectance
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHL	Hippuryl-Histidyl-Leucine
His	Histidine
HPLC	High performance liquid chromatography
H_{tot}	Total number of peptide bonds in the protein
IC_{50}	Concentration required to inhibit the activity by 50%.
ICES	International Council for the Exploration of the Sea
IDF	International Dairy Federation
Ile	Isoleucine
Indo	Indomethacine
IS	Internal standard
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
kDa	kiloDaltons
kV	kiloVolts
L*	CIELAB lightness/darkness value
LA	Lactic acid
LDL	Low density lipoprotein
Leu	Leucine
λ_{max}	Wavelength at an absorption maximum

Lys	Lysine
M	Molarity
MA	Malic acid
MAG	Monoacylgylcerols
meqv	Milliequivalents
Met	Methionine
mM	milliMolar
MW	Molecular weight
N	Normality
NaOH	Sodium hydroxide
NF	Non fractionated hydrolysates
NMR	Nuclear magnetic resonance
OPA	o-phthaldialdehyde
ORAC	Oxygen radical absorbance capacity
Pa	Pascal
PAO	Plasma amine oxidase
PARs	Protease-activated receptors
PF4	Platelet factor 4
PG E_1	Prostaglandin E_1
pH	Power of Hydrogen
Phe	Penylalanine
PL	Phospholipids
ppm	Parts per million
Pro	Proline
PUFA	Poly unsaturated fatty acids
PV	Peroxide value
ρA	The slope of the increase in absorbance
rpm	Rotation per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Seconds
Ser	Serine

SSAO	Semicarbazide-sensitive amine oxidase
T	Temperature
TA	Tartaric acid
TAC	Total allowable catches
TAG	Triacylgylcerols
TFA	Tri-fluoroacetic acid
Thr	Threonine
TLC	Thin layer chromatography
TPTZ	2,4,6-Tris(2-pyridyl)-1,3,5-triazine
Trp	Tryptophan
TSB	Tryptone Soy Broth
Tyr	Tyrosine
USA	United States of America
US$	US Dollar
v	Volume
Val	Valine
VE	Viscoelastic
w	Weight
WHO	World Health Organisation
δ	Phase shift
Φ	Volume fraction
μm	Micrometers (micron)
μM	Micromolar

Table of Contents

ABSTRACT __________ **i**

DECLARATION __________ **ii**

ACKNOWLEDGEMENTS __________ **iv**

List of Abbreviations __________ **v**

Table of Contents __________ **1**

List of Figures __________ **7**

List of Tables __________ **11**

Chapter 1 – Introduction __________

1.1. Fish and fish processing industries __________ **13**

1.1.1. Classification, distribution and characteristics of fish species __________ 13

1.1.2. Nutritional properties of fish __________ 17

1.1.3. Waste from the fish processing industry __________ 18

1.2. Potential functional and bioactive compounds from fish waste __________ **21**

1.2.1. Collagen and gelatine __________ 21

1.2.2. Fish protein hydrolysates (FPH) __________ 35

1.2.3. Fish oils __________ 52

1.3. Functional foods __________ **57**

1.4. Proposed study __________ **58**

1.4.1. Problem statement __________ 58

1.4.2. Research hypothesis __________ 59

1.4.3. Objectives of the study __________ 59

Chapter 2 - Materials and methods __________

2.1. Materials 61

2. 2. Characterisation of fish waste 62

2.2.1. Proximate analysis of fish waste 62

2.2.2. Determination of the pH of fish waste 62

2.3. Extraction of fish gelatines 63

2.3.1. Skin and head gelatines 63

2.3.2. Bone gelatine 64

2.4. Characterisation of fish gelatines 67

2.4.1. Yield of gelatine extraction 67

2.4.1. Proximate analysis of fish gelatines 67

2.4.2. Determination of the pH of fish gelatines 67

2.4.3. Colour measurement of fish gelatines 67

2.4.4. Turbidity measurement of fish gelatines 68

2.4.5. Protein pattern of fish gelatines 68

2.4.7. Amino acid analysis of fish gelatines 69

2.4.8. Spectroscopic analyses of fish gelatines 70

2.5. Analysis of functional properties of fish gelatines 71

2.5.1. Foaming properties 71

2.5.2. Emulsifying properties 71

2.5.3. Protein solubility 72

2.5.4. Rheological characterisation 74

2.5.5. Textural properties 75

2.6. Enzymatic hydrolysis of fish waste 76

2.6.1. Enzymatic hydrolysis of gelatine 76

2.6.2. Enzymatic hydrolysis of mackerel viscera 77

2.7. Characterisation of FPH 80

2.7.1. Degree of hydrolysis 80

2.7.2. Protein and peptide pattern of FPH 81

2.8. Screening for bioactivity of FPH 82

2.8.1. Antioxidant activity 82

2.8.2. Angiotensin I-converting enzyme (ACE) inhibitory activity 84

2.8.3. Semicarbazide-sensitive amine oxidase (SSAO) inhibitory activity 85

2.8.4. Platelet aggregation inhibitiory activity 86

2.8.5. Effect of FPH on microbial growth 88

2.9. Fractionation of bioactive FPH 89

2.9.1. HPLC analysis 90

2.9.2. Preparative HPLC analysis 90

2.9.3. Determination of peptide content of fractionated FPH 91

2.9.4. Mass spectrometric determination of the amino acid sequence 92

2.10. Mackerel oil extraction procedures 92

2.10.1. Chemical extraction 92

2.10.2. Physical extraction 93

2.10.3. Enzymatic extraction 93

2.10.4. Alkaline and enzymatic extraction 94

2.11. Characterisation of mackerel oils 96

2.11.1. Yield of oil extraction 96

2.11.2. Clarification of the oil samples 96

2.11.3. Physicochemical properties of mackerel oils 96

2.11.4. Fourier Transform Infra-Red (FTIR) analysis of mackerel oils 97

2.11.5. Composition and distribution of neutral and polar lipids 97

2.11.6. Gas chromatographic analysis of fatty acids 99

2.12. Screening for bioactivity of mackerel oils 100

2.12.1. Antioxidant activity of fish oils 100

2.12.2. Fractionation of mackerel oils 100

2.13. Statistical analyses **101**

Chapter 3 - Fish waste

3.1. Characterisation of fish waste **102**

3.1.1. Quantification of fish waste 102

3.1.2. Proximate analysis of fish waste 103

3.1.3. pH determination of fish waste 105

3.2. Fish waste as potential resource for food ingredients **107**

3.3. Conclusions **108**

Chapter 4 - Fish gelatines

4.1. Extraction and characterisation of fish gelatines **109**

4.1.1. Yield of gelatine extraction 109

4.1.2. pH values of fish gelatines 112

4.1.3. Proximate analysis of fish gelatines 113

4.1.4. Colour analysis of fish gelatines 115

4.1.5. Turbidity analysis of fish gelatines 118

4.1.6. Protein pattern of fish gelatines 121

4.1.7. Amino acid composition fish gelatines 123

4.1.8. Spectroscopic analyses of fish gelatines 127

4.2. Functional properties of fish gelatines **140**

4.2.1. Foaming capacity and stability 140

4.2.2. Emulsifying activity and stability 144

4.2.3. Protein solubility 152

4.2.4. Rheological properties 157

4.2.5. Textural properties 167

4.3. Conclusions **173**

Chapter 5 - Fish protein hydrolysates

5.1. Preparation and characterisation of FPH 175

5.1.1. Degree of hydrolysis of mackerel gelatines 176

5.1.2. Degree of hydrolysis of mackerel viscera 179

5.1.3. Protein and peptide patterns of FPH 180

5.2. Screening for bioactivity 184

5.2.1. Antioxidant activity 184

5.2.2. ACE inhibitory activity 189

5.2.3. Semicarbazide-sensitive amine oxidase SSAO inhibitory activity 192

5.2.4. Platelet aggregation inhibitory activity 194

5.2.5. Effect of FPH on microbial growth 196

5.3. Fractionation of bioactive FPH 200

5.4. Screening for bioactivity of fractionated FPH 206

5.4.1. Mackerel skin gelatine hydrolysates 206

5.4.2. Mackerel viscera hydrolysates 209

5.5. Selection of fractions with highest bioactivity 211

5.6. Peptide sequence analysis 216

5.7. Screening for bioactivity of blue whiting gelatine hydrolysates 219

5.8. Conclusions 220

Chapter 6 - Fish oils

6.1. Extraction and characterisation 221

6.1.1. Yield of extraction 221

6.1.2. Physicochemical and colour analysis of mackerel oils 223

6.1.3. FTIR analysis of mackerel oils 228

6.1.4. Chromatographic characterisation of mackerel oils 230

6.2. Antioxidant activity of mackerel oils ________________________________ **236**

6.3. Conclusions ____ **241**

Chapter 7 - General conclusions ________________________________**242**

Chapter 8 - References ____**244**

Appendices ____ **300**

List of Figures

Figure 1.1 Species distribution map for mackerel 14

Figure 1.2 Atlantic mackerel (*Scomber scombrus*) 15

Figure 1.3 Species distribution map for blue whiting 16

Figure 1.4 Blue whiting (*Micromesistius poutassou*) 17

Figure 1.5 Process flow diagram for the filleting of fish 19

Figure 1.6 Triple helical structure of collagen 22

Figure 1.7 Conversion of collagen, through heating and cooling, to a gelatine network 34

Figure 1.8 The peroxidase linked continuous assay for amine oxidase enzymes 47

Figure 1.9 Platelet aggregation scheme 50

Figure 1.10 Schematic representation of EPA and DHA 53

Figure 1.11 Schematic representation of a gas chromatograph 56

Figure 2.1 Map of FAO major fishing areas 61

Figure 2.2 Fish bones after pre-treatment 65

Figure 2.3 Schematic overview of the procedures used in this research for extraction of gelatine from fish heads, skins and bones. 66

Figure 2.4 Extraction process for oils from mackerel heads and skins. 95

Figure 4.1 Yield of gelatine extraction 110

Figure 4.2 Colour of mackerel head gelatines, pre-treated with different organic acids, and commercial fish and bovine gelatines 117

Figure 4.3 SDS-PAGE patterns of fish gelatines 122

Figure 4.4 FTIR spectra of gelatines obtained from mackerel and blue whiting using different pre-treatment 131

Figure 4.5 FTIR spectra of gelatine from mackerel heads, pre-treated with citric acid, and commercial fish and bovine gelatines. 134

Figure 4.6 ^{1}H NMR spectra of gelatines obtained from mackerel and blue whiting using different pre-treatment 136

Figure 4.7 Relative foaming capacity (FC) of fish gelatines 141

Figure 4.8 Relative foaming stability (FS) of fish gelatines....142

Figure 4.9 Relative foaming capacity (A) and stability (B) of commercial fish and bovine gelatines 143

Figure 4.10 Relative emulsifying capacity index (EAI) of gelatines from mackerel at different protein concentrations (0.2 %, 0.1 % and 0.05 %, w/v)....145

Figure 4.11 Relative emulsifying capacity index (EAI) of gelatines from blue whiting at different protein concentrations (0.2%, 0.1% and 0.05%, w/v)....146

Figure 4.12 Relative emulsifying stability index (ESI) of gelatines from mackerel at different protein concentrations (0.2%, 0.1% and 0.05%, w/v)....148

Figure 4.13 Relative emulsifying stability index (ESI) of gelatines from blue whiting at different protein concentrations (0.2%, 0.1% and 0.05%, w/v)....149

Figure 4.14 Relative emulsifying capacity index (EAI) and emulsifying stability index (ESI) of commercial fish and bovine gelatines at different protein concentrations (0.2%, 0.1% and 0.05%, w/v) 151

Figure 4.15 Relative solubility of fish gelatines in the pH range of 2 - 12....153

Figure 4.16 Relative solubility of fish gelatines as function of NaCl concentration....155

Figure 4.17 Relative solubility of commercial fish and bovine gelatines 156

Figure 4.18 Viscoelastic properties, including the storage (G') and loss modulus (G'') as well as the phase angle (δ) during the cooling (a, b and c) and heating ramps (d, e and f) of gelatines extracted from mackerel heads (A), skins (B) and bones (C) 158

Figure 4.19 Viscoelastic properties, including the storage (G') and loss modulus (G'') as well as the phase angle (δ) during the cooling (a, b and c) and heating ramps (d, e and f) of gelatines extracted from blue whiting heads (A), skins (B) and bones (C)....161

Figure 4.20 Viscoelastic properties, including the storage (G') and loss modulus (G'') as well as the phase angle (δ) during the cooling (a, b and c) and heating ramps (d, e and f) of commercial bovine and fish gelatines....162

Figure 4.21 Elastic modulus (G′) at 5 °C under frequency sweep of fish gelatines....165

Figure 4.22 Scanning electron microscopy (× 1500) of gelatine gels 171

Figure 4.23 Scanning electron microscopy (× 1500) of mackerel head gelatine gels 173

Figure 5.1 Evolution of the enzymatic hydrolysis as a function of time 175
Figure 5.2 Evolution of the degree of hydrolysis using pepsin for mackerel gelatine as a function of hydrolysis time 176
Figure 5.3 Mackerel head (A) and skin (B) gelatines after the heating process 177
Figure 5.4 Evolution of the degree of hydrolysis for mackerel viscera as a function of hydrolysis time 179
Figure 5.5 SDS-PAGE patterns of mackerel head (A, C & E) and skin (B, D & F) gelatine hydrolysates 181
Figure 5.6 SDS-PAGE patterns of mackerel viscera hydrolysates 183
Figure 5.7 The DPPH scavenging activity of mackerel gelatine hydrolysates at different hydrolysis times 185
Figure 5.8 The reducing power (FRAP) of mackerel gelatine hydrolysates, prepared using pepsin, at different hydrolysis times 186
Figure 5.9 The DPPH scavenging activity of mackerel viscera hydrolysates at different hydrolysis times 187
Figure 5.10 The reducing power (FRAP) of mackerel viscera hydrolysates at different hydrolysis times 188
Figure 5.11 The ACE inhibitory activity of mackerel gelatine hydrolysates at different concentrations 190
Figure 5.12 The ACE inhibitory activity of mackerel viscera hydrolysates at different concentrations 191
Figure 5.13 The platelet aggregation inhibitory activity of mackerel gelatine hydrolysates......... 195
Figure 5.14 Zone of inhibition obtained in anti-bacterial disc diffusion assay 199
Figure 5.15 RP (C_{18}) HPLC chromatograms of mackerel hydrolysates 203
Figure 5.16 RP (C_{18}) preparative HPLC chromatograms of mackerel hydrolysates 204
Figure 5.17 Peptide content of mackerel skin gelatines (A) and viscera (B) fractionated hydrolysates. 205
Figure 5.18 The platelet aggregation inhibitory activity of fractionated mackerel skin gelatine hydrolysates 208

Figure 5.19 RP-HPLC C_{18} chromatograms of fractions from the bioactive hydrolysates............213

Figure 5.20 IC_{50} values for the SSAO inhibitory activity of the bioactive fractions.....................214

Figure 5.21 IC_{50} values for the ACE inhibitory activity of the bioactive fractions.......................215

Figure 5.22 MS/MS spectra of the molecular ions present in mackerel skin gelatine fractions....218

Figure 5.23 The DPPH scavenging activity of blue whiting gelatine hydrolysates prepared from heads and skins at different hydrolysis times 219

Figure 6.1 Mackerel oils extracted with different procedures..221

Figure 6.2 Extraction yields of mackerel head (i) and skin (ii) oils as affected by the extraction procedure 222

Figure 6.3 Colour of mackerel head and skin oils as affected by the extraction procedure. 227

Figure 6.4 FTIR spectra of mackerel head (A) and skin (B) oils extracted using various procedures. 229

Figure 6.5 Typical gas chromatogram of lipid classes from mackerel oil....................................230

Figure 6.6 HPLC/ELSD chromatogram of mackerel oil extracted using the Folch method.........232

Figure 6.7 Gas chromatograms of fatty acid methyl esters (FAME)..233

Figure 6.8 DPPH scavenging activity of mackerel head (i) and skin (ii) oils extracted using various procedures...237

Figure 6.9 HPLC/ELSD chromatograms of mackerel head oils 238

Figure 6.10 Separation of oil fraction by thin layer chromatography (TLC) 239

Figure 6.11 DPPH scavenging activity of fractionated mackerel head oil extracted after hydrolysis with Alcalase 240

Figure 7.1 An optimised protocol for extracting bioactive compounds from mackerel waste......243

List of Tables

Table 1.1 The IUPAC EC classification of enzymes. 36

Table 1.2 Example of commercial proteases used for hydrolysing fish proteins. 38

Table 1.3 Advantages and disadvantages of the ORAC, DPPH and FRAP assays for determining antioxidant activity. 43

Table 2.1 Specificity of proteolytic enzymes. 76

Table 2.2 Conditions used for the enzymatic hydrolysis of mackerel gelatine. 77

Table 2.3 Conditions used for the enzymatic hydrolysis of mackerel viscera. 79

Table 3.1 Percentage (w/w) of waste from mackerel and blue whiting. 102

Table 3.2 Proximate analysis of mackerel and blue whiting per tissue type. 104

Table 3.3 pH of mackerel and blue whiting per tissue type. 106

Table 4.1 pH values of fish gelatines. 112

Table 4.2 Proximate analysis of gelatines. 114

Table 4.3 Colour measurement of gelatines from mackerel and blue whiting. 116

Table 4.4 Turbidity of fish gelatines. 119

Table 4.5 Average amino acid composition (g amino acid / 100 g gelatine) of gelatines from mackerel tissues. 124

Table 4.6 Average amino acid composition (g amino acid / 100 g gelatine) of gelatines from blue whiting tissues. 125

Table 4.7 Assignment of FTIR spectra of gelatines from mackerel and blue whiting. 129

Table 4.8 Rheological parameters of gelatines from mackerel and blue whiting. 163

Table 4.9 Gel strength of gelatines from mackerel, blue whiting and commercial sources. 169

Table 5.1 The SSAO inhibitory activities (%) of fish protein hydrolysates. 193

Table 5.2 The enhanced growth when probiotic strains were supplemented with 100 µg of fish protein hydrolysates for 24 h compared to control (water). 197

Table 5.3 Bioactivity level of fish protein hydrolysates. 201

Table 5.4 Fractionation of mackerel skin gelatine hydrolysates and screening for bioactivities of corresponding fractions. 207

Table 5.5 Fractionation of mackerel viscera hydrolysates and screening for the bioactivities of corresponding fractions. 210

Table 5.6 Bioactivity level of different fractions from mackerel skin gelatine and viscera hydrolysates. 212

Table 6.1 Colour and quality parameters of oils from mackerel. 224

Table 6.2 Lipid classes of mackerel head and skin oils (from GC measurements). 231

Table 6.3 Fatty acid composition (% of total peak area) of mackerel oils extracted from heads and skins using various procedures. 235

Chapter 1 – Introduction

1.1. Fish and fish processing industries

Fishing and the fish processing industries are important economic sectors in the world. According to the Food and Agriculture Organisation (FAO), the global fisheries production (catch and aquaculture) in 2008 was estimated to be around 110 million tonnes, with a global catch exceeding 76 million tonnes (FAO, 2010). Ireland contributed with 293,732 tonnes of the production, of which 240,610 tonnes were caught fish (FAO, 2010). In Ireland, the marine fishing industry represents an important source of economic activity, particularly to the coastal communities where it is based, providing 6,000 direct jobs. Moreover, 10,000 jobs onshore are dependent on catches from Irish vessels (Irish Fisheries Board (BIM), 2010). The Irish fishing industry is growing steadily with a significantly high export of fish products. The total Irish seafood sales on both domestic and export markets in 2008 was estimated to be €730 million (BIM, 2010).

1.1.1. Classification, distribution and characteristics of fish species

Fish can be classified, depending on their lipid level, as lean or fatty fish. Lean fish store lipids in the liver whereas fatty fish store lipids in fat cells in their body (Huss, 1995). Many factors can affect the fat level in fish, such as species, sex, age, season and diet (Tzikas *et al.,* 2007).

1.1.1.1. Oily fish (Mackerel)

Pelagics species, such as Atlantic mackerel (*Scomber scombrus*), are the largest source of fish oils (Colin, 1992). The fat level in mackerel depends on the capture season. Aubourg *et al.* (2005) reported that mackerel caught in May contained the lowest lipid content, while those caught in November had the highest lipid content.

Mackerel belongs to the family of *Scombridae*, and it is abundant in cold and temperate shelf areas (Figure 1.1), such as the North Atlantic Ocean (including the Baltic Sea), Eastern Atlantic (including the Mediterranean and the Black seas) and Western Atlantic Oceans, from Labrador to Cape Lookout (Collette & Nauen, 1983).

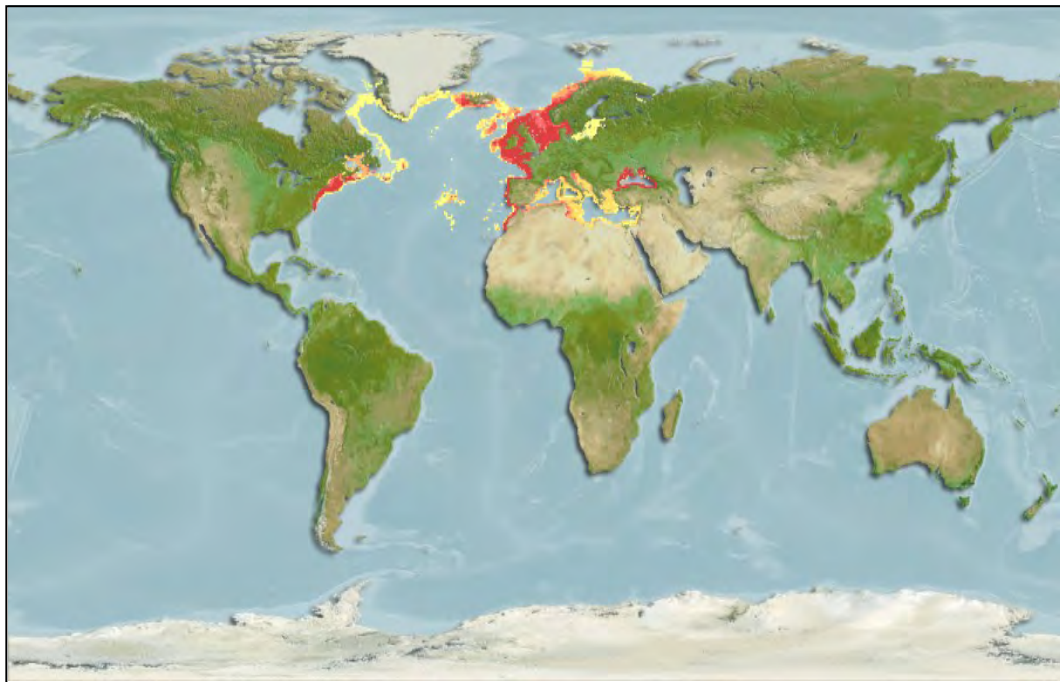


Figure 1.1 Species distribution map for mackerel (FAO FishFinder, 2010).
Red = Certain, Yellow = Uncertain.

Mackerel has oblique to near vertical markings on the back, with relatively little undulating and the belly is unmarked (Figure 1.2). The maximum length is 50 cm, but the common length of mackerel is 30 cm and females grow bigger than males. Mackerel is a typical zooplankton and small fish feeder (Collette & Nauen, 1983).

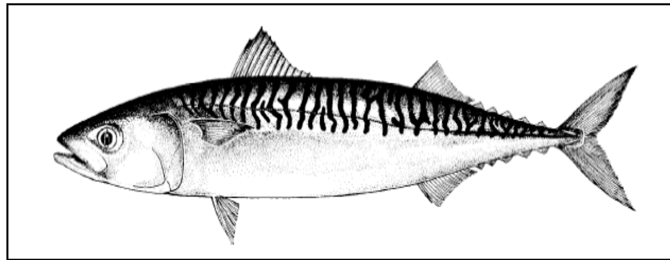


Figure 1.2 Atlantic mackerel (*Scomber scombrus*) (Collette & Nauen, 1983).

The world catch of mackerel declined from 1,092,759 tonnes in 1975 to 609,845 tonnes in 2008 (FAO, 2010), due to the massive overfishing. Based on the actual stocks and with the aim to better manage these and sustain the fish species, every country within the European Union (EU) is allowed to catch a certain amount of fish, called the quota, which represents a portion of the Total Allowable Catches (TACs). The mackerel stock was harvested unsustainably, and as a protective measure, Norway, EU and the Faroe Islands agreed "*For 2000 and subsequent years, the Parties agreed to restrict their fishing on the basis of a TAC consistent with a fishing mortality in the range of 0.15 - 0.20 for appropriate age groups as defined by the International Council for the Exploration of the Sea (ICES), unless future scientific advice requires modification of the fishing mortality rate*" (ICES, 2006). An increase in the capture of mackerel is estimated for the next few years due to recovery of the heavily exploited stock.

1.1.1.2. Lean fish (Blue whiting)

Blue whiting (*Micromesistius poutassou*) is a typical lean species and belongs to the *Gadidae* family along with cod and haddock. Blue whiting is distributed in the North Atlantic (Figure 1.3), from the Barents Sea south through the eastern Norwegian Sea, around Iceland, through the eastern Atlantic and in the western Mediterranean (Cohen *et al.,* 1990).

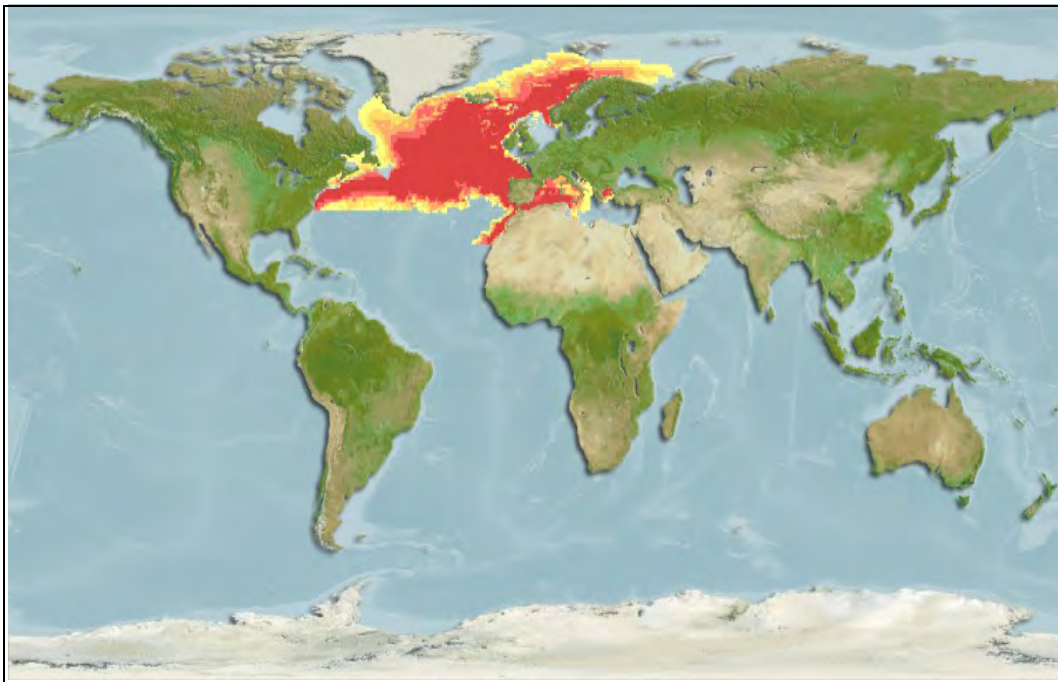


Figure 1.3 Species distribution map for blue whiting (FAO FishFinder, 2010). Red = Certain, Yellow = Uncertain.

Blue whiting has a blue-grey colour on the back, paler on the sides, shading to white on the belly and can reach 50 cm in total length but the common length is from 15 to 30 cm (Figure 1.4). Blue whiting feed mostly on small crustaceans but large individuals also prey on small fish and cephalopods (Cohen *et al.,* 1990).

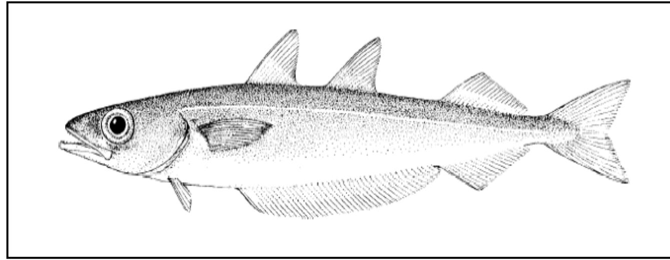


Figure 1.4 Blue whiting (*Micromesistius poutassou*) (Bini, 1969).

Blue whiting is an under-utilised fish with a global catch estimated to be 1,283,711 tonnes in 2008 (FAO, 2010).

1.1.2. Nutritional properties of fish

Fish represent a valuable source of proteins and nutrients, and its consumption is high in many countries. It is estimated that the total food supply available from fisheries to be slightly higher than 16 kilos per year for each of the world's inhabitants (Farmer, 2003). Many studies showed that fish consumption is associated with reduction in blood pressure (Connor, 2000) and risk of coronary heart disease (He *et al.,* 2004). These health benefits are associated with the consumption of fish oil containing polyunsaturated long chain omega-3 and omega-6 fatty acids (Lara *et al.,* 2007).

Fish contain all the essential amino acids and lysine, methionine and cysteine being present in significant levels (Ababouch, 2005). These amino acids play a major role in maintaining health and vitality (Usydus *et al.,* 2009). The amino acid composition of fish muscles is also associated with the functional properties of fish protein. It was reported that lysine and threonine exhibit high

water solubility making them interesting for technological reasons (Taskaya *et al.,* 2009). The amino acid composition of fish flesh is similar to that of a hen's egg and the consumption of fish together with products of plant origin which are poor in some amino acids (lysine, threonine), enables not only a complete utilisation of plant protein, but also improves the content of the diet (Bykowski & Dutkiewicz, 1996).

1.1.3. Waste from the fish processing industry

Several products from fish processing are available on the market, including fresh, frozen or marinated fillets, canned fish, fish meal, fish oil or other fish products, such as surimi. Fish are processed either offshore (on board of the fishing vessel) or onshore (processing plants). The processing of fish includes various unit operations starting with the pre-treatment followed by filleting, trimming of fillets, packing and storage.

In general, the pre-treatment of the fish involves the removal of ice, washing, grading according to size and de-heading. Fillets are processed either manually or by mechanical filleting machines. Bones are removed from the fillet. Before packing, the fillets are checked and those that do not have the standard quality are discarded. The final product is packed and kept either in ice if intended to be sold fresh or frozen for longer duration. Figure 1.5 shows a general process flow diagram for the filleting of fish.

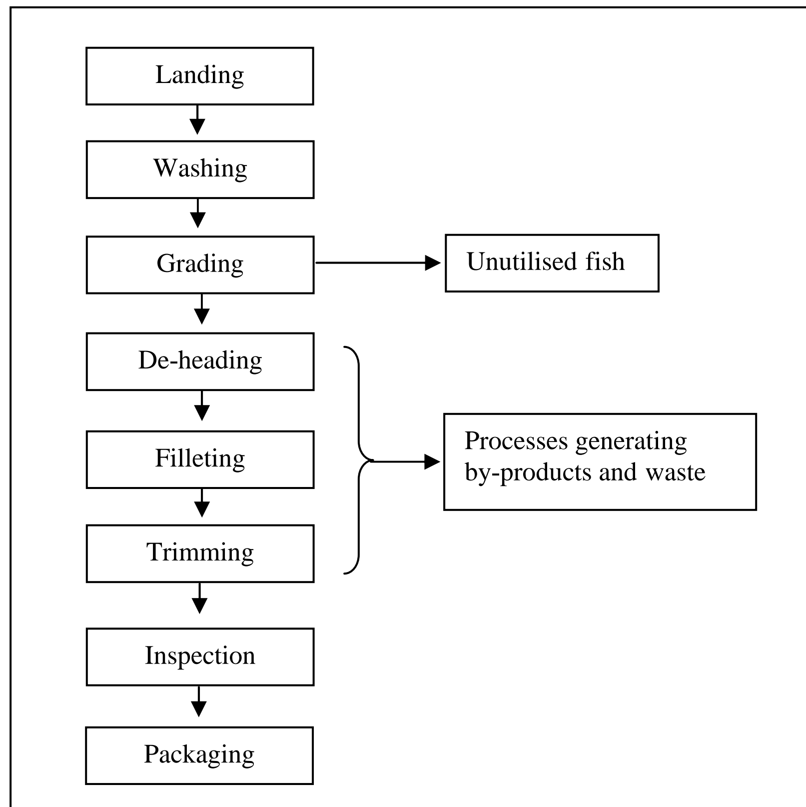


Figure 1.5 Process flow diagram for the filleting of fish (adapted from Bykowski & Dutkiewicz, 1996).

The fish processing industry generates significant amounts of by-products and waste, generally composed of viscera, heads, cut-offs, bone and skin. In fact there is a difference between the terms by-product and waste. By-products are products that are not regarded as ordinary saleable products (fillet, round, eviscerated or beheaded fish), but which can be recycled after treatment. Waste is any product that cannot be used for feed or value added products but which have to be composted, burned or destroyed (Rustad, 2003).

It is estimated that 25 % of the global fish production is regarded as waste and is discarded (Shahidi, 1994) or in the best case scenario processed into fish oil, fishmeal or pet food (Kim & Mendis, 2006). Fish waste is costly to dispose of and is typically discarded overboard in case of onboard processing or buried to landfill for the on-shore processing. In both cases and due to its high content of organic matter, fish waste disposal may cause severe health and environmental issues and is an increasing cost for the whole seafood industry (Jespersen *et al.,* 2000). The European Union legislation (Regulation (EC) No.1774/2002) restricts the disposal of animal by-products not intended for human consumption. Therefore more sustainable alternatives are needed. According to this regulation, animal wastes must be treated depending on the category to which they belong. Three categories were defined; category 1 includes high risk wastes (such as infected animals, international catering waste, etc.) which must not be used under any circumstances and have to be disposed of by incineration. Category 2 refers to high risk animal by-products (diseased animals, manure and digestive tract content) which can be used as compost or for biogas production after sterilisation. Category 3 includes low risk material (parts of slaughtered animals fit for human consumption, fresh by-products from fish, etc.). Wastes in category 3 can be ensiled, composted or disposed of by other means such as recycling.

Recycling fish waste is of interest from an environmental point of view by reducing the organic contaminant charge. Recent advances in fish waste management have resulted in their examination as a source of ingredients with a potential application to the food industry. Many studies have found an important number of bioactive compounds remaining in fish and shellfish waste (reviewed

by Kim *et al.,* 2008). Fish waste remains a source of bioactive materials such as collagen and gelatine, peptides and fish oil containing long chain polyunsaturated fatty acid (ω-3) (Shahidi, 1994).

1.2. Potential functional and bioactive compounds from fish waste

1.2.1. Collagen and gelatine

Collagen is the most abundant protein of animal origin, representing approximately 30 % of total animal protein (Muyonga *et al.,* 2004). Collagens provide strength and resilience to tissue resulting in the maintenance of the architecture of tissues. Collagens are also involved in several important functions such as in early development and organogenesis as well as in the regulation of cell behaviour (Kielty *et al.*, 1993; Kivirikko, 1993; Prockop & Kivirikko, 1995). Collagens have several variants, with type I, II, III and V being most abundant and well studied. Type I collagen is found in all connective tissue, including bones and skins while type II is usually found in cartilage. Type II collagen has a lower protein content (65 % - 70 %) than type I collagen (Van Der Rest & Garrone, 1991; Pihlajaniemi & Rehn, 1995; Grant, 2007). Type III collagen depends on the age of the animal, with skins from young animals containing up to 50 %. This content is reduced to 5 - 10 % for older animals. The other collagen types exist only in very low amounts and are mostly organ-specific (Schrieber & Gareis, 2007). The denaturation of collagen, by partial hydrolysis, produces gelatine. Type I collagen, which contains no cysteine, is used in the manufacture of gelatine. The traces of cysteine found in some gelatine are mainly due to the

presence of type III collagen in the raw material used for the extraction (Cole, 2000).

The basic structure of collagen is composed of three coiled polypeptides (Figure 1.6), made up of long chains of amino acids connected by peptide bonds (Ockerman & Hansen, 1988; Ward & Courts, 1977). Each collagen polypeptide has a significant number (334 repetitive units) of repeating triplets (Gly-X-Y). However, some short chains of 15 - 26 amino acids do not conform to this structure only at the N- and C-terminal ends (Schrieber & Gareis, 2007). In general, X and Y can be any amino acids, however, proline (Pro) has a preference for the X position and hydroxyproline (Hyp) for the Y position (Baily & Light, 1989; Johnston-Banks, 1990; Te Nijenhuis, 1997; Ogawa *et al.,* 2003).

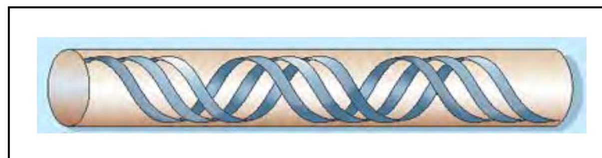


Figure 1.6 Triple helical structure of collagen (Taton, 2001).

The coiling of three collagen helices in a right-handed direction forms tropocollagen. Tropocollagen can form fibrils (triple-stranded helix structure) of approximately 300 nm in length and 8.6 nm in height through the association in either lateral or longitudinal directions (Djabourov, 1988; Tosh *et al.,* 2003). Collagen fibrils are maintained and stabilised by covalent cross-links between tropocollagen molecules (Neklyudov *et al.,* 2003).

Many studies showed that fish collagens consist of two α chain variants, which are normally designated as α_1 and α_2 (Gómez-Guillén *et al.,* 2002; Nagai *et al.,* 2002). Fish collagens vary widely in their amino acid composition depending on the fish species. Glycine is the major amino acid in collagen, usually present as one-third of the total amino acids. The contents of imino acids (proline and hydroxyproline) vary significantly among fish species (Balian & Bowes, 1977; Gudmundsson & Hafsteinsson, 1997; Poppe, 1992). Hydroxyproline and hydroxylysine are formed after the enzymatic oxidation of proline and lysine, respectively (Kotch *et al.,* 2008). These amino acids play an important role in the stabilisation of the helix which is achieved mainly through hydrogen bonds between C=O and N-H groups of the amino acids (Tosh *et al.,* 2003). Type I collagen does not contain tryptophan or cysteine, and tyrosine and histidine are present in very low content (Muyonga *et al.,* 2004).

The temperature in which the fish lives affects the amount of imino acids, especially hydroxyproline, as well as the thermal stability of the collagens (Kimura *et al.,* 1988). Hence collagens extracted from cold water fish have a lower content of hydroxyproline and exhibit lower thermal stability than those from warm water fish. This could be explained by the fact that hydroxyproline is involved in inter-chain hydrogen bonding, which stabilises the triple helical structure of collagen (Darby & Creighton, 1993).

1.2.1.1. Extraction and preparation of gelatine

Gelatine is a biopolymer produced by extraction and hydrolysis of fibrous, insoluble collagen. In terms of nutritional properties, gelatine is not a complete food protein due to the lack of the essential amino acid tryptophan and to the low level of methionine. The amino acid analysis of gelatine is variable depending on raw the material and process used (Stevens, 1992).

The industrial process of gelatine manufacture involves either an acid or alkaline pre-treatments followed by extraction with warm water. Heat denaturation easily converts collagen into gelatine. Further clarification steps include filtration, concentration, drying and milling (Schrieber & Gareis, 2007). The acid or alkali pre-treatment of raw material is necessary to cleave the cross-links. As the animal age increases, collagen structure evolves and becomes extremely stable with the formation of cross links. Gelatine prepared by the acid process is called type A gelatine and contains 18.5 % nitrogen, whereas that prepared by the alkaline process is called type B gelatine and has only about 18 % nitrogen due to the loss of amide groups (Eastoe & Leach. 1958; Zhou & Regenstein, 2005).

The method of manufacture greatly affects the physicochemical properties of the gelatine (Montero & Gómez-Guillén, 2000). The heat treatment hydrolyses the non-covalent bonds, some intermolecular bonds, some intramolecular bonds and few peptide bonds of collagen. These changes result in conversion of the tri-stranded collagen structure to a more amorphous form called gelatine (Dai *et al.,* 2006). If the temperature of gelatine is lowered below a certain critical value, all parts of collagen rich in proline and hydrolxyproline partially recover some of

their structure. Since many molecules are usually involved, a three dimensional structure is produced and it is responsible for the gel observed at low temperature. These gels are affected by the temperature and the molecular weight distribution in the collagen (Veis, 1965).

Pig skin, cattle bone and hide are generally the main sources of commercial gelatines and the amount of gelatine used worldwide in the food industry is increasing annually due to global demand (Montero & Gómez-Guillén, 2000). The global annual production of gelatine is around 326,000 tonnes, with pig skin-derived gelatine accounting for the highest (46 %) followed by gelatines from bovine hides (29.4 %), bones (23.1 %), and other sources (1.5 %) (GME, 2008). The estimated world usage of gelatine is 200,000 tonnes/year (Badii & Howell, 2006).

For some cultural, religious and ethnic reasons, pig gelatine is prohibited for use (Gómez-Guillén *et al.,* 2007). Fish gelatine gained interest following the outbreak of bovine spongiform encephalopathy (BSE) (Eysturskard *et al.,* 2009). In spite of being inferior to mammalian gelatines, in terms of functional properties (Cho *et al.,* 2005), fish gelatines are considered to be a suitable alternative and can satisfy the increasing demand for halal and kosher food products (Arnesen & Gildberg, 2007). Sources for fish collagen can be fish skins, bones, scales or connective tissues (Kim & Mendis, 2006).

1.2.1.2. Industrial application of gelatine

Gelatine has different applications in the food industry. It is widely used in the confectionery industry as a gelling agent. During cheese manufacture, casein usually loses its emulsifying properties after fermentation and the use of gelatine compensates for this loss and prevents the creaming of milk fat. Gelatine can stop the separation of phases in casein gels, which is observed during pasteurisation. Another major role of gelatine in the dairy industry consists of stabilising and enhancing the casein gel's texture, destroyed due to stirring process, especially in fruit yoghurt and yoghurt drinks (Schrieber & Gareis, 2007). In ice creams and whipped deserts, gelatine is added as a foam stabiliser. It also influences the size and the distribution of ice crystals and decreases the melting rate of water in case of temperature fluctuation during storage. Gelatine is used in the aspic products to adjust the firmness. It improves the juice holding ability, the taste and subsequent slicing. The beverage sector depends largely on gelatines for the clarification and the removal of unwanted colour, bitterness, odour and off-flavours.

The new trend in consuming healthy foods has resulted in increased production of low fat products. Gelatine is usually added to low fat margarine to prevent syneresis and phase separation. Its ability to act as a fat emulsifier enhances the margarine structure.

Gelatine has also an important application in the pharmaceutical industry for the manufacture of capsules. Other uses of gelatine include: thickener for syrups and liquid dosage, gel forming agent for dental preparations and an excipient for the manufacture of granules and tablets (Schrieber & Gareis, 2007).

1.2.1.3. Functional properties of gelatine

The main challenge of the modern food industry is to produce novel high quality foods using a limited range of ingredients (Gerrard, 2006). Proteins represent one of the major molecules that influence the properties of food. In addition to being nutritionally important, proteins impart a variety of physical characteristics to foods, generally referred to as functional properties (Kinsella, 1982). Functionality is defined as the physical and chemical properties, apart from nutritional attributes, that influence the behaviour of proteins in food systems during processing, storage, preparation and consumption (Kinsella, 1982; Cheftel *et al.,* 1985). Protein functional properties include foam forming ability, emulsification, solubility, viscosity and gelling properties (Motoki & Kumazawa, 2000; Kuraishi *et al.,* 2000). The functional properties of proteins are intimately related to their molecular structure and interactions (Damodaran, 1996; Kinsella & Phillips, 1989).

Foaming properties

Foams are complex two-phase colloidal systems which contain, at least initially, a continuous liquid phase and a gas phase dispersed as bubbles or air cells. The dispersed phase volume is large in comparison to the continuous phase. The surface hydrophobicity represents the main factor controlling the foaming properties. The presence of certain food components such as salts, sugars and lipids affect the foaming properties by altering both the protein and the viscosity of the continuous phase (Glaser *et al.,* 2007). High surface energy created by large air/water interfacial surface area and substantial density differences between

phases render these dispersions thermodynamically unstable (German & Phillips, 1994).

The properties of foams determine their industrial applications. In the food industry, the prediction of foaming properties has a significant impact on the processing and the quality of some products (Exerowa & Kruglyakov, 1998). Several food products are usually converted into foams before being further processed. The drying step of milk, coffee and egg powder depends largely on their previous foaming stage (Exerowa & Kruglyakov, 1998).

Emulsifying properties

An emulsion is a system containing two immiscible liquid phases, one of which is dispersed in the other as droplets varying between 0.1 and 50 μm in diameter. The phase present in the form of droplets is called the dispersed phase while the matrix in which the droplets are dispersed is called the continuous phase (Nawar, 1985). The formation of small dispersed droplets is associated with an increase in the interfacial area between the two liquids which means a decrease in the droplet diameter. From a physicochemical point of view, emulsions are thermodynamically unstable systems as a result of the large positive energy at the interface of the two liquids. Physical destabilisation mechanisms of emulsions include: oil droplet size variation processes, such as flocculation and coalescence, and particle migration phenomena, like sedimentation and creaming (Comas *et al.,* 2006).

In the food industry, emulsifiers are used to reduce the interfacial tension between the oil and water phases resulting in decreasing the free energy and stabilising the emulsion. Emulsifiers are also able to form a protective coating

around the oil droplets leading to prevention of coalescence phenomenon (Kasapis *et al.,* 2009). The manufacture of mayonnaise, margarine, salad creams, desserts and beverages represent the major markets for food emulsifiers (Boom, 2007; Tadros, 2009).

Solubility

The solubility of a protein under a given set of conditions is the thermodynamic state of equilibrium between protein/protein and protein/solvent interactions. It is related to free energy change arising from interaction of hydrophobic and hydrophilic residues on the protein surface with the surrounding solvent (Damodaran, 1996). Factors influencing the protein solubility include the properties of both protein and solvent, the concentration, pH of the medium, the ionic strength, the ratio of protein to solvent volume and the temperature (Lee *et al.,* 2003; Sikorski, 2001). Changes in pH alter the attractive and repulsive forces of proteins and their ability to associate with water. The isoelectric pH is defined as the pH at which a particular molecule carries no net electrical charge resulting in minimal hydration and swelling (Cheftel *et al.,* 1985).

Solubility characteristics are very useful in practice for determining optimum conditions for the extraction and purification of proteins from natural sources and separation of protein fractions. Also the solubility behaviour under various conditions provides a prediction of the potential application of proteins.

Rheology

Rheology is defined as the science of the deformation and flow of matter. It is the study of the behaviour in which materials respond to applied stress or strain. The material properties that govern the specific way in which these deformation or flow behaviours occur are called rheological properties which include elastic, viscous and viscoelastic properties (Steffe, 1996).

Elasticity

A material is called elastic if it deforms under stress but then returns to its original shape when the stress is removed. Usually, when a solid on an immovable surface is loaded with a weight it responds with a deformation that can be characterised by a reduction in its initial height dimension. In the case of an elastic deformation, the strain is completely reversible. That means when the stress is taken away, the strain (deformation) goes back to zero. The elastic regime is characterised by a linear relationship between stress and strain, denoted linear elasticity (Van Vliet, 1999).

Viscosity

Viscosity is defined as the internal friction of a fluid or its tendency to resist flow (Bourne, 2002). Unlike solids, fluids cannot support their own weight, and are incapable of holding any shape. They must be contained in a vessel or surface depression, otherwise, they will flow under the shear stress caused by their own weight. Therefore, the way in which liquids deform to an applied stress is to flow (continuous deformation) (Van Vliet, 1999).

When there is a linear relationship between shear stress and resulting shear rate, the flow behaviour is called ideal viscous or Newtonian. Fluids with that behaviour are named Newtonian fluids. If there is no linear relationship between shear stress and shear rate the flow is called non-Newtonian flow behaviour. The flow behaviour of non-Newtonian fluids depends on the stress conditions to which the fluids are subjected (Van Vliet, 1999).

Many factors affect the viscosity. An inverse relationship between the viscosity and the temperature is usually observed. Higher concentrations and the molecular weight of a solute increase the viscosity in a nonlinear manner (Bourne, 2002). During the food formulation, fluid and semi-fluid foods are pumped and mixed as part of the processing. The study and measurement of viscosity allow the optimisation of these unit operations.

Viscoelasticity

Viscoelasticity is the property of materials that exhibit both viscous and elastic characteristics when undergoing deformation. When a material is subjected to a shear stress, it starts to flow very slowly, and will show a slowly increasing deformation. If the shear stress is released back to zero again, the deformation will slowly recover to its initial value. An ideal elastic solid material would show an instant deformation and instant recovery of that deformation, like the behaviour of a Hookean spring. On the other hand, an ideal viscous liquid would flow (continuous deformation), but never recover to its initial zero deformation. Therefore, when a material shows both viscous and elastic behaviours it is called a viscoelastic material (Rao, 1999; Lucas *et al.,* 2002).

There are different instruments that can be used to measure the mechanical and viscoelastic response of materials. Common instruments that are capable of measuring fundamental rheological properties of semi-solid foods may be divided into two categories: rotational and capillary-tube type. Instruments which measure both viscosity and yield stress of a material are called rheometers; viscometers refer to devices that only measure viscosity (Steffe, 1996). Rotational instruments may be operated in the steady shear or oscillating modes. Some rotational instruments function in the controlled stress mode facilitating the collection of creep data and the analysis of materials at very low shear rates.

With a rheometer running under oscillating mode, both viscous and elastic properties of a material can be measured simultaneously. When the amplitude of the oscillation is appropriately chosen so as not to be too high, the sample material can be subjected to an oscillating shear stress without breakage of any molecular structure within the sample. There is a mathematical equation to describe these behavioural properties. These types of mathematical factors are called complex numbers. By using the complex shear modulus, both the viscous and the elastic properties of a material can be characterised by measuring the phase shift δ only (Ferry, 1980).

The complex shear modulus:

$$G^* = G' + G''$$ **Equation 1.1**

With G’ for the elastic component, and G’’ for the viscous component.

The phase shift is simply:

$$Tan\ \delta = G''/G'$$ **Equation 1.2**

A material with ideal elastic properties has a viscous component G''= 0.

A material with ideal viscous properties has no elastic component, so G' = 0.

Gelation

Gelation is an important functional property. Many factors affect the gelation process. Usually thermal treatment is required, cooling is also necessary, as well as acidification. Hydrophobic interactions are enhanced at high temperatures and the formation of hydrogen bonds is favoured during cooling (Te Nijenhuis, 1997).

The mechanisms and the interactions leading to the formation of the networks of gels are not fully understood, but most of the studies agree on the fact that proteins has to be denaturated and unfolded prior to the step of the ordered state of protein-protein interaction and aggregation (Figure 1.7). The balance between protein-protein and protein-solvent interactions and attractive and repulsive forces between adjacent polypeptide chains lead to the formation of the gels (Djabourov, 1988).

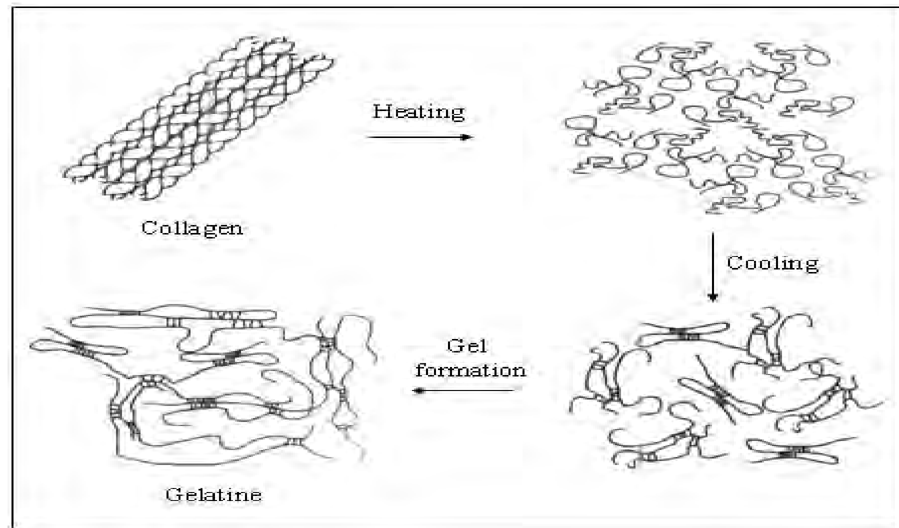


Figure 1.7 Conversion of collagen, through heating and cooling, to a gelatine network (Schrieber & Gareis, 2007).

Johns & Courts (1977) reported that the process of conversion of collagen to gelatine involves the hydrolysis of lateral non peptide bonds, disruption of hydrogen bonds and hydrolysis of peptide bonds. Fish collagen has less cross-links than mammalian collagen and thus it requires only the disruption of hydrogen bonds to break the cross-links. The pH, the temperature and the concentration are the major factors affecting the gelling properties of protein (Zayas, 1997).

Many foods are based on colloidal gels structure such as yoghurts, cheeses, desserts and sausages. Industrially processed foods are very complex products obtained after formulation of several ingredients. The incompatibility between these food ingredients and gelation leads to phase separation and various gel structures. Hence, the study of the gelation mechanism as well as the gel properties of these ingredients enables the prediction of the final texture of the semisolid food (Hermansson, 2007).

1.2.2. Fish protein hydrolysates (FPH)

Proteins are polymers composed of different amino acids linked by peptide bonds. All amino acids can be classified depending on their interaction with water as non-polar (hydrophobic) or polar (hydrophilic) amino acids. Differences among the side chains of amino acids affect their physical and bioactive properties and play an important role in stabilising the conformations of proteins (Horton *et al.,* 1993).

Fish protein hydrolysates are proteins chemically or enzymatically hydrolysed into peptides of varying sizes (Rustad, 2003). The enzymatic hydrolysis *in vitro* is an effective approach for the recovery of bioactive peptides as well as the improvement of the functional and nutritional properties of protein from under-utilised fish and fish by-products (Šližytė *et al.,* 2005).

1.2.2.1. Classification of enzymes and preparation of FPH

The Enzyme Commission (EC) developed a numerical nomenclature for enzymes, using a four-digit number, based on the reactions they catalyse and their specific substrate. Table 1.1 presents the six different categories that have been defined. Proteases are enzymes that cleave the peptide bonds and hence belong to the third category in the IUPAC EC classification. Proteases are divided into endopeptidases and exopeptidases. Endopeptidases, which include serine proteases, cysteine proteases, aspartic proteases and metallo proteases, cleave the polypeptide chain at specific peptide bonds. However, exopeptidases hydrolyse amino acid either at the N terminus (aminopeptidases) or at the C terminus (carboxypeptidases).

Table 1.1 The IUPAC EC classification of enzymes.

First EC number	Classification	Enzymatic reaction
1	Oxidoreductases	Oxidation/reduction
2	Transferases	Transfer of chemical group
3	Hydrolases	Hydrolytic bond cleavages
4	Layses	The cleavage of bonds by reactions other than hydrolysis
5	Isomerases	Change in arrangements of atoms in molecules
6	Liages	The formation of bonds

Adapted from Enzyme Nomenclature, NC-IUBMB, Academic Press, New York, (1992).

Several proteolytic enzymes can be used for the hydrolysis of fish processing waste (Simpson *et al.,* 1998) which can be from microbial, plant or animal sources (Table 1.2). These enzymes should be food grade and if they are from microbial origin, the producing organism has to be non-pathogenic (Bhaskar & Mahendrakar, 2008). Various digestive enzymes are naturally present in fish viscera (Shahidi & Kamil, 2001). Enzymes from the digestive tract play an important role in the autolysis (self-digestion) of un-eviscerated fish. The major autolytic enzymes are cathepsin, chymotrypsin, trypsin, cacboxypeptidase, calpain, collagenase and TMAO demethylase and are either localised in the pyloric caecae or in the stomach wall (Huss, 1988). Autolysis of proteins results in the formation of free amino acids and other nitrogenous compounds that liberate NH_3, CO_2 and volatile compounds upon decomposition (Sen, 2005; Shukla, 2010). These endogenous enzymes can be used in the hydrolysis process and a number of enzymes have been successfully isolated from fish processing discards (Venugopal & Shahidi, 1995). However, differences among fish species, seasons, type and amount of enzymes make the endogenous hydrolysis rate difficult to control (Sikorski & Naczk, 1981; Aspmo *et al.,* 2005). Hence, due to

the complexity of controlling the hydrolysis process by the endogenous enzymes, the use of purified enzymes is preferred. The enzymatic hydrolysis under controlled conditions can improve the functional properties of protein (Quaglia & Orban, 1990). Hydrolysis modifies the molecular size of peptides as well as their hydrophobic and polar groups (Adler-Nissen, 1986; Kristinsson & Rasco, 2000b). These changes affect the functional properties and the uses of hydrolysates as food ingredients (Kristinsson & Rasco, 2000b).

Table 1.2 Example of commercial proteases used for hydrolysing fish proteins.

Type of proteases	Name	Source	Type of activity	References
Serine proteases	Trypsin	Bovine/ porcine pancreas	Endopeptidase	Pavlisko & Coppes, 1999; Pavlisko *et al.,* 1999; Jun *et al.,* 2004; Je *et al.,* 2005b; Mendis *et al.,* 2005a; Qian *et al.,* 2007; Bougatef *et al.*, 2008.
	Chymotrypsin	Bovine/ porcine pancreas	Endopeptidase	Jun *et al.,* 2004; Je *et al.,* 2005b; Mendis *et al.,* 2005a; Jung *et al.,* 2006; Qian *et al.,* 2007; Bougatef *et al.,* 2008.
	Alcalase	*Bacillus licheniformis*	Endoproteinase	Liceaga-Gesualdo & Li-Chan, 1999; Kristinsson & Rasco, 2000a; Liaset *et al.,* 2000; Sathivel *et al.,* 2003; Jun *et al.,* 2004; Aspmo *et al.,* 2005; Je *et al.,* 2005b; Sathivel *et al.,* 2005; Qian *et al.,* 2007; Bougatef *et al.,* 2008.
	Protex 6L	*Bacillus licheniformis*	Endoproteinase	Sathivel *et al.,* 2005.
Cysteine proteases	Papain	Papaye	Broad specificity	Jun *et al.,* 2004; Aspmo *et al.,* 2005; Je *et al.,* 2005b; Qian *et al.,* 2007.
	Bromelain	Ananas	Broad specificity	Aspmo *et al.,* 2005.
	Actinidin	Kiwifruit	Endoproteinase	Aspmo *et al.,* 2005.
Aspartic proteases	Pepsin	Bovine/ porcine pancreas	Endoproteinase	Pavlisko *et al.,* 1999; Liaset *et al.,* 2000; Je *et al.,* 2004; Jun *et al.,* 2004; Mendis *et al.,* 2005a; Aspmo *et al.,* 2005; Je *et al.,* 2005b; Qian *et al.,* 2007; Bougatef *et al.,* 2008; Yoshie-Stark *et al.,* 2009.
Metallo proteases	Neutrase 0.5L	*Bacillus subtilis*	Endoproteinase	Liaset *et al.,* 2000; Gilmartin & Jervis, 2002; Jun *et al.,* 2004; Aspmo *et al.,* 2005; Je *et al.,* 2005b; Sathivel *et al.,* 2005; Šližytė *et al.,* 2005; Qian *et al.,* 2007.
Crude extract	Flavourzyme	*Aspergillus oryzae*	Endo- and exopeptidases	Kristinsson & Rasco, 2000a; Gilmartin & Jervis, 2002; Sathivel *et al.,* 2005; Šližytė *et al.,* 2005; Klompong *et al.,* 2007; Thiansilakul *et al.,* 2007; Samaranayaka & Li-Chan, 2008.
	Pancreatin	Bovine/ porcine pancreas	Broad specificity	Yoshie-Stark *et al.,* 2009.
	Corolase 7089	*Bacillus subtilis*	Endoproteinase	Kristinsson & Rasco, 2000a; Gilmartin & Jervis, 2002.
	Corolase PN-L	*Aspergillus sojae*	Endo- and exopeptidases	Kristinsson & Rasco, 2000a.
	Protamex	*Bacillus subtilis*	Endo- and exopeptidases	Liaset *et al.,* 2003; Aspmo *et al.,* 2005.
	Validase	*Bacillus subtilis*	Endopeptidase	Gilmartin & Jervis, 2002; Samaranayaka & Li-Chan, 2008.
	Pronase	*Streptomyces griseus*	Endo- and exopeptidases	Jun *et al.,* 2004.
	Umamizyme	*Aspergillus oryzae*	Endo- and exopeptidases	Guérard *et al.,* 2002.

Commercial crude enzymes usually contain a mixture of proteases having both endo and exopeptidase activities. Microbial enzymes are widely used due to their relatively low cost (Leclerc *et al.,* 2002; Prioult *et al.,* 2005). The price of these preparations depends on the isolation procedures and the purity of the extract. However, if intended to be used in food, these preparations must meet the purity levels described by the *Food Chemicals Codex*.

1.2.2.2. Industrial application, nutritional and bioactive properties of FPH

Fish protein hydrolysates are mainly used as fertiliser (Benjakul & Morrissey, 1997) or as animal feed, due to the bitter flavour and fishy odour associated with the hydrolysis (Picot *et al.,* 2006). Recent advances in the enzymatic hydrolysis of fish waste and by-products resulted in hydrolysates with better organoleptic, functional and nutritional properties that can be used as food ingredients (Gildberg, 1993; Pacheco-Aguilar *et al.,* 2008).

Fish protein hydrolysates have an excellent solubility at a high degree of hydrolysis (Shahidi *et al.,* 1995). Fish protein hydrolysates, prepared by controlled enzymatic digestion, are among the best protein hydrolysates due to the cleavage of specific peptide bonds and the release of peptides with better functional and nutritional properties (Kristinsson & Rasco, 2000b). Several parameters affect the composition and the physicochemical properties of the hydrolysates. These factors include the proteolytic enzymes, enzyme to substrate ratio (E/S), hydrolysis time, pH, temperature and water to raw material ratio (He *et al.,* 2006; Mullaly *et al.,* 1995).

Peptides isolated from various fish protein hydrolysates have shown numerous bioactivities such as antihypertensive, antithrombotic, immuno-modulatory and antioxidant activities (Kim & Mendis, 2006). Amarowicz & Shahidi (1997) and Kim *et al.* (2001) reported that many protein hydrolysates possess antioxidant activities. Mendis *et al.* (2005a) have demonstrated that the enzymatic hydrolysis of hoki (*Johnius belengerii*) skin gelatines with trypsin released an antioxidant peptide and the peptide sequence His-Gly-Pro-Leu-Gly-Pro-Leu (797 Da) acted as a strong radical scavenger. Many studies on the hydrolysis of fish by-products showed that the hydrolysates have the ability to inhibit the angiotensin converting enzyme (ACE). He *et al.* (2007) found that marine food proteins represent a potential material for the production of Angiotensin I-converting enzyme (ACE) inhibitory peptides by proteolysis. Je *et al.* (2004) reported a novel ACE inhibitory peptide from Alaska pollack frame protein hydrolysates. When bigeye tuna dark muscle were hydrolysed by alcalase, neutrase, pepsin, papain, chymotrypsin and trypsin, an ACE inhibitory activity was detected and the peptide derived from tuna dark muscle hydrolysis was Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro with an IC_{50} of 21.6 μM (Qian *et al.*, 2007).

Picot *et al.* (2006) studied the anti-proliferative activity of fish protein hydrolysates on human breast cancer cell lines and they observed that numbers of hydrolysates from blue whiting, cod, plaice and salmon were identified as significant growth inhibitors on the two cancer cell lines used in their study. Previous research discovered that the epidermis and the epidermal mucous of different fish species have antifungal and antibacterial properties (Ljima, 2003;

Richards *et al.,* 2001). Patat *et al.* (2004) reported that hemocyte histone proteins of shrimp had an antimicrobial activity, representing a natural defence mechanism for these organisms.

1.2.2.3. Screening for bioactivity of FPH

'*Bioactive compounds are essential and non-essential compounds that occur in nature, are part of the food chain, and can be shown to have an effect on human health*' (Biesalski *et al.,* 2009). Many food constituents contain polysaccharides, polypeptides, amino acids, fatty acids and phenolic compounds with bioactive properties. These substances may have antitumor, antioxidant, blood fat reducing, anti-inflammatory, anti-hypertensive and antimicrobial functions (Bomford, 1989; Goto *et al.,* 1993).

Antioxidant activity

Highly reactive free radicals and oxygen species are formed in biological systems in response to a wide variety of stimuli. Nucleic acids, proteins, lipids or DNA can be oxidised by the free radicals and initiate degenerative disease (Higdon & Frei, 2003). An antioxidant has the ability to scavenge free radicals. After hydrolysis, some food proteins, including fish protein and gelatine, may act as antioxidant molecules by scavenging free radicals (peroxide, hydroperoxide or lipid peroxyl) which inhibit the oxidative mechanisms that lead to degenerative diseases (Mendis *et al.,* 2005b; Martinez *et al.,* 2005; Guérard *et al.,* 2003).

Various methods have been used to monitor and compare the antioxidant activity of foods. The main methods for the evaluation of the antioxidant potency are based on either an electron or a hydrogen atom transfer (Huang *et al.,* 2005).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a rapid, simple and inexpensive method to estimate the *in vitro* antioxidant capacity of food. DPPH is mainly used to evaluate the ability of compounds to scavenge free radicals. The DPPH is a stable radical with one electron delocalised over the molecule. This delocalisation gives a deep purple colour with absorption maxima at 517 nm in an ethanol solution. The decrease in absorption at 517 nm per time is correlated with the scavenging activity. The reaction between DPPH and the antioxidant can be summarised as follows:

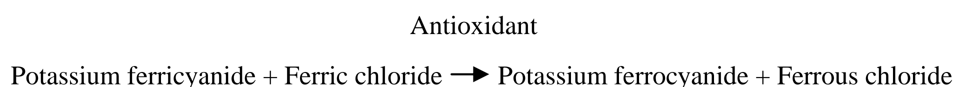
$$DPPH^{\cdot}\ (purple) + RH \rightarrow DPPHH\ (yellow) + R$$

Equation 1.3

where RH is the antioxidant.

In terms of hydrogen donating ability, the higher the discolouration, the better the scavenging potential (Oktay *et al.,* 2003). The antioxidant capacity in this assay depends on the chemical structure of the antioxidant (Vattem & Shetty, 2006).

The ferric reducing antioxidant power (FRAP) assay is another *in vitro* method that depends on the transfer of an electron. The FRAP assay is based on a redox reaction and employs ferric chloride ($FeCl_3$) as the oxidant (Benzie & Strain, 1996). The ferrous (Fe^{2+}) ion produced from the redox reaction forms a coloured product with 2,2’ dipyridyl according to the following reaction:



Equation 1.4

The antioxidant activity is estimated following the increase of absorbance at 595 nm, and compared to a standard antioxidant compound.

The oxygen radical absorbance capacity (ORAC) method consists of measuring the decrease in the fluorescence of a protein due to the loss of its conformation as result of oxidative damage caused by peroxyl radicals ($ROO^{\cdot}$). The ORAC assay, particularly that employing fluorescein as target molecule, is widely used. This *in vitro* method was applied to evaluate the antioxidant activity of different fruits, including berries (Ou *et al.,* 2001; Wu *et al.,* 2004).

A comparison between the advantages and disadvantage of these assays is presented in Table 1.3.

Table 1.3 Advantages and disadvantages of the ORAC, DPPH and FRAP assays for determining antioxidant activity.

Method	Mechanism	Advantage	Disadvantage
ORAC	Hydrogen atom transfer	▪ Allows the study of reaction kinetic. ▪ Biologically relevant. ▪ Automated and standardised.	Cost and time consuming.
DPPH FRAP	Electron transfer	▪ Inexpensive. ▪ Easy to use. ▪ Quick assays.	High sensitivity to light and temperature.

So far, no official standardised method for the determination of the antioxidant activity was proposed (Zulueta *et al.,* 2009). Therefore, the use of various methods for the evaluation of the antioxidant activity is recommended (Frankel & Meyer, 2000).

Artificial antioxidants, such as butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) are widely used in the food manufacture due to economical reasons (Xiu-Qin *et al.,* 2009). However, the growing consumer tendency to buy food products with natural antioxidants and the strict legislation create new challenges for the whole food sector. FPH with antioxidant activities can serve as possible natural antioxidants and may replace the synthetic preservatives.

Anti-hypertensive activity

Angiotensin-I converting enzyme (ACE), a dipeptidylcarboxypeptidase, converts the inactive decapeptide, angiotensin I, into a potent vasoconstrictor, the octapeptide angiotensin II. Angiotensin II is known to be a strong vasopressor. Other physiological functions of ACE include regulation of fluid and salt balance in mammals, regulation of local levels of other endogenous peptides, such as enkephalins and substance P, as well as inactivation of bradykinin (Ondetti *et al.,* 1977). The inhibition of ACE activity reduces angiotensin II level, but increases bradykinin and enkephalins levels, leading to lowering of blood pressure (Koike *et al.,* 1980).

The ability of a peptide to inhibit ACE is linked to its amino acid composition and its primary sequence. The presence of proline and some aromatic amino acids (Trp, Tyr and Phe) in C-terminal position seems to increase the ability of the peptide to inhibit ACE (Ariyoshi, 1993; Saito *et al.,* 1994; Yamamoto & Takano, 1999; Mizuno *et al.,* 2004). It has also been shown that peptides containing an Arg residue in the C-terminal position would be good ACE inhibitors (Maruyama *et al.,* 1989; Meisel & Schlimme, 1994).

The most utilised protocol for screening the angiotensin-I converting enzyme (ACE) activity was developed by Cushman & Cheung (1971). This assay is based on the hydrolysis of hippuryl-histidyl-leucine (HHL) by ACE to hippuric acid (HA) and histidyl-leucine as products. The HA is extracted into ethyl acetate and quantified by measuring the absorbance at 228 nm using a spectrophotometer or HPLC. However, there is a critical problem associated with the possible contamination of HA with ethyl-acetate which absorbs strongly at 228 nm giving wrong estimation of the ACE activity (Wu *et al.,* 2002).

Another simple and robust assay uses N-[3-(2-furyl) acryloyl]-L-phenylalanyl- glycylglycine (FA-PGG) as substrate for the ACE (Vermeirssen *et al.,* 2002). The FA-PGG is hydrolysed into furylacryloylphenylalanine (FA-P) and glycylglycine (GG) resulting in lowering the absorbance at 340 nm.

Sentandreu & Toldra (2006) proposed a fluorescent assay for angiotensin-I converting enzyme (ACE) based in the hydrolysis of a fluorescent substrate (*O*-aminobenzoylglycyl-pnitro-phenylalanyl-proline) by the action of ACE which then measured by a fluorometer.

The detection of novel bioactive peptides with antihypertensive activity, released by enzymatic hydrolysis of food proteins, can open a door for replacing synthetic inhibitors known to have possible side effects.

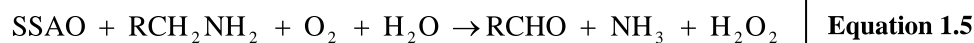
Anti-inflammatory activity

Inflammation is significant in various pathological conditions (Kerschensteiner *et al.,* 2008). The proliferation of parenchymal microglia and the recruitment of monocytes are very substantial during pathological response (Flugel *et al.,* 2001; Ladeby *et al.,* 2005; Djukic *et al.,* 2006; Ajami *et al.,* 2007).

In addition to being responsible for local pathologies (Montagna *et al.,* 2000), inflammation, can be a major risk in various diseases. Inflammation is usually involved in metabolic disorders (diabetes), autoimmune diseases (pemphigus) and blood dyscrasias (leukemia). Mast cells, neutrophils and macrophages play a major role in inflammatory disorders and activation of microglial cells and also contribute to the development of inflammatory diseases (Perioli *et al.,* 2008). The study and treatment of the inflammatory diseases has attracted lot of interest in recent years.

Semicarbazide-sensitive amine oxidase (SSAO) is a common name for enzymes that catalyse the oxidative deamination of primary aromatic and aliphatic amines. SSAO is present either in tissue-bound or soluble (plasma) forms in most mammalians (O'Sullivan *et al.,* 2004). Unlike the plasma amine oxidase (PAO), the copper-containing semicarbazide-sensitive amine oxidase (SSAO) enzymes are very sensitive to inhibition by carbonyl group reagents. Semicarbazide is a potent inhibitor for SSAO due to the presence of a 2, 4, 5-trihydroxyphenylalanine (6-hydroxydopa) quinine as a redox cofactor (Houen, 1999). SSAO are resistant to the acetylenic monoamine oxidase inhibitors (clorgyline, l-deprenyl and pargyline).

SSAO catalyses the oxidative deamination of primary amines, according to the overall reaction:



$$SSAO + RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

Equation 1.5

The production of hydrogen peroxide can be measured by a spectrophotometer following the production of quinoneimine dye as described by Holt *et al.* (1997). In this reaction, 4-aminoantipyrine acts as the proton donor in the peroxidase reaction and then condenses with vanillic acid to produce a quinoneimine dye that absorbs at λ_{max} of 498 nm (Figure 1.8).

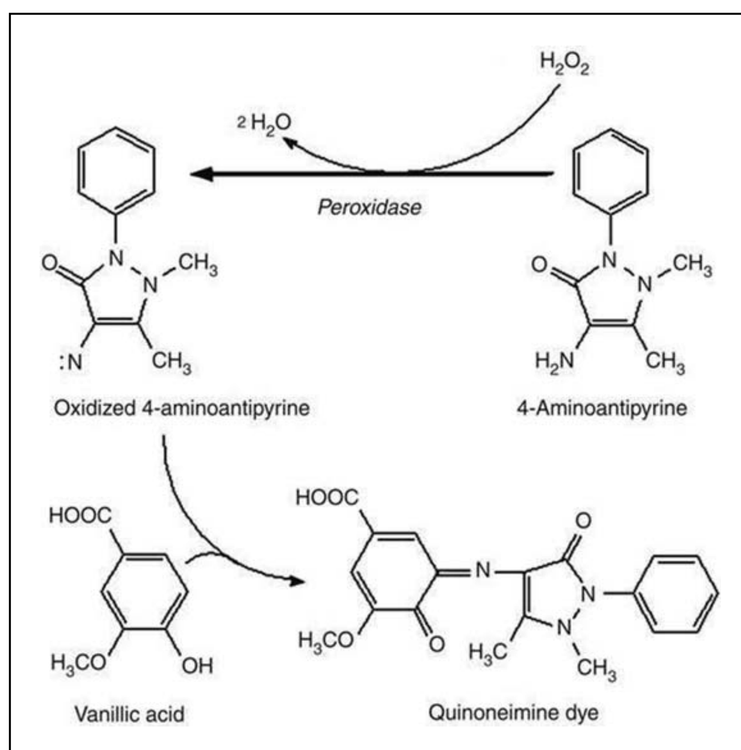


Figure 1.8 The peroxidase linked continuous assay for amine oxidase enzymes (Holt *et al.,* 1997).

SSAO appears to be multifunctional depending on the tissue in which it is expressed (O'Sullivan *et al.,* 2004). SSAO was reported to stimulate the glucose transport in adipocytes (Enrique-Tarancón *et al.,* 1998). Different activities of SSAO were associated with various diseases such as Alzheimers (Ferrer *et al.,* 2002), cancer (Lyles, 1996), cardiac disease (Boomsma *et al.,* 2000), diabetes (Karádi *et al.,* 2002) as well as inflammatory diseases (Kurkijärvi *et al.,* 1998). Additionally, SSAO is involved in lymphocyte trafficking inducing inflammation (Smith *et al.,* 1998). Hence, the discovery of natural bioactive compounds that inhibit SSAO represents an emerging target for developing new anti-inflammatory drugs.

Antithrombotic activity

Platelets are small anucleate cell fragments of the larger hematopoietic precursor, the megakaryocyte (Petersen *et al.,* 2010). Platelets circulate close to the endothelial cell surface as individual entities without interacting with any other cell types (Ruggeri, 2003). Their primary role in hemostasis is to maintain the integrity of the endothelium in order to prevent blood loss (Maguire *et al.,* 2004). The disruption, rupture and erosion of the atherosclerotic plaque cause thrombus formation, i.e., the clotting of blood inside the vessels (Friedewald *et al.,* 2007; Giugliano *et al.,* 2007; Piazza *et al.,* 2008). Vascular damage, stimulation of platelets and activation of the coagulation cascade are the factors contributing to thrombosis which causes high rates of morbidity and mortality (Piazza *et al.,* 2008). Thrombosis is a serious pathological issue and a major cause of stroke and atherosclerosis (Tolleson *et al.,* 2003; Bridgman *et al.,* 2006; Cuisset

et al., 2006; Giugliano *et al.,* 2007; Rdzanek *et al.,* 2007). Suppression or inhibition of platelet activity may decrease incidence of the disease (Shimizu *et al.,* 2009).

The platelet aggregometry test is a conventional *in vitro* analysis and is performed in anticoagulated blood. In this assay, the platelet aggregation is induced by various agonists, such as thrombin and collagen, and then measured (Goto *et al.,* 1999). However, this technique is time consuming and needs large volumes of sample (Sun *et al.,* 2001) making it unsuitable for screening antithrombotic agents.

Platelets contain two types of granules, dense and α granules. Dense granules mainly consist of serotonin, ADP, ATP and PF4, while α granules contain large proteins (Reed *et al.,* 2000). The activation of platelets induces the migration of α granules, dense granules and lysosomes to the cell surface as well as their discharge (Fukami *et al.* 2001) causing the change in shape of the platelet cytoskeleton and the activation of the major platelet receptors (Figure 1.9). Platelet activation is enhanced by agonists such as thrombin and collagen (Maguire *et al.,* 2004). The released ADP can be detected by luminescence using luciferase/luciferin (Sun *et al.,* 2001).

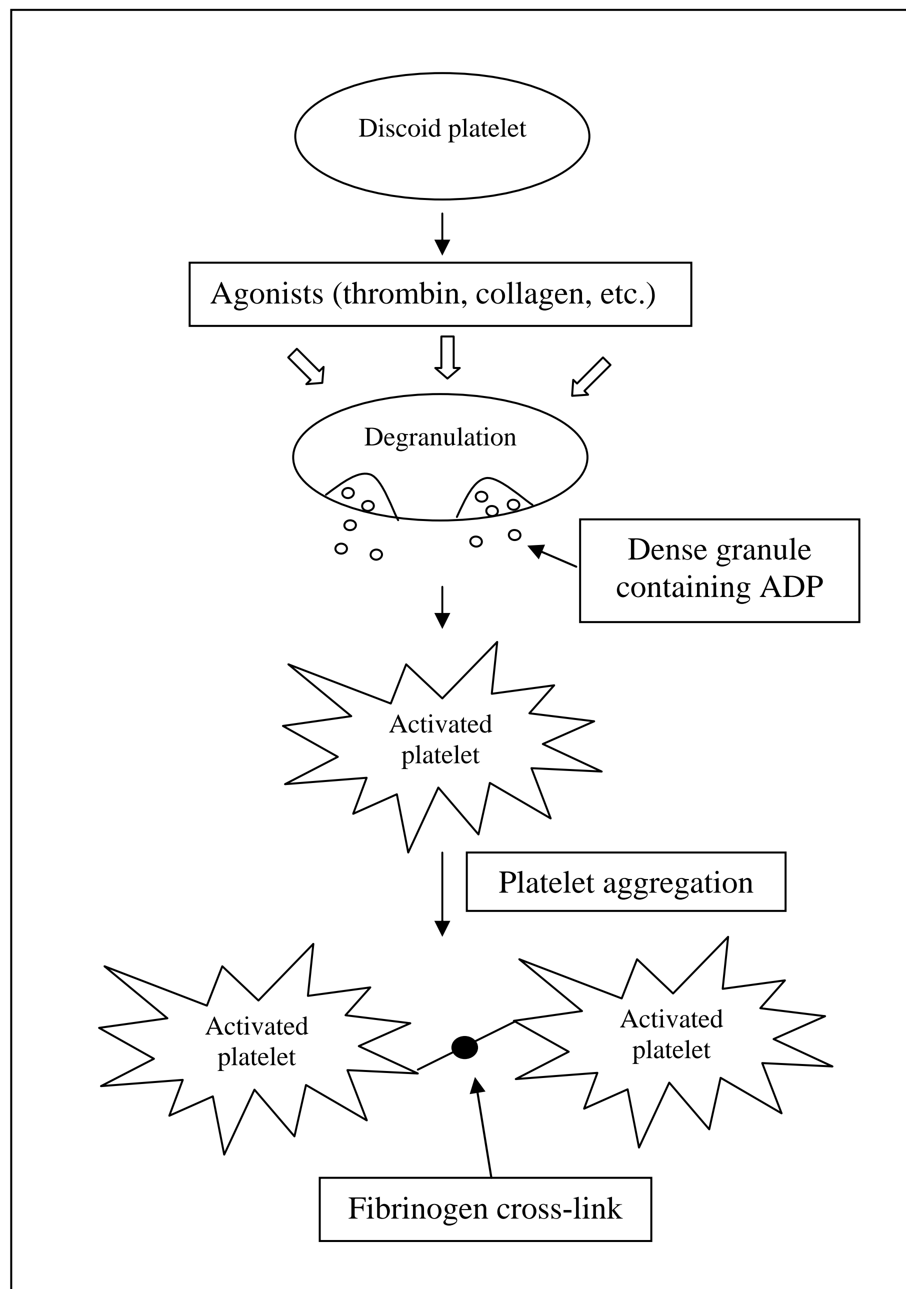


Figure 1.9 Platelet aggregation scheme (adapted from Woulfe *et al.*, 2001).

Low cost bioactive peptides from fish waste could be a promising way for inhibiting ADP-induced platelet aggregation and reducing the formation of thrombosis.

Antimicrobial activity

Several microorganisms, including Gram positive and Gram negative bacteria, as well as fungi, represent the major causes of various human infections. According to the World Health Organisation (WHO), 30 % of people in industrialised countries suffer from a food-borne disease each year. At least 2.1 million people died from diarrhoeal diseases worldwide in 2000, due to contamination of food and drinking water (WHO, 2002). Salmonellosis was the most widely occurring bacterial food-borne disease, with more than 20 million cases and an estimated death toll of 200,000 worldwide each year (Boyle *et al.,* 2007).

There is still demand to develop methods to control food-borne diseases. The worldwide trend is to minimise the incidence of food-borne diseases while reducing negative and side effects on human health (Bautista-Banos *et al.,* 2006). The new approach in the Western society is to minimise or ban the use of synthetic additives with priority given to the safety, quality and shelf-life of the food (Bautista-Banos *et al.,* 2006). Although effective antimicrobials have been developed over the years, new natural products have to be screened for antimicrobial activities due to the ability of microorganism to acquire resistance to the presently available antimicrobials (Chopra, 2007).

Different assays were proposed to screen the ability of a chemical or a natural compound to eliminate microorganisms. The disc diffusion and the microtitre plate-based assays are the most common ones. In the disc diffusion assay, discs are soaked with the active compound and the susceptibility of an organism to the sample would result in a zone of inhibition in the plate, with an

inhibition zone related to the amount of bioactive compound present (Selvakumar *et al.,* 1999). The need of a less time consuming assay, has resulted in the development of the microtitre plate-based assay. In this assay, the optical density, turbidity or absorbance are usually monitored to determine the biomass growth and death rates (Archer *et al.,* 1996; Nayak *et al.,* 2002; Lopez-García *et al.,* 2003). The microtitre plate-based assay can be used to screen for antimicrobial activity of natural products (Devienne & Raddi, 2002).

1.2.3. Fish oils

Fatty acids are biological molecules that occur as components of lipids. They are present, mainly as phospholipids and glycolipids in membranes or as triacylglycerols in plants seeds, fish oils and animal fat (Dobson, 2008). Lipids are classified into two main groups, neutral and polar lipids. Neutral lipids or non-polar lipids include triacylgylcerols (TAG), diacylgylcerols (DAG), monoacylgylcerols (MAG), and sterols. Polar lipids contain free fatty acids (FFA), phospholipids (PL) and sphingolipids (Jobling *et al.,* 2002). Triacylgylcerols are the main fraction of fish oil and they are formed by fatty acid chains with various lengths and degree of unsaturation. The difference between fish oils and oils from plant or animal sources is the unique composition of fatty acids. Fish oils contain high levels of long chain polyunsaturated fatty acids (PUFA), including the pentaunsaturated eicosapentaenoic acid (EPA; 20:5, ω-3) and the hexaunsaturated docosahexaenoic acid (DHA; 22:6, ω-3) (Figure 1.10). PUFA are also found in other sources such as seed oils and macro and microalgae (Mishra *et al.,* 1993).

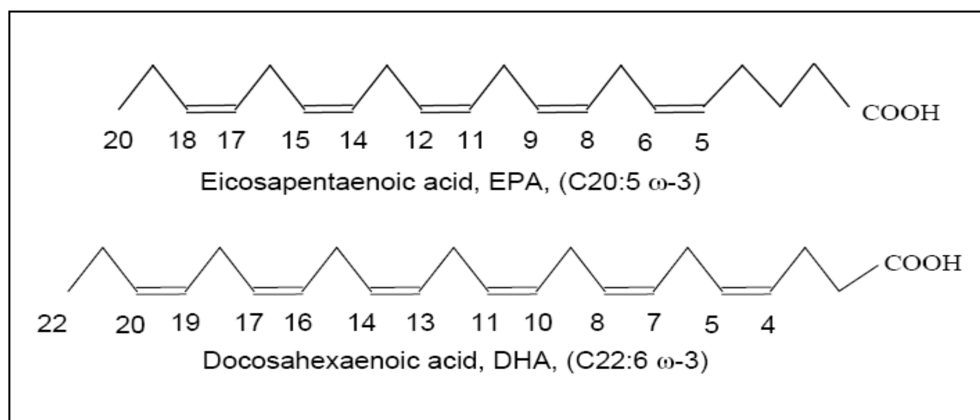


Figure 1.10 Schematic representation of EPA and DHA (Mishra *et al.,* 1993).

The fatty acid content in fish oils varies among the fish species, season, food habitat, body tissue and organs (McGill & Moffat, 1992). Oily fish include mackerel, salmon, sardine and tuna.

1.2.3.1. Role of omega-3 (ω-3) fatty acids in health

Many investigations have been conducted to study the role of omega-3 fatty acids on human health following the work of Dyerberg & Bang (1979) on the diet of Eskimos population. This diet was rich in sea food and was linked to the possible reduction of heart diseases. After many epidemiological, clinical and experimental studies, omega-3 fatty acids have been reported to be very beneficial for humans. These fatty acids protect against the development of breast and prostate cancer (Terry *et al.,* 2003) and limit tumour cell proliferation (Roynette *et al.,* 2004). Fernandez *et al.* (1999) showed that fish consumption increased the protection against the risk of digestive tract cancers. Omega-3 fatty acids also decrease the contents of triacylglycerols, low density lipoproteins (LDL) and cholesterol in the human serum resulting in possible prevention of coronary heart

diseases (Harris, 2005). The consumption of eggs enriched with omega-3 fatty acids was associated with a significant 16 to 18 % decrease in serum triglycerides (Bovet *et al.,* 2007). Geelen *et al.* (2005) reported that the consumption of ω-3 fatty acids decreased heart rate by 2.1 beats/min, which significantly reduced the risk of sudden death. The main active fractions of fish oil, EPA and DHA, were documented to have anti-inflammatory properties (Hamazaki *et al.,* 2006). In addition, omega-3 fatty acids inhibited the aggregation of blood platelets, the damage of blood vessels and protected against hypertension, hypertrigyceridemia and autoimmune disorders (Uauy & Valenzuela, 2000).

1.2.3.2. Extraction of fish oil

As fish oils are very susceptible to oxidation (Stołyhwo *et al.,* 2006), it is very important to reduce the contact with light and oxygen to minimise their oxidation. Oils in general are easily extracted by solvents. For analytical purposes, the chloroform-methanol method proposed by Folch *et al.* (1957) and later modified by Bligh & Dyer (1959), to take into account the amount of water in the sample, have been found to be the most efficient in terms of extracting total lipid from foodstuffs (Dobson, 2008). However, due to the risk of chlorinated solvents which are believed to be carcinogenic agents (Aryee & Simpson, 2009), other processes were developed for the extraction of fish oil. Hirata *et al.* (1993) proposed the fractionation by high-speed centrifugation. Moffat *et al.* (1993) used solvent extraction at low temperature, in which fish oil droplets were rapidly solidified in liquid nitrogen then extracted with acetone at - 60 °C. Extraction of fish oil by fractionation through supercritical fluid (Davarnejad *et al.,* 2008), as

well as wet and steam rendering methods, including cooking, pressing and centrifugation (Chantachum *et al.,* 2000) were also employed. Recently, enzymatic hydrolysis has been introduced in the extraction of fish oil in order to increase the extraction yield and to obtain oils with higher concentrations of ω-3 fatty acids (Linder *et al.,* 2005; Šližytė *et al.,* 2005; Mbatia *et al.,* 2010).

1.2.3.3. Analysis of fish oil

Fatty acids are generally analysed as methyl esters, volatile derivatives of fatty acids. The derivatisation of fatty acids can be achieved either by acidic or alkaline reagents. Acidic reagents include sulfuric acid, hydrochloric acid, boron trifluoride and acetyl chloride in methanol (Schuhardt & Lopes, 1988; Meier *et al.,* 2006). Alkaline reagents include potassium hydroxide/methanol mixture and sodium methoxide (Berdeaux *et al.,* 1998; Pérez-Serradilla *et al.,* 2007). The only difference between these two reagents is that the acidic reagents will form methyl esters from both esterified fatty acids and free fatty acids, while the alkaline reagents catalyse the formation of methyl esters from esterified fatty acids only and do not react with free fatty acids. Both of these reagents can be used for the derivatisation of long-chain PUFA with no adverse effects (Dobson, 2008).

Fatty acids are usually analysed by gas chromatography (GC) which gives a profile of all the fatty acid components. Gas chromatography-flame ionisation detection (GC-FID) is one of the most widely used analytical techniques in the field of lipid analysis (McNair & Miller, 1998). This is mainly due to the fact that GC is simple, fast, high sensitive and reproducible. The gas chromatograph is basically composed of an injector, a column and a detector (Figure 1.11).

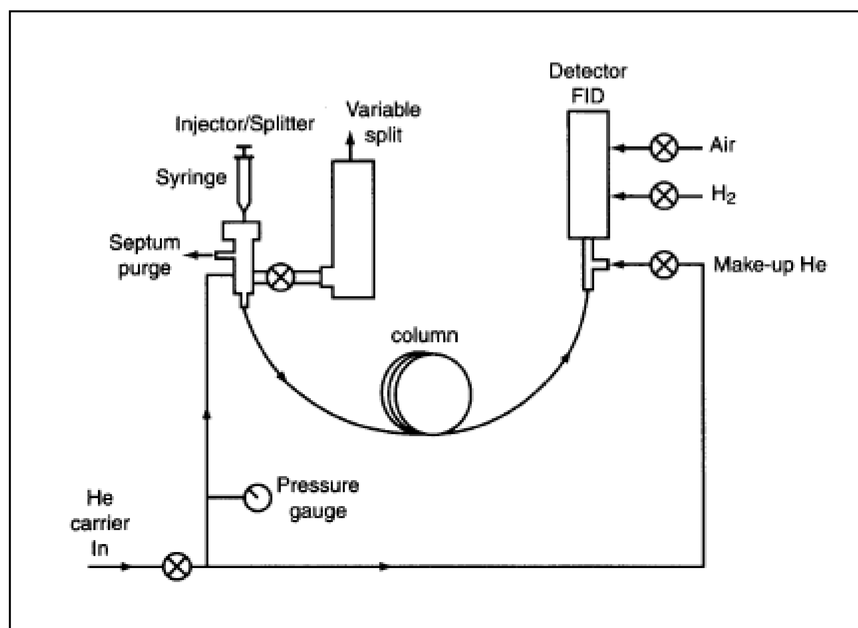


Figure 1.11 Schematic representation of a gas chromatograph (McNair & Miller, 1998).

The vaporisation of the sample takes place in the injector. High temperatures convert the sample from liquid state to gas. The sample enters the column and it is carried by a mobile phase which is usually an inert gas such as helium or hydrogen. The separation of the components of the sample occurs in the column and the affinity of the analytes to the stationary phase controls the overall separation process. GC columns contain a thin layer of non-volatile substance which is either coated into the wall in the case of capillary columns or coated into an inert solid in the case of packed columns (Kiston *et al.,* 1996).

Once the separation is completed, the analyte enters a detector and a signal is registered. In the case of the flame ionisation detector (FID), the analyte is burned in an oxygen/hydrogen flame that causes the formation of ions and electrons. These ions are collected and a current is detected, amplified and then

converted to a signal. The Flame ionisation detectors are considered to be universal detectors with wide range of linearity (McNair & Miller, 1998).

Other techniques such as gas chromatography-mass spectrometry (GC-MS) (Roach *et al.,* 2000) and high-performance liquid chromatography (HPLC) (Ostrowska *et al.,* 2000) are also used for analysing oils.

1.3. Functional foods

Food and diet were regarded as source of nutrients, capable of suppressing the feeling of hunger and giving a sense of satisfaction. However, recently the concepts that diet may play a beneficial role in reducing the risk of some diseases as well as promoting health beyond simply providing nutrients has emerged (Roberfroid, 2000).

The concept of functional food varies between countries and there is no legislative definition of the term functional food. On July 16, 2003, the European Commission proposed a harmonised regulation on nutrition and health claims made on foods, including dietary supplements (Commission of the European Communities, 2003). On May 16, 2006 the European Parliament concluded the second reading of the Health and Nutrition Claims Legislation (Regulation (EC) No. 1924/2006). This regulation aims to provide an assurance to consumers that only standardised nutritional claims or specifically authorised health claims may be carried on food these health claims can be ‘‘function claims’’ and ‘‘reduction of disease risk claims’’. However, medicinal claims relating to cure, prevention or treatment of disease remain the remit of medicines legislation (Regulation (EC) No. 1924/2006). In the EU, each member country has its own direction at present.

For Ireland, the Food Safety Authority of Ireland (FSAI) is responsible for the enforcement of all aspects of food legislation, including food labelling (FSAI, 2008).

In Europe, the market of functional food is still growing. In 2006, the European functional food sales were estimated to be around 16 billion US$ (Kotilainen *et al.,* 2006). The most important market for functional food in Europe is shared between Germany, France, the United Kingdom and the Netherlands (Mäkinen-Aakula, 2006; Bech-Larsen & Scholderer, 2007). However, sales of functional foods are rising in new emerging markets such as Hungary, Poland and Russia (Benkouider, 2004). The demand on functional foods in Europe varies between regions and countries. For instance, Central and Northern European countries consume more functional foods than the Mediterranean countries, where natural and fresh are considered better for health (Van Trijp, 2007).

1.4. Proposed study

1.4.1. Problem statement

The seafood processing industry produces an enormous quantity of waste as a result of the increased consumption of fish (caught and farmed). Under the new legislations, the established processes and disposal techniques of animal waste have been either banned or became inefficient in terms of cost. Therefore, new sustainable methods for treating fish waste need to be found.

Fisheries waste is usually transformed into low economic value products (Kim & Mendis, 2006). This waste can generate profits if directed to human consumption. In addition, the isolation of bioactive compounds from fish waste

represents the most beneficial and economical process. Hence, the development of new methods to extract bioactive compounds from fish processing waste will be of great benefit for the fishery industry and the environment by reducing the hazardous biological materials associated with their disposal.

In Ireland, both oily and lean fish are commercially available and consumed, leading to an important amount of waste. However, the insufficient and the inconsistent volumes of waste is still a challenge for the development of high value products (Pfeiffer, 2003). Research into maximising the use of Irish waste and the development of protocols suitable for both oily and lean fish species are still needed.

1.4.2. Research hypothesis

Based on recent studies showing that fish waste represents a source of valuable compounds (Binsi *et al.,* 2009; Davarnejad *et al.,* 2008; Kim *et al.,* 2008) a research hypothesis was proposed:

Waste from both oily and lean fish species (mackerel and blue whiting, respectively), could be suitable for the isolation of possible functional and bioactive compounds.

1.4.3. Objectives of the study

The main objective of this project was to extract and characterise functional and bioactive compounds from fish waste. Mackerel and blue whiting, models for oily and white fish, respectively, were investigated.

The aims were

- To investigate the physicochemical composition and the quality of the fish waste and to target possible functional and bioactive molecules.
- To extract gelatines from mackerel and blue whiting using different pre-treatments, and to characterise and study their functional and bioactive properties.
- To prepare hydrolysates/peptides from the fish waste using commercial proteases, screen their bioactivity and to purify and characterise the bioactive peptides.
- To develop methods for the extraction of mackerel oil, to determine the fatty acid composition by chromatographic techniques and to recover bioactive oil fractions.

Chapter 2 - Materials and methods

2.1. Materials

Atlantic mackerel (*Scomber scombrus*) caught in early March 2007, were kindly provided by Bord Iascaigh Mhara (BIM, Ireland). Blue whiting (*Micromesistius poutassou*) caught in January 2008, were provided by Donegal Seafood. Both fish were caught in the Area 27 (Atlantic, Northeast) (Figure 2.1), kept in ice and delivered the next day to the laboratory. Upon arrival at the laboratory in the Dublin Institute of Technology (DIT), the fish were devided in batches and kept in the freezer at - 20 °C until use.

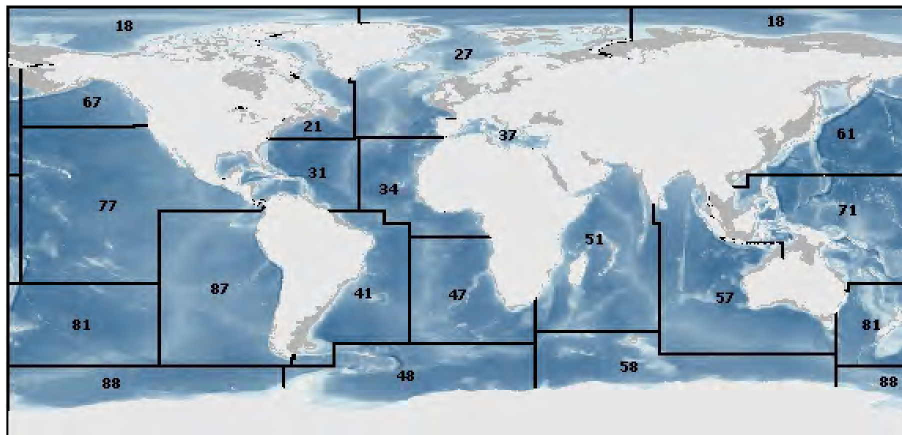


Figure 2.1 Map of FAO major fishing areas (Carocci, 2008).

Area 18 (Arctic Sea); Area 21 (Atlantic, Northwest); Area 27 (Atlantic, Northeast); Area 31 (Atlantic, Western Central); Area 34 (Atlantic, Eastern Central); Area 37 (Mediterranean and Black Sea); Area 41 (Atlantic, Southwest); Area 47 (Atlantic, Southeast); Area 48 (Atlantic, Antarctic); Area 51 (Indian Ocean, Western); Area 57 (Indian Ocean, Eastern); Area 58 (Indian Ocean, Antarctic and Southern); Area 61 (Pacific, Northwest); Area 67 (Pacific, Northeast); Area 71 (Pacific, Western Central); Area 77 (Pacific, Eastern Central); Area 81 (Pacific, Southwest); Area 87 (Pacific, Southeast); Area 88 (Pacific, Antarctic).

Fish samples (~ 20 Kg) were thawed overnight at room temperature. Fish heads were separated from the fish by cutting with a knife and ground using an Officine meat mincer model 12 mec (Officine C.G.T., Milan, Italy). Fish skins were manually removed from the fillet using a knife and cut manually into squares (2 cm^2) using scissors. Fish viscera were collected and ground using an Officine meat mincer. Fish bones and scales were cut manually into small pieces

(1 to 2 cm length) using scissors. The different fish tissues were divided into batches and kept in the freezer at - 20 °C until use. All chemicals used were of analytical grade.

2. 2. Characterisation of fish waste

2.2.1. Proximate analysis of fish waste

The proximate analysis of the samples (heads, skins, bones, viscera and flesh) was carried out according to the procedures of the Association of Official Analytical Chemists (AOAC, 2000). Crude protein content was determined by the Kjeldahl method (with a nitrogen conversion factor of 6.25) using an automatic Kjeldahl system (Gerhardt, Bonn, Germany). Moisture was determined by using the air oven (Qualivac, Greenfield Oldham, UK) until a constant weight was reached (at 100 °C for 18 h). Ash was determined by incineration in a muffle furnace (Carbolite, Bamford Sheffield, England) at 550 °C for 4 h. Lipid content was determined according to the Bligh and Dyer method (Bligh & Dyer, 1959). Each of these analyses was done in triplicate, for each tissue type, and repeated for 3 batches.

2.2.2. Determination of the pH of fish waste

The pH of different fish samples (heads, skins, viscera, bones or flesh) was determined according to the method described by Lima Dos Santos *et al.* (1981). Solutions of 10 % (w/v) of each sample were prepared with distilled water, then homogenised using an Ultra-Turrax homogenizer, model T 25 (IKA Works, Inc. Staufen, Germany) at 10,000 rpm for 1 min. The mixture was filtered using Whatman No.4 filter paper (Whatman, Maidenstone, England). The pH of the

filtrate was measured using an Orion pH meter Model 420A (Orion Research Inc, Beverly, MA. USA).

2.3. Extraction of fish gelatines

Gelatine extraction procedure was carried out according to Gomez-Guillén & Montero (2001) with some modifications. The method consists of a mild acid swelling step in 0.05 M of an organic acid and subsequent gelatine extraction in distilled water at 45 °C overnight. The modifications were mainly the volume of acid and base used and the duration of the pre-treatment.

2.3.1. Skin and head gelatines

The fish material (1.5 Kg of skins or heads) were treated with 0.1 N NaOH to remove non-collagenous proteins and pigments (in a ratio of 1/3, w/v, for 30 min and repeated 3 times) and washed with distilled water each time until reaching a neutral pH. Different organic acids, acetic, citric, lactic, malic or tartaric acid were added at 0.05 M individually to separate amounts of cleaned material (250 g) in the ratio of 1/3 (w/v) for 4 h. The material was washed under running water from a tap and the gelatine was extracted overnight at 45 °C with distilled water (in a ratio of 1/3, w/v). All extraction steps were done under continuous stirring at 150 rpm. Extracted gelatine was filtered using a Whatman No.4 filter paper (Whatman, Maidenstone, England) with a particle size retention of 20 - 25 µm. Gelatine was then evaporated under vacuum at 45 °C using a Büchi Rotavapor model R-210 fitted with a Büchi temperature-controlled water bath model B-491 and Büchi vacuum system model V-700 (Büchi UK Ltd., Oldham, UK) and freeze dried.

2.3.2. Bone gelatine

Different pre-treatment methods were used to extract gelatines from mackerel and blue whiting bones.

2.3.2.1. Pre-treatment of fish bones

Chemical pre-treatment (alkaline treatment):

Bones (250 g) used for gelatine extraction were treated with 0.1 N NaOH in a ratio of 1/3 (w/v) for 30 min and repeated 3 times to eliminate non-collagenous proteins and fat.

Enzymatic pre-treatment (hydrolysis of the frames):

Two hundred and fifty grams of fish bones were mixed with 0.1 M phosphate buffer (pH 8) in a ratio of 1/3 (w/v) then heat treated in a microwave oven model R-244 (Sharp Electronics Ltd, Uxbridge, UK) for 5 min to inactivate the endogenous enzymes. After cooling down, Flavourzyme (≥ 500 units/g) produced by DSM Nutritional Products, Inc. (Kaiseraugst, Switzerland) or Alcalase (≥ 2.4 units/g) produced by Novozyme Co. (Copenhagen, Denmark) and supplied by Sigma (Dublin, Ireland) were added in an enzyme/substrate ratio of 0.1 % (v/w). The frames were hydrolysed for 4 h at 50 °C with continuous shaking at 150 rpm. After hydrolysis the samples were heat treated in a microwave oven model R-244 (Sharp Electronics Ltd, Uxbridge, UK) for 5 min to inactivate the enzymes. The mixture was allowed to cool down and filtered through a sieve to separate the bones from the protein hydrolysates. The clean bones (Figure 2.2) were collected and demineralised.

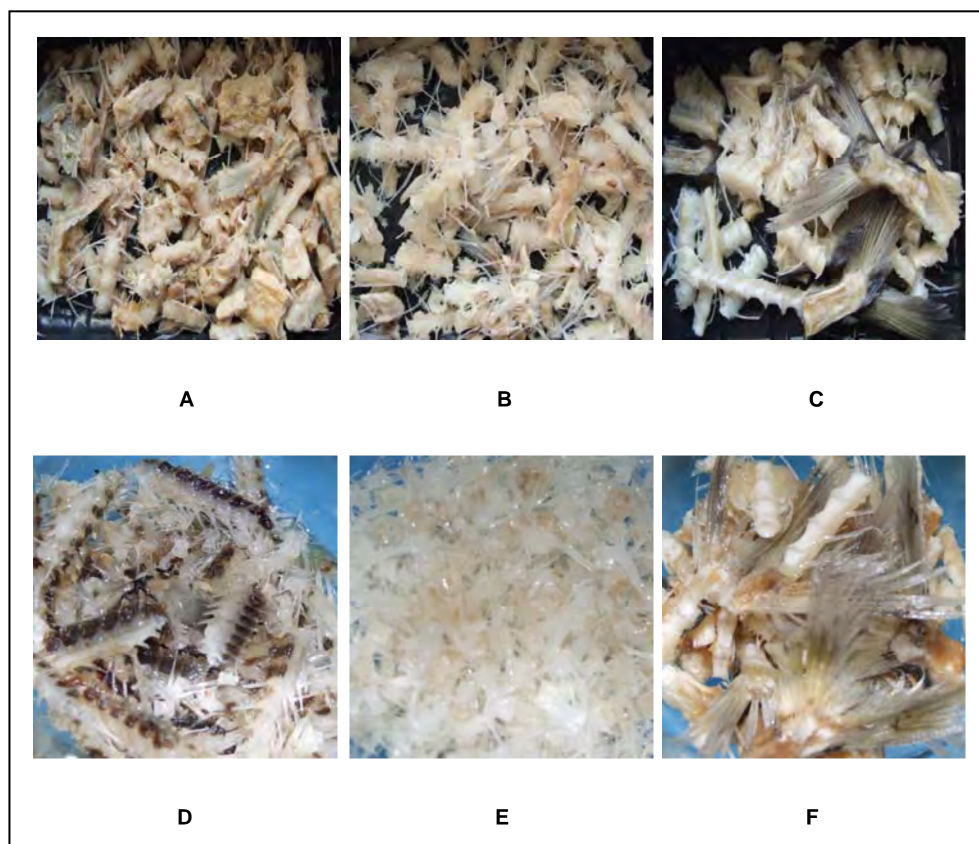


Figure 2.2 Fish bones after pre-treatment. Mackerel bone pre-treated with Flavourzyme (A), Alcalase (B) and sodium hydroxide (C), blue whiting bone pre-treated with Flavourzyme (D), Alcalase (E) and sodium hydroxide (F).

2.3.2.2. Demineralisation of fish bones and extraction of gelatine

Fish bones were demineralised using 0.25 N HCl (1/3, w/v), at room temperature until the bones did not have any hard cores (overnight). The demineralised bones were washed under running water from a tap to remove the acid. Gelatine extraction was then carried out as described for heads and skins.

The extraction of gelatine from fish wastes is summarised in Figure 2.3.

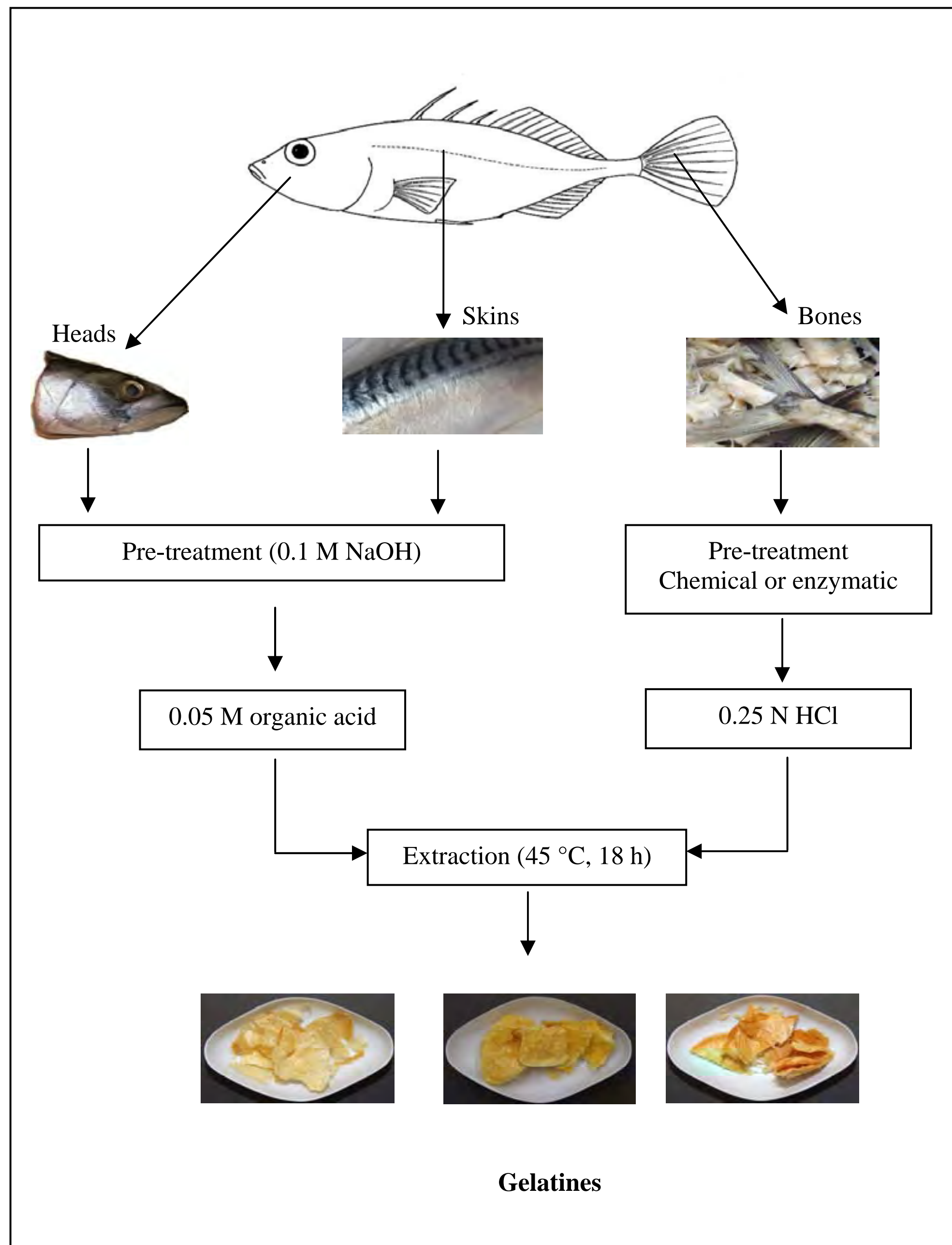


Figure 2.3 Schematic overview of the procedures used in this research for extraction of gelatine from fish heads, skins and bones.

2.4. Characterisation of fish gelatines

2.4.1. Yield of gelatine extraction

Gelatine extraction yield was calculated as grams (g) of dry gelatine per 100 g of clean fish material (Giménez *et al.,* 2005).

2.4.1. Proximate analysis of fish gelatines

The proximate analysis was carried out according to the procedures of the Association of Official Analytical Chemists (AOAC, 2000) as previously described (Section 2.2.1). Crude protein was calculated based on a nitrogen conversion factor of 5.4 for gelatines (Eastoe & Eastoe, 1952).

2.4.2. Determination of the pH of fish gelatines

The pH was determined according to the British Standard Institution method (BSI, 1975). Gelatine solutions (1 %, w/v) were prepared by dissolving 1 g of gelatine in 100 mL of distilled water at 60 °C then filtering the solution through Whatman No.4 filter paper (Whatman, Maidenstone, England). The pH of the filtrate was measured using an Orion pH meter Model 420A (Orion Research Inc, Beverly, MA. USA).

2.4.3. Colour measurement of fish gelatines

The colour of gelatine was determined using a CIELAB Colorquest XE colorimeter (Hunterlab, Virginia, USA). The CIELAB colour space is organised in a cube form. The L* axis has a maximum of 100, representing perfect reflection, and a minimum of zero, which represents black. The a* and b* axes

have no specific numerical limits. Positive and negative a* values represents red and green colour, respectively. Positive and negative b* values represents yellow and blue colour, respectively. Gelatine solutions were prepared to a concentration of 1 % (w/v, on a protein basis) using distilled water at 60 ºC, then 25 mL was transferred into the transparent glass cell (dimension 2.5 cm × 2.5 cm × 5 cm). The parameters measured were L* (lightness value), a* (green-red) and b* (blue-yellow).

2.4.4. Turbidity measurement of fish gelatines

The turbidity of the gelatine samples was measured using a DR/2000 Spectrophotometer (Hach Co., Loveland, CO, USA). Gelatine was first dissolved in distilled water at 60 ºC to 1 % (w/v, on a protein basis) solution, then 25 mL of sample was transferred into the transparent glass cell (dimension 2.5 cm × 2.5 cm × 5 cm) and the absorption was read at 450 nm. The instrument was calibrated with formazin standards prepared from a 4000 NTU stock solution (Hach Co., Loveland, CO, USA) and the turbidity was expressed as formazin turbidity units (FTU).

2.4.5. Protein pattern of fish gelatines

The electrophoresis procedure was carried out according to Gómez-Guillén *et al.* (2002) with minor modifications which included using a stronger resolving gel. Gelatine solutions (5 mg/mL) were made in distilled water at 60 ºC and then diluted to a final concentration of 2 mg/mL with sample buffer containing β-mercaptoethanol (Sigma, Dublin, Ireland). Gelatine samples were heated to 85 ºC for 10 min to denature the proteins. Samples and molecular weight

marker (10 µL each) were loaded onto SDS-PAGE having a 4 % stacking gel and 10 % resolving gel according to Laemmli (1970), the analysis was run in an Atto Dual Mini-slab Size Electrophoresis System AE-6450 (Atto Corporation, Tokyo, Japan) at a constant current of 25 mA/gel. The gel was immersed in a fixative solution prepared with distilled water, methanol and acetic acid (50:40:10, v/v/v) for 1 hour, and then protein bands were stained with Coomassie Brilliant Blue R250. The gel was de-stained using a mixture of isopropanol, acetic acid and distilled water (12:10:78, v/v/v).

The molecular weight marker (Sigma, Dublin, Ireland) contained a lyophilised mixture of six proteins: bovine carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase B from rabbit (97.4 kDa), β-galactosidase from *E. coli* (116 kDa) and myosin from rabbit muscle (200 kDa).

2.4.7. Amino acid analysis of fish gelatines

The amino acid profile was determined according to the method of Giménez *et al.* (2005). Briefly, 10 µg of gelatine was hydrolysed for 24 h at 110 °C, with 6 M HCl containing 0.1 % phenol. Norleucine (Sigma, Madrid, Spain) was added as an internal standard. The amino acid composition was analysed using a cation exchange Biochrom 20 amino acid analyzer (Pharmacia Biotech, Ltd., Cambridge, England) with postcolumn derivatisation with ninhydrin. The amino acids were detected and quantified at 440 and 570 nm. Results were averaged and presented as grams (g) per 100 g gelatine. Analyses were run by Dr. Javier Varela at the Centro de Investigacion Biologicas (CIB), Madrid, Spain.

2.4.8. Spectroscopic analyses of fish gelatines

2.4.8.1. Fourier Transform Infra-Red (FTIR) analysis

Freeze dried gelatines (approximately 2 mg) were mixed with 100 mg potassium bromide (KBr) then transferred to a hydraulic press to prepare small discs. The discs were placed in the FTIR spectrometer Model Avatar 360 (Thermo-Nicolet, Madison, Wisconsin, USA). FTIR spectra (32 scans) were analysed in the absorbance mode, between 400 and 4000 cm^{-1} with a resolution of 2 cm^{-1}. The spectra were analysed using Omnic 5.0 software (Thermo-Nicolet, Madison, Wisconsin, USA).

2.4.8.2. Nuclear magnetic resonance (NMR) analysis

Proton NMR (^1H NMR) spectra were performed on freeze dried gelatine samples; approximately 200 mg of each gelatine sample were dissolved in 2 mL of D_2O and mixed by shaking. An aliquot of this mixture was transferred into a 5 mm NMR sample tube and analysed by a Bruker Avance 500 MHz spectrometer equipped with 4 mm ^1H HR-MAS probe, at 300 ºK and 10 KHz and using a pre-saturation sequence for water suppression. Data were acquired using Bruker Topsin software version 2.1 (Bruker UK Limited, Coventry, UK). Resonance assignments were based on chemical shifts. Analyses were run by Dr. Padraig McLoughlin at the Food Research Centre (Teagasc), Ashtown, Ireland.

2.5. Analysis of functional properties of fish gelatines

2.5.1. Foaming properties

Foaming properties including foaming capacity (FC) and foam stability (FS) were determined by the method of Fernandez & Macarulla (1997). Gelatine solutions were prepared in 50 mM phosphate buffer at pH 7.5 to a final concentration of 0.3 % (w/v, in protein content). Five millilitres of each sample were homogenised with an Ultra-Turrax homogenizer, model T 25 (IKA Works, Inc. Staufen, Germany) at 23,000 rpm for 1 min. FC was calculated as the percent increase in volume of the protein dispersion upon mixing, while FS was estimated as the percentage of foam remaining after 15 min.

2.5.2. Emulsifying properties

The emulsifying properties of gelatine samples were determined by the method of Pearce & Kinsella (1978) with some modifications regarding the amount of gelatine solutions and oil used to prepare the emulsion. Different concentrations of gelatine solution were used. Gelatines were first dissolved in 50 mM phosphate buffer containing 0.3 M NaCl at pH 7.5 to the final concentration of 0.05 %, 0.1 %, or 0.2 % (w/v, in protein content). Then 2.0 mL of pure sunflower oil was mixed with 8.0 mL of each gelatine solution. The mixture was shaken in a plastic tube at 25 °C and homogenised with an Ultra-Turrax homogenizer, model T 25 (IKA Works, Inc. Staufen, Germany) at 23,000 rpm for 1 min. An aliquot (50 µL) of emulsion was diluted in 5 mL sodium dodecyl sulfate (SDS) solution (0.1 %, w/v) and the absorbance was measured at 500 nm. In order to estimate the emulsion stability, the emulsions were left for 15 min at

25 °C and then 50 µL of the emulsion were diluted in 5 mL SDS solution (0.1 %, w/v) and the absorbance was measured at 500 nm. The emulsifying activity and emulsion stability were expressed as indices.

The emulsifying activity index was defined as:

$$EAI(m^2/g) = \frac{2 \times 2.303}{C \times \phi \times 10^4} \times A_{500} \times Dilution$$ **Equation 2.1**

Where; A_{500} represents the absorbance at 500 nm, C the protein concentration (g/mL) before emulsification and Φ the oil volume fraction (v/v) of the emulsion (i.e., the volume of emulsion droplets divided by the total volume of the emulsion, $\Phi = 0.2$).

The emulsion stability index (ESI) was calculated as the ratio of the turbidity measured at 500 nm of the emulsion at time zero (A_0) and after 15 minutes (A_{15}) (Agyare *et al.,* 2009).

$$ESI(\%) = 100 \times \frac{A_{15}}{A_0}$$ **Equation 2.2**

2.5.3. Protein solubility

2.5.3.1. Effect of pH on gelatine solubility

The solubility of gelatines was determined according to the method of Montero *et al.* (1991). Gelatine samples were first dissolved in distilled water to a final concentration of 0.3 % (w/v, protein content). Eight millilitres of each gelatine solution were added to individual glass test tubes and the pH was adjusted to end points ranging from 2.0 to 12.0 with either 1 M HCl or 1 M NaOH

using a pH meter. The final volume was then adjusted to 10 mL with distilled water having the same pH as the gelatine solution. Samples were centrifuged at 9,000 × g for 15 min at 5 ºC. Protein content of the clear supernatant was determined according to the Biuret assay as described by Gornall *et al.* (1949) and using bovine serum albumin (BSA) as a reference protein (Sigma-Aldrich, Inc., Dublin, Ireland). A calibration curve was constructed (in the concentration range of 2.5 - 25 mg/mL) with an equation ($R^2 = 0.998$):

$$\text{Protein (mg/mL)} = 33.7 \times \text{Abs} - 0.3$$ **Equation 2.3**

The Relative solubility was calculated in comparison with that obtained at the pH rendering highest solubility.

2.5.3.2. Effect of NaCl on gelatine solubility

Gelatines were dissolved in 50 mM phosphate buffer at pH 7.5 to a final concentration of 0.6 % (w/v, protein content). Five millilitres of gelatine solution were mixed with 5 mL of NaCl in 50 mM phosphate buffer at pH 7.5 at various concentrations (0 %, 2 %, 4 %, 6 %, 8 %, 10 % and 12 % (w/v)). The mixture was stirred continuously at 5 °C for 30 min, followed by centrifugation at 9,000 × g for 15 min at 5 ºC. Protein content of the clear supernatant was determined according to the Biuret assay as described by Gornall *et al.* (1949) and using bovine serum albumin (BSA) as a reference protein (Sigma-Aldrich, Inc., Dublin, Ireland). Relative solubility was calculated in comparison with that obtained at the NaCl concentration rendering highest solubility.

2.5.4. Rheological characterisation

A stress amplitude sweep test was performed to determine the viscoelastic (VE) range. The VE is defined as the domain below a strain threshold value where the sample structure is preserved and the elastic (G') and viscous (G'') moduli show a constant high plateau. Once the amplitude of the deformation exceeds the threshold value, the structure of the sample is irreversibly destroyed (Di Giuseppe *et al.,* 2009). The dynamic viscoelastic behaviour (DVB) and the frequency sweep tests of gelatine samples were performed according to the method described by Binsi *et al.* (2009). A controlled stress rheometer (Bohlin C-VOR, Malvern Instruments Ltd., Malvern, UK) was set to perform oscillation with a stress of 1.0 Pa and a frequency of 1 Hz, using 5.5 cm parallel plate geometry with a gap of 200 μm between plates.

The viscoelastic properties of gelatine solutions (6.67 %, w/v) were measured in the temperature range of 25 - 5 °C and 5 - 25 °C, with heating/cooling rate at 1 °C/min. After completing the cooling process, gelatines were kept for 10 min at 5 °C before starting the heating process. The elastic modulus (G′), viscous modulus (G″) and Tan δ (G″/G′) values were measured as a function of temperature. The frequency sweep tests were performed at 5 °C and the elastic modulus (G') was obtained as a function of frequency (varying from 0.2 to 5.2 Hz).

2.5.5. Textural properties

2.5.5.1. Gel strength

The gel strength was determined according to Gómez-Guillén *et al.* (2002) with some modifications as described herein. The gelatine gels (6.67 %, w/v) were matured at 10 °C for 16 - 18 h. The gel strength was determined using samples having 3.5 cm diameter and 1.5 cm height on an Instron Universal Testing Machine model 3300 (Instron Ltd., High Wycombe, England) and using a static load cell of 500 Newtons equipped with a flat-faced cylindrical probe (diameter of 1.27 cm). The test was run at a penetration speed of 1 mm/s. Gel strength was expressed as maximum force (g) obtained at 4 mm penetration depth on the gelatine gels.

2.5.5.2. Cryo-scanning electron microscopy (Cryo-SEM)

Cryo-scanning electron microscopy (Cryo-SEM) was used to observe the effects of the pre-treatment as well as the source on the extracted gelatines. Cryo-SEM analysis was performed according to the method described by Stuart & Panitch (2009) with minor modifications in the preparation of gelatine gels. Gelatine samples (6.67 %, w/v) were first frozen by immersion in Slush Nitrogen (- 210 °C). Samples were then fractured, warmed (at - 94.5 °C, 10^{-5} Torr vacuum, for 15 min to sublime the water), gold coated and viewed in the cold-stage scanning electron microscopy (JEOL JSM-5410, Tokyo, Japan) at an acceleration voltage of 15 kV. Five micrographs per sample were obtained.

2.6. Enzymatic hydrolysis of fish waste

Gelatines extracted from fish heads and skins together with mackerel viscera were hydrolysed using commercial proteases (pepsin, trypsin, chymotrypsin, Alcalase and Flavourzyme). Table 2.1 shows the specificity of these enzymes and the reaction type.

Table 2.1 Specificity of proteolytic enzymes.

Enzyme	Specificity *
Pepsin	Preferential cleavage: hydrophobic, preferably aromatic
Trypsin	Cleaves very specifically Arg+, Lys+
α-Chymotrypsin	Preferential cleavage: Tyr+, Trp+, Phe+, Leu+
Alcalase	Hydrolysis of proteins with broad specificity for peptide bonds, and a preference for a large uncharged residue in P1. Hydrolyses peptide amides.
Flavourzyme	Hydrolysis of proteins with broad specificity. Does not hydrolyse peptide amides

* Collected from Braunschweig Enzyme Database (BRENDA) and Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB).

2.6.1. Enzymatic hydrolysis of gelatine

Gelatines extracted from mackerel heads and skins, after pre-treatment with citric acid, were dissolved at a concentration of 1 % (w/v) in 0.1 M of the appropriate buffer (phosphate or glycine buffer). Samples were heat treated in a microwave oven model R-244 (Sharp Electronics (UK) Ltd, Uxbridge, UK) for 2 min to inactivate any endogenous enzymes. Three enzymes (pepsin, trypsin and chymotrypsin) were used to hydrolyse gelatine in an enzyme/gelatine ratio of 1 % by weight. Different buffers were used: phosphate buffer, pH 8, for trypsin and

chymotrypsin and glycine buffer, pH 2, for pepsin. The optimum temperature was 37 ºC for all enzymes. Four time intervals were chosen: 1, 2, 6 and 24 h. After hydrolysis, samples were heat treated in a microwave oven model R-244 (Sharp Electronics Ltd, Uxbridge, UK) for 2 min to inactivate the enzymes. The mixture was allowed to cool down and then centrifuged in a refrigerated centrifuge model 2K15 (Sigma, Osterode, Germany) at 13,500 × g for 10 min at 5 ºC. The hydrolysates were collected and stored at - 20 °C until further investigations. All experiments were performed in duplicate. The conditions used for the hydrolysis are summarised in Table 2.2.

Table 2.2 Conditions used for the enzymatic hydrolysis of mackerel gelatine.

Enzymes	Source	Enzymatic activity	E/S	pH	T (°C)	Buffer
Pepsin	Porcine gastric mucosa	800 - 2,500 units/mg	1 %	2	37	0.1 M Glycine
Trypsin	Porcine pancreas	1,000 - 2,000 units/mg	1 %	8	37	0.1 M Na_2HPO_4-NaH_2PO_4
Chymotrypsin	Bovine pancreas	≥ 50 units/mg	1 %	8	37	0.1 M Na_2HPO_4-NaH_2PO_4

2.6.2. Enzymatic hydrolysis of mackerel viscera

The frozen Atlantic mackerel (*Scomber scombrus*) were thawed overnight at room temperature, and then gutted manually, viscera including all the internal organs (such as kidney, stomach, intestines, pyloric caeca, heart, liver, gonads and gall bladder) were collected and then ground using an Officine meat mincer (Officine cgt, Milan, Italy). The processed viscera were divided in batches and transferred immediately into plastic bags and stored at - 20 °C until further use.

Different assays were designed in these experiments by varying the time of hydrolysis and the type of enzyme. Three time periods (2, 6, and 24 h) and six different enzymes (endogenous enzymes, Flavourzyme, Alcalase, pepsin, trypsin or chymotrypsin) were chosen. The frozen ground viscera were thawed at 4 °C, samples of 150 g each were divided into flasks and 0.1 M of the appropriate buffer solution was added in the proportion of 1:3 (w/v). For all experiments, except the one using endogenous enzymes, samples were heated in a microwave oven model R-244 (Sharp Electronics Ltd, Uxbridge, UK) for 5 min to inactivate the endogenous enzymes. Glycine buffer (pH 2) was used for pepsin and phosphate buffer (pH 8) was applied for the rest of the enzymes.

The enzymatic hydrolysis started when the temperature of the mixture reached 50 °C (for the proteases and autolysis) or 37 °C (for pepsin, trypsin and chymotrypsin) by adding the enzymes. The hydrolysis proceeded for the designed period of time with continuous shaking at 150 rpm followed by heat inactivation of enzymes in a microwave oven model R-244 (Sharp Electronics Ltd, Uxbridge, UK) for 5 min. After cooling down, the hydrolysed viscera were centrifuged in a refrigerated centrifuge 2K15 (Sigma, Osterode, Germany) at 13,500 × g for 10 min at 5 ºC. Three fractions were obtained: the sludge (un-hydrolysed protein), the protein hydrolysates and an oily phase or emulsion. The protein hydrolysates were collected and subjected to a second centrifugation at 7,500 × g for 5 min at 5 °C to remove fat and clarify the samples more. The fish protein hydrolysates (FPH) were concentrated under vaccum and kept in the freezer at - 20 ºC until further investigations. All experiments were performed in duplicate. The conditions used for the hydrolysis are summarised in Table 2.3.

Table 2.3 Conditions used for the enzymatic hydrolysis of mackerel viscera.

Enzymes	Source	Enzymatic activity	E/S	pH	T (°C)	Buffer
Endogenous enzymes	Mackerel viscera	…	…	8	50	0.1 M Na_2HPO_4-NaH_2PO_4
Flavourzyme	*Aspergillus oryzae*	≥ 500 units/g	0.1 %	8	50	0.1 M Na_2HPO_4-NaH_2PO_4
Alcalase	*Bacillus licheniformis*	≥ 2.4 units/g	0.1 %	8	50	0.1 M Na_2HPO_4-NaH_2PO_4
Trypsin	Porcine pancreas	1,000 - 2,000 units/mg	0.1 %	8	37	0.1 M Na_2HPO_4-NaH_2PO_4
α-Chymotrypsin	Bovine pancreas	≥ 50 units/mg	0.1 %	8	37	0.1 M Na_2HPO_4-NaH_2PO_4
Pepsin	Porcine gastric mucosa	800 - 2,500 units/mg	0.1 %	2	37	0.1 M Glycine

2.7. Characterisation of FPH

2.7.1. Degree of hydrolysis

The degree of hydrolysis (DH) is defined as the ratio of the number of peptide bonds broken with respect to the total number of bonds per unit weight and was determined following the method of Nielsen *et al.* (2001). This method is based on the fact that the α-amino groups released by hydrolysis react with OPA and dithiothreitol (DTT) to form a compound that absorbs strongly at 340 nm.

The o-phthaldialdehyde (OPA) reagent was prepared as follow: 7.62 g disodium tetraborate decahydrate and 200 mg sodium dodecyl sulfate (SDS) were dissolved in 150 mL deionised water (first solution). The reagents were completely dissolved before continuing. 160 mg 97 % o-phthaldialdehyde (OPA) was dissolved in 4 mL ethanol then transferred to the first solution. 176 mg 99 % dithiothreitol (DTT) was added to the solution which was made up to 200 mL with deionised water. The serine standard was prepared by dissolving 50 mg serine in 500 mL deionised water. To 3 mL of OPA reagent, 400 µL of serine standard or sample solution (at concentration of 1 mg/mL) was added. The homogenate was mixed by inversion and held for exactly 2 min before being read at 340 nm (Milton Roy Spectonic 1201, NY, USA). Blanks were prepared with 400 µL of deionised water. The DH was calculated as follows:

$$\text{DH}\,(\%) = \left(\frac{H}{H_{tot}}\right) \times 100$$ **Equation 2.4**

$$H = (Serine - NH_2 - \beta)/\alpha$$ **Equation 2.5**

$$\text{Serine - NH}_2 = \frac{\text{Abs}_{\text{sample}} \text{ - Abs}_{\text{blank}}}{\text{Abs}_{\text{standard}} \text{ - Abs}_{\text{blank}}} \times 0.9516 \times 0.1 \times 100/\ (\text{X} \times \text{P})$$ **Equation 2.6**

Where: Abs represents the absorbance at 340 nm; *Serine-NH*$_2$ = meqv serine NH_2/g protein; X = g sample; P = % protein in sample; 0.1 is the sample volume in litre (L).

For fish α = 1, β = 0.40 and H_{tot} = 8.6.

For gelatine α = 0.796, β = 0.457 and H_{tot} = 11.1 (Adler-Nissen, 1986).

2.7.2. Protein and peptide pattern of FPH

Tricine SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which is based on Tricine-Tris buffer system, was performed essentially as described by Schägger (2006). Hydrolysates were prepared as 2 mg/mL (w/v, in protein content) in distilled water at 60 ºC and diluted to a final concentration of 1 mg/mL with sample buffer containing β-mercaptoethanol (Sigma, Dublin, Ireland) then heated to 85 ºC for 10 min. Gels were prepared according to Laemmli (1970) in 4 % stacking gel and 15 % resolving gel. SDS-PAGE gels were loaded with 10 µL of protein hydrolysates and molecular weight markers (low molecular weight peptide marker and high molecular weight proteins both supplied by Sigma, Dublin, Ireland).

The analysis was run in an Atto Dual Mini-slab Size Electrophoresis System AE-6450 (Atto Corporation, Tokyo, Japan) at a constant current of 25 mA/gel. Gels were then silver stained according to Sørensen *et al.* (2002). The gels were fixed in 40 % methanol, 10 % acetic acid and 50 % water for 20 min,

washed 3 times for 10 min in 30 % ethanol, followed by incubation for 1 min in 1.25 mM sodium thiosulfate. After washing 3 times in water, gels were stained for 20 min in 0.2 % silver nitrate, 0.02 % formaldehyde and 0.03 mM sodium thiosulfate and then washed briefly in water. The gel was developed until the bands appeared (5 - 10 min) in 280 mM sodium carbonate and 0.05 % formaldehyde and finally stopped with 5 % acetic acid.

The low molecular weight peptide marker contained a mixture of peptides: myoglobin (16,950 Da), myoglobin I + II (14,440 Da), myoglobin I + III (10,600 Da), myoglobin I (8,160 Da), myoglobin II (6,210 Da) and myoglobin III (2,510 Da).

2.8. Screening for bioactivity of FPH

2.8.1. Antioxidant activity

2.8.1.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The scavenging activities of the gelatine and viscera hydrolysates were measured using the stable radical DPPH, according to the method of Yen & Wu (1999) with some modifications in the volume of reagents used for the assay. A 0.5 mL volume of each sample (1 mg/mL in distilled water) was added to 0.5 mL of 60 μM methanolic solution of DPPH. The mixture was shaken, incubated for 30 min in the dark at room temperature. Then the absorbance was measured using a spectrophotometer (Milton Roy Spectonic 1201, NY, USA) at 517 nm. The percentage of inhibition of radical scavenging activity was calculated as follows:

$$\% \text{ DPPH radical scavenging activity} = \left(\frac{A_{517} \text{ (Control)} - A_{517} \text{ (Sample)}}{A_{517} \text{ (Control)}} \right) \times 100$$

Equation 2.7

Where; A_{517} (Control) is the absorbance of reference solution containing only DPPH and water and A_{517} (Sample) is the absorption of the DPPH solution with sample after 30 min. Methanol was used as a blank. A lower absorbance represented a higher DPPH scavenging activity.

2.8.1.2. Reducing power

The reducing ability of fish protein hydrolysates was measured using the ferric reducing antioxidant power (FRAP) assay according to Benzie & Strain (1996). The FRAP reagent was freshly prepared by mixing 2.5 mL of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2.5 mL of 20 mM $FeCl_3.6H_2O$, and 25 mL of 30 mM acetate (pH 3.6). The mixture was warmed to 37 °C. An aliquot of sample (100 µL) was mixed with 900 µL of FRAP solution and incubated for 30 min in the dark at 37 °C. The absorbance was measured at 595 nm, high absorbance of the reaction mixture indicates high reducing power. A calibration curve was plotted using Trolox as a standard (in the concentration range of 0.05 - 0.25 mM) and the results were expressed as mM Trolox equivalent ($R^2 = 0.996$):

$$\text{mM Trolox equivalent} = 0.25 \times \text{Abs} + 0.03$$

Equation 2.8

2.8.2. Angiotensin I-converting enzyme (ACE) inhibitory activity

The screening for the angiotensin converting enzyme inhibitory activity was based on the hydrolysis of N-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycylglycine (FA-PGG) which represents a substrate for angiotensin converting enzyme (Bunning *et al.,* 1983).

The assay was performed based on the method described by Vermeirssen *et al.* (2002) as modified by Shalaby *et al.* (2006). Buffers were made according to Hou *et al.* (2003) and the volumes were reduced to fit into microtitre plate wells. FA-PGG was dissolved at a concentration of 1.75 mM in 50 mM-Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl. The ACE solution 0.25 units/mL was freshly prepared by adding purified water to a vial containing 0.25 units of enzyme (Sigma, Dublin, Ireland). The assay was performed in a 96-well, clear, flat-bottomed polystyrene plate (Corning Costar, Sigma, Dublin, Ireland). Ten microlitres of ACE solution and 10 µL of sample were placed separately in the well at room temperature. Using an eight channel pipette, 150 µL of pre-heated (37 °C, 15 min) substrate solution (FA-PGG) was added quickly to each well and the reaction started. The microtitre plate was immediately transferred to the PowerWave microplate reader (BioTek, Bedforshire, UK). Enzyme activity at 37 °C was based on the initial linear rate of change in absorbance at 340 nm, recorded every 3 min for 30 min.

The control contained all reaction components but water instead of the sample. Blanks with no enzyme (substituted by water) or with no substrate (substituted by 50 mM Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl) were used. The ACE activity was expressed as the slope of the decrease in absorbance

at 340 nm (ρA) and the ACE inhibition (%) was calculated from the ratio of the slope in the presence of sample to the slope obtained without added sample, according to the formula:

$$\% \text{ ACE inhibition} = [1 - (\rho\text{A}_{\text{inhibitor}} / \rho\text{A}_{\text{control}})] \times 100$$ **Equation 2.9**

The IC_{50} values (concentration required to achieve 50 % inhibition) were determined from plots of % ACE inhibition versus sample concentration.

2.8.3. Semicarbazide-sensitive amine oxidase (SSAO) inhibitory activity

The activity of SSAO was determined following the production of hydrogen peroxide at 490 nm, according to the method of Holt *et al.* (1997), in the presence of 5 mM benzylamine and 10 µg/mL SSAO. The SSAO was purified from bovine blood and was kindly provided by the Department of Biochemistry, Trinity College, Dublin, Ireland. The assay buffer was prepared by dissolving 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in purified water to a final concentration of 100 mM. Different salts were added to the HEPES solution to reach the indicated concentrations: KCl (10 mM), $CaCl_2$ (4 mM) and $MgCl_2$ (2.8 mM). The pH of the buffer was adjusted to 7.4.

The chromogenic solution for the detection of SSAO was prepared by mixing horseradish peroxidase (4 units/mL) and 4-aminoantipyrine (500 mM) and vanillic acid (1 mM) in physiological HEPES buffer. The assay was performed in a 96-well, clear, flat-bottomed polystyrene plate (Corning Costar, Sigma, Dublin, Ireland). Seventy five microlitres of SSAO solution together with 75 µL of sample

(1 mg/mL) and 75 µL of HEPES buffer were placed in the well at room temperature and incubated for 0 or 2 h before adding the substrate. Using an eight channel pipette, 75 µL of pre-heated (37 °C, 15 min) substrate solution (benzylamine) was added quickly to each well and the reaction started. The microtitre plate was immediately transferred to the PowerWave microplate reader (BioTek, Bedforshire, UK). Enzyme activity was evaluated at 37 °C and was based on the initial linear rate of change in absorbance at 490 nm, recorded every 3 min for 30 min.

The control contained all reaction components but buffer instead of sample. Blanks with no enzyme (substituted by HEPES buffer) or with no substrate (substituted by water) were used. The SSAO activity was expressed as the slope of the increase in absorbance at 490 nm (ρA) and the SSAO inhibition (%) was calculated from the ratio of the slope in the presence of sample to the slope obtained without added sample, according to the formula:

$$\% \text{ SSAO inhibition} = [1 - (\rho A_{\text{inhibitor}} / \rho A_{\text{control}})] \times 100$$

Equation 2.10

The IC_{50} values (concentration required to achieve 50 % inhibition) were determined from plots of % SSAO inhibition versus sample concentration.

2.8.4. Platelet aggregation inhibitiory activity

2.8.4.1. Platelet preparation

Blood was drawn from the antecubital veins of healthy volunteers, free from any medications (nonsteroidal anti-inflammatory drugs) for 2 weeks, and

mixed with 15 % (v/v) acid citrate dextrose (ACD: 38 mM citric acid, 75 mM sodium citrate, 124 mM dextrose). Washed platelets were prepared as described previously by Stephens *et al.* (1998) and Martin *et al.* (2003). Platelet rich plasma was collected from the anticoagulated blood after centrifugation at 150 × g for 10 min at room temperature. The pH was then adjusted to 6.5 with ACD. Prostaglandin E_1 (PGE_1) was added to prevent activation of the platelets. The platelets were then obtained from the platelet rich plasma after spinning at 720 × g for 10 min at room temperature. The platelet pellet was resuspended in buffer A (130 mM NaCl, 10 mM sodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 3 mM KCl, 10 mM Tris [Tris(hydroxymethyl)-aminomethane], pH 7.4), adjusted to 3×10^5/µL, and supplemented with 1.8 mM $CaCl_2$ just prior to the assay to reactivate the platelets.

2.8.4.2. ADP secretion assay

Washed platelets were prepared and resuspended at concentrations of 3×10^5/µL in buffer A and 10 µL $CaCl_2$ (1.8 mM) was added just prior to the assay. Two assays were designed, with or without the synthetic agonist thrombin receptor-activating peptide (TRAP, synthesised by the Center for Synthesis and Chemical Biology, Department of Pharmaceutical and Medicinal Chemistry, Royal College of Surgeons in Ireland). Aliquots (70 µL) of platelets with 10 µL buffer A (or 1 µM TRAP) and 10 µL sample were dispensed into a 96-well, flat-bottomed, microtitre white isoplates. The plate was incubated at 37 °C for 3 min under vigorous shaking in an orbital mode at medium speed.

ADP secretion was measured using Chrono-Lume reagent according to the manufacturer's protocol. After incubation, 10 μL Chronolume Luciferin-Luciferase reagent (Labmedics Limited, Manchester, UK) was added quickly. The plate was shaken for 10 sec and measurements of the luminescence were performed using a Victor2 V Multilabel Counter (PerkinElmer, Wellesley, MA, USA). Platelet modulators included the known inhibitory pharmacological agents indomethacin (20 μM) and aspirin (200 μM). Inhibition of platelet aggregation inhibition was estimated in terms of ADP released.

2.8.5. Effect of FPH on microbial growth

2.8.5.1. Effect on probiotic strains

The assay was performed according to the method described by Huebner *et al.* (2007) with minor modifications in the concentration of the substrate. *Lactobacillus plantarum* ATCC 8014 (Microbiologics, Minnesota, USA) and *Bifidobacterium breve* ATCC 15700 (Medical supply Co. Ltd., Dublin, Ireland) were grown overnight at 37 °C in MRS broth (supplemented with 0.05 % L-cysteine hydrochloride for *Bifidobacterium*). The assay was performed by adding 1 % (v/v) of the overnight culture of each probiotic strain (serially diluted to 10^6 cells/mL) to tubes containing MRS broth with 5 % (v/v) of hydrolysates (10 mg/mL of protein content). The cultures were incubated at 37 °C under anaerobic conditions (anaerobic jar) for *Bifidobacterium* and atmospheric conditions for *Lactobacillus*. Samples were enumerated on MRS agar (supplemented with 0.05 % L-cysteine hydrochloride for *Bifidobacterium breve*). Sterile water (instead of hydrolysates) was used as the blank. Three replicates were performed and the

assays were duplicated. The microbial growth was estimated as the increase in bacterial colonies after 24 h compared to the blank and was reported as Log_{10} (cfu/mL).

2.8.5.2. Antimicrobial activity

The assay for antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was conducted using the disc diffusion method. *Staphylococcus aureus* and *Escherichia coli* cultures were grown in Nutrient Broth (Biokar Diagnostics, Beauvais, France) and *Pseudomonas* cultures in Tryptone Soy Broth (TSB, Biokar Diagnostics, Beauvais, France). Hydrolysates were prepared at a protein concentration of 10 mg/mL (w/v). Agar plates (Nutrient Agar and Tryptone Soy Agar) were prepared, allowed to set and dry at room temperature. Bacterial cultures were diluted in either Nutrient Broth or TSB to give a count of approximately 10^6 cfu/mL. Aliquots (100 µL) of freshly prepared inoculum of the organism under study were spread in the agar plates. Sterile disc papers (8 mm of diameter) were soaked in the sample and placed in the agar plates, which were incubated at 37 °C for 24 h. Sterile water was used as the blank. A transparent ring around the paper disc indicates antibacterial activity. Three replicates were performed and the assays were duplicated.

2.9. Fractionation of bioactive FPH

Hydrolysates from mackerel skin gelatine (digested with pepsin for 24 h) and hydrolysates from mackerel viscera (digested with Flavourzyme for 24 h) were ultrafiltrated with a 3000 Da cut-off filter (Vivaspin, Sartorius Stedim Ltd.,

Dublin, Ireland) and the bioactivity assays were then performed on the filtrates and their concentrates. The active fractions were further concentrated by a Speed-Vac concentrator and fractionated by a preparative HPLC (Section 2.9.2).

2.9.1. HPLC analysis

Separation and analysis of peptides from the protein hydrolysates (injection volume of 10 µL) was carried out using a reverse-phase Waters Spherisorb ODS-2 C_{18} column (150 mm × 4.6 mm, 5 µm) on a Waters (Milford, MA, USA) HPLC system equipped with a UV-Vis detector. The mobile phases consisted of A: water and B: acetonitrile (both containing 0.1 % TFA), delivered at a constant flow rate of 1 mL/min with a linear gradient of 5 - 95 % of B over a period of 40 min. The column temperature was maintained at 20 ºC and the elution positions of the peptides were determined following the absorbance at 215 nm. Instrument control and data acquisition and analysis were performed using the Empower 2 Enterprise (Build 2154) software.

2.9.2. Preparative HPLC analysis

The fractionation of hydrolysates from mackerel skin gelatine (digested with pepsin for 24 h) and hydrolysates from mackerel viscera (digested with Flavourzyme for 24 h) was achieved by reverse-phase small scale preparative HPLC system. All preparative separations were performed on a Varian ProStar Preparative HPLC system (Varian, Inc., California, USA) that includes the binary ProStar HPLC pumps, a ProStar 335 diode array detector and a Varian 701 fraction collector. Separation of peptides (injection volume of 10 mL) was achieved by a reverse-phase Phenomenex Luna C18 (2) column (100 × 21.2 mm,

5 µm, 100 Å). The mobile phases consisted of A: water and B: acetonitrile (both containing 0.1 % TFA), delivered at a constant flow rate of 10 mL/min with a linear gradient of 5 - 95 % of B over a period of 40 min. The elution of the peptides was determined following the absorbance at 215 nm and the column temperature was maintained at 20 ºC using a ProStar 510 column oven. Galaxie software version 1.9.3.2 (Varian, Inc., California, USA) was used to control the system and for the data analysis.

2.9.3. Determination of peptide content of fractionated FPH

The quantification of peptides present in the fish protein hydrolysates was assessed by the o-phthaldialdehyde (OPA) spectrophotometric assay described by Church *et al.* (1983) with minor modifications in the volume of reagents used. The OPA solution was prepared by combining 25 mL of 100 mM sodium tetraborate, 2.5 mL of 20 % SDS, 40 mg of OPA in 1 mL methanol and 100 µL of β-mercaptoethanol to a total volume of 50 mL. To assay the peptide content, 50 µL of the hydrolysates was directly added to 950 µL of OPA solution. The solution was mixed by inversion, incubated for 2 min at room temperature and the absorbance at 340 nm was measured in a spectrophotometer (Milton Roy Spectonic 1201, NY, USA). A calibration curve using Leu-Gly peptide as standard (Sigma, Dublin, Ireland) was constructed in the concentration range of 0.1 - 1 mg/mL and used for the quantification of peptide content ($R^2 = 0.996$):

$$\text{Peptides (mg/mL)} = 0.78 \times \text{Abs} + 0.01 \quad \textbf{Equation 2.11}$$

where Abs refers to the absorbance at 340 nm.

2.9.4. Mass spectrometric determination of the amino acid sequence

The determination of the molecular mass and the peptide sequence were performed using electrospray ionisation-mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS). ESI-MS data were acquired on Waters TQD tandem quadrupole mass spectrometer on positive ion mode. Sample (10 µL) was first mixed with 100 µL acetonitrile, 90 µL water and 10 µL 0.1 % formic acid and then injected with a flow rate of 1 µL/min. The peptide sequence was determined by MS/MS experiments using 8 - 10 eV collision energy and Argon as collision gas.

2.10. Mackerel oil extraction procedures

Different methods were developed and used in order to extract oils from mackerel heads and skins. Mackerel heads were ground in an Officine meat mincer (Officine cgt, Milan, Italy) and mackerel skins were removed from the fish by a knife, cut manually into squares (~ 2 cm^2) by scissors then homogenised using a Waring laboratory blender (AGB, Dublin, Ireland) for 2 min.

2.10.1. Chemical extraction

2.10.1.1. Solvent extraction (hexane)

The fish material (150 g of mackerel heads or skins) was mixed with hexane (1/3, w/v) and stirred for 15 min at room temperature, then filtered using a Whatman No.4 filter paper (Whatman, Maidenstone, England). The extraction was repeated twice. The solvent was evaporated under vacuum at 45 °C. The collected oil was centrifuged in a refrigerated centrifuge 2K15 (Sigma, Osterode, Germany) at 13,500 × g for 15 min at 5 °C.

2.10.1.2. Alkaline extraction (NaOH)

During the gelatine extraction, an initial alkaline treatment step was required in order to remove the oil and non-collagenous proteins. This alkaline solution was collected and put in a closed glass bottle covered with aluminium foil and left overnight undisturbed in the fridge at 4 °C. Three phases were obtained, sludge at the bottom, liquid phase in the middle and an oily/emulsion phase on the top. The oily phase was collected and centrifuged in a refrigerated centrifuge 2K15 (Sigma, Osterode, Germany) at 13,500 × g for 10 min at 5 °C.

2.10.2. Physical extraction

The oil from the mackerel heads and skins were extracted according to the procedure of Turon *et al.* (2005). The fish material (150 g of mackerel heads or skins) were mixed with water (1/3, w/v) and heated at 80 °C for 30 min under continuous stirring at 150 rpm. The mixture was left undisturbed in a closed bottle and in dark for 2 h at room temperature in order to separate the liquid phases. The upper phase was drained and the oil was separated from the water by centrifugation in a refrigerated centrifuge 2K15 (Sigma, Osterode, Germany) at 13,500 × g for 15 min at 5 °C.

2.10.3. Enzymatic extraction

The method was performed according to Linder *et al.* (2005) with some modifications. The fish material (150 g of mackerel heads or skins) and 0.1 M phosphate buffer at pH 8 were mixed in the proportion of 1/3 (w/v). The mixture was then heat treated in a microwave oven model R-244 (Sharp Electronics Ltd.,

Uxbridge, UK) for 5 min to inactivate the endogenous enzymes. The enzymatic hydrolysis started when the temperature of the mixture reached 50 °C by adding 0.1 % (v/w) Alcalase (Sigma, Dublin, Ireland). The hydrolysis proceeded for 2 h under continuous shaking using a Gallenkamp orbital incubator (AGB, Dublin, Ireland). To stop the hydrolysis, the mixture was heat treated in a microwave oven model R-244 (Sharp Electronics Ltd., Uxbridge, UK) for 5 min. After cooling down, the hydrolysed material was centrifuged in a refrigerated centrifuge 2K15 (Sigma, Osterode, Germany) for 10 min at 13,500 × g at 5 °C. Three fractions were obtained: the sludge, the protein hydrolysates and an oily phase on the top. The oil was collected and further analysed.

2.10.4. Alkaline and enzymatic extraction

The recovery of fish oil by centrifugation after pre-treating the fish material with an alkaline solution was not very effective since most of the oil present was emulsified with proteins. In order to release the oil, a hydrolysis of the collected oily phase from alkaline pre-treated material was performed. The pH was adjusted to 8 using 3 N HCl then heat treated for 5 min in a microwave oven model R-244 (Sharp Electronics Ltd., Uxbridge, UK) to inactivate any endogenous enzymes. The hydrolysis started when the temperature reached 50 °C by adding 0.1 % (v/v) Alcalase and it proceeded for 4 h under continuous shaking using a Gallenkamp orbital incubator (AGB, Dublin, Ireland) and without further adjustment of pH. After hydrolysis the mixture was heat treated for 5 min in a microwave oven model R-244 (Sharp Electronics Ltd., Uxbridge, UK). The oil

was recovered by centrifugation in a refrigerated centrifuge 2K15 (Sigma, Osterode, Germany) at 13,500 × g for 10 min at 5 °C.

The procedures for extracting oils from mackerel heads and skins are summarised in Figure 2.4.

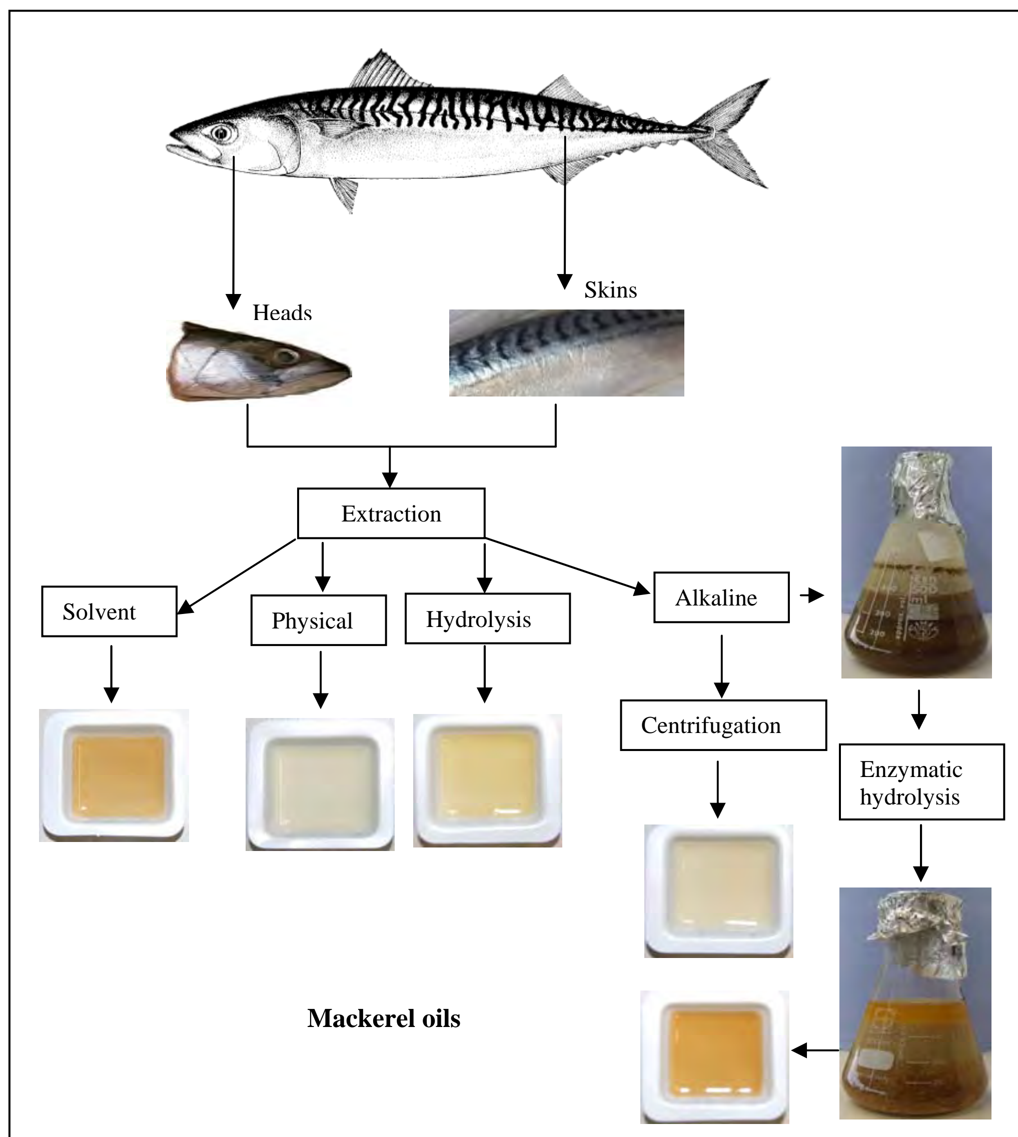


Figure 2.4 Extraction process for oils from mackerel heads and skins.

2.11. Characterisation of mackerel oils

2.11.1. Yield of oil extraction

The yield of recovered oil was calculated as:

$$\text{Yield}\,(\%) = 100 \times \frac{\text{Weight of extracted oil (g)}}{\text{Weight of oil in the initial sample (g)}}$$ **Equation 2.12**

The weight of oil in the initial sample was determined using the oil content as measured by the proximate analysis.

2.11.2. Clarification of the oil samples

A fraction of oil (1 mL) was mixed with 5 mL hexane, vortexed for 1 min and then passed through a bed of sodium sulphate anhydrous (4 g) and washed twice with 5 mL hexane. Sodium sulphate anhydrous retains the water and all other impurities. Hexane was evaporated under a steam of nitrogen to obtain the clean oil.

2.11.3. Physicochemical properties of mackerel oils

The acid value (Method 969.17), peroxide value (Method 965.33) and iodine value (Method 993.20) were determined according to AOAC Official Method (AOAC, 2000).

2.11.4. Fourier Transform Infra-Red (FTIR) analysis of mackerel oils

Infrared spectra were obtained using an Avatar 360 FTIR spectrometer equipped with a removable Smart Multi-Bounce HATR sample compartment (Thermo-Nicolet, Madison, Wisconsin, USA). FTIR spectra (32 scans) were analysed in the absorbance mode, between 400 and 4000 cm^{-1} with a resolution of 2 cm^{-1}. The spectra were analysed using Omnic 5.0 software (Thermo-Nicolet, Madison, Wisconsin, USA). Before collecting background spectrum and analysing samples, the HATR crystal was carefully cleaned with hexane to remove previous samples and any impurities.

2.11.5. Composition and distribution of neutral and polar lipids

2.11.5.1. Gas chromatographic analysis of triglycerides

The analysis of total TAG in fish oils was performed according to the method described by Fontecha *et al.* (1998). Oil samples (~ 50 mg) were dissolved in 0.45 mL of 2/1 (v/v) Cholorfom/methanol then 0.2 μL was injected into the gas chromatograph. The triglyceride analyses were performed on an Autosystem Gion 4072042 gas chromatograph (Perkin-Elmer, Beaconsfield, UK) equipped with an automatic injector (split/splitless) and programmed temperature. A capillary column (30 m × 0.22 mm i.d. × 0.20 μm film thickness), supplied by Restek (Bellefonte, PA, USA), Rtx®-65TG (35 % dimethyl, 65 % diphenyl polysiloxane) was used.

Experimental chromatographic conditions were as follows: the initial temperature (220 °C) was raised to 320 °C at a rate of 15 °C/min and then to 355 °C at a rate of 7 °C/min and then held at this temperature for 20 min. The injector and detector temperatures were 355 and 370 °C, respectively. The pressure at the top of the column was 25 psi, the split ratio was 1: 4 and the carrier gas was helium. The flow rate was 0.8 mL/min.

2.11.5.2. Determination of lipid classes

Separation of neutral and polar lipids from fish oil was performed on a HPLC (Shimadzu Vp Series, Duisburg, Germany) coupled with an evaporative light scattering (ELSD) detector (SEDERE. SEDEX 85 model, Alfortville Cedex, France) using filtered air at 60 °C as the nebulising gas at a pressure of 3.5 bar. A 250 nm × 4.6 mm Zorbax Rx-SIL column (Agilent Tecnhnologies, Palo Alto, CA, USA) with 5 µm particle diameter was used. The elution program was a linear gradient of A: 99/1 (v/v) Iso-octane: tetrahydrofuran; B: 87.5/12/0.5 (v/v/v) chloroform: methanol: 1 M formic acid (pH 3); C: 28/60/12 (v/v/v) chloroform: methanol: 1 M formic acid (pH 3). The flow rate was 1 mL/min and the injection volume was 10 µL. The column was equilibrated at 40 °C. Retention times of the compounds of interest were tested by injection of internal standards, diluted in chloroform. Oils extracted from heads and skins using the Folch method (Folch *et al.,* 1957) were used as reference oils since this method was believed to extract total lipids (including the phospholipids).

2.11.6. Gas chromatographic analysis of fatty acids

2.11.6.1. Methylation of the oil samples

Fatty acid methyl esters (FAMEs) were prepared using potassium hydroxide as described by International Standard method ISO-IDF (ISO, 2002). Briefly, 25 mg oil sample and 200 μL of hexane were mixed and vortexed. Then 50 μL of 2 N KOH (in methanol) was added and vortexed for 1 min. The mixture was allowed to react for 5 min in dark. After that 125 mg of sodium bisulfate monohydrate ($NaHSO_4 \bullet H_2O$) was added to stop the reaction. The sample was centrifuged for 5 min at 9,000 × g at 5 ºC. The supernatant was carefully collected and diluted with 250 μL of hexane.

2.11.6.2. Fatty acid determination and quantification

FAMEs were analysed according to the method reported by Rodríguez-Alcalá *et al.* (2009) on an Agilent gas chromatography unit (model 6890N, Palo Alto, CA, USA) equipped with a flame ionisation detector. Fatty acids were separated using a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.20 μm film thickness, Chrompack, Middelburg, Netherlands). The column was held at 100 °C for 1 min after injection, temperature-programmed at 7 °C/min to 170 °C, held at 170 °C for 55 min, and then temperature-programmed at 10 °C/min to 230 °C and held at the final temperature for 33 min. Helium was the carrier gas with a split ratio of 1:20. The injector temperature was set at 250 °C and the detector temperature was set at 270 °C. Injection volume was 0.5 μL. A standard containing a mixture of 37 FAMEs from Supleco (Sigma-Aldrich, Dublin, Ireland) was used to obtain the response factors (RF) and the retention

times of fatty acid methyl esters. For quantitative purposes, tridecanoic acid (C13; 1.24 mg/mL) was added as an internal standard. Duplicate determinations were carried out.

2.12. Screening for bioactivity of mackerel oils

2.12.1. Antioxidant activity of fish oils

DPPH radical-scavenging activity of fish oil was determined by the method of Amarowicz *et al.* (2004). An aliquot of fish oil (100 µL) was mixed with 1.4 mL of methanol and then added to 1.5 mL of DPPH solution (60 µM in MeOH). The mixture was shaken vigorously, incubated for 30 min in the dark at room temperature and the absorbance was measured using a spectrophotometer (Milton Roy Spectonic 1201, NY, USA) at 517 nm. The DPPH scavenging activity was calculated as per Equation 2.7.

2.12.2. Fractionation of mackerel oils

The separation of the neutral and polar fractions was performed according to the method described by International Union of Pure and Applied Chemistry (IUPAC, 1987). The separation of oil fractions, based on adsorption chromatography, was achieved in a glass column (20 cm × 10 mm i.d.) using 5 g Silica gel (particle size 0.063 - 0.200 mm). Fish oil (1 g) was first dissolved in 10 mL of elution solvent prepared by mixing 90 % petroleum ether and 10 % diethyl ether (v/v) and then introduced onto the Silica column. The elution of non-polar compounds was carried out with 60 mL of the elution solvent. The polar compounds were eluted with 50 mL of diethyl ether. The flow rate was adjusted

to 1.5 mL/min. The elution solvents were evaporated and the fractions were collected.

Thin layer chromatography (TLC) was used to check the efficiency of the separation. Ten percent solutions of crude fish oil, non polar and polar fractions were prepared in chloroform then 5 µL of each solution were loaded on the TLC plate of Silica gel (10 × 20 cm plate, 0.2 mm thickness). The plates were eluted with a mixture of petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v), sprayed with 50 % sulphuric acid (in ethanol) and dried at 130 °C for 15 min to visualise the lipid fractions.

2.13. Statistical analyses

ANOVA (Multifactor and one-way) was used to find differences between treatments. Means were compared by significant difference (LSD) test, at a significance level of $p < 0.05$ using the Statgraphics Centurion XV software (version 15.1.02; StatPoint, Inc., Virginia, USA). Three independent trials were carried out.

The curve fit and the value of IC_{50} of were obtained with the aid of the computer software GraphPad Prism, version 5.00 (GraphPad Software, San Diego, USA).

Chapter 3 - Fish waste

3.1. Characterisation of fish waste

3.1.1. Quantification of fish waste

The average weight of whole mackerel was higher than that of blue whiting reflecting the typical difference in size of these two species. Average weights of 326 g and 69 g for mackerel and blue whiting, respectively, were reported by Toppe *et al.* (2007).

On average, the quantity of waste material was similar in both fish, a slightly higher percentage of total waste was observed with the blue whiting (Table 3.1).

Table 3.1 Percentage (w/w) of waste from mackerel and blue whiting.

	Mackerel	**Blue whiting**
Weight (g)	277.0±94.7^{b}	116.8±3.7^{a}
% Head	16.6±0.9^{a}	22.7±1.7^{b}
% Bone	8.5±0.7^{a}	8.9±1.5^{a}
% Viscera	10.5±0.4^{a}	9.4±1.1^{a}
% Skin	17.0 ±3.0^{a}	17.5±2.0^{a}
% Total waste	52.2±5.0^{a}	58.5±6.4^{a}

Values were given as mean ± standard deviation. Different letters in the same row indicate significant ($p < 0.05$) differences between fish.

According to Leu *et al.* (1981), the average edible portion of mackerel is about 53.5 % (w/w). They reported the distribution of different components of mackerel to be as follows: heads constituted 17.1 %; bones, fins and tails constituted 8.4 %; skins constituted 10.3 %, and viscera constituted 10.9 % of the

whole mackerel. An average edible portion of blue whiting was estimated to be around 58.2 % (Bykov, 1983).

During the processing of both mackerel and blue whiting in this research, a significant amount of waste was obtained (52.2 % and 58.8 %, respectively). This waste mainly constituted skins and heads (35 - 40 %) and viscera and bones at lower amounts (~ 18 %). The traditional way to treat fish processing waste was to use them as low value fish meal and fertiliser (Nagai & Suzuki, 2000). Fish viscera were typically discarded overboard or dumped to landfill. However, the European Directive 1999/31/EC on the landfill of waste (Council Directive, 1999) forbids and restricts the disposal of untreated organic waste not untended for human consumption. The development of new sustainable processes for optimal use of fish waste may represent a new approach to lower the disposal cost and increase profit. Hence, the abundant low quality biological waste from mackerel and blue whiting can be converted into potential value added products depending on their nutritional quality and chemical composition.

3.1.2. Proximate analysis of fish waste

The proximate analysis gives a profile of the model fish helping to direct the screening. The proximate composition of different tissues from mackerel indicated a protein content ranging from 16.0 to 19.8 % and moisture content varying from 64.6 to 77.6 %. The fat content was high in all tissues except for bones and viscera which was around 5 % (Table 3.2). Similar composition was reported for the white and dark muscle of mackerel (*Scomber scombrus*) with a protein content of 18.9 and 17.3 %, a moisture content of 71.5 and 68.1 %, a fat

content of 9.1 and 14.1 % and an ash content of 1.2 and 1.1 % for white and dark muscles, respectively (Leu *et al.,* 1981).

Reported data for the fillet of chub mackerel (*Scomber japonicus*) showed a higher protein content of 21.4 %, a higher moisture content of 71.8 % and an ash content of 2.3 % (Mbarki *et al.,* 2009). These differences may be due to the variation among species.

Table 3.2 Proximate analysis of mackerel and blue whiting per tissue type.

	% Moisture		% Ash		% Protein		% Lipid	
	M	**BW**	**M**	**BW**	**M**	**BW**	**M**	**BW**
Flesh	65.4^{aA} ±0.4	82.3^{bB} ±0.3	1.7^{aA} ±0.6	1.1^{aA} ±0.1	19.8^{bB} ±0.3	16.7^{aA} ±0.4	17.9^{cB} ±0.5	0.7^{aA} ±0.1
Head	65.3^{aA} ±0.3	77.9^{bB} ±1.1	4.3^{bA} ±0.3	6.0^{bA} ±1.2	16.3^{aA} ±0.1	15.7^{aA} ±0.8	14.3^{bB} ±0.1	0.8^{aA} ±0.3
Skin	64.6^{aA} ±0.2	80.2^{bB} ±0.2	2.3^{aB} ±0.2	1.1^{aA} ±0.1	18.6^{bA} ±0.1	18.0^{bA} ±0.1	13.7^{bB} ±0.5	0.6^{aA} ±0.4
Bone	64.9^{aA} ±0.1	64.2^{aA} ±1.5	8.9^{cA} ±0.6	16.0^{cB} ±0.7	19.8^{bA} ±0.1	19.5^{bA} ±0.3	5.5^{aB} ±0.9	0.8^{aA} ±0.1
Viscera	77.6^{bB} ±0.9	64.6^{aA} ±8.8	1.4^{aA} ±0.6	1.6^{aA} ±0.4	16.1^{aA} ±0.1	16.9^{aA} ±0.3	4.9^{aA} ±0.5	8.6^{bB} ±0.2

M: Mackerel, BW: Blue whiting. Values given as mean ± standard deviation. Different lower case letters, in the same column, indicate significant differences ($p < 0.05$) between tissue type. Different upper case letters in the same row, within the same parameter, indicate significant differences ($p < 0.05$) between fish.

The proximate composition of different tissues from blue whiting indicated a protein content ranging from 15.7 to 19.5 % and moisture content varying from 64.2 to 82.3 %. The fat content was less than 1 % for all the tissue type except for viscera (Table 3.2). Very similar results were reported by Oehlenschläger *et al.* (2008) for blue whiting fillet. In their study, the moisture content was 82.1 %, the protein content was 16.8 % and the ash was 1.5 %.

Brennan & Gormley (1999) studied the quality of under-utilised deep-water fish species, including blue whiting. The proximate analysis of the flesh of blue whiting was similar to the present results except for the protein content which was low in their study (9.8 %). De Lurdes *et al.* (1998) reported similar values of moisture content (76.1 %), protein content (16.9 %) but higher lipid content (2.7 %) for blue whiting. Yoshie-Stark *et al.* (2009) studied the composition of Southern blue whiting (*Micromesistius australis*) muscle and they reported a moisture content of 79.4 %, protein content of 18.6 %, ash 1.2 % and a fat level of 0.8 %, these results were also in agreement with the present results. The ash content is considerably higher for fish bone and head due to the high content of minerals.

3.1.3. pH determination of fish waste

The pH values of the different tissue of mackerel and blue whiting are presented in Table 3.3. The pH value of the mackerel tissue ranged from 5.5 to 6.1 which were in agreement with the values of 6.2 and 6.1 for chub mackerel (*Scomber japonicus*) reported by Goulas & Kontominas (2007) and Metin *et al.* (2001), respectively. The pH values of blue whiting in this study were also higher

than the pH of 5.3 as reported by De Lurdes *et al.* (1998). Cheow *et al.* (2007) reported pH values of 6.6 and 6.59 for Sin croaker and Shortfin scad skin, respectively. Parisi *et al.* (2002) reported a pH 6.88 for European sea bass muscle and Sigholt *et al.* (1997) also reported a pH 6.7 for Atlantic salmon muscle.

Table 3.3 pH of mackerel and blue whiting per tissue type.

Raw material	Mackerel	Blue Whiting
Head	6.1 ± 0.01^{bA}	$6.3\pm0.\ 01^{aA}$
Viscera	5.5 ± 0.02^{aA}	6.3 ± 0.03^{aB}
Skin	$5.7\pm0.\ 01^{aA}$	$6.6\pm0.\ 03^{bB}$
Flesh	$5.6\pm0.\ 03^{aA}$	$6.3\pm0.\ 01^{aB}$
Bones	$5.8\pm0.\ 03^{aA}$	$6.2\pm0.\ 04^{aB}$

Values given as mean ± standard deviation. Different lower case letters, in the same column, indicate significant differences ($p < 0.05$) between tissue type. Different upper case letters, in the same row, indicate significant differences ($p < 0.05$) between fish.

Results showed that both fish had lower pH values than the typical pH of live fish (~ 7.0) and blue whiting having higher pH values than mackerel. Being an oily fish, mackerel is very prone to deterioration (Boran *et al.,* 2006). After catch, glycolysis takes place converting glycogen to pyruvic acid and later to lactic acid (Abbas *et al.,* 2008). This results in decreasing the pH and affects the texture of fish muscle. The low pH values, observed with mackerel raw material, suggest that the autolysis has taken place.

3.2. Fish waste as potential resource for food ingredients

In 2008, the annual catch of mackerel and blue whiting in Ireland was around 45,000 and 23,000 tonnes, respectively (FAO, 2010). Therefore, mackerel processing would produce around 7,500 tonnes of heads and skins and 3,800 tonnes of bones. Blue whiting processing, would produce about 5,200 tonnes of heads, 4,000 tonnes of skins and 2,000 tonnes of bones. In addition, a further 10 % of the annual catch of mackerel and blue whiting are discarded as undersized, damaged and low quality fish (Pierce *et al.,* 2002).

Previous studies on fish composition showed that the collagen content in skins was as high as 80 % of the total protein (Gundmundsson & Hafsteinsson, 1997; Kołodziejska *et al.,* 2008). The collagen level in fish heads and bones was around 35 % of the total protein (Kołodziejska *et al.,* 2008). Thus, around 1,100 and 580 tonnes of collagen or gelatine could be recovered every year in Ireland from mackerel and blue whiting skins, respectively. The annual catch and processing of both fish could produce around 430 and 290 tonnes of gelatine from mackerel and blue whiting heads, respectively. Fish bones would also generate around 260 and 140 tonnes of gelatine from mackerel and blue whiting, respectively.

Mackerel oils have a high nutritional value due to the high amount of PUFA (Zuta *et al.,* 2003). Mackerel heads and skins had similar oil content (~ 14 %). However, these raw materials are usually discarded during the processing. In light of the annual catch (45,000 tonnes) mackerel heads and skins present opportunities for recovering PUFA with significant amounts (~ 1,000 tonnes).

Visceral wastes are usually regarded as low quality raw material. Results from this study showed a high level of protein remaining in the fish viscera (Table 3.2). Hydrolysis of fish viscera to obtain possible bioactive peptides might be an option in order to add value to this waste. Although both fish viscera had similar protein content (~ 16 %), previous studies showed that blue whiting viscera are heavily infested by *Anisakis simplex*. This round worm nematode parasite (*Anisakis simplex*) is usually found as larva in great numbers in the gut cavity and in the flesh, particularly in the belly flaps (Cruz *et al.,* 2007). These parasites commonly infest blue whiting and other species such as whiting and herring. If ingested, the live larvae may pose a serious health problem (*anisakidosis*) to consumers. In addition to that allergic problems may arise from the products excreted/secreted or released by the larvae (Vidacek *et al.,* 2009). For these reasons and in order to avoid the risk of concentrating the allergens, only mackerel viscera were used as source for protein hydrolsayes.

3.3. Conclusions

The high protein content in mackerel and blue whiting heads, bones and skins, make them a potential source for extracting gelatines.

Mackerel head and skin would be suitable for the recovery of oils, possibly high in polyunsaturated fatty acids.

Mackerel viscera could be converted into protein hydrolysates which could enhance their bioactive properties.

Chapter 4 - Fish gelatines

4.1. Extraction and characterisation of fish gelatines

4.1.1. Yield of gelatine extraction

A general procedure to extract gelatine consists of an acid pre-treatment which solubilises the collagen but does not alter its triple helix (very low concentration of acid) and then application of heat treatment which cleaves hydrogen and non-covalent bonds that converts the triple helix configuration of collagen into coiled conformation resulting in a gelatinous state (Djabourov *et al.,* 1993). In this study 5 different organic acids (0.05 M) were used: acetic, citric, lactic, malic and tartaric acid. Figure 4.1 shows the yield of gelatine extracted from different tissues of mackerel and blue whiting (i.e., head, skin and bone).

The yield of gelatine was expressed as a percentage on a wet weight basis. No significant ($p > 0.05$) differences were observed in the extraction yields for mackerel head and skin gelatines. Only slight differences were observed depending on the organic acid used. Mackerel heads pre-treated with citric acid (Figure 4.1A) showed higher gelatine extraction yield (3.7 %), followed by lactic and malic acids (3.5 %). Mackerel heads pre-treated with acetic and tartaric acids had the lowest gelatine extraction yields (3.3 %). For mackerel skins, citric and malic acids gave the highest yields (3.3 %) and the lowest were observed after pre-treatment with acetic and lactic acids (Figure 4.1C).

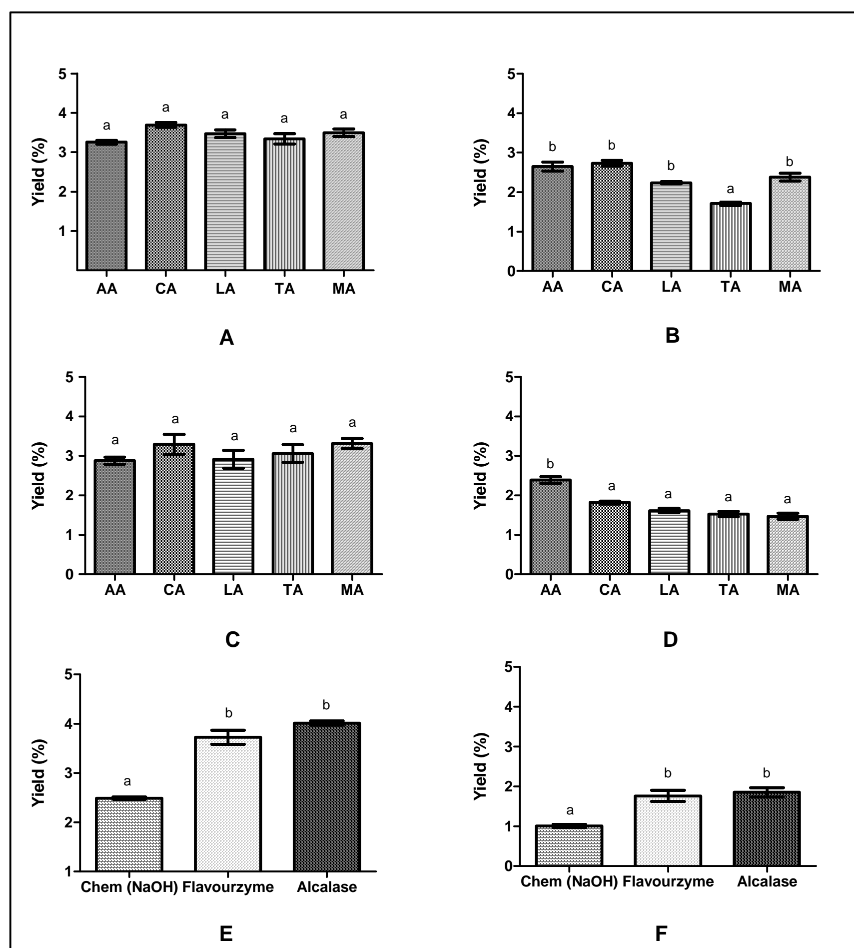


Figure 4.1 Yield of gelatine extraction. Gelatines from mackerel heads (A), gelatine from blue whiting heads (B), gelatines from mackerel skin (C), gelatine from blue whiting skin (D), gelatines from mackerel bones (E) and gelatine from blue whiting bones (F). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase. Different letters, within the same graph, indicate significant ($p < 0.05$) differences between pre-treatments.

The yield varied depending on the organic acid used for blue whiting. Gelatine extracted from blue whiting heads after pre-treatment with tartaric acid showed significantly ($p < 0.05$) the lowest yield (1.7 %), no significant differences were observed for the rest of acids (Figure 4.1B). The highest extraction yield for

blue whiting skins was observed with acetic acid (2.3 %) which was statistically higher ($p < 0.05$) than that obtained by the other acids (Figure 4.1D).

Giménez *et al.* (2005) examined the ability of lactic acid compared to acetic acid to prepare gelatine from Dover sole (*Solea vulgaris*) skins and the yield using lactic acid was slightly lower than that using acetic acid. Gelatines yields from salmon and cod skins as reported by Arnesen & Gildberg (2007) were 39.7 % and 44.8 %, respectively (on a dry weight basis). Also Arnesen & Gildberg (2006) extracted gelatine from cod head and they were able to recover 47.5 % of the total protein.

Regardless of fish, gelatine extracted from chemically pre-treated bone, showed the lowest yield (Figure 4.1E & F). A yield of 2.5 % and 1.0 % were observed for mackerel and blue whiting respectively. Bones pre-treated enzymatically generated significantly ($p < 0.05$) highest yields (~ 3.8 % and 1.8 %, for mackerel and blue whiting, respectively). Gildberg *et al.* (2002) reported that the major part of muscle protein and collagen in the cod backbone fraction can be recovered by a combination of gentle enzymatic hydrolysis and chemical extraction.

The difference between the yields, which depended on the nature of the organic acid, could also be due to losing collagen during the washing step (Cheow *et al.,* 2007). The type of acid affects the pH of the extraction solution, which affects the breaking of non-covalent bonds in the collagen cross-links of the raw material (Gómez-Guillén & Montero, 2001). Hence suitable mild acid pre-treatment is essential for gelatine extraction (Giménez *et al.,* 2005).

4.1.2. pH values of fish gelatines

The pH of gelatines from the mackerel and blue whiting are presented in Table 4.1.

Table 4.1 pH values of fish gelatines.

Fish	Tissue	Treatment	pH
Mackerel	**Heads**	**AA**	6.2±0.3[c]
		CA	4.4±0.1[a]
		LA	4.9±0.1[b]
		MA	5.0±0.2[b]
		TA	4.3±0.1[a]
Blue whiting	**Heads**	**AA**	5.1±0.2[a]
		CA	4.8±0.2[a]
		LA	5.1±0.1[a]
		MA	5.3±0.1[a]
		TA	5.0±0.1[a]
Mackerel	**Skins**	**AA**	4.4±0.1[c]
		CA	3.5±0.1[b]
		LA	3.5±0.2[b]
		MA	3.3±0.2[b]
		TA	2.8±0.1[a]
Blue whiting	**Skins**	**AA**	4.7±0.2[c]
		CA	3.4±0.1[b]
		LA	3.8±0.2[b]
		MA	3.5±0.1[b]
		TA	2.8±0.1[a]
Mackerel	**Bones**	**NaOH**	1.7±0.2[a]
		Enz (A)	1.7±0.1[a]
		Enz (B)	1.7±0.1[a]
Blue whiting	**Bones**	**NaOH**	3.4±0.2[b]
		Enz (A)	2.2±0.1[a]
		Enz (B)	2.2±0.1[a]
Commercial fish	-	-	5.3±0.1
Commercial bovine	-	-	5.4±0.1

Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; NaOH: alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase. The controls used were commercial fish and bovine gelatines. Values given as mean ± standard deviation. Different letters, within the same raw material, indicate significant ($p < 0.05$) differences between pre-treatments.

The pH values varied from 1.77 to 3.39 for bone gelatines and from 3.38 to 6.28 for head and skin gelatines. Gundmundsson & Hafsteinsson (1997) reported that pH of gelatine from cod skins varied between 2.7 and 3.9, and Grossman & Bergman (1992) reported a value of 3.77 for gelatine from tilapia. The low pH value observed in bone gelatines may be due to the use of the hydrochloric acid in the demineralisation step.

4.1.3. Proximate analysis of fish gelatines

The proximate composition of gelatine was found to vary with the type of tissue examined and the fish type (Table 4.2). All gelatine samples had low moisture content and no significant ($p > 0.05$) differences were observed. Gelatines extracted from fish heads and skins were generally low in ash, the calculated values were lower than the recommended maximum of 2.6 % (Jones, 1977). Ash content higher than 2.6 % may affect the functional properties of gelatine and may increase the risk of the presence of toxic heavy metals (aluminium, arsenic, cadmium, mercury and lead) (Nabrzyski, 2007). Gelatines from fish bones had higher ash content, and were in agreement with values of bone gelatine of young and adult Nile perch (Muyonga *et al.,* 2004). The lipid content of extracted gelatines was less than 1 % indicating that the pre-treatment process was efficient in removing oils.

Table 4.2 Proximate analysis of gelatines.

Fish	Tissue	Treatment	% Moisture	% Ash	% Protein	% Lipid
Mackerel	**Heads**	**AA**	7.7±1.4[a]	1.6±0.5[a]	89.4±1.8[b]	0.8±0.1[a]
		CA	8.1±2.8[a]	1.8±0.7[a]	87.4±1.7[b]	0.7±0.2[a]
		LA	9.0±1.5[a]	1.9±0.1[a]	88.5±1.4[b]	0.9±0.1[a]
		MA	8.9±1.9[a]	1.7±0.1[a]	82.1±2.2[a]	0.8±0.1[a]
		TA	9.1±1.6[a]	1.7±0.8[a]	83.3±0.8[a]	0.9±0.2[a]
Blue whiting	**Heads**	**AA**	9.8±1.3[a]	0.8±0.2[b]	88.5±0.8[a]	-
		CA	9.3±0.6[a]	1.9±0.3[c]	87.8±3.2[a]	-
		LA	8.4±2.0[a]	0.4±0.1[a]	89.9±3.0[a]	-
		MA	8.3±1.5[a]	0.4±0.1[a]	89.7±3.4[a]	-
		TA	9.1±0.4[a]	1.2±0.3[b]	88.1±0.6[a]	-
Mackerel	**Skins**	**AA**	10.3±1.8[a]	0.8±0.1[a]	86.2±1.3[a]	0.7±0.1[a]
		CA	9.0±1.9[a]	0.7±0.2[a]	87.2±2.8[a]	0.9±0.2[a]
		LA	10.2±1.7[a]	0.8±0.1[a]	85.0±2.7[a]	0.7±0.2[a]
		MA	8.7±1.6[a]	0.5±0.2[a]	87.2±2.0[a]	0.9±0.3[a]
		TA	8.9±1.2[a]	1.0±0.2[a]	85.9±1.9[a]	0.8±0.1[a]
Blue whiting	**Skins**	**AA**	10.2±1.2[a]	1.9±0.2[a]	84.4±1.7[a]	-
		CA	10.2±1.7[a]	1.7±0.5[a]	84.1±3.2[a]	-
		LA	9.6±0.9[a]	1.9±0. 6[a]	81.9±1.1[a]	-
		MA	9.3±0.8[a]	1.8±0.2[a]	82.1±3.7[a]	-
		TA	10.7±1.0[a]	1.6±0.4[a]	83.6±0.5[a]	-
Mackerel	**Bones**	**NaOH**	9.4±2.3[a]	11.8±1.5[a]	72.6±2.9[a]	-
		Enz (A)	8.9±1.7[a]	13.9±1.4[a]	72.5±3.6[a]	-
		Enz (B)	9.3±2.8[a]	9.3±1.3[a]	73.1±3.2[a]	-
Blue whiting	**Bones**	**NaOH**	9.5±1.2[a]	11.6±1.8[a]	73.7±3.7[a]	-
		Enz (A)	10.2±0.7[a]	12.4±2.0[a]	72.0±1.0[a]	-
		Enz (B)	10.1±0.8[a]	12.2±1.9[a]	71.2±1.5[a]	-

Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; NaOH: alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase. Values given as mean ± standard deviation. Different letters in the same column, within the same raw material, indicate significant ($p < 0.05$) differences between pre-treatments.

For all gelatines, the protein content was high. Gelatines extracted from mackerel heads after pre-treatment with acetic, citric and lactic acids showed significantly ($p < 0.05$) higher protein content (89.4 %, 87.4 % and 88.5 %, respectively) than tartaric and malic acid pre-treated gelatines. The pre-treatment did not affect ($p > 0.05$) the protein content of gelatines extracted from mackerel skins and bones nor gelatines extracted from blue whiting heads, skins and bones. Muyonga *et al.,* (2004) reported that the protein content of gelatines derived from skins and bones of young Nile perch were 88.8 % and 83.3 %, respectively. Whereas, gelatines derived from skins and bones of adult Nile perch had protein contents of 88.0 % and 78.4 %, respectively. Additionally, gelatine from skins of Bigeye snapper and Brownstripe red snapper had protein contents of 87.9 % and 88.6 %, respectively (Jongjareonrak *et al.,* 2006).

4.1.4. Colour analysis of fish gelatines

The colour of gelatines (Table 4.3 and Figure 4.2) were significantly ($p < 0.05$) affected by the tissue type and the organic acid used in the extraction process. The lightness (L* value) of gelatines obtained from mackerel heads using malic acid was significantly ($p < 0.05$) higher than those extracted with the other acids. In case of mackerel skin gelatines, lactic acid showed the highest L*. For the blue whiting, tartaric and malic acids showed significantly ($p < 0.05$) the highest L* values for heads while tartaric and citric acids yielded highest values for skin gelatines. Mackerel bones pre-treated with Alcalase and blue whiting bones pre-treated with sodium hydroxide showed the highest L*.

Table 4.3 Colour measurement of gelatines from mackerel and blue whiting.

Fish	Tissue	Treatment	L*	a*	b*
Mackerel	**Heads**	**AA**	41.5±0.4^{a}	-1.3±0.1^{a}	1.1±0.1^{c}
		CA	42.4±0.6^{a}	-1.3±0.1^{a}	-0.1±0.1^{a}
		LA	41.6±0.5^{a}	-1.3±0.2^{a}	0.4±0.1^{b}
		MA	41.6±0.6^{a}	-1.4±0.1^{a}	0.5±0.1^{b}
		TA	43.6±0.7^{a}	-1.3±0.1^{a}	0.1±0.1^{a}
Blue whiting	**Heads**	**AA**	34.9±1.0^{a}	-0.0±0.2^{b}	0.1±0.3^{c}
		CA	35.6±0.7^{a}	-0.2±0.1^{b}	-0.5±0.2^{b}
		LA	36.0±0.1^{a}	-0.5±0.0^{a}	-0.3±0.1^{b}
		MA	38.5±0.8^{b}	-0.6±0.1^{a}	-1.0±0.1^{a}
		TA	36.1±0.5^{a}	-0.5±0.1^{a}	-0.4±0.1^{b}
Mackerel	**Skins**	**AA**	51.9±0.6^{a}	-1.6±0.1^{a}	0.2±0.1^{c}
		CA	52.4±2.2^{a}	-1.1±0.2^{a}	-0.8±0.2^{a}
		LA	56.3±0.9^{b}	-1.5±0.1^{a}	-0.3±0.1^{b}
		MA	50.1±0.5^{a}	-1.3±0.2^{a}	-0.6±0.0^{a}
		TA	53.2±1.7^{a}	-1.2±0.1^{a}	-0.3±0.0^{b}
Blue whiting	**Skins**	**AA**	43.8±0.9^{c}	-0.1±0.1^{c}	-0.5±0.3^{c}
		CA	40.0±0.3^{b}	-0.5±0.1^{b}	-2.3±0.2^{a}
		LA	35.3±0.4^{a}	-0.1±0.0^{c}	-1.9±0.1^{a}
		MA	41.1±0.4^{b}	-0.4±0.1^{b}	-1.3±0.1^{b}
		TA	40.1±1.3^{b}	-0.7±0.0^{a}	-1.7±0.1^{a}
Mackerel	**Bones**	**NaOH**	49.9±0.9^{b}	-1.2±0.0^{a}	-2.7±0.1^{a}
		Enz (A)	34.6±0.4^{a}	-0.2±0.1^{b}	-2.1±0.1^{b}
		Enz (B)	32.6±0.3^{a}	-0.5±0.0^{b}	-2.0±0.1^{b}
Blue whiting	**Bones**	**NaOH**	56.0±0.6^{b}	-2.0±0.1^{a}	-2.1±0.1^{a}
		Enz (A)	37.1±0.6^{a}	-0.4±0.1^{b}	-1.2±0.1^{b}
		Enz (B)	35.2±0.6^{a}	-0.2±0.2^{b}	-0.2±0.1^{c}

Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; NaOH: alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase. Values given as mean ± standard deviation. Different letters in the same column, within the same raw material, indicate significant ($p < 0.05$) differences between pre-treatments.

The lightness of gelatine can be either appreciated or not depending on the industrial application. For instance, in the production of mallows, the colour of the gelatine solution is less important than it is in fruit gummies (Schrieber & Gareis, 2007).

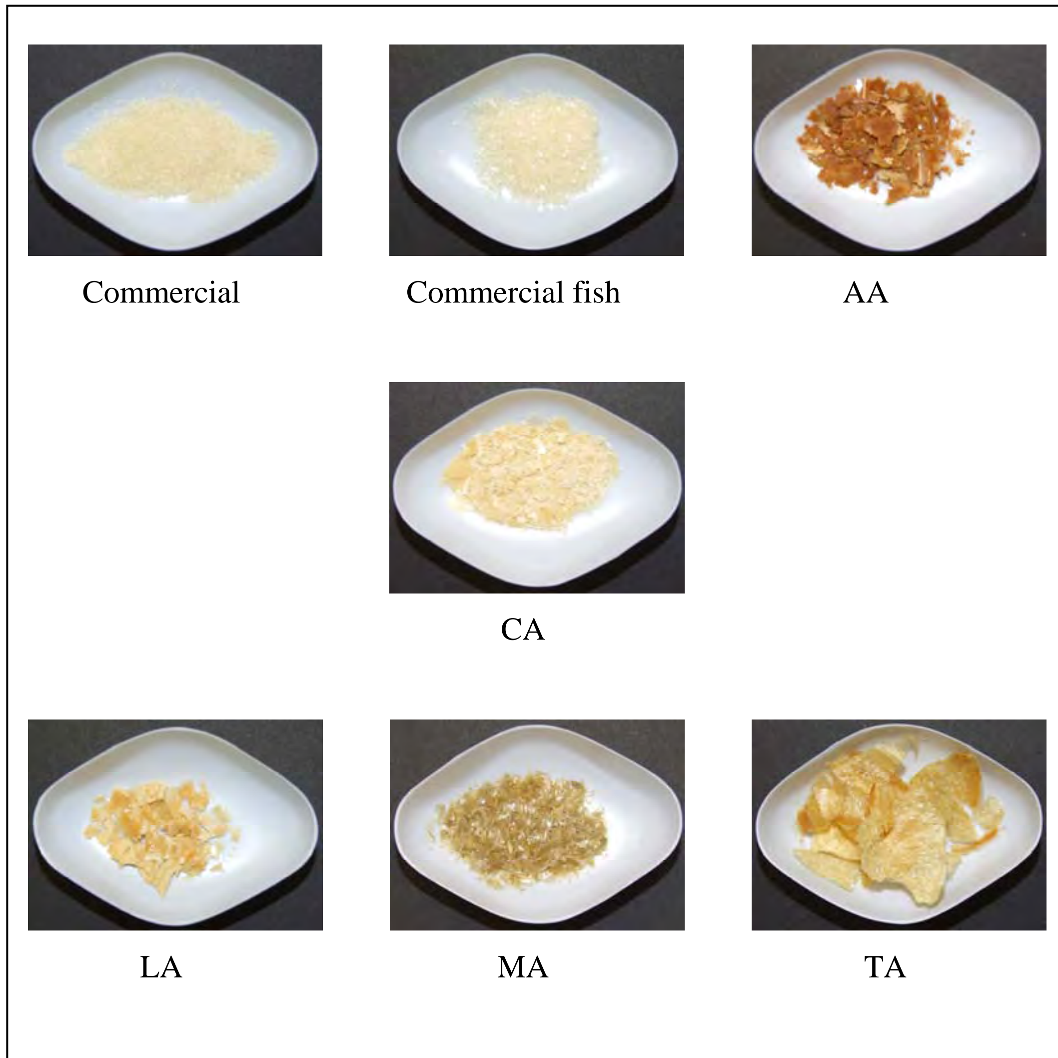


Figure 4.2 Colour of mackerel head gelatines, pre-treated with different organic acids, and commercial fish and bovine gelatines. Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; MA: malic acid and TA: tartaric acid.

All gelatines extracted after pre-treatment with acetic acid had significantly ($p < 0.05$) highest b* values, which indicated a higher yellowish colour (Table 4.3). Although all acids were at the same concentration (0.05 M), the pH of acetic acid solution was higher (3.1) than the pH of malic (2.3), lactic (2.3), citric (2.2) and tartaric (2.2) acids. It seems that the pH significantly ($p < 0.05$) affects the colour of the gelatine. Zhang *et al.* (2007) reported that pre-treatments at acidic solution prior to the extraction resulted in transparent gelatine, whereas pre-treatments at basic solution resulted in dark-coloured gelatine from Channel Catfish (*Ictalurus punctatus*). For industrial applications that require gelatine with less dark colour, deceasing the extraction time might be beneficial, since long reaction times favours the Maillard reaction between protein and traces of carbohydrates in the raw material (Schrieber & Gareis, 2007).

4.1.5. Turbidity analysis of fish gelatines

The effects of pre-treatments on turbidity of mackerel and blue whiting gelatines are shown in Table 4.4. Turbidity was significantly ($p < 0.05$) affected by both the pre-treatment used and the source of gelatine.

Table 4.4 Turbidity of fish gelatines.

Fish	Tissue	Treatment	Turbidity (FTU)
Mackerel	**Heads**	**AA**	121±12.5[a]
		CA	126±18.1[a]
		LA	176±3.2[b]
		MA	134±15.0[a]
		TA	129±14.3[a]
Blue whiting	**Heads**	**AA**	63±3.5[a]
		CA	84±1.6[b]
		LA	172±6.6[d]
		MA	99±6.2[c]
		TA	79±11.4[b]
Mackerel	**Skins**	**AA**	249±20.9[b]
		CA	295±13.3[c]
		LA	320±5.0[c]
		MA	247±21.5[b]
		TA	224±6.6[a]
Blue whiting	**Skins**	**AA**	346±29.8[b]
		CA	322±7.7[b]
		LA	442±7.3[c]
		MA	334±20.5[b]
		TA	228±9.8[a]
Mackerel	**Bones**	**NaOH**	339±16.9[b]
		Enz (A)	270±8.3[a]
		Enz (B)	260±7.2[a]
Blue whiting	**Bones**	**NaOH**	204±1.6[b]
		Enz (A)	115±7.7[a]
		Enz (B)	119±3.3[a]

Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; NaOH: alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase. Values given as mean ± standard deviation. Different letters in the same column, within the same raw material, indicate significant ($p < 0.05$) differences between pre-treatments.

The range of turbidity values recorded in this study was very wide (60 - 440 FTU). Gelatines extracted from mackerel and blue whiting after pre-treatment with lactic acid showed significantly ($p < 0.05$) higher turbidity values (Table 4.4). These results were in agreement with those reported by Giménez *et al.* (2005) for gelatine from Dover sole (*Solea vulgaris*). These authors found that the use of lactic acid induced the solubilisation of material with a high degree of aggregation and as a consequence higher turbidity values were obtained.

During the preparation of gelatines, the deamidation of asparagine to aspartate and glutamine to glutamate takes place. However, the conversion of protein amide groups to carboxyl groups during the deamidation of asparagine and glutamine amide residues affects both the charge and the hydrophobicity of gelatine by introducing negatively charged carboxyl groups (Lindner & Helliger, 2001). This leads to a decrease in the isoelectric point (Miwa *et al.,* 2010). It was reported that the gelatine type A or acid-processed gelatine has an isoelectric point that can vary from 6.5 to 9.0 whereas gelatine type B or alkaline-processed gelatines has an isoelectric points found over a narrower pH range, typically 4.8 - 5.0 (Johnston-Banks, 1990; Foegeding *et al.,* 1996). When the pH reaches the isoelectric point, large dipoles of protein molecules attract themselves through the counter charged domain and there is no electrostatic repulsion between neighbouring molecules. As a consequence, protein molecules tend to precipitate (Milewski, 2001) resulting in higher turbidity.

4.1.6. Protein pattern of fish gelatines

The electrophoretic (SDS-PAGE) profiles of the various gelatine preparations are shown in Figure 4.3. Mackerel and blue whiting gelatines had slightly different molecular weight profiles. The gel electrophoresis showed the presence of the three bands that correspond to gelatine, one β chain and two α chains (α_1 upper and α_2 lower). These three major chains are characteristics of gelatine from type I collagen. Similar SDS-PAGE patterns were observed for other fish species (Gómez-Guillén *et al.,* 2002). Reported data showed that the typical relative molecular weights of α chains were in the range of 100 - 120 kDa and those for β chain were in the range of 200 - 250 kDa (Kim & Park, 2004; Ogawa *et al.,* 2003; Zhang *et al.,* 2007).

Slight differences were observed in the relative mobility of the β and α-chains among gelatines prepared with the different organic acids from same fish material. This indicated that the organic acid slightly affected the molecular weight of the gelatine.

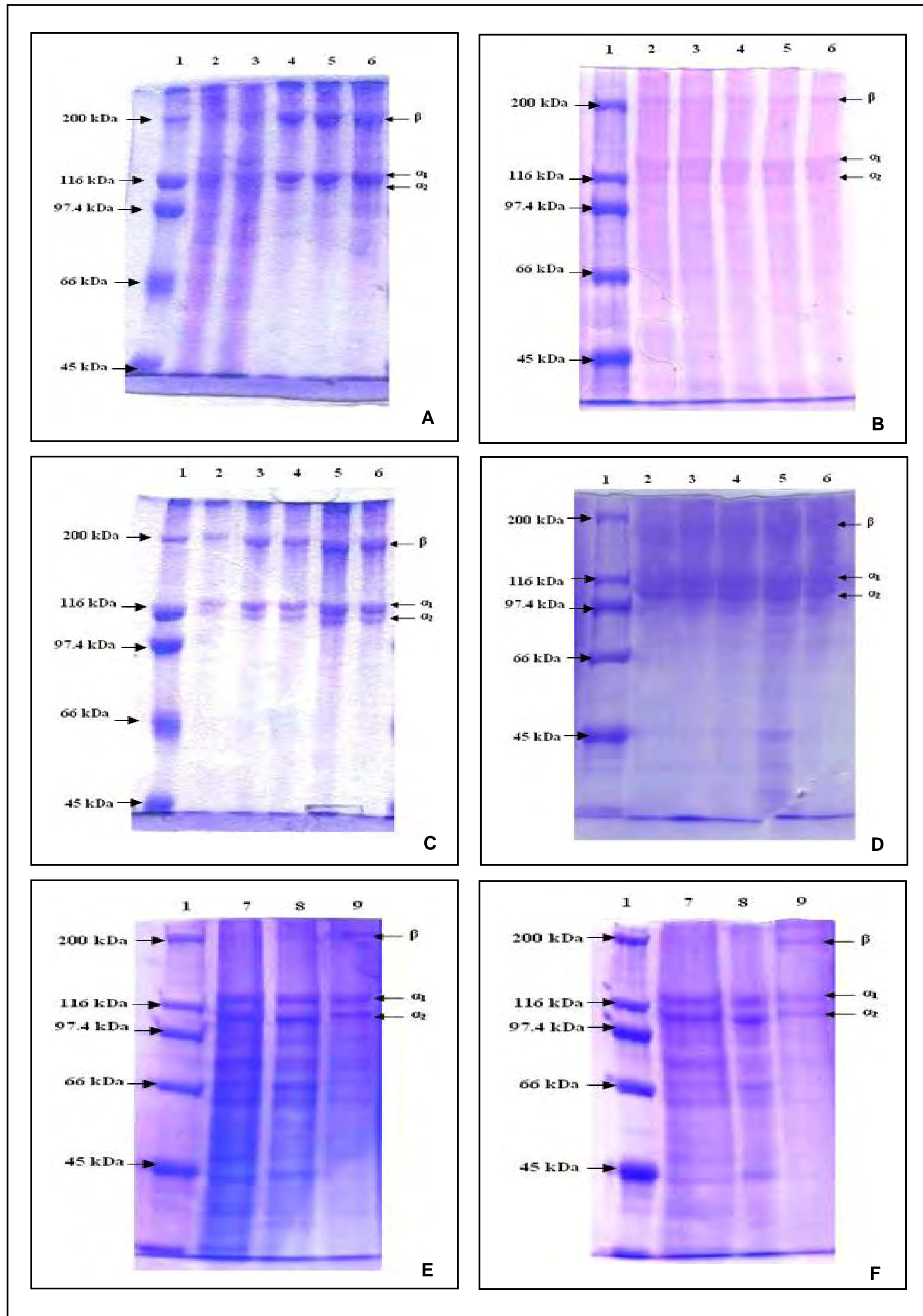


Figure 4.3 SDS-PAGE patterns of fish gelatines. Mackerel heads (A), blue whiting heads (B), mackerel skins (C), blue whiting skins (D), mackerel bones (E) and blue whiting bones (F). Lane 1: Molecular weight marker (MW. 30,000 - 200,000); lane 2: Acetic acid-extracted gelatine; lane 3: Lactic acid-extracted gelatine; lane 4: Citric acid-extracted gelatine; lane 5: tartaric acid-extracted gelatine; lane 6: Malic acid-extracted gelatine; lane 7: gelatine from bone pre-treated with Flavourzyme; lane 8: gelatine from bone pre-treated with Alcalase; lane 9: gelatine from bone pre-treated with NaOH.

The presence of low molecular weight proteins was observed in gelatines extracted after pre-treatment with acetic and lactic acid for mackerel heads (Figure 4.3A, lane 1 & 2), gelatines from blue whiting skin pre-treated with tartaric acid (Figure 4.3D, lane 5) and fish bone gelatines (Figure 4.3E & F). This may indicate a partial hydrolysis of gelatine during extraction (Giménez *et al.,* 2005).

The β-chain seems to be lower or entirely absent in bone gelatine extracted after enzymatic pre-treatment (Figure 4.3E & F, lane 7 & 8), which could be due to the combined effect of enzymatic and chemical hydrolysis of the gelatines as a consequence of the demineralisation step. It is known that a low pH can favour a maximum extraction rate but is detrimental to the physical properties because it produces more degradation and proliferation of lower molecular weight peptides (Johnston-Banks, 1990).

4.1.7. Amino acid composition fish gelatines

The average amino acid compositions of mackerel and blue whiting gelatines, expressed as percentage by weight of total amino acids, are shown in Tables 4.5 and 4.6, respectively.

Table 4.5 Average amino acid composition (g amino acid / 100 g gelatine) of gelatines from mackerel tissues.

	Heads					Skins					Bones		
	AA	**CA**	**LA**	**TA**	**MA**	**AA**	**CA**	**LA**	**TA**	**MA**	**NaOH**	**Enz (A)**	**Enz (B)**
Asp	5.6	5.6	5.3	5.5	5.5	5.5	6.3	6.3	5.8	6	6.1	7.8	8.1
Thr	2.5	2.5	2.4	2.4	2.5	2.5	2.7	2.7	2.7	2.6	2.8	3.7	3.8
Ser	5.1	4.9	4.6	5.2	4.8	4.4	5	4.8	5.1	5.1	4.8	4.4	4.6
Glu	10.1	9.7	9.4	10.0	9.6	9.1	9.2	9.6	10.2	9.7	9.8	13.3	13.5
Gly	24.6	24.9	25.1	25.1	24.5	23.5	23.9	25.3	24.8	24.1	21.3	11.6	11.4
Ala	9.6	9.0	9.8	9.2	9.4	8.3	8.9	9.4	9.1	9.1	9.0	7.3	7.0
Cys	1.0	1.7	1.4	1.4	1.3	0.6	1.2	1.2	0.9	1.3	0.8	1.2	1.2
Val	1.7	1.9	2.3	1.8	1.8	1.6	2	1.9	2	1.9	2.3	3.7	4.0
Met	1.9	2.0	3.0	1.9	1.9	1.8	2	2.1	1.9	1.8	2.1	2.6	2.6
Ile	1.0	1.0	1.5	1.0	1.0	0.9	1.1	1.1	1.2	1.1	1.3	2.5	3.0
Leu	2.8	2.9	3.3	2.8	2.9	2.7	3.3	3.2	3.2	3.1	3.7	6.0	6.2
Tyr	0.7	0.7	0.7	0.7	1.0	0.6	0.9	0.8	0.9	0.8	1.1	2.3	2.7
Phe	2.2	2.3	2.5	2.3	2.2	2.2	2.4	2.6	2.4	2.2	2.6	3.1	3.3
His	0.6	0.7	0.6	0.7	0.7	0.6	0.9	0.9	0.8	0.9	1.2	2.3	2.3
Lys	3.4	3.5	3.5	3.5	3.5	2.9	3.3	3.5	3.6	3.6	4.1	7.0	6.7
Arg	8.3	8.5	8.4	8.7	8.5	7.7	8.4	8.7	8.6	8.5	8.4	7.5	7.3
Pro	11.4	11.0	10.0	10.9	11.4	14.8	11.4	10.4	10.6	11.3	10.3	7.7	6.9
Hyp	7.5	7.1	6.4	7.0	7.8	10.3	7.1	5.7	6.4	6.8	8.3	6.0	5.4

Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; NaOH: alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase.

Table 4.6 Average amino acid composition (g amino acid / 100 g gelatine) of gelatines from blue whiting tissues.

	Heads					Skins					Bones		
	AA	**CA**	**LA**	**TA**	**MA**	**AA**	**CA**	**LA**	**TA**	**MA**	**NaOH**	**Enz (A)**	**Enz (B)**
Asp	6.8	8.0	8.3	8.0	9.9	6.2	6.6	6.7	7.1	7.9	6.5	8.4	8.7
Thr	3.2	3.4	3.7	3.6	3.7	2.8	3	3.1	2.9	3.1	3	3.3	2.9
Ser	5.7	5.2	5.2	5.4	4.9	5.3	5.5	5.6	5.5	5	5.6	5.4	5.3
Glu	11.1	13.6	13.6	13.7	13.2	10.4	10.9	10.5	10.7	14.5	10.5	11.7	10.9
Gly	19.6	14.3	13.1	14.2	11.9	20.1	20.5	21	20.7	12.1	20.7	17.3	20
Ala	8.8	7.7	7.4	7.5	7.1	8.4	8.5	8.9	8.7	6.3	9.1	8.3	8.8
Cys	0.0	0.2	0.2	0.2	0.3	0	0	0	0	0	0.1	0.2	0.2
Val	2.6	3.1	3.5	3.2	3.6	2.1	2.2	2.1	2.1	2.3	2.3	2.8	2.5
Met	2.5	2.7	3.0	2.7	2.8	2.4	2.3	2.2	2.4	3.1	2.4	2.7	2.5
Ile	1.6	2.2	2.7	2.4	2.7	1.3	1.4	1.3	1.2	1.6	1.4	2	1.6
Leu	3.3	5.1	5.7	5.1	6.0	2.9	3	2.6	2.8	3.6	2.9	4.1	3.4
Tyr	0.9	1.8	2.2	1.8	2.4	0.6	0.6	0.4	0.6	1	0.7	1.5	1.1
Phe	2.6	2.7	3.0	2.8	3.0	2.2	2.5	2.3	2.4	3.8	2.4	2.7	2.4
His	1.5	1.8	1.9	1.9	2.0	1.3	1.3	1.2	1.3	1.8	1.6	1.8	1.6
Lys	4.5	6.3	6.6	6.2	7.0	3.5	3.6	3.6	3.6	4.8	3.7	4.8	4.1
Arg	8.6	8.3	8.1	8.2	8.0	8	10.3	8.5	8.3	13.4	8.6	8.4	8.8
Pro	9.3	7.4	6.8	7.0	6.2	11.7	10.6	10.5	10.2	10.6	9.7	8.4	9.4
Hyp	7.6	6.3	4.9	6.0	5.4	10.9	7.2	9.6	9.4	5.4	8.5	6.1	5.7

Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; NaOH: alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase.

All mackerel and blue whiting gelatines, regardless of the source and the pre-treatment, had glycine as the major amino acid, followed by proline, glutamic acid, alanine, arginine and hyroxyproline. The glycine content of all gelatines ranged from 20 % to 25 %, except for blue whiting head gelatines and mackerel and blue whiting bone gelatines extracted after the enzymatic pre-treatments (11 % to 14 %). Unlike blue whiting gelatines, all mackerel gelatines had very low content of tyrosine and histidine. These results were in agreement with those found by Kim & Park (2004) for acid-soluble collagen from Pacific whiting and collagen from Tilapia skins (Grossman & Bergman, 1992). Tryptophan and cysteine are usually absent in collagens and gelatines. No tryptophan was found in any gelatines. However, all extracted gelatines, except those from blue whiting skins, contained different levels of cysteine. This might indicate a contamination by non-collagenous proteins in the extraction process (Morimura *et al.,* 2002).

Differences in amino acid content, due to the pre-treatment, were observed mainly in imino acid (proline and hydroxyproline) levels. Collagen from cold-water fish species contains 16 - 18 % imino acids (Gilsenan & Ross-Murphy, 2000; Gundmundsson & Hafsteinsson, 1997; Norland, 1990). Gelatines extracted from both fish skins, regardless of the pre-treatment, had higher imino acid content (16.1 % - 25.1 %) than those from heads (11.6 % - 19.2 %). This was mainly due to the initial amount of collagen in fish skins and heads. The amount of imino acids plays a major role in the rheological properties of gelatines and affects the functional properties of gelatine. Gelatines with low proline and hydroxyproline level usually show lower melting point and weaker gel network

(Gilsenan & Ross-Murphy, 2000; Johnston-Banks, 1990). The higher the imino acid content, the more stable the helices are (Wong, 1989).

Acetic acid pre-treatment of fish heads and skins favoured the extraction of gelatines with higher proline and hydroxyproline levels. From this study, the imino acid content of gelatine extracted after pre-treatment with acetic acid were 18.9 % for mackerel head gelatines, 25.1 % for mackerel skin gelatines, 16.9 % for blue whiting head gelatines and 22.6 % for blue whiting skin gelatines. The effect of acetic acid was more prominent for skins of both fish as shown by the higher imino acid content compared to head gelatines. The chemical pre-treatment of fish bones has a positive effect on the level of imino acid of gelatines (~ 18.4 %).

4.1.8. Spectroscopic analyses of fish gelatines

4.1.8.1 FTIR analysis of fish gelatines

Proteins exhibit absorption bands in the infrared spectra associated with their characteristic amide group. There are nine characteristic bands of the amide groups of protein chains which are named amides A, B, I, II …VII (Stuart, 2004). These absorptions are mainly due to C=O stretching, C-N stretching, N-H stretching and O-C-N bending (Bandekar, 1992; Fabian & Mantele, 2002). Amide I band mainly results from the C=O stretching vibration. Amide II results from the N-H bending vibration coupled to the C-N stretching vibration. The amide III band is usually found in the region from 1250 to 1350 cm^{-1} and amide A is mainly due to the N-H stretching (Chittur, 1998).

Mackerel and blue whiting gelatines were analysed using FTIR. The absorption bands in the FTIR spectra were situated in the amide band region (Figure 4.4). Amide I and amide II bands of fish gelatines appeared around 1650 and 1540 cm^{-1} (Table 4.7). Amide I and amide II of gelatine from bovine skin were also observed at 1700 - 1600 cm^{-1} and 1560 - 1500 cm^{-1} as reported by Yakimets *et al.* (2005). Differences among gelatines were observed in the relative intensity of the peaks as well as in amide frequencies. These differences could be attributed to the effect of organic acid and the pre-treatment used in the extraction procedure and they are an indication of degradation of collagen (Muyonga *et al.,* 2004). The degradation of polypeptide chain in gelatine increases the -OH stretching and bending observed at 3400 and 1650 cm^{-1}, respectively. Since both the amide I band and the -OH bending absorb the infra red light at 1650 cm^{-1}, higher degradation would increase the absorption intensity of the amide I band (Susi *et al.,* 1971). The denaturation of collagen affects the amide frequency. A shift of the amide II band, from 1550 to 1530 cm^{-1}, is observed when the collagen helix is converted to the disordered form found in gelatine (Brodsky-Doyle *et al.,* 1975).

The peaks of amide I and II of gelatine from mackerel heads extracted after pre-treatment with malic acid were at higher frequencies (1653 cm^{-1} and 1560 cm^{-1}, respectively) than peaks of amide I and II from gelatine extracted after pre-treatment with acetic, citric, lactic and tartaric acids (Table 4.7). High frequencies of amine I and II were also observed for gelatines extracted from mackerel and blue whiting skins pre-treated with acetic acid, blue whiting heads pre-treated with tartaric acid and gelatines from both fish bones pre-treated

chemically (Table 4.7). The higher frequencies for amide I and II of these gelatines suggest a higher degree of molecular order and better stability (Payne & Veis, 1988).

Table 4.7 Assignment of FTIR spectra of gelatines from mackerel and blue whiting.

		A vibration		Amide I		Amide II		Amide III	
Pre-treatment		**M**	**BW**	**M**	**BW**	**M**	**BW**	**M**	**BW**
Heads	**AA**	3373.8	3304.8	1651.1	1653.5	1541.0	1541.1	1237.3	1240.9
	CA	3398.2	3426.4	1648.4	1651.6	1541.0	1539.3	1238.7	1262.9
	LA	3424.7	3357.5	1647.2	1652.3	1534.3	1540.7	1236.8	1241.4
	TA	3412.1	3421.4	1637.8	1655.3	1538.6	1543.1	1240.5	1237.9
	MA	3332.5	3400.1	1653.0	1649.4	1560.0	1538.6	1239.1	1240.9
Skins	**AA**	3430.3	3303.7	1654.7	1654.5	1560.7	1584.2	1237.4	1243.7
	CA	3411.5	3413.6	1654.1	1652.0	1542.3	1536.3	1239.5	1232.4
	LA	3446.4	3348.9	1644.6	1653.0	1539.7	1544.0	1239.3	1285.2
	TA	3435.7	3428.2	1651.2	1652.9	1540.5	1541.1	1235.3	1239.1
	MA	3400.3	3429.5	1651.2	1634.2	1539.8	1533.9	1239.7	1238.7
Bones	**NaOH**	3435.7	3397.8	1654.9	1652.0	1545.0	1547.6	1249.0	1238.6
	Enz (A)	3396.0	3397.3	1652.3	1649.9	1541.1	1539.8	1234.7	1233.1
	Enz (B)	3407.2	3391.4	1652.7	1650.6	1542.5	1545.8	1234.2	1233.6

M: Mackerel, BW: Blue whiting. Pre-treatments: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; NaOH: alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase.

Gelatine extracted from mackerel heads after pre-treatment with lactic acid and gelatines from blue whiting head pre-treated with citric, malic and tartaric as well as blue whiting bone gelatines pre-treated with enzymes had the lowest amide I, II and III intensities. This may indicate a high degree of deamidation of asparagine and glutamine. The pH, temperature and ionic strength are the main factors involved in the deamidation of amino acid (Tyler-Cross & Schirch, 1991) which result in the alteration of the triple helix (Freiss & Lee, 1996).

A noticeably higher peak around 2930 cm^{-1}, corresponding to the $-CH_2$ stretching band, was observed for gelatine extracted from mackerel heads after pre-treatment with lactic acid (Figure 4.4A), gelatines from blue whiting heads (Figure 4.4B) and gelatines from blue whiting bone pre-treated with enzymes (Figure 4.4F). This peak was less predominant and slightly merged with the amide A band for the rest of gelatines, which may indicate a better stability of the components of these gelatines (Kemp, 1987).

Infrared absorption of proteins around 1200 cm^{-1}, 1500 cm^{-1}, 1700 cm^{-1}, and 3500 cm^{-1} are typical for the amide III, II, I and A vibrational modes, respectively (Hayashi & Mukamel, 2008). C=O and N-H bond stretching are mainly present in the amide I and A regions, respectively. The C-N stretching and H-N-C bending usually appear in the amide II and III frequencies (Hayashi & Mukamel, 2008).

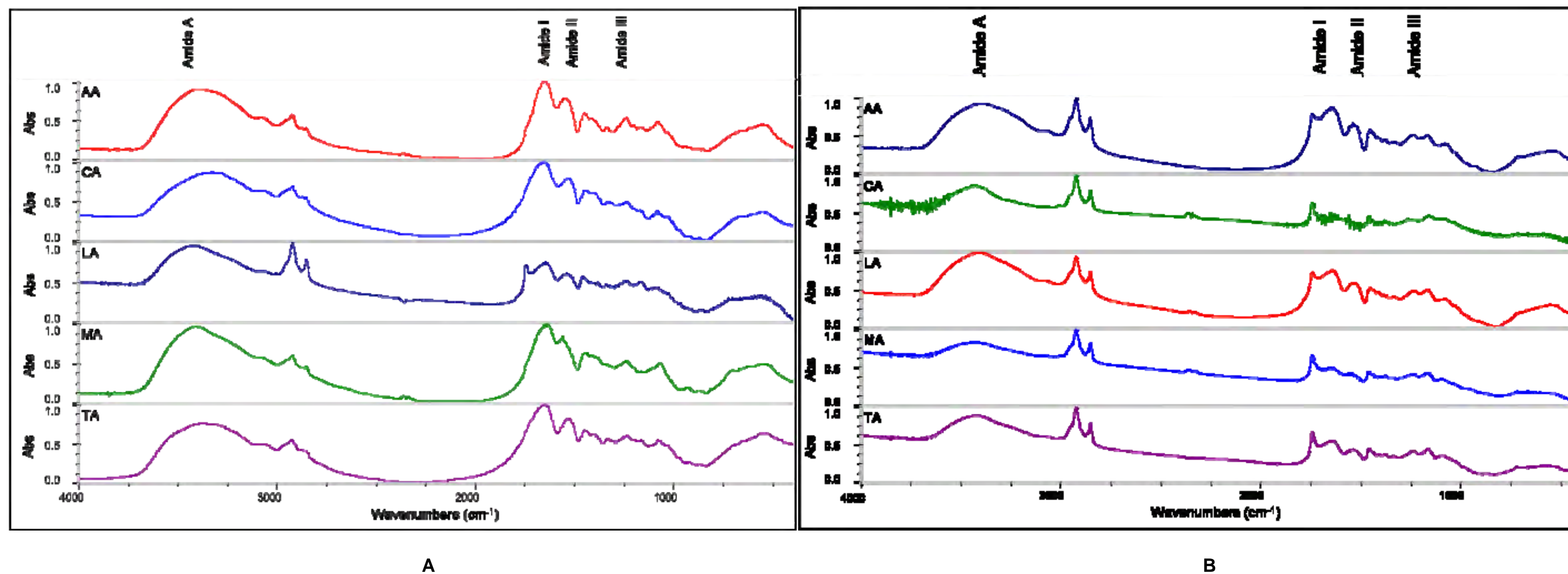


Figure 4.4 FTIR spectra of gelatines obtained from mackerel and blue whiting using different pre-treatment. (A) gelatines from mackerel heads and (B) gelatines from blue whiting heads. Pre-treatment: A: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid and MA: malic acid.

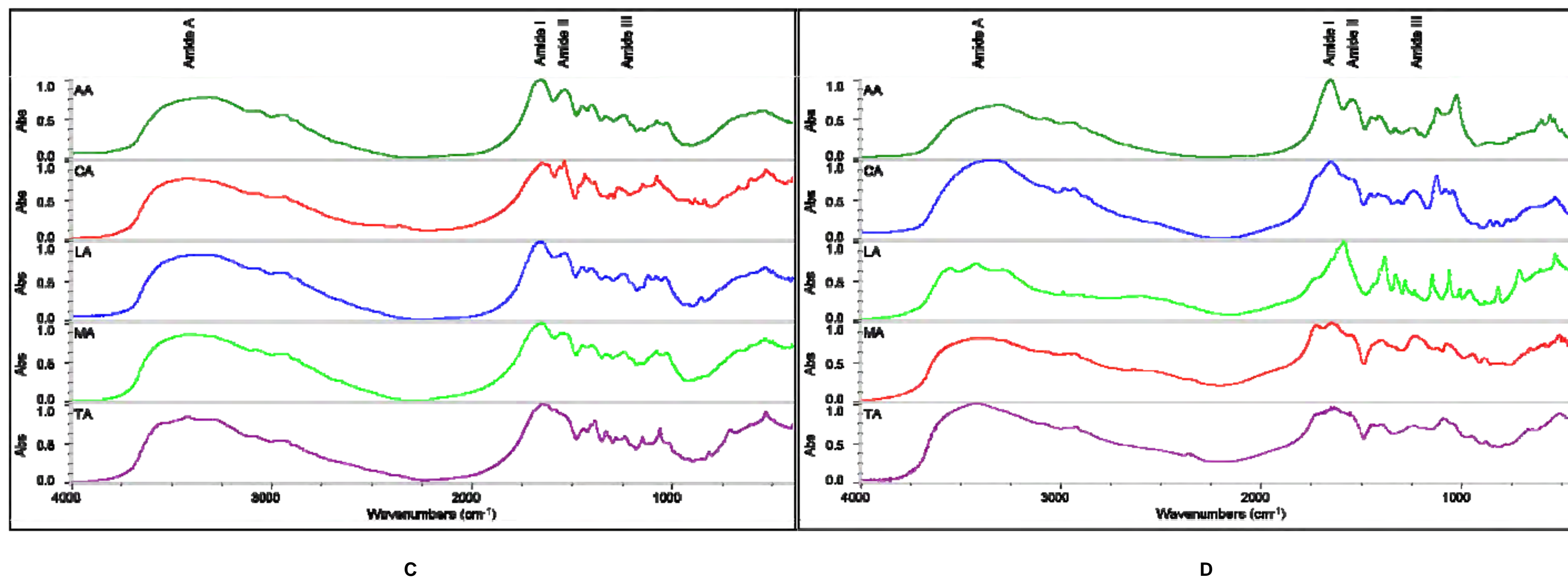


Figure 4.4 (continued) FTIR spectra of gelatines obtained from mackerel and blue whiting using different pre-treatment. (C) gelatines from mackerel skins and (D) gelatines from blue whiting skins. Pre-treatment: A: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid and MA: malic acid.

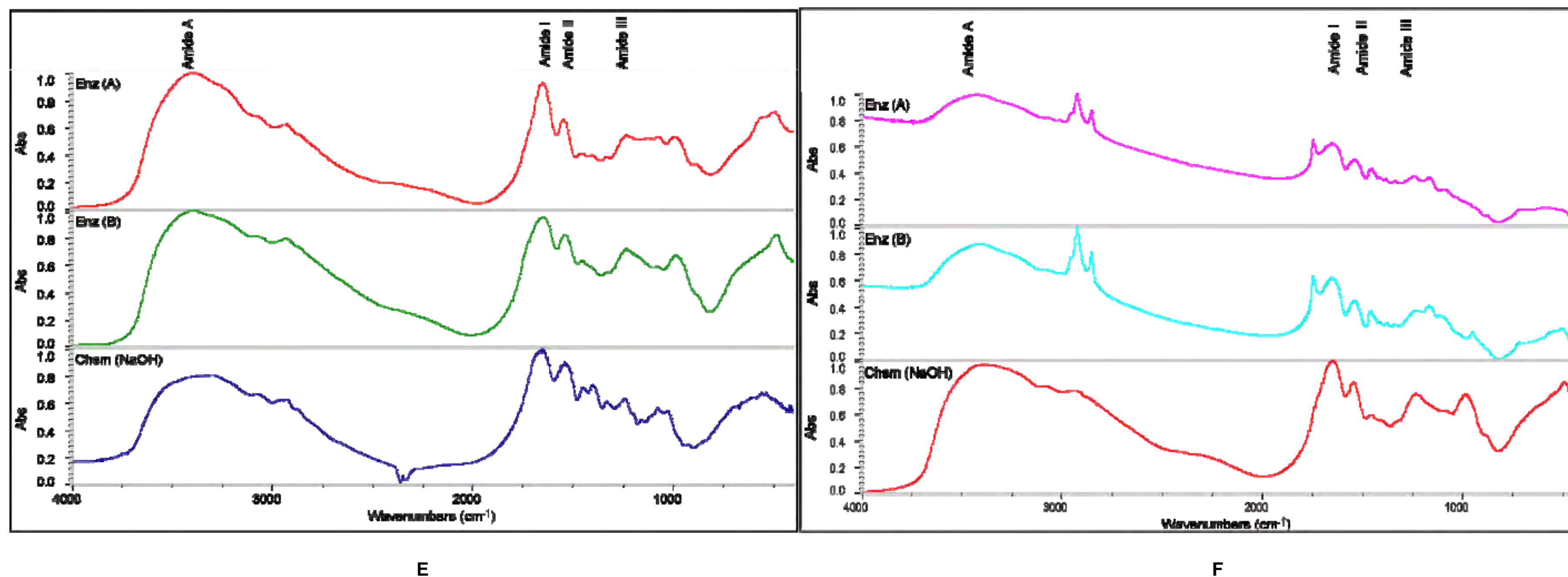


Figure 4.4 (Continued) FTIR spectra of gelatines obtained from mackerel and blue whiting using different pre-treatment. (E) gelatines from mackerel bones and (F) gelatines from blue whiting bones. Pre-treatment: Chem (NaOH): alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase.

The extracted gelatines from mackerel and blue whiting showed very similar FTIR spectra to the commercial cold water fish gelatine, but slightly different than the commercial bovine gelatine which had higher intensities in amide I, II and III (Figure 4.5).

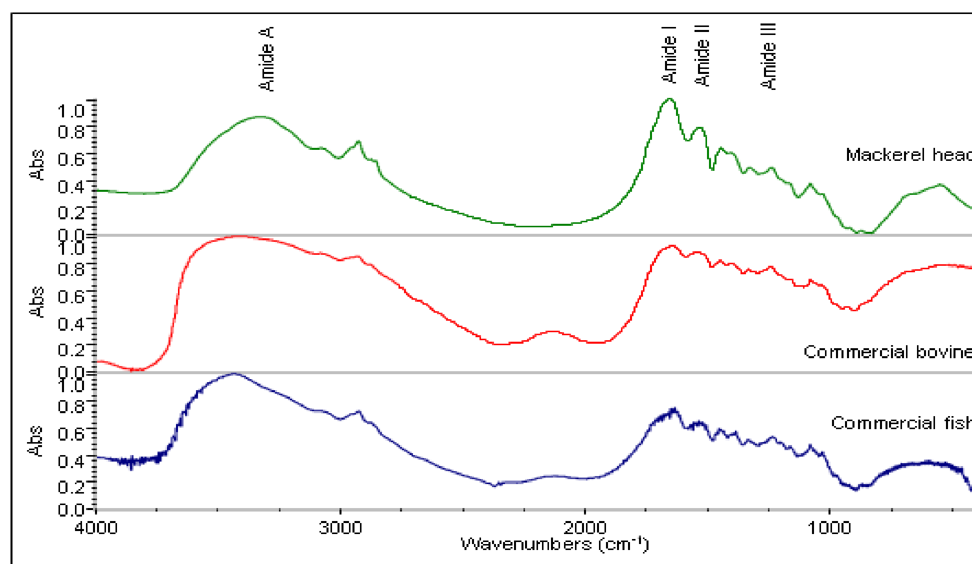


Figure 4.5 FTIR spectra of gelatine from mackerel heads, pre-treated with citric acid, and commercial fish and bovine gelatines.

Amide I bands are characteristic of α helices in protein (Haris and Chapman, 1994). The change in the intensity and the frequency of the amide I band in protein indicates a change of structures from α helices to a random coil (Susi & Byler, 1988; Yang *et al.,* 1985). Hence, differences observed in amide I band of gelatines obtained after using the organic acids suggested that these acids might affect the helix coil structure of gelatine fish. The amide II band is generally considered to be much more sensitive to hydration than to secondary structure change (Wellner *et al.,* 1996). In this study, FTIR spectra analysis was performed in the dry state and the changes in the amide II band might be related to possible alterations in protein secondary structure induced by organic acids.

4.1.8.2. NMR analysis of fish gelatines

Figure 4.6 shows the ^{1}H NMR spectra of gelatines extracted from mackerel and blue whiting. The spectra showed a large number of overlapping signals which could be due to partial degradation of gelatine caused by the organic acid used in the extraction. According to Williamson & Asakura (1997), in denatured proteins, most nuclei resonate at frequencies similar to those observed in small peptides, and therefore show a considerable degree of overlap and a lack of spectral dispersion.

In this study, the NMR spectral domains were identified as amino acids. A large number of signals with considerable overlap are present in the upfield region. The chemical shifts from 0.5 to 1.5 ppm, represent aliphatic protons that are attached to carbon atoms of valine, leucine and isoleucine. The region from 1.5 to 3.0 ppm, represents protons on aliphatic carbon atoms of arginine, leucine, lysine, proline, glycine, asparagine and aspartic acid. The region between 3.2 and 4 ppm represents the resonance signals of the α-CH of the amino acids. The last portion of the ^{1}H NMR spectrum represents the aromatic region (7 - 8 ppm) and represents phenylalanine.

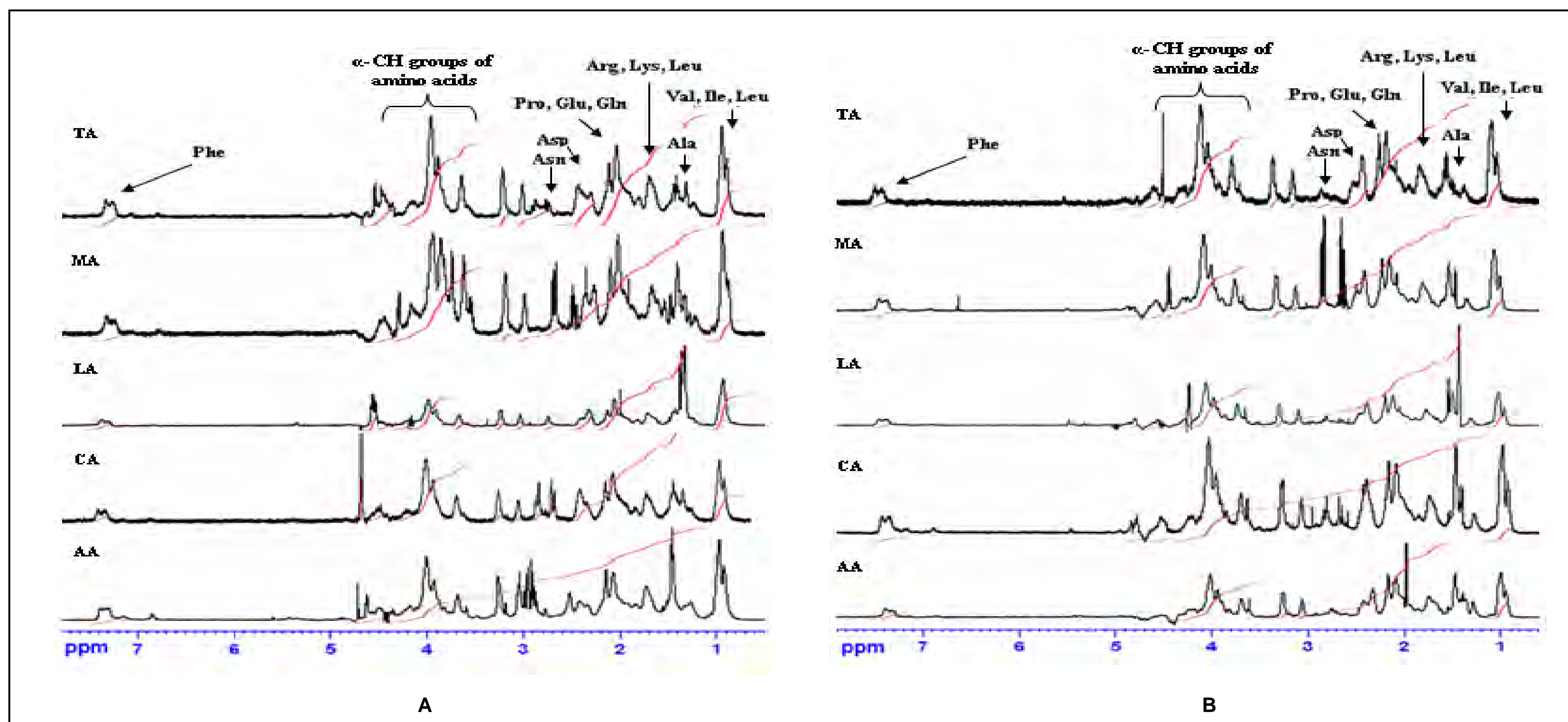


Figure 4.6 ^{1}H NMR spectra of gelatines obtained from mackerel and blue whiting using different pre-treatment. (A) Gelatines from mackerel heads and (B) gelatines from blue whiting heads. Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid and MA: malic acid

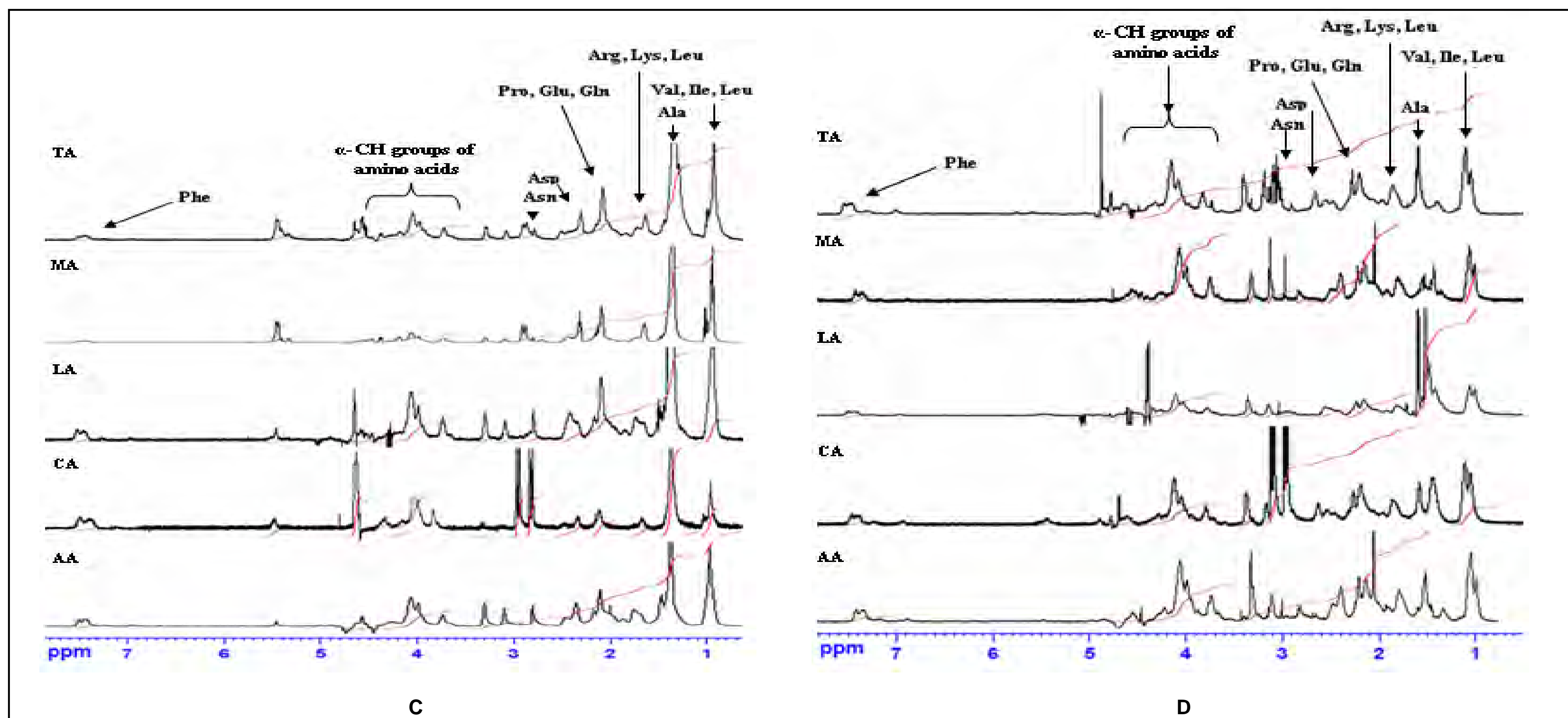


Figure 4.6 (Continued) ^{1}H NMR spectra of gelatines obtained from mackerel and blue whiting using different pre-treatment**.** (C) gelatines from mackerel skins and (D) gelatines from blue whiting skins. Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid and MA: malic acid.

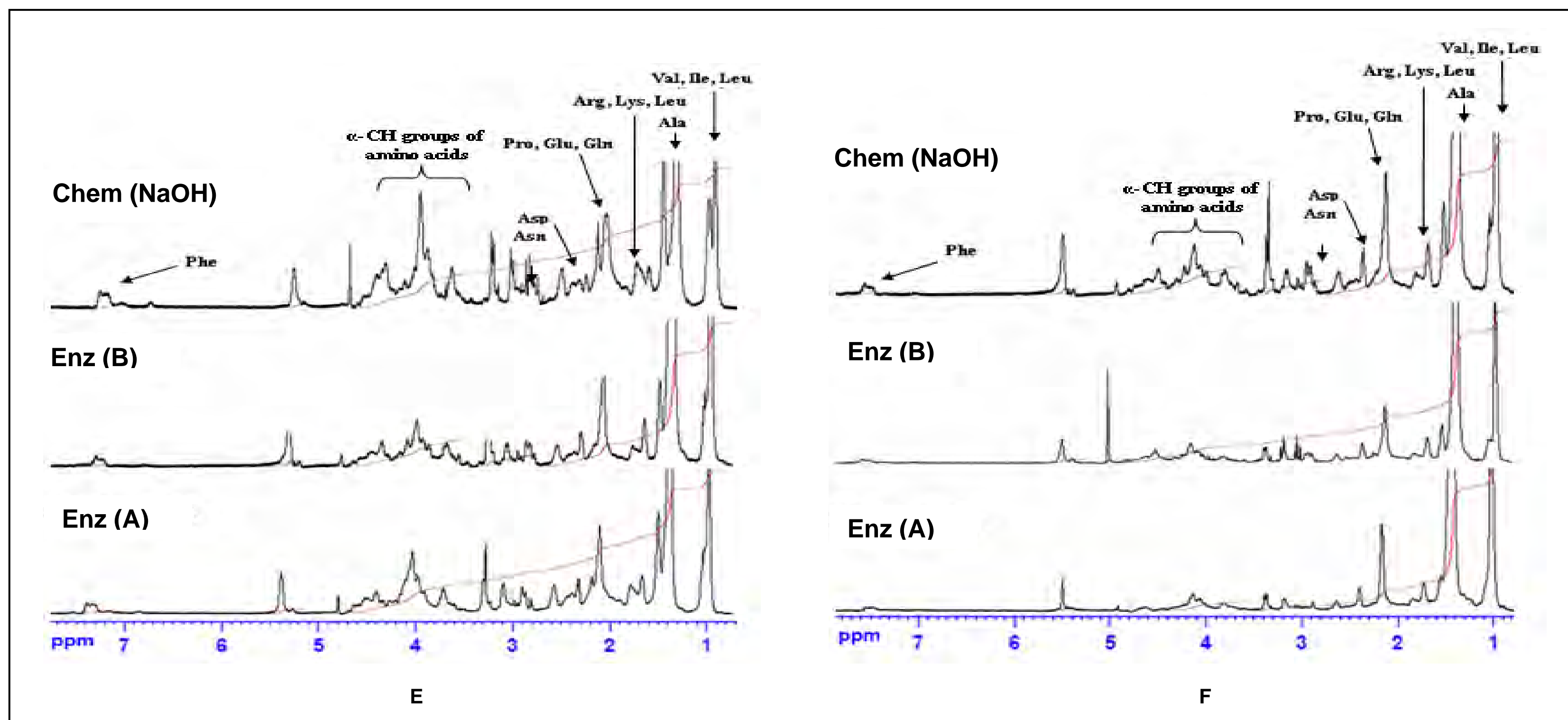


Figure 4.6 (Continued) ^{1}H NMR spectra of gelatines obtained from mackerel and blue whiting using different pre-treatment. (E) gelatines from mackerel bones and (F) gelatines from blue whiting bones. Pre-treatment: Chem (NaOH): alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase.

^{1}H NMR was used to trace the presence of the organic acid used in the extraction of gelatines from fish heads and skins. Acetic acid (AA) generated a chemical shift at 2.00 ppm, citric acid (CA) had chemical shifts at 3.02 and 2.84 ppm (Figure 4.6A, B, C & D). Lactic acid (LA) had chemical shifts at 4.09 and 1.33 ppm, tartaric acid (TA) showed a chemical shift at 4.31 ppm and malic acid (MA) had chemical shifts at 2.35, 2.65 and 4.26 ppm (Figure 4.6A, B, C & D).

For both fish bones gelatines, ^{1}H NMR spectra showed that the intensities of the peaks from enzymatically pre-treated bones were significantly lower than those from chemically pre-treated bones (Figure 4.6E & F), which might indicate a higher degree of degradation of gelatine. This result was correlated with the electrophoretic analysis where both fish bone gelatines extracted after the enzymatic pre-treatment showed lower β-chain than bone gelatines extracted after the chemical pre-treatment (section 4.1.6).

4.2. Functional properties of fish gelatines

The study of functional properties of the extracted gelatines is important to determine their potential use in food products.

4.2.1. Foaming capacity and stability

The foaming capacity (FC) of gelatines, extracted from mackerel and blue whiting, varied significantly ($p < 0.05$) depending on the source and the pre-treatment. Regardless of the organic acid used, mackerel head gelatines had slightly higher FC than blue whiting head gelatines (Figure 4.7A & D). However, mackerel skin gelatines showed lower FC than Blue whiting skin gelatines (Figure 4.7B & E). Gelatines from both fish bones had high FC (Figure 4.7C & F), regardless of the pre-treatment used.

Several factors affect the foaming properties such as pH, temperature, concentration, ionic strength, volume of continuous phase and homogenisation time and speed (Kinsella, 1981). In this research, high FC values were observed with gelatines containing some low molecular weight peptides (i.e., gelatines extracted from mackerel heads, blue whiting skins and both fish bones) as indicated by the SDS-PAGE analyses (see section 4.1.6). Since all gelatines were analysed at the same experimental conditions, the differences observed for FC might be attributed to the molecular weight distribution of gelatines. The adsorption to the air-water interface which reduces the surface tension and increases the FC, was probably quicker in these gelatines due to the presence of small sizes peptides (Phillips *et al.,* 1994; Wilde & Clark, 1996).

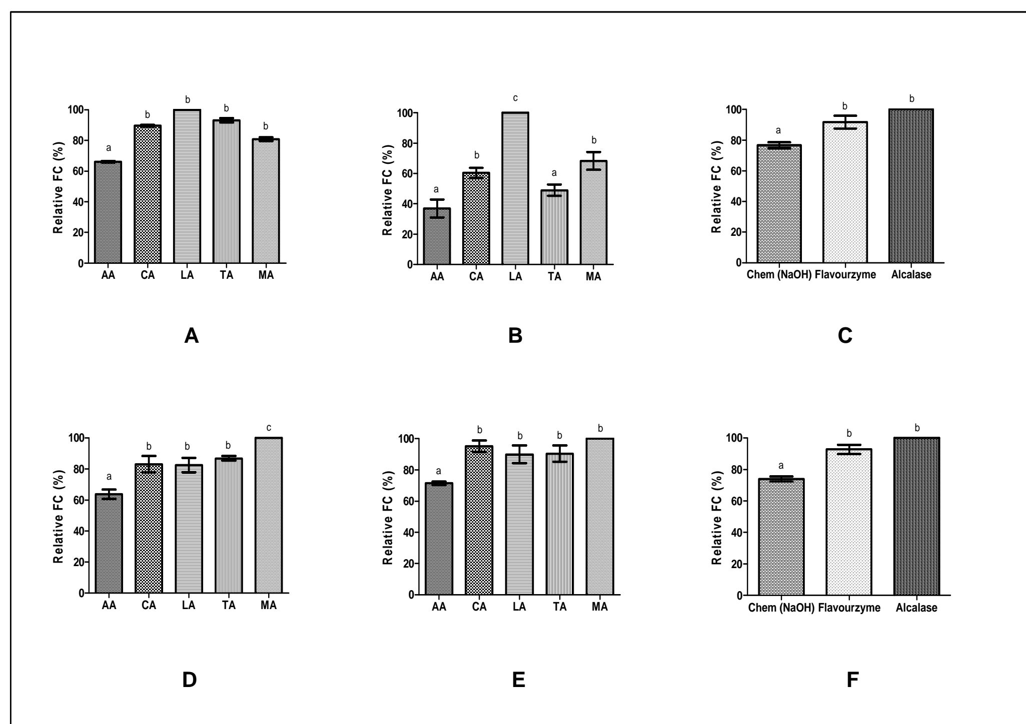


Figure 4.7 Relative foaming capacity (FC) of fish gelatines. Gelatines from mackerel heads (A), gelatines from mackerel skins (B), gelatines from mackerel bones (C), gelatines from blue whiting heads (D), gelatines from blue whiting skins (E) and gelatines from blue whiting bones (F). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase. Different letters, within the same graph, indicate significant ($p < 0.05$) differences between pre-treatments for gelatines from same fish raw material.

The pre-treatment of mackerel heads and skins with lactic acid as well as blue heads and skins with malic acid resulted in gelatines with higher FC. Gelatines extracted from mackerel and blue whiting bones after the enzymatic pre-treatment showed significantly ($p < 0.05$) higher FC than that extracted after the chemical pre-treatment. This may be due to the higher amount of less polar amino acid residues (Ala, Val, Ile, Leu, Met, Phe and Cys) in these gelatines. The foaming agent, having an amphiphilic property, adsorbs at the air-water interface and orients itself in such a way that the lipophilic group is oriented towards the

non-polar phase and the hydrophilic group towards the aqueous phase. This phenomenon reduces the surface tension allowing the formation of the foam (Liceaga-Gesualdo & Li-Chan, 1999). It was reported that hydrophobic amino acids control the adsorption of protein to the air-water interface (Foegeding *et al.,* 2006). Hence, an increase of surface hydrophobicity may have resulted in an increase of peptide adsorption and higher FC.

The foaming stability of gelatines extracted from mackerel, regardless of the pre-treatment used, was significantly ($p < 0.05$) higher than gelatines extracted from blue whiting (Figure 4.8).

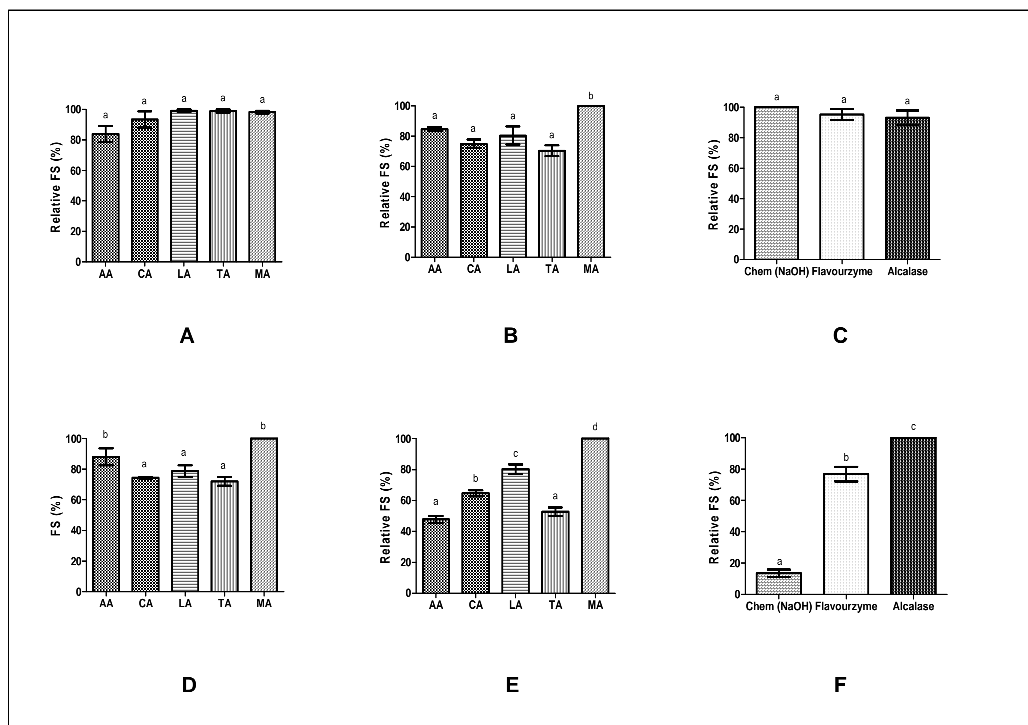


Figure 4.8 Relative foaming stability (FS) of fish gelatines. Gelatines from mackerel heads (A), gelatines from mackerel skins (B), gelatines from mackerel bones (C), gelatines from blue whiting heads (D), gelatines from blue whiting skins (E) and gelatines from blue whiting bones (F). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase. Different letters, within the same graph, indicate significant ($p < 0.05$) differences between pre-treatments for gelatines from same fish raw material.

The lower FS observed with gelatines from blue whiting skins compared to those from blue whiting heads can be due to the lower percentage of negatively charged amino acid (Asp and Glu). The Asp and Glu content in blue whiting skin gelatines (16.6 - 22.4 %) was lower than the content of Asp and Glu in blue whiting head gelatines (17.9 - 23.1 %). The increase of negatively charged amino acid can also explain the difference among the FS observed with blue whiting bone gelatines. The enzymatic pre-treated bones had 20 % negatively charged amino acids compared to 17 % from chemically pre-treated bones. The larger the number of negatively charged amino acids prevented the neutralisation of charge in gelatine molecules and enhanced the FS.

Commercial fish and bovine gelatines showed very high FC and FS (Figure 4.9) indicating a better ability to form stable foam.

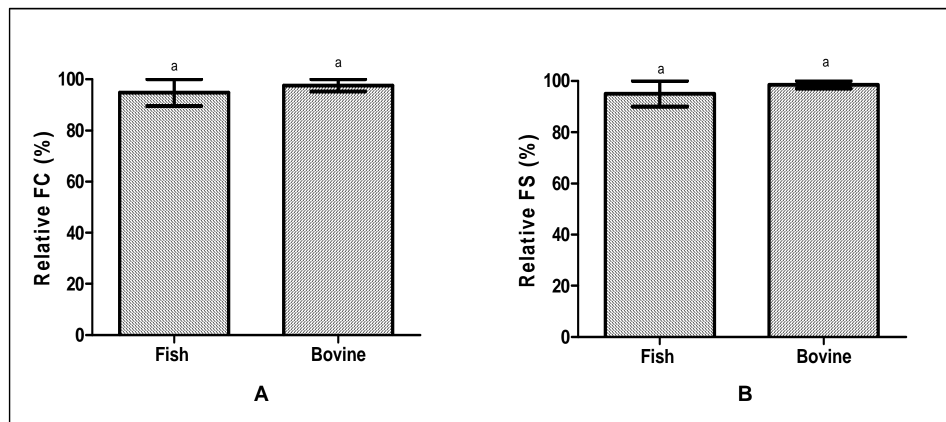


Figure 4.9 Relative foaming capacity (A) and stability (B) of commercial fish and bovine gelatines. Different letters, within the same graph, indicate significant ($p < 0.05$) differences between gelatines.

4.2.2. Emulsifying activity and stability

Emulsion activity index (EAI) and emulsion stability index (ESI) were measured at different protein concentrations (0.05, 0.1 and 0.2 %).

4.2.2.1. Emulsifying activity

The emulsifying activity index (EAI), a measurement of the area of interface stabilised per unit weight of protein (m^2/g), relates the ability of a protein to coat an interface (Pearce & Kinsella, 1978). Results showed that, regardless of the source and the pre-treatment, increasing the concentration of gelatine solution decreased the EAI (Figures 4.10 & 4.11). Similar results were reported by Binsi *et al.* (2009) for bigeye snapper skin gelatines. The higher value of EAI observed for lower concentration in the research reported here may be due to the higher degree of unfolding of protein during the emulsifying process (Kinsella, 1976). The protein concentration is an important parameter that affects the emulsifying activity. Low protein concentration favours higher EAI, due to the ability of the protein to diffuse and adsorb at the oil-water interface (Cheftel *et al.,* 1985). While at high protein concentration, the diffusion is limited as a result of the activation energy barrier (Phillips, 1981).

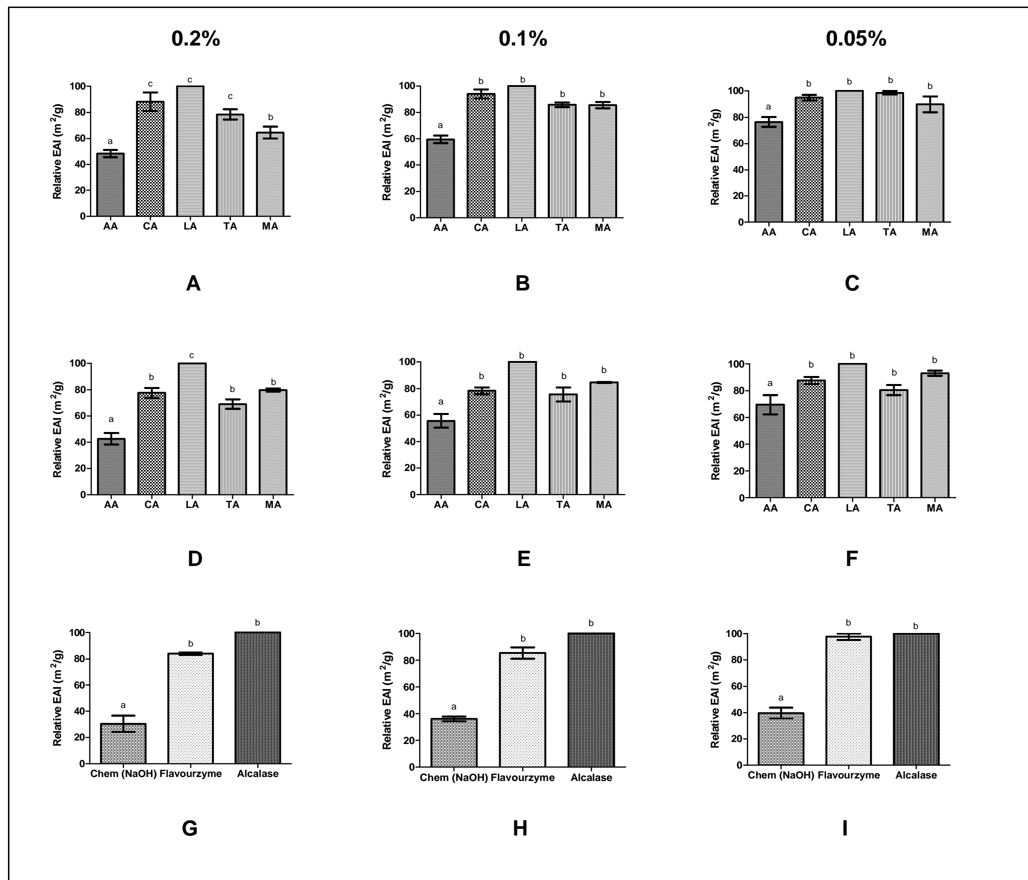


Figure 4.10 Relative emulsifying capacity index (EAI) of gelatines from mackerel at different protein concentrations (0.2 %, 0.1 % and 0.05 %, w/v). Gelatines from mackerel heads (A, B and C), gelatines from mackerel skins (D, E and F) and gelatines from mackerel bones (G, H and I). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase. Different letters, within the same graph, indicate significant ($p < 0.05$) differences between pre-treatments for gelatines from same fish raw material.

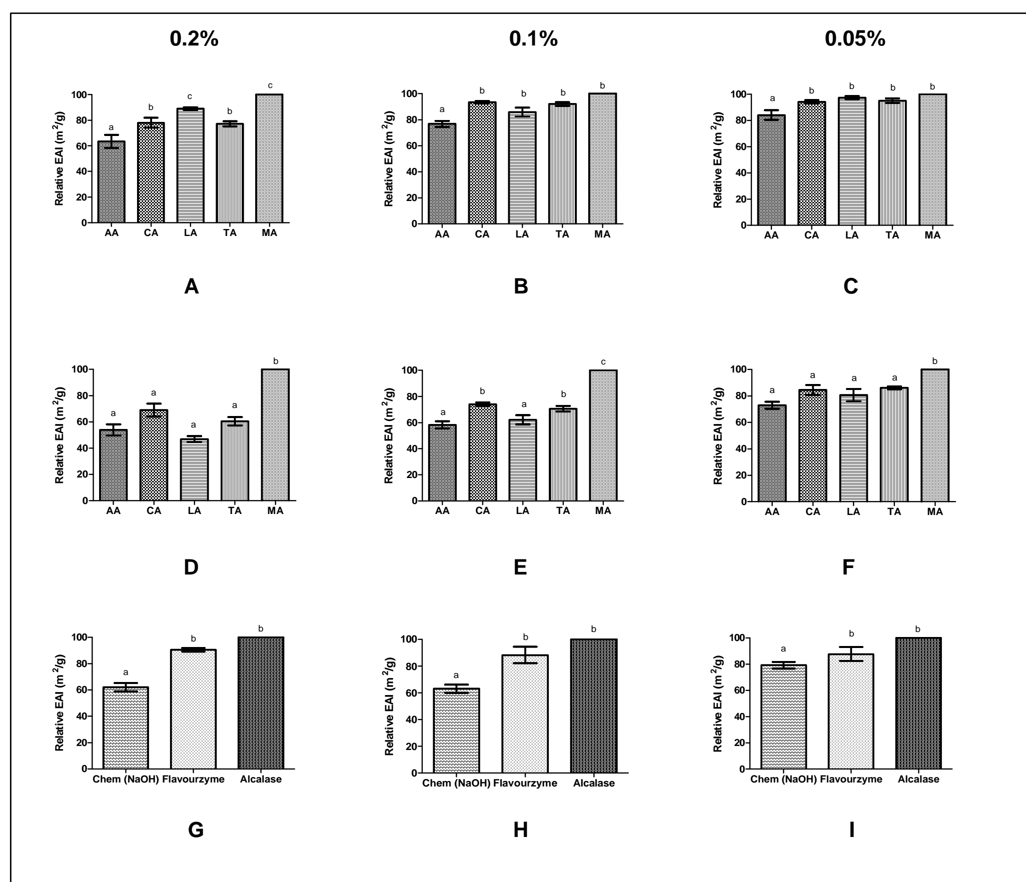


Figure 4.11 Relative emulsifying capacity index (EAI) of gelatines from blue whiting at different protein concentrations (0.2%, 0.1% and 0.05%, w/v). Gelatines from blue whiting heads (A, B and C), gelatines from blue whiting skins (D, E and F) and gelatines from blue whiting bones (G, H and I). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase. Different letters, within the same graph, indicate significant ($p < 0.05$) differences between pre-treatments for gelatines from same fish raw material.

At 0.05 % (w/v) (the protein concentration which showed the highest EAI for all gelatines), acetic acid pre-treatment of mackerel and blue whiting heads and skins resulted in gelatines with significantly ($p < 0.05$) lowest EAI (Figures 4.10 & 4.11). This can be due to their lower content in hydrophobic amino acid residues (20.9 % and 18.7 % for mackerel head and skin gelatines, respectively) and (23.3 % and 20.9 % for blue whiting head and skin gelatines, respectively). The enzymatic pre-treatment of mackerel and blue whiting bones resulted in significantly ($p < 0.05$) higher EAI. Gelatines from fish bones, pre-treated enzymatically, had a very high content in hydrophobic amino acid residues (~ 29.4 % and 24.4 % for mackerel and blue whiting, respectively). According to Rahali *et al.* (2000), sites for the anchorage of peptides in the interfacial layer mostly depend on the alternative distribution of hydrophobic and charged amino acids. When large amount of hydrophobic amino acids is present, the interaction between the protein and lipid is increased. This leads to lower interfacial tension and higher emulsion activity (Caessens *et al.,* 1999).

4.2.2.2. Emulsifying stability

For all gelatines (Figures 4.12 & 4.13), regardless of the source and the pre-treatment, a positive correlation between the protein concentration and the ESI was found (increasing the concentration of gelatine solutions increased the ESI). Similar results were previsouly observed for whey proteins (Hung & Zayas, 1991). High protein concentrations result in higher viscosity of the disperson. This usually leads to a better emulsion stability probably by reducing the coalescence rate (Sajjadi, 2007).

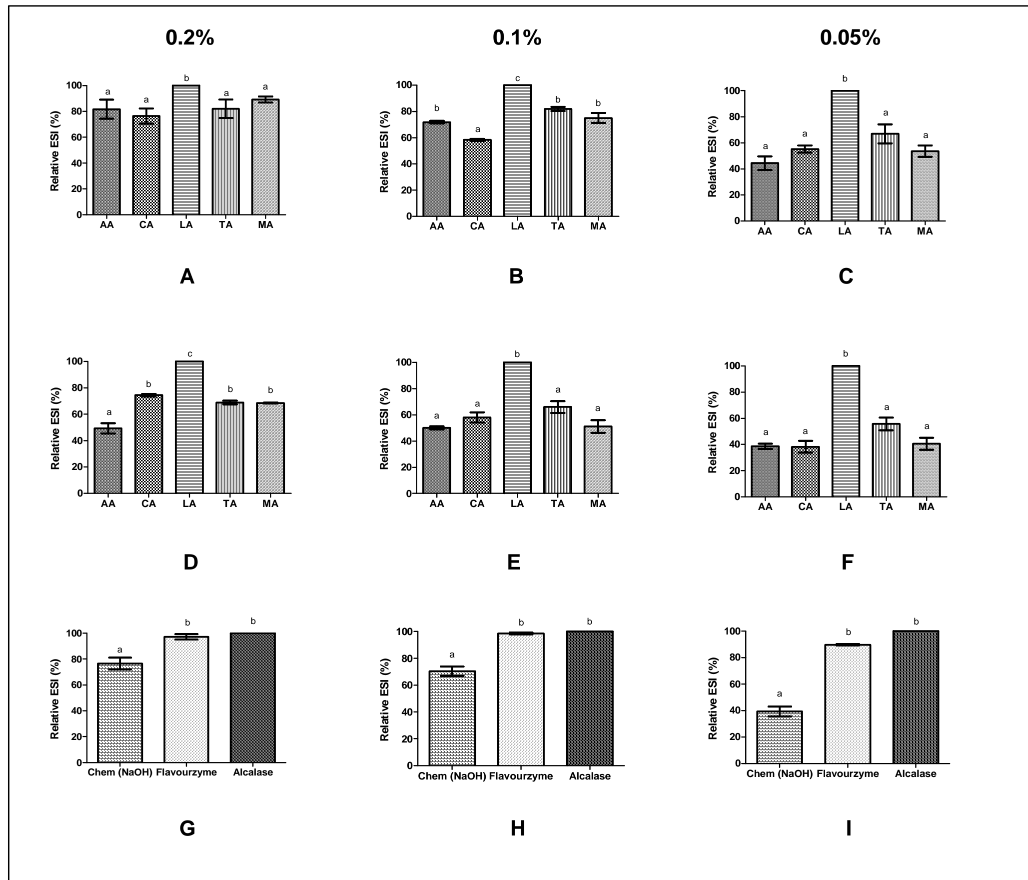


Figure 4.12 Relative emulsifying stability index (ESI) of gelatines from mackerel at different protein concentrations (0.2%, 0.1% and 0.05%, w/v). Gelatines from mackerel heads (A, B and C), gelatines from mackerel skins (D, E and F) and gelatines from mackerel bones (G, H and I). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase. Different letters, within the same graph, indicate significant ($p < 0.05$) differences between pre-treatments for gelatines from same fish raw material.

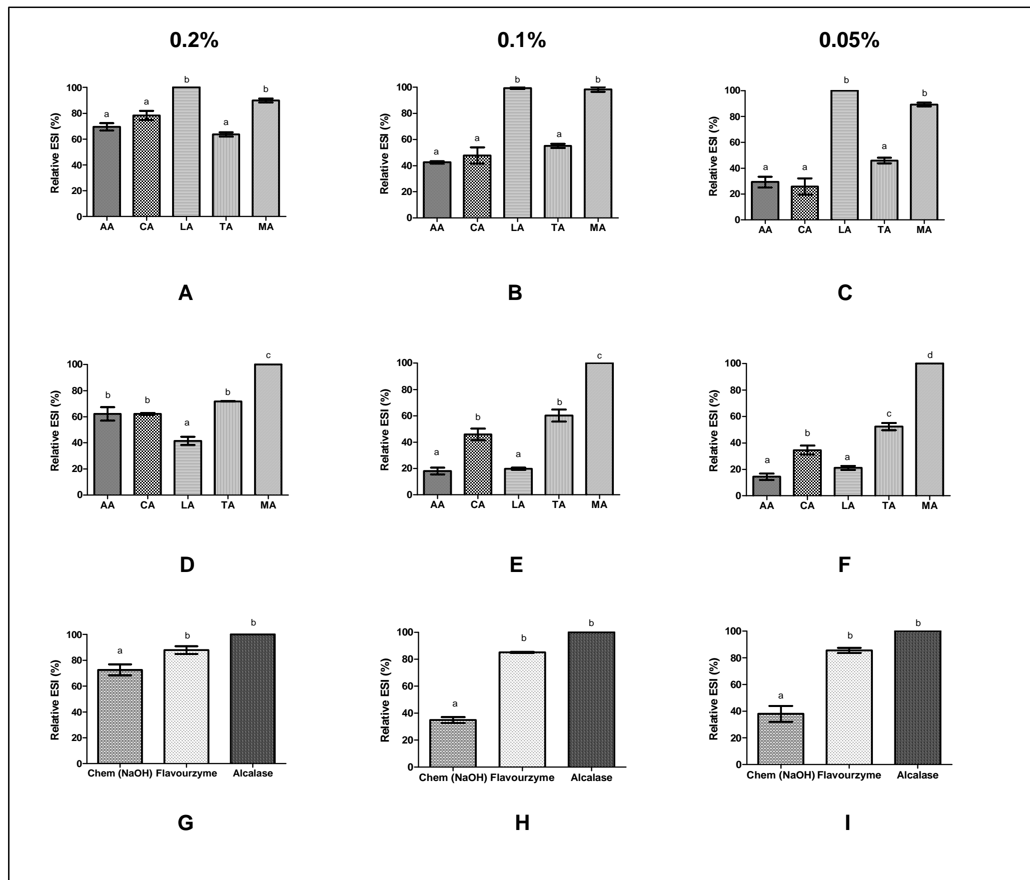


Figure 4.13 Relative emulsifying stability index (ESI) of gelatines from blue whiting at different protein concentrations (0.2%, 0.1% and 0.05%, w/v). Gelatines from blue whiting heads (A, B and C), gelatines from blue whiting skins (D, E and F) and gelatines from blue whiting bones (G, H and I). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase. Different letters, within the same graph, indicate significant ($p < 0.05$) differences between pre-treatments for gelatines from same fish raw material.

At 0.2 % (w/v) (the protein concentration which showed the highest ESI for all gelatines), gelatines extracted from mackerel heads and skins after pre-teratment with lactic acid showed significantly ($p < 0.05$) higher ESI. Gelatines extracted from blue whiting heads after pre-treatment with lactic and malic acids and gelatines extracted from blue whiting skins after pre-treatment with malic acid showed significantly ($p < 0.05$) higher ESI values. As in foaming properties, the enzymatic pre-treatment of mackerel and blue whiting bones resulted in significantly ($p < 0.05$) higher ESI. Mechanisms of emulsification process of gelatines are correlated to the adsorption ability at the surface of freshly formed oil droplets during homogenisation and formation of a protective membrane that prevents droplets coalescence. When less hydrophobic amino acids are present, adsorption of relatively polar amino acids takes place to surround the interface, resulting in destabilising the emulsion (Agyare *et al.,* 2009). This was mainly observed with acetic acid pre-treated mackerel head and skin and blue whiting head gelatines, lactic acid pre-treated blue whiting skin gelatines and chemically pre-treated mackerel and blue whiting bone gelatines.

Commercial fish and bovine gelatines had high EAI at all protein concentrations, with the highest observed at the lowest protein content (0.05 %). High ESI were observed at concentrations of 0.2 % and 0.1 %. Commercial bovine gelatine had slightly higher ESI than commercial fish gelatine (Figure 4.14).

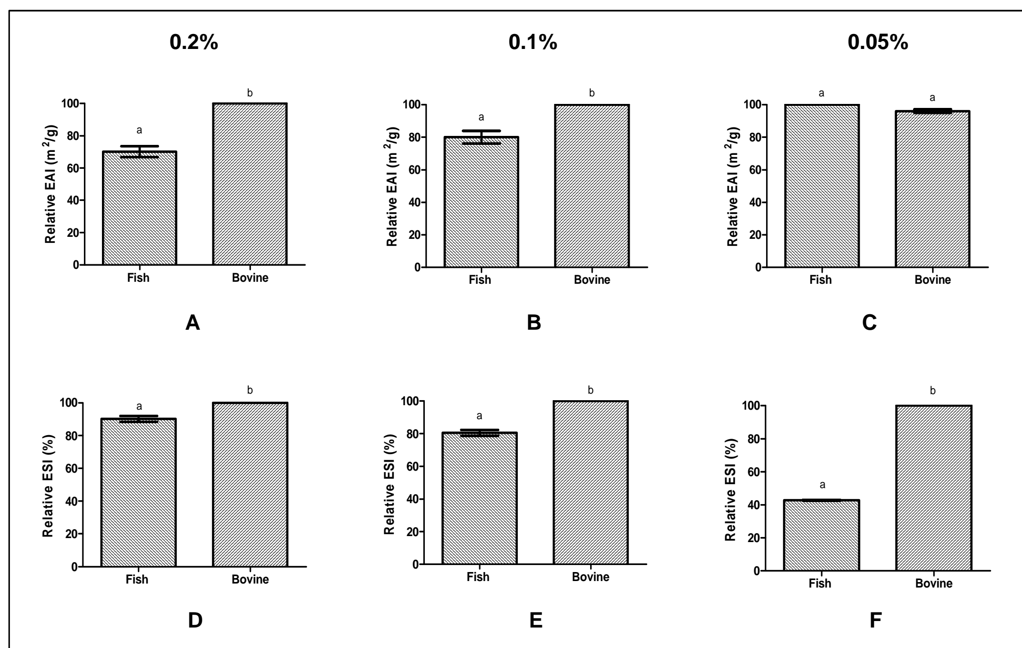


Figure 4.14 Relative emulsifying capacity index (EAI) and emulsifying stability index (ESI) of commercial fish and bovine gelatines at different protein concentrations (0.2%, 0.1% and 0.05%, w/v). EAI: A, B & C and ESI: D, E & F. Different letters, within the same graph, indicate significant ($p < 0.05$) diffrences between gelatines.

The analysis of the emulsifying properties showed that the higher content in less polar amino acid in some fish gelatine (due to the pre-treatment used) resulted in better hydrophilic/hydrophobic distribution of the amino acids. This enhanced the exposure of hydrophobic amino acid residues and improved the emulsifying properties of gelatines.

4.2.3. Protein solubility

4.2.3.1. Effect of pH

The relative solubility of gelatines was measured in the pH range of 2 to 12. Solubility of mackerel and blue whiting gelatines at different pH is shown in Figure 4.15. All the gelatines, regardless of the acid used in the pre-treatment, showed similar pH behaviour. The solubility was higher at low pH, with a maximum at pH 2 to 4. The lowest solubility was observed close to neutral pH. Similar results were also reported by Kittiphattanabawon *et al.* (2005) for collagen from skin and bone of bigeye snapper. Aewsiri *et al.* (2008) found that the lowest solubility of gelatines from pre-cooked tuna fin was observed at pH 6.

The solubility of blue whiting head and skin gelatines were slightly higher than those from mackerel head and skin. These differences in solubility might result from the differences in amino acid compositions, sequence, molecular weight, conformation and the content of polar and non-polar groups in amino acids (Zayas, 1997). For instance, blue whiting head and skin gelatines had higher polar amino acid residues ((16.0 % - 17.4 %) and (14.5 % - 19.6 %) for head and skin, respectively) than mackerel head and skin gelatines ((14.1 % - 16.1 %) and (14.0 % - 17.8 %) for head and skin, respectively). Similarly, the non polar amino acid residues in blue whiting head and skin gelatines ((22.3 % - 27.9 %) and (19.8 % - 21.7 %), respectively) were higher than those from mackerel head and skin gelatines ((20.9 % - 24.5 %) and (18.7 % - 22.3 %), respectively).

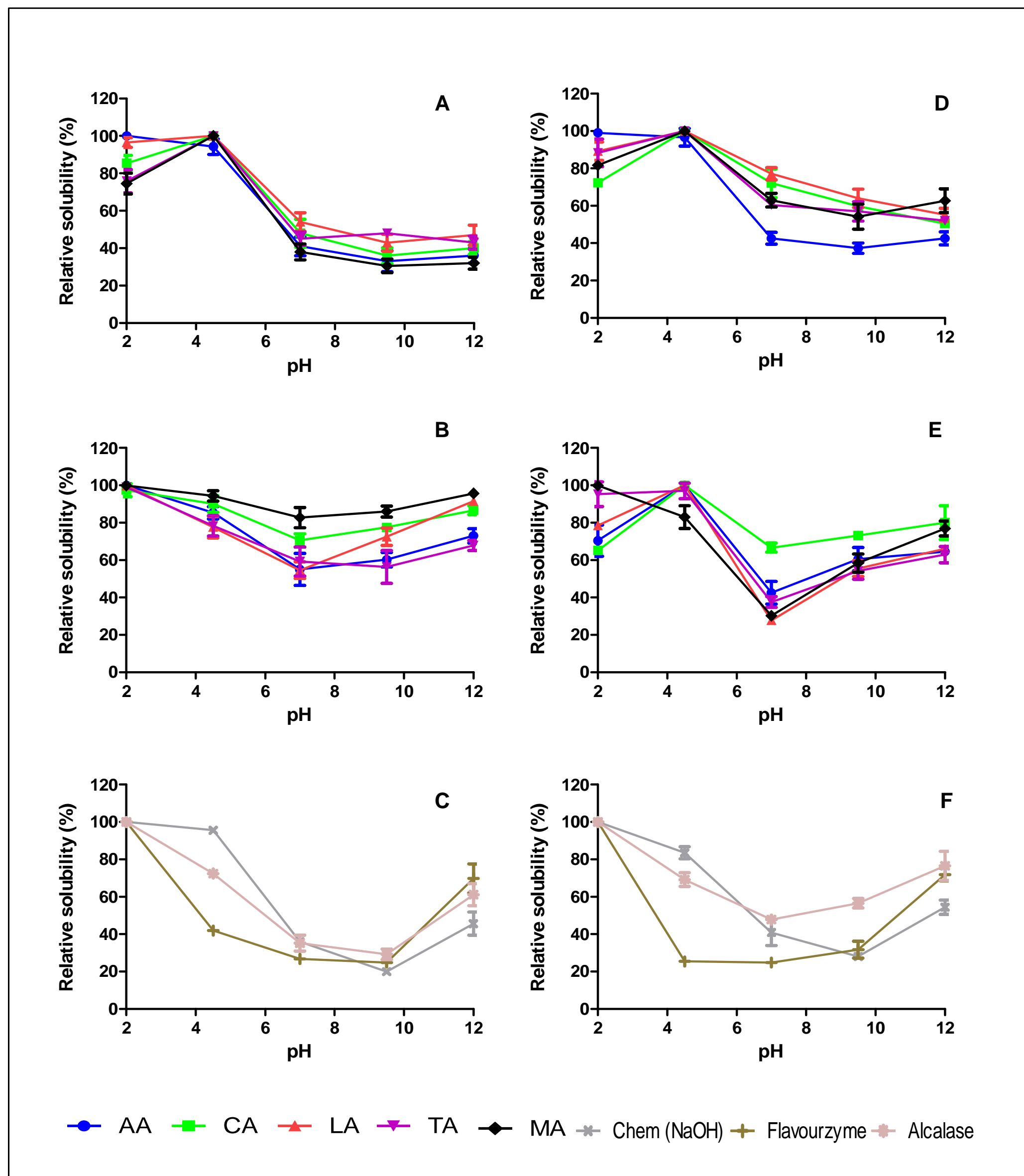


Figure 4.15 Relative solubility of fish gelatines in the pH range of 2 - 12. Gelatines from mackerel heads (A), gelatines from mackerel skins (B), gelatines from mackerel bones (C), gelatines from blue whiting heads (D), gelatines from blue whiting skins (E) and gelatines from blue whiting bones (F). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase.

The higher solubility of fish bone gelatines pre-treated with enzymes compared to gelatines from bone pre-treated chemically, could be due to the presence of low molecular weight peptides formed during the extraction process (See section 4.1.6). The hydrolysis generally releases peptides with more polar residues that may interact with water molecule through hydrogen bonds and as result an increase in solubility (Gbogouri *et al.,* 2004) (See section 4.1.7).

4.2.3.2. Effect of ionic strength

Gelatine solubility decreased with increased ionic strength (Figure 4.16). Gelatines showed a gradual decrease of solubility from 0 to 2 % then a sharp decease was observed from 2 to 3 %. These results were in agreement with previous studies for collagen from trout skin (Monetro *et al.,* 1991) and hake skin (Monetro *et al.,* 1999). The decrease in solubility with the increase of NaCl concentration was probably due to the increase of the hydrophobic interactions and to the competition of ionic salts for water (Vojdani, 1996).

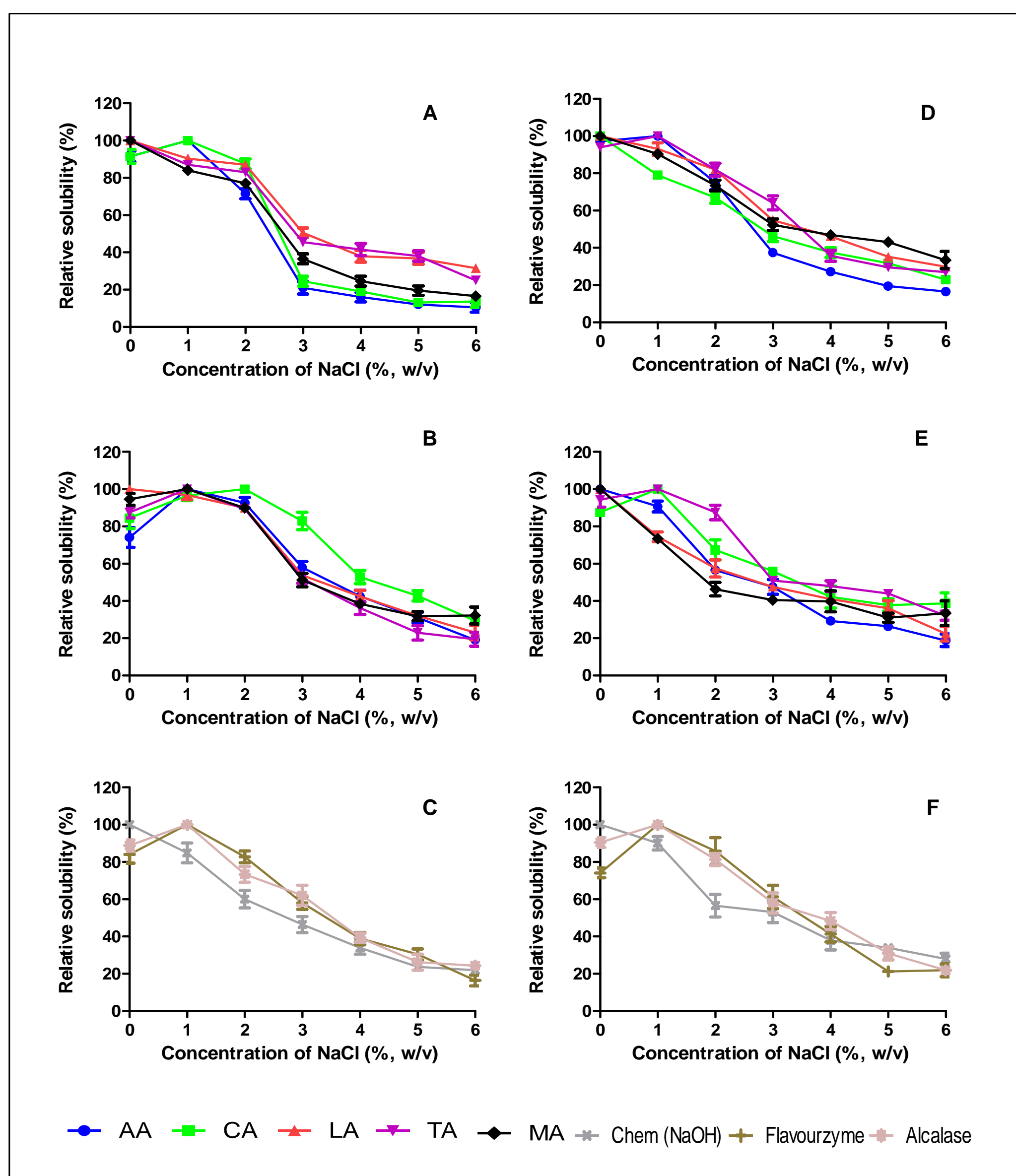


Figure 4.16 Relative solubility of fish gelatines as function of NaCl concentration. Gelatines from mackerel heads (A), gelatines from mackerel skins (B), gelatines from mackerel bones (C), gelatines from blue whiting heads (D), gelatines from blue whiting skins (E) and gelatines from blue whiting bones (F). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Flavourzyme and Alcalase.

Commercial fish and bovine gelatines showed similar solubility behaviour compared to the extracted gelatines from mackerel and blue whiting. In the pH range of 2 - 12, commercial fish gelatine was more soluble than commercial bovine gelatine. The increase of NaCl concentration resulted in lowering the solubility of both gelatines (Figure 4.17)

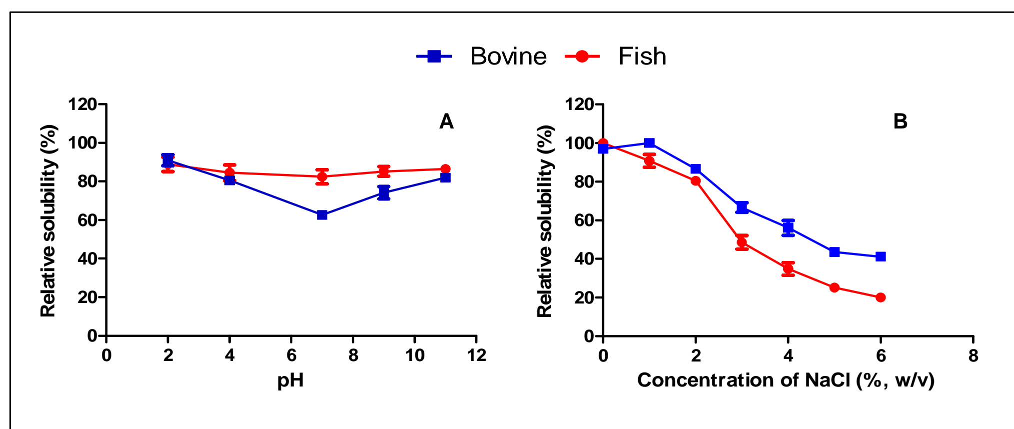


Figure 4.17 Relative solubility of commercial fish and bovine gelatines. (A) Solubility in the pH range 2 - 12. (B) Solubility as function of NaCl concentration.

Gelatines extracted from mackerel heads after pre-treatment with lactic and tartaric acids showed higher solubility (from pH 6 and at NaCl concentrations higher than 3 %), compared to gelatines extracted after pre-treatment with citric, lactic and tartaric acids. Similar results were observed for gelatines from mackerel skins pre-treated with lactic and malic acids, gelatines from blue whiting heads pre-treated with citric, lactic and malic acids, as well as gelatines from blue whiting skins pre-treated with citric and malic acids. This may be an indication of low degree of cross-linking of these gelatines (Kittiphattanbawon *et al.,* 2005).

At neutral pH, gelatines from mackerel and blue whiting heads, regardless of the organic acid used, exhibited a lower solubility than gelatines extracted from mackerel and blue whiting skins. This could be possibly due to a lower degree of protein-H_2O interactions via the polar hydroxyl groups (OH) from Ser, Thr, Tyr and Hyp amino acids which were less abundant in fish head gelatines (14.1 -16.1 % and 16.0 -17.4 %, for mackerel and blue whiting, respectively) compared to fish skin gelatines (14.0 -17.8 % and 14.5 -19.6 %, for mackerel and blue whiting, respectively)

4.2.4. Rheological properties

4.2.4.1. Dynamic viscoelastic behaviour (DVB)

Figure 4.18 shows the viscoelastic properties, including the storage (G') and loss modulus (G'') as well as the phase angle (δ) during the cooling (a, b and c) and heating ramps (d, e and f) of gelatines extracted from mackerel heads (A), skins (B) and bones (C).

In the cooling process (i.e., from 25 ºC to 5 ºC), the elastic modulus (G') of mackerel head and skin gelatines increased rapidly between 10 and 15 ºC, representing the transition from solution to gel state (Figure 4.18A (a) & B (a)). Slight differences on the increase rate were observed. Similar behaviour was observed for the viscous modulus (G'') with a gradual increase (Figure 4.18A (b) & B (b)).

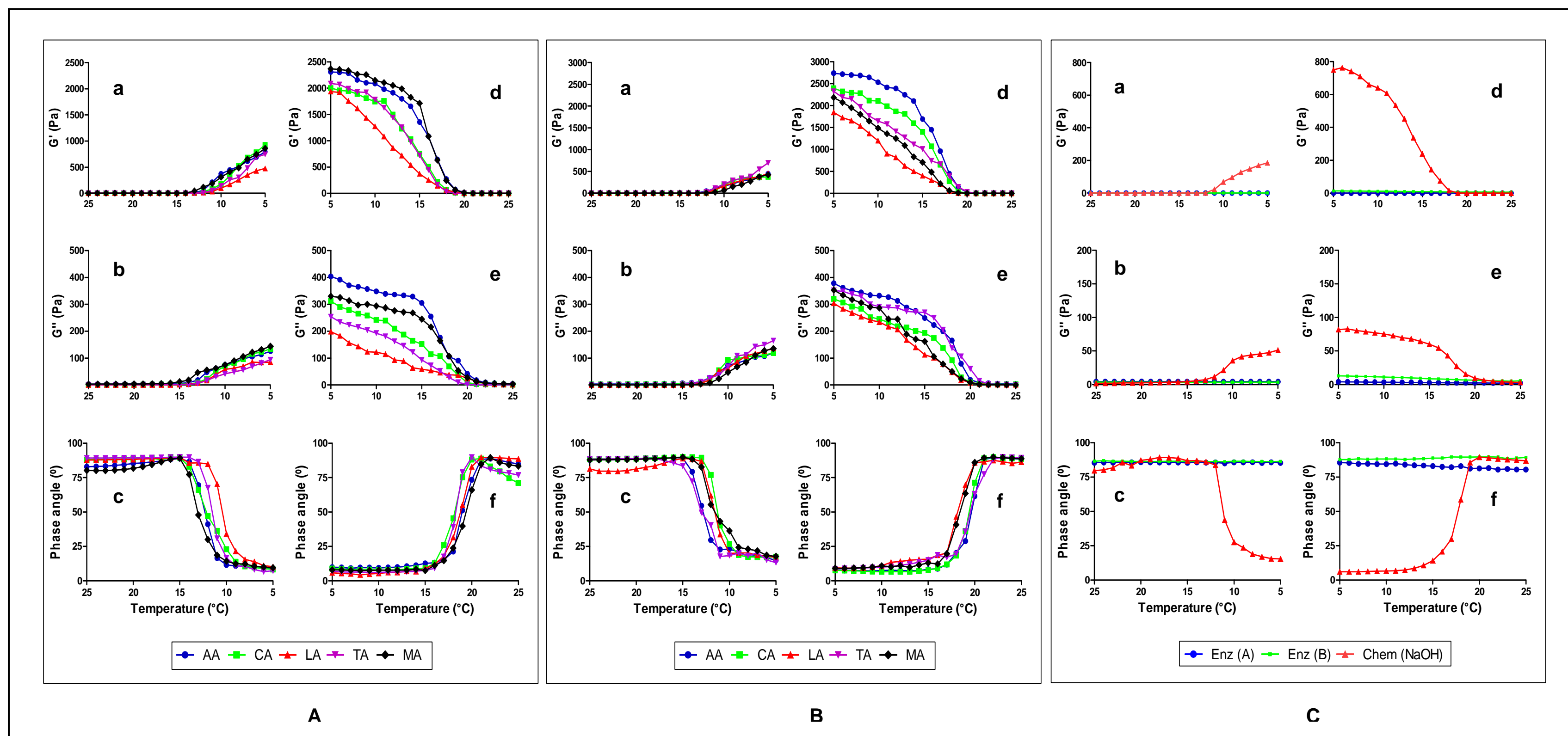


Figure 4.18 Viscoelastic properties, including the storage (G') and loss modulus (G'') as well as the phase angle (δ) during the cooling (a, b and c) and heating ramps (d, e and f) of gelatines extracted from mackerel heads (A), skins (B) and bones (C). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase.

As observed from the heating ramp (i.e., from 5 ºC to 25 ºC), the elastic modulus (G') of gelatine extracted from mackerel heads after pre-treatment with lactic acid, decreased gradually from 8 ºC showing that this solution started to melt. The elastic modulus (G') of the rest of gelatines decreased slowly around 10 - 11 ºC then a rapid decrease was observed between 15 ºC and 20 ºC representing the transition from gel to solution (Figure 4.18A (d)). The rate of decrease was very low for gelatines extracted using acetic and malic acids while the highest rate was observed for gelatine extracted after pre-treatment with lactic acid.

For mackerel skin gelatines, lactic, malic and tartaric pre-treated gelatines started to melt rapidly as soon as the heating began. Gelatines extracted after pre-treatment of mackerel skins with acetic and citric showed a gradual decrease from 5 ºC to 15 ºC then a higher rate was observed from 15 ºC and 20 ºC (Figure 4.18B (d)). The viscous modulus (G'') showed similar behaviour but the decrease was gradual (Figure 4.18A (e) & B (e)). Mackerel bone gelatines showed different rheological properties depending on the pre-treatment used. The enzymatic pre-treatment of bones resulted in gelatines with no gelling ability. For both heating and cooling ramps, no differences in elastic and viscous modulus were observed.

In both processes (cooling and heating) the phase angle showed similar profiles. All gelatines had a low phase angle at low temperature which indicates acceptable gelling ability (Gómez-Guillén *et al.,* 2005).

Figure 4.19 shows the viscoelastic properties, including the storage (G') and loss modulus (G'') as well as the phase angle (δ) during the cooling (a, b and c) and heating ramps (d, e and f) of gelatines extracted from blue whiting heads (A), skins (B) and bones (C). Gelatines from blue whiting showed a similar rheological behaviour to mackerel gelatines. Differences in behaviour were only observed in the type of acid used and the range of melting and gelling temperatures which was lower and narrower than mackerel gelatines. Blue whiting gelatines started to become gels in the range of 10 - 5 °C as shown by the increase in elastic modulus (Figure 4.19A (a) & B (a)). The transition from gel to solution was achieved in the temperature range of 10 - 15 °C (Figure 4.19A (d) & B (d)). Gelatines extracted from blue whiting heads and skins pre-treated with acetic and tartaric acids showed better rheological behaviour than the rest of the gelatines. Similar to mackerel bone gelatines, blue whiting bone gelatine pre-treated with sodium hydroxide had better rheological properties than enzymatic pre-treated bone gelatines (Figure 4.19C).

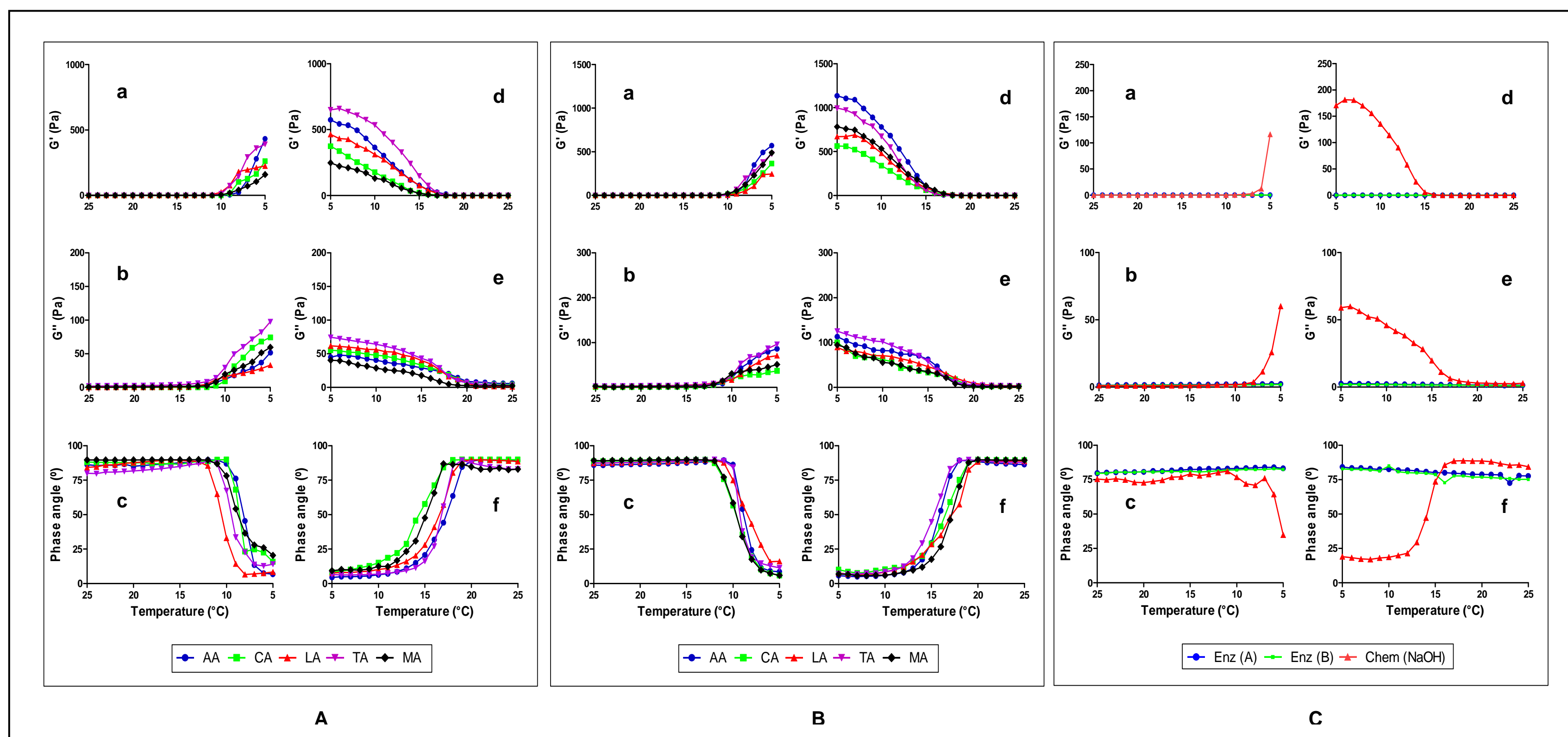


Figure 4.19 Viscoelastic properties, including the storage (G') and loss modulus (G'') as well as the phase angle (δ) during the cooling (a, b and c) and heating ramps (d, e and f) of gelatines extracted from blue whiting heads (A), skins (B) and bones (C). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; Chem (NaOH): alkaline; Enz (A): Flavourzyme and Enz (B): Alcalase.

Commercial bovine gelatine had higher viscoelastic properties than commercial fish gelatines (Figure 4.20), which resulted in considerably higher gelling and melting points than fish gelatines. Commercial fish gelatine showed rheological properties similar to gelatine extracted from mackerel skins using acetic acid.

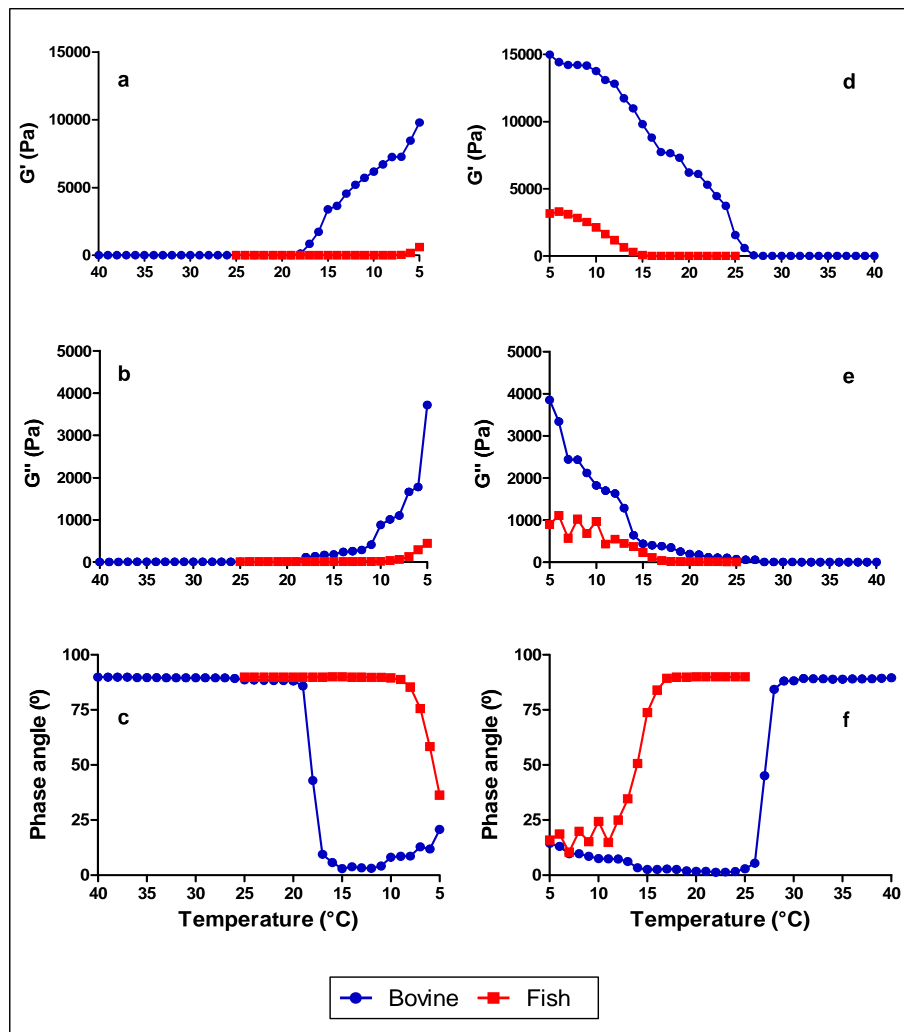


Figure 4.20 Viscoelastic properties, including the storage (G') and loss modulus (G'') as well as the phase angle (δ) during the cooling (a, b and c) and heating ramps (d, e and f) of commercial bovine and fish gelatines.

For all gelatines, the differences among the values of G′ at 5 °C during the cooling and heating process could be due to the maturation of gelatines at 5 °C for 10 min before starting the heating process. The slight differences in the transition curves during the melting and gelling processes among gelatines resulted in slight differences in gelling and melting temperatures of these gelatines. In this study, the gelling temperatures varied from 10 ºC to 12.9 ºC for gelatines from mackerel and in the range of 5.6 ºC to 10 ºC for gelatines from blue whiting. The melting temperatures ranged from 17.3 ºC to 20.4 ºC and 13.4 ºC to 17.4 ºC for gelatines from mackerel and blue whiting, respectively (Table 4.8).

Table 4.8 Rheological parameters of gelatines from mackerel and blue whiting.

	Pre-treatment	**Gelling temperature**		**Melting temperature**	
		M	**BW**	**M**	**BW**
Heads	**AA**	12.2	9	19.4	17
	CA	11.6	9.3	19.1	16.8
	LA	10.2	10	18.4	17.4
	TA	11.8	9.1	18.9	16.6
	MA	11.4	9.6	18.9	16.8
Skins	**AA**	12.9	9	20.4	16
	CA	11.9	9.9	19.8	17.2
	LA	12.2	9	18.4	17
	TA	11.8	9.9	18.9	17.1
	MA	11.9	9.4	19.1	17.3
Bones	**NaOH**	10	5.6	17.3	13.4

M: Mackerel, BW: Blue whiting. Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid and NaOH: alkaline.

Several studies showed that the melting temperatures for fish gelatines vary from 15 ºC to 32 ºC. Rigby (1968) reported 15 ºC for cod; Kimura & Ohno (1987) mentioned 16.8 ºC for Alaska pollack. Kimura *et al.* (1988) reported 32.5

ºC for carp, 29.3 ºC for eel and 19.4 ºC for chum salmon. The melting temperatures in the present study were lower than 26.1 ºC for common mackerel as reported by Kimura *et al.* (1988). This could be due to the variation among the species, the temperature of the habitat, the extraction procedure, the pH and the concentration of gelatines (Rigby, 1968).

Previous results (Section 4.1.7) found that fish gelatines had different contents of imino acids (proline and hydroxyproline). These differences may explain the slight difference in gelling and melting temperatures for gelatines extracted from both fish. Comparing both fish gelatines, it was observed that mackerel had better rheological properties than blue whiting. For both fish, regardless of organic acid or pre-treatment used, skin gelatines had better rheological properties followed by head gelatines. Bone gelatines had poor rheological properties compared to head and skin gelatines. These differences could be due to the differences among the fish species, raw material, pre-treatments used, amino acid content and the molecular weight distribution of gelatines.

4.2.4.2. Frequency sweep

The effect of the frequency on the elastic (G') modulus was studied (Figure 4.21). These analyses were carried out to verify the rheological behaviour of the gelatines and to assess the strength of the gel network. For all gelatine samples, a slight dependence of G′ values on the frequency was observed indicating a relatively weak gelatine gel. These results were similar to those observed for cod gelatines (Gilsenan & Ross-Murphy, 2000).

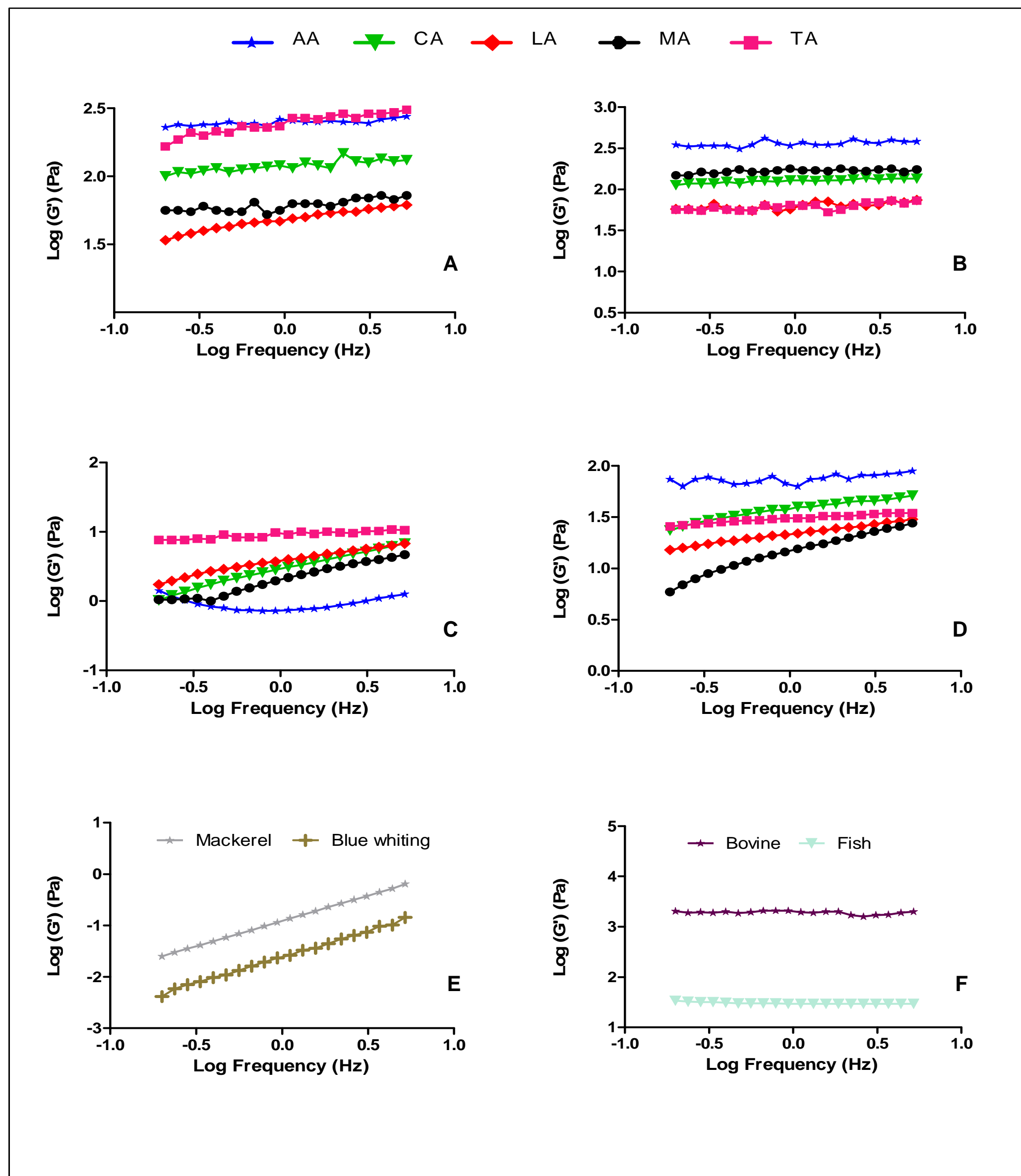


Figure 4.21 Elastic modulus (G′) at 5 ºC under frequency sweep of fish gelatines. Gelatines from mackerel heads (A), gelatines from mackerel skins (B), gelatines from blue whiting heads (C), gelatines from blue whiting skins (D), gelatines from fish bones pre-treated chemically (E) and commercial fish and bovine gelatines (F). Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid and MA: malic acid.

The slope of G' values as a function of frequency varied among the gelatines depending on the source and the pre-treatment used. For mackerel head gelatines, the lowest slope was observed with gelatine extracted after pre-treatment with acetic acid (0.04) followed by gelatine extracted after pre-treatment with malic and citric acids (0.08) and the highest slopes were seen for gelatines prepared after pre-treatment with tartaric (0.16) and lactic (0.17) acids (Figure 4.21A). All gelatine gels from mackerel skins showed better textural stability as proven by the low slope varying from 0.04 to 0.07 (Figure 4. 21B).

As seen in Figure 4.21C, blue whiting head gelatines extracted after pre-treatment with citric, lactic and malic were dependent on frequency, G' increased by increasing the frequency. All blue whiting skin gelatines, except those pre-treated with acetic acid, were dependent on the frequency (Figure 4.21D). Malic acid pre-treated gelatines were the most affected by the frequency (slope 0.44) followed by citric and lactic acid extracted gelatines (slope 0.2). Both fish bone gelatines extracted after the chemical pre-treatment were very dependent on the frequency as shown in Figure 4.20E. The calculated slopes were 0.99 and 1.02 for mackerel and blue whiting bone gelatines, respectively. Although commercial bovine gelatine was stronger than commercial fish gelatine, both gels were very stable to the change in frequency (Figure 4.21F).

The low slopes observed with mackerel head gelatines (pre-treated with acetic and malic acids), all mackerel skin gelatines as well as blue whiting head and skin gelatines (pre-treated with acetic and tartaric acids) are indicative of a good gel network and better stability of this gel when subjected to shear forces.

4.2.5. Textural properties

4.2.5.1. Gel strength

Gel strength is one of the most important physical properties of gelatine (Cheow *et al.,* 2007). Gelatine is a biological polymer that forms thermo-reversible gels. Even after formation, the structure of the gel is not static and continues to evolve and change because of the instability of low energy interactions within the gel network (Tosh *et al.,* 2003). The gel formation is a complex process, in which the gel develops and changes during the storage time. Different mechanisms are associated with the gel strengthening during maturation. Mainly, the regeneration of helical structures (between collagen peptide chains) and the formation of hydrogen bonds between hydroxylated amino acids and incorporated water molecules (Haug *et al.,* 2004).

Results showed that the gel strength of fish gelatines was affected significantly ($p < 0.05$) by the pre-treatment. The gel strength of the various gelatine preparations, after overnight maturation at 10 ˚C, is presented in Table 4.9. Gelatines extracted from mackerel heads after pre-treatment with acetic, citric and malic acids showed significantly ($p < 0.05$) high gel strength (71 - 76 g) in comparison to those extracted with lactic and tartaric acids, corresponding with gelatines that have the highest imino acid contents. Acetic acid pre-treated mackerel skins gave gelatine with the highest gel strength (80 g); citric and malic acids provided gels with the next highest gel strengths (73 - 76 g). Gelatines from blue whiting heads pre-treated with tartaric acid showed significantly ($p < 0.05$) highest gel strength (29 g) while the lowest were observed with citric, lactic and malic acid pre-treated heads. Gelatines extracted from blue whiting skins using

malic acid showed the lowest gel strength (22 g) and lactic and acetic acid extracts of the same blue whiting fraction showed the highest (~ 38 g). Similar gel strengths (~ 45 g) were observed with bone gelatines for both fish. The enzymatic pre-treatement of bones gave gelatines with no gel forming ability which could be due to the hydrolysis of the gelatine during the extraction process. As expected commercial bovine gelatine had higher gel strength than commercial fish gelatine and all extracted gelatines from mackerel and blue whiting.

Gelatines with a gel strength of 108 g for salmon and 71 g for cod skins were reported by Arnesen & Gildberg (2007). Gelatines with low gel strength had in fact low imino acid content (Section 4.1.7). According to Arnesen & Gildberg (2002) the low hydroxyproline content in fish gelatine resulted in gelatines with low gel strength compared to mammalian gelatines. Interestingly, it seems that the organic acid affects the properties of gelatines depending on the source (i.e., mackerel or blue whiting). Good gel strengths were observed for mackerel head and skin gelatines when using acetic, citric or malic acids in the pre-treatment. For blue whiting, acetic, lactic or tartaric acid pre-treatment of heads or skins improved the gel strength.

Table 4.9 Gel strength of gelatines from mackerel, blue whiting and commercial sources.

Fish	Tissue	Treatment	Gel strength (g)
Mackerel	**Heads**	**AA**	70.4±1.4[b]
		CA	71.4±7.2[b]
		LA	41.8±1.4[a]
		MA	76.0±0.7[b]
		TA	54.6±2.2[a]
Blue whiting	**Heads**	**AA**	21.9±2.2[a]
		CA	17.8±0.7[a]
		LA	18.4±1.4[a]
		MA	15.8±0.7[a]
		TA	29.6±1.4[b]
Mackerel	**Skins**	**AA**	80.2±1.4[b]
		CA	76.4±0.7[b]
		LA	43.3±0.7[a]
		MA	71.1±3.6[b]
		TA	49.4±1.4[a]
Blue whiting	**Skins**	**AA**	38.2±0.7[b]
		CA	26.0±3.6[a]
		LA	37.2±1.1[b]
		MA	22.8±7.8[a]
		TA	32.1±2.1[a]
Mackerel	**Bones**	**NaOH**	45.4±0.7
Blue whiting	**Bones**	**NaOH**	46.4±0.7
Commercial fish	**-**	**-**	84.6±2.4
Commercial bovine	**-**	**-**	249.3±5.6

Pre-treatment: AA: acetic acid; CA: citric acid; LA: lactic acid; TA: tartaric acid; MA: malic acid; NaOH: alkaline. Values given as mean ± standard deviation. Different letters, within the same raw material, indicate significant ($p < 0.05$) differences between pre-treatments.

In addition to the difference in the imino acids content, differences among the gel strength of gelatines from mackerel heads could be due to the differences in pH. The gel strength might be dependent on the isoelectric point and could also be controlled by adjusting the pH (Gudmundsson & Hafsteinsson, 1997).

4.2.5.2. Microscopic characterisation of gelatines by cryo-scanning electron microscopy (cryo-SEM)

Micrographs (Cryo-SEM) of various gelatines were carried out to investigate the gel microstructure. Rheological analyses conducted in this research showed that, regardless of the pre-treatment, gelatines from mackerel were better than blue whiting. It was also observed that acetic acid pre-treated gelatines exhibited better rheological properties. Hence, gelatines extracted from mackerel and blue whiting heads and skins after pre-treatment with acetic acid were chosen for comparative reasons.

Cryo-SEM images showed that commercial bovine gelatine had a honeycomb structure with thin stranded protein network and large number of interconnected pores. These pores were very small and uniform (Figure 4.22E). However, commercial fish gelatine showed larger voids (Figure 4.22A) indicating a relatively weak nature of the gel. The higher the number of small interconnected pores, the stronger the gel (Wangtueai & Noomhorm, 2009).

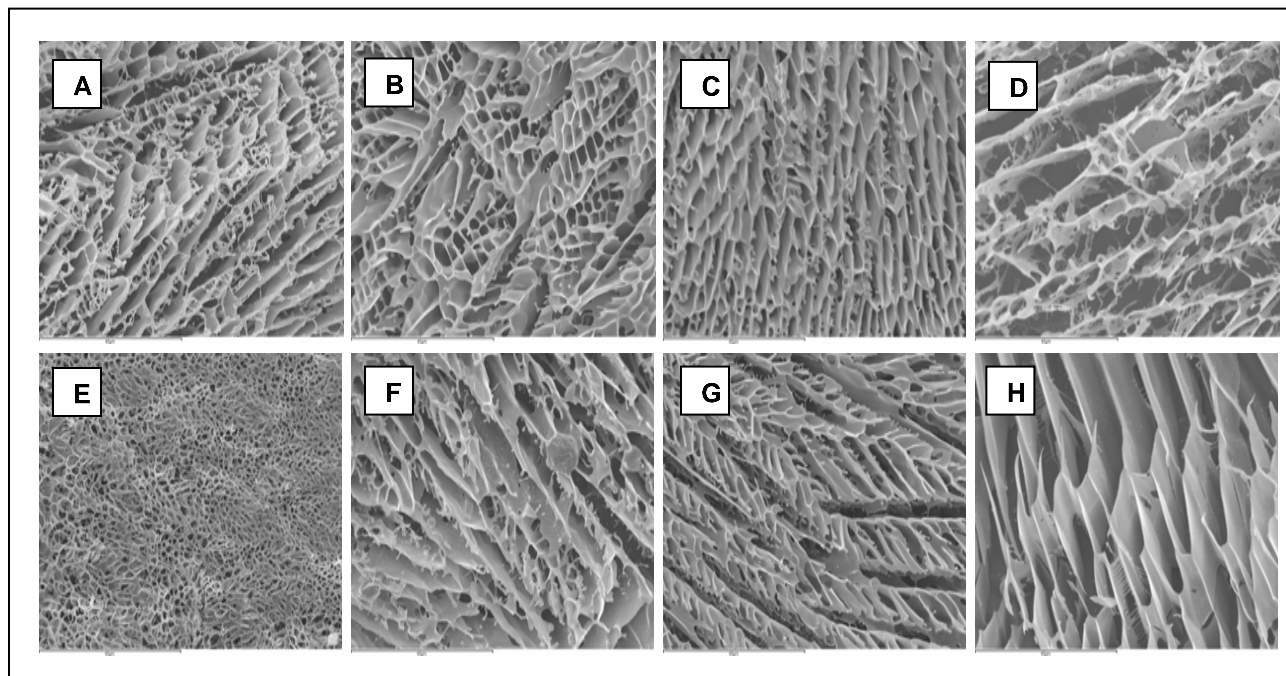


Figure 4.22 Scanning electron microscopy (× 1500) of gelatine gels. Commercial fish gelatine (A), gelatine from mackerel heads pre-treated with acetic acid (B), gelatine from mackerel skins pre-treated with acetic acid (C), gelatine from mackerel bones pre-treated with sodium hydroxide (D), commercial bovine gelatine gel (E), gelatine from blue whiting heads pre-treated with acetic acid (F), gelatine from blue whiting skins pre-treated with acetic acid (G) and gelatine from mackerel bones pre-treated with Flavourzyme (H).

Mackerel head and skin gelatines (Figure 4.22B & C, respectively) showed a higher number of interconnected small pores than blue whiting head and skin gelatines (Figure 4.22F & G, respectively). The protein matrices of mackerel head and skin gelatines were lacy and very similar. Gelatine from mackerel bones pre-treated with sodium hydroxide (Figure 4.22D) showed also a lacy network with small interconnected pores. However, the enzymatic pre-treatment of mackerel bones with Flavourzyme produced protein strands with less interconnections and larger void size in the protein matrix (Figure 4.22H).

The organic acid affected the rheological properties of the extracted gelatines. This was also confirmed by the microstructure of gelatines (Figure 4.23). Differences were observed for gelatines extracted from mackerel heads after pre-treatment with acetic, citric and lactic acids (different gel strength, from higher to lower). Mackerel head gelatines extracted after pre-treatment with lactic acids showed very poor rheological and textural properties. From the micrographs (Figure 4.23C), it was clear that these gelatines have non-connected big voids compared to gelatines extracted after pre-treatment with acetic and citric acids (Figure 4.23A & B, respectively).

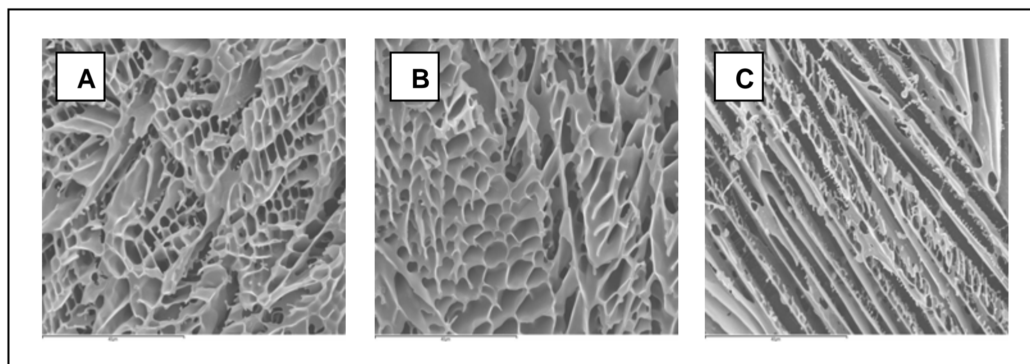


Figure 4.23 Scanning electron microscopy (× 1500) of mackerel head gelatine gels. Gelatines extracted after pre-treatment with acetic acid (A), citric acid (B) and lactic acid (C).

The CryoSEM results showed that the microstructures were highly related to the gel strength values, where denser strands (bovine gelatine and gelatines extracted from mackerel head and skin after pre-treatment with acetic acid) represented higher gel strength than the looser strands such as in the case of mackerel head gelatines pre-treated with lactic acid and mackerel bone gelatines extracted after the enzymatic pre-treatment.

4.3. Conclusions

The pre-treatment methods applied to gelatine extraction affected the yield, colour, turbidity, amino acid profiles as well as functional properties of mackerel and blue whiting gelatines. pH and ionic strength, which varies depending on the type of acid used, are important for the functional effectiveness of the extraction. Citric acid pre-treatment of mackerel heads and skins produced gelatines with the highest yield (~ 3.5 %, w/w). Acetic acid pre-treatment, however, showed the highest yield for blue whiting heads and skins (~ 2.5 %, w/w). The chemical pre-treatment of fish bones gave the lowest yield.

For both fish, and in order to increase lightness and clarity of the gelatines, the use of acetic and lactic acids to assist extraction should be avoided.

The protein solubility of all gelatines was greater at acidic pH than at higher pH values. Functionality tests indicated that the emulsifying and foaming properties varied significantly ($p < 0.05$) between gelatines depending on the pre-treatment and the type of tissue used.

Rheological and textural analyses varied significantly depending on the source and the pre-treatment used in the extraction. Mackerel exhibited stronger and more stable gels than blue whiting. Acetic acid pre-treatment of mackerel and blue whiting heads and skins produced better gelatines. The enzymatic pre-treatment of fish bones negatively affected the rheological behaviour of gelatines. The differences among gelatines were correlated to the difference in the molecular weight distribution and to the variation in the imino acids (proline and hydroxyproline) level of gelatines.

Chapter 5 - Fish protein hydrolysates

5.1. Preparation and characterisation of FPH

Gelatines extracted from heads and skins of mackerel after pre-treatment with citric acid were hydrolysed with pepsin, trypsin and chymoptrypsin for, 1, 2, 6 and 24 h, while mackerel viscera were hydrolysed with endogenous enzymes, pepsin, trypsin, chymoptrypsin, Alcalase and Flavourzyme for 2, 6 and 24 h. It was observed that as the hydrolysis proceeded, the turbidity decreased and the solution became clear (Figure 5.1).

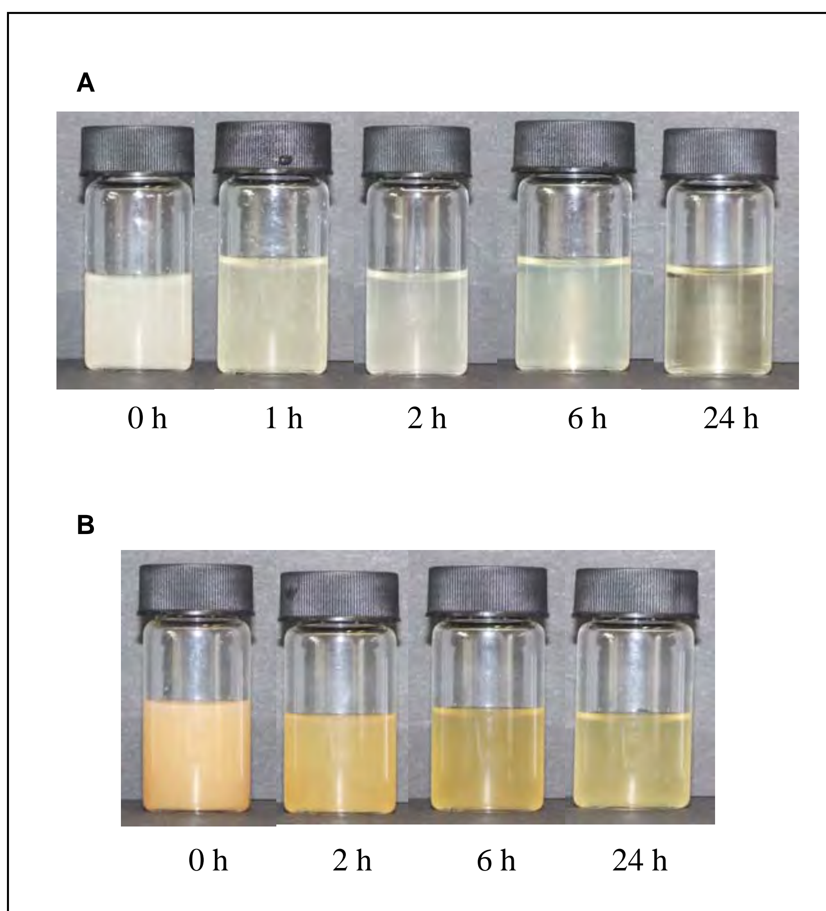


Figure 5.1 Evolution of the enzymatic hydrolysis as a function of time. A: Mackerel skin gelatine hydrolysed with pepsin. B: Mackerel viscera hydrolysed with Flavourzyme.

5.1.1. Degree of hydrolysis of mackerel gelatines

Gelatines extracted from heads and skins of mackerel after pre-treatment with citric acid were hydrolysed using three digestive enzymes, trypsin, chymotrypsin and pepsin. As discussed in section 4.1.1, mackerel heads and skins pre-treated with citric acid produced gelatines with the highest yields. Therefore, the use of citric acid for the preparation of gelatine would be advantageous at industrial scale. The low cost of citric acid, its safety and its use as food additive make this choice of acid very reasonable. Primary screening of the hydrolysates showed that pepsin hydrolysis was the most efficient method, confirmed by the increase in the degree of hydrolysis (DH). The DH of mackerel skin and head gelatines during hydrolysis with pepsin is shown in Figure 5.2.

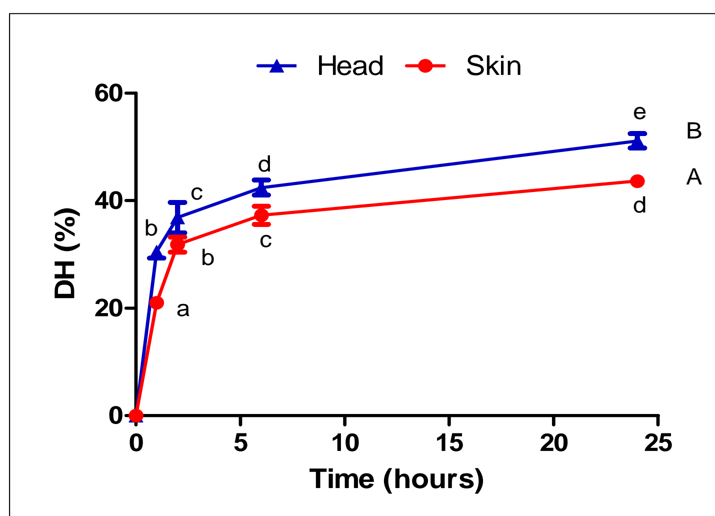


Figure 5.2 Evolution of the degree of hydrolysis using pepsin for mackerel gelatine as a function of hydrolysis time. Different lower case letters indicate significant ($p < 0.05$) differences between hydrolysis times. Different upper case letters indicate significant ($p < 0.05$) differences between sources.

Pepsin hydrolysed mackerel head and skin gelatines in a similar way (similar shape of hydrolysis curves). The rate of hydrolysis increased rapidly during the initial 120 min of hydrolysis and decreased thereafter. The rate of mackerel head gelatine hydrolysis was slightly higher than skin gelatine hydrolysis. It is known that pepsin is specific to hydrophobic amino acids and preferentially the aromatic ones (Keil, 1992). From the amino acid composition of mackerel head and skin gelatines extracted after pre-treatment with citric acid (Table 4.5, section 4.1.7), it was observed that both gelatines had similar hydrophobic amino acid (Ala, Cys, Val, Met, Ile, Leu, Tyr and Phe) content (22 %) with 3 % aromatic residues (Tyr and Phe). However, mackerel skin gelatine at 1 % (w/v, in 0.1 M glycine buffer at pH 2) was more turbid than head gelatine solution prepared at the same conditions (Figure 5.3). This might be due to the presence of aggregated proteins (Benjwal *et al.,* 2006), probably produced during the heating process (during the inactivation of endogenous enzymes). Following the aggregation, proteins lose their solubility and flocculate. Thus, the thermal aggregation of skin gelatine may have slightly reduced the peptic activity compared to that observed with head gelatine as result of reduced exposure of hydrophobic residues.

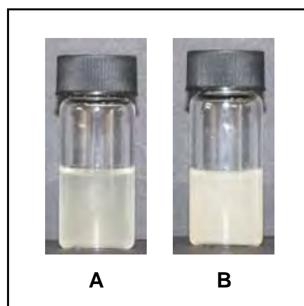


Figure 5.3 Mackerel head (A) and skin (B) gelatines after the heating process. Gelatine solutions were prepared as 1 % (w/v) in 0.1 M glycine buffer at pH 2.

The kinetics of the hydrolysis curves were typical of those previously published for fish protein hydrolysis (Hevia *et al.,* 1976; Baek & Cadwallader, 1995; Onodenalore & Shahidi, 1996). After 24 h, mackerel head gelatine hydrolysates had slightly higher DH (51 %) than mackerel skin gelatine hydrolysates (44 %).

Trypsin and chymotrypsin had lower hydrolytic activity compared to pepsin in this study under the experimental conditions. The calculated degree of hydrolysis was very low and did not increase during the hydrolysis period (24 h). The very limited effect of these two enzymes on mackerel head and skin gelatines, extracted after pre-treatment with citric acid, might be due to the structure of these gelatines which had a high level of cross-links as indicated by their lower solubility (Section 4.2.3.2). The broader specificity of pepsin made it more efficient than trypsin or chymotrypsin. The low pH associated with the optimum medium for pepsin, may have denaturated the gelatine and increased its susceptibility to hydrolysis (Sathe & Venkatachalam, 2007).

Mendis *et al.* (2005a) were able to hydrolyse hoki skin gelatine also with trypsin and chymotrypsin. Sztuka & Kołodziejska (2008) reported that unmodified fish gelatine films were almost completely hydrolysed by trypsin but pepsin hydrolysis was only about 60 %. Also the degree of hydrolysis for gelatines from skins of jumbo squid was 97 %, 87 % and 56 % for trypsin, chymotrypsin and pepsin hydrolysates, respectively (Mendis *et al.,* 2005b). These differences, compared to the present results, could be due to the variation in the gelatines, the extraction methods and the experimental conditions.

5.1.2. Degree of hydrolysis of mackerel viscera

Figure 5.4 shows the kinetics of hydrolysis for mackerel viscera after hydrolysis with the endogenous enzymes, pepsin, trypsin, chymotrypsin, Flavourzyme and Alcalase.

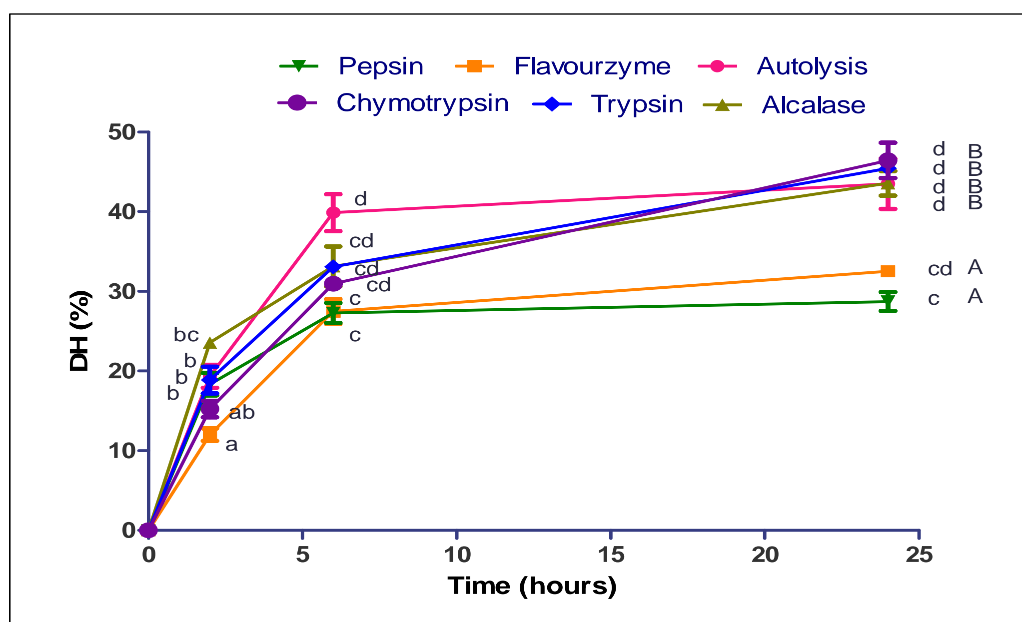


Figure 5.4 Evolution of the degree of hydrolysis for mackerel viscera as a function of hydrolysis time. Different lower case letters indicate significant ($p < 0.05$) differences between hydrolysis times. Different upper case letters indicate significant ($p < 0.05$) differences between enzymes.

The degree of hydrolysis was proportional to the time of incubation. The kinetics of the DH curves for all enzymes were similar. The degree of hydrolysis increased rapidly in the first 6 h and then the rate of reaction decreased. Pepsin and Alcalase showed significantly ($p < 0.05$) lower DH (28 and 32 %, respectively) than the other enzymes. At the end of the hydrolysis process, the degree of hydrolysis varied between 28 and 46 %.

All the enzymes were added at the same enzyme/substrate (E/S) ratio, in a weight (w/w) basis. Trypsin and chymotrypsin showed higher DH than pepsin suggesting that, in the present conditions, mackerel viscera had less available hydrophobic amino acid residues. The DH of Flavourzyme was lower than the DH of Alcalase which might be due to the fact that Flavourzyme does not hydrolyse peptide amide resulting in lower DH. In spite of being added at the same ratio, the activities and specificities of all the enzymes were different (Table 2.1 & Table 2.3), this may explain the difference in the DH among the various enzymes.

5.1.3. Protein and peptide patterns of FPH

5.1.3.1. Mackerel head and skin gelatines

Figure 5.5 shows the peptide profile of mackerel head and skin gelatine hydrolysates with pepsin, trypsin and chymotrypsin at different periods of time (0, 1, 2, 6 & 24 h).

The SDS-PAGE was used to study the molecular weight distribution of gelatines before and after hydrolysis. Results showed that high molecular weight proteins in intact gelatines were degraded into smaller peptides. For pepsin, most bands corresponding to subunits of un-hydrolysed gelatines (lane 2) disappeared after 2 h of hydrolysis for mackerel head gelatines (Figure 5.5A) and after 6 h for mackerel skin gelatines (Figure 5.5B). This might be due to the difference in the gelatines, which resulted in lower hydrolysis rate in the first 6 hours. After 24 h, it was observed that the majority of high molecular weight proteins (200 - 100 kDa) were hydrolysed into peptides with lower molecular weight (~ 17 kDa).

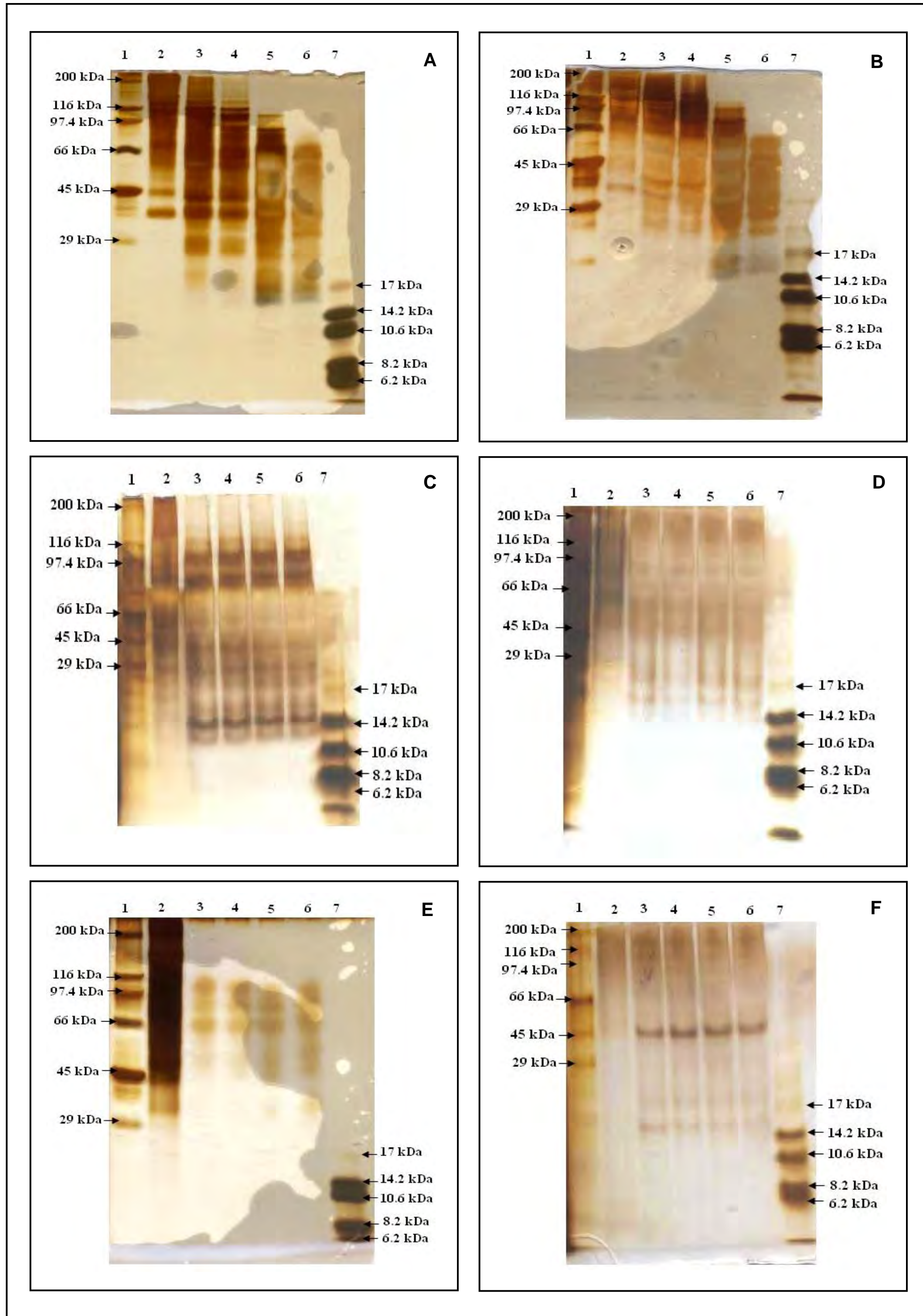


Figure 5.5 SDS-PAGE patterns of mackerel head (A, C & E) and skin (B, D & F) gelatine hydrolysates. (A & B) pepsin hydrolysates, (C & D) trypsin hydrolysates and (E & F) chymotrypsin hydrolysates. Lane 1: molecular weight marker (MW. 30,000 - 200,000); lane 2: 0 h hydrolysis; lane 3: 1 h hydrolysis; lane 4: 2 h hydrolysis; lane 5: 6 h hydrolysis; lane 6: 24 h hydrolysis; lane 7: low molecular weight peptides marker (MW. 2,500 - 17,000).

For head and skin gelatines, trypsin and chymotrypsin hydrolysates (Figure 5.5C, D, E & F) did not show variation between the peptide bands over the hydrolysis time. SDS-PAGE was mainly used for the detection of low molecular weight peptides. The result showed no distinct low molecular weight peptides other than some faint smearing that appeared in the 200 - 17 kDa region. These results were consistent with the determined degree of hydrolysis, indicating a limited hydrolysis of gelatine by trypsin and chymotrypsin.

5.1.3.2. Mackerel viscera

Figure 5.6 shows the peptide profile of mackerel viscera hydrolysates prepared with several enzymes for different periods of time (0, 2, 6 & 24 h).

The SDS-PAGE results showed that as the hydrolysis proceeded, high molecular weight proteins observed in intact viscera (Figure 5.6, lane 2) are broken down into smaller peptides. After 2 h and 6 h of hydrolysis with an average DH of 15 % and 30 %, respectively, few visible bands (in the range of 200 - 45 kDa) and a cluster in the low molecular weight region, were observed (Figure 5.6, lane 3 & 4). At the end of hydrolysis at 24 h (average DH of 50 %), a distinctive visible band, less than 6 kDa, was visible (Figure 5.6, Lane 5).

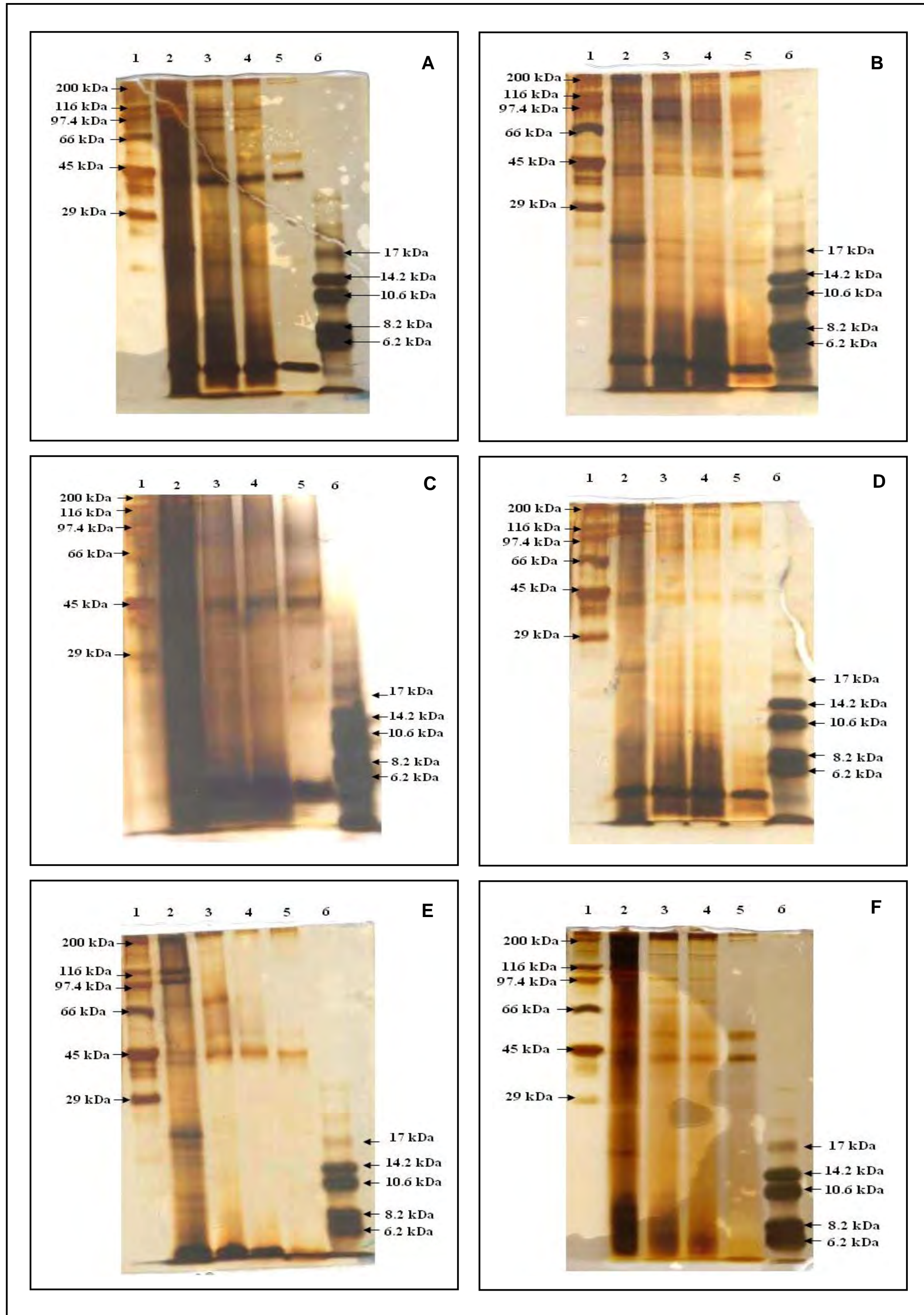


Figure 5.6 SDS-PAGE patterns of mackerel viscera hydrolysates. (A) Flavourzyme hydrolysates, (B) trypsin hydrolysates, (C) pepsin hydrolysates, (D) hydrolysates from autolysis, (E) chymotrypsin hydrolysates and (F) Alcalase hydrolysates. Lane 1: molecular weight marker (MW. 30,000 - 200,000); lane 2: 0 h hydrolysis; lane 3: 2 h hydrolysis; lane 4: 6 h hydrolysis; lane 5: 24 h hydrolysis; lane 6: low molecular weight peptides marker (MW. 2,500 - 17,000).

Fish viscera represent a cheap source of digestive proteases (Pavlisko *et al.,* 1997b; Simpson & Haard, 1999), mainly phosphorylases, lipases, cathepsins and gut enzymes (Mukundan *et al.,* 1985). Mackerel viscera were found to contain several proteolytic enzymes (Castillo-Yanez *et al.* 2005; Kishimura *et al.,* 2006). Despite the efficiency of endogenous enzymes in hydrolysing mackerel viscera, this process was not preferable due to possible variation among fish viscera according to season, sex and habitat (Venugopal, 2008).

5.2. Screening for bioactivity

5.2.1. Antioxidant activity

5.2.1.1. Mackerel gelatine hydrolysates

The *in vitro* antioxidant activity of the gelatine hydrolysates was measured using the DPPH and FRAP assays. Figure 5.7 shows the DPPH scavenging activities of gelatines from mackerel heads and skins after hydrolysis with pepsin, trypsin and chymotrypsin for 24 h. Hydrolysates from mackerel head and skin gelatines obtained after hydrolysis with pepsin for 24 h possessed strong ability to quench the DPPH radical. No significant difference ($p > 0.05$) between the antioxidant activities of mackerel head and skin gelatines hydrolysates were observed. The scavenging effect for the hydrolysates increased by increasing the hydrolysis time, and reached the highest activity (80 % reduction of DPPH) at the longest period of hydrolysis (24 h) for pepsin-digested mackerel skin gelatines. DPPH screening showed that hydrolysates from mackerel head and skin gelatines obtained from trypsin and chymotrypsin had lower antioxidant activities than those prepared using pepsin.

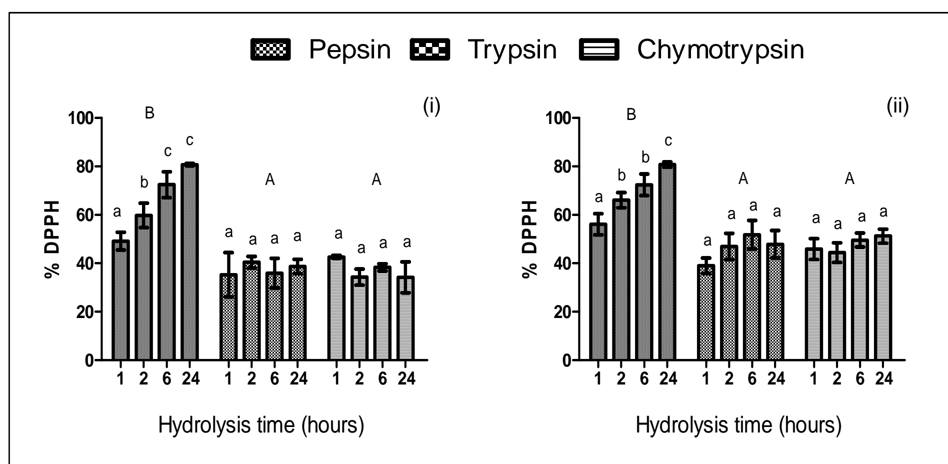


Figure 5.7 The DPPH scavenging activity of mackerel gelatine hydrolysates at different hydrolysis times. Gelatine hydrolysates from: (i) mackerel heads and (ii) mackerel skins. Different lower case letters indicate significant ($p < 0.05$) differences between hydrolysis times. Different upper case letters indicate significant ($p < 0.05$) differences between enzymes.

For both gelatines, trypsin and chymotrypsin hydrolysates showed lower antioxidant activities compared to pepsin hydrolysates and their antioxidant activities did not increase by increasing the hydrolysis time. It was also observed that there was a positive correlation between the degree of hydrolysis using pepsin and the antioxidant activity with DPPH ($R^2 = 0.96$ for head gelatine & $R^2 = 0.90$ for skin gelatine).

When testing the *in vitro* antioxidant activity by FRAP assay (Figure 5.8) both head and skin gelatines hydrolysates had similar activity values. The antioxidant activity of the hydrolysates increased with increasing hydrolysis time, with hydrolysates at 6 and 24 h having the highest activity (~ 0.125 mM Trolox equivalent).

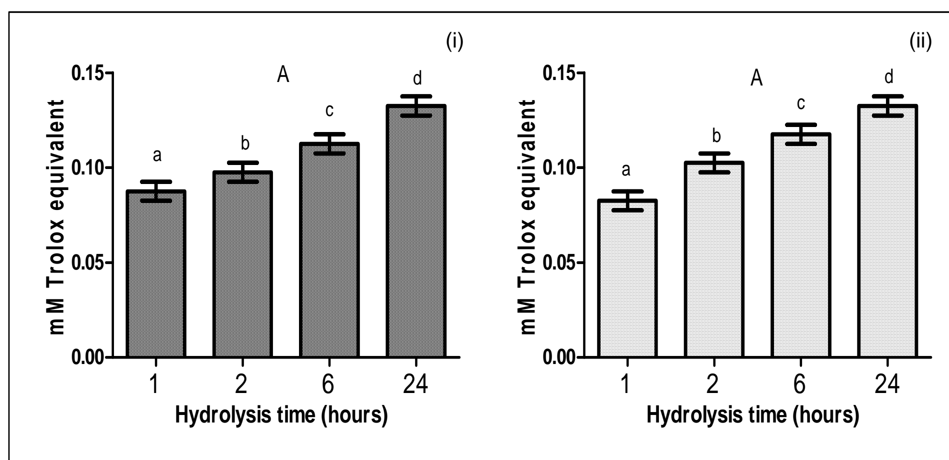


Figure 5.8 The reducing power (FRAP) of mackerel gelatine hydrolysates, prepared using pepsin, at different hydrolysis times. Gelatine hydrolysates from: (i) mackerel heads and (ii) mackerel skins. Different lower case letters indicate significant ($p < 0.05$) differences between hydrolysis times. Different upper case letters indicate significant ($p < 0.05$) differences between sources.

A positive correlation between the degree of hydrolysis and the antioxidant activity with FRAP was found (R^2 = 0.92 for head gelatine & R^2 = 0.94 for skin gelatine) was observed.

The ability of peptides to act as an antioxidant (especially for lipids and fatty acids oxidation) seems to be linked to the presence of certain amino acids (Tyr, Met, His, Lys and Trp) that possess the ability to bind metal ions. The amino acid composition, the primary sequence and conformation of peptides are factors that can explain their antioxidant activity (Pena-Ramos & Xiong, 2001; Elias *et al.,* 2006). In addition, fish and marine gelatines have also proven to be good sources of antioxidant peptides, such as jumbo squid (*Dosidicus gigas*) skin gelatine (Mendis *et al.,* 2005a), jumbo flying squid (*Dosidicus eschrichitii Steenstrup*) skin gelatine (Lin & Li, 2006) and hoki (*Johnius belengerii*) skin gelatine (Mendis *et al.,* 2005a).

5.2.1.2. Mackerel viscera hydrolysates

The *in vitro* antioxidant activity estimated by the DPPH and the FRAP assays of mackerel viscera hydrolysates are presented in Figure 5.9 and Figure 5.10, respectively. All hydrolysates showed high DPPH radical scavenging activity. However, at all the hydrolysis times, hydrolysates obtained with pepsin had significantly ($p < 0.05$) lower antioxidant activity than hydrolysates prepared with the rest of enzymes. Regardless of the enzyme, the lowest DPPH radical scavenging activity was observed after 2 h of hydrolysis for all hydrolysates and no significant ($p > 0.05$) differences were observed between the antioxidant activity at 6 and 24 h of hydrolysis (Figure 5.9).

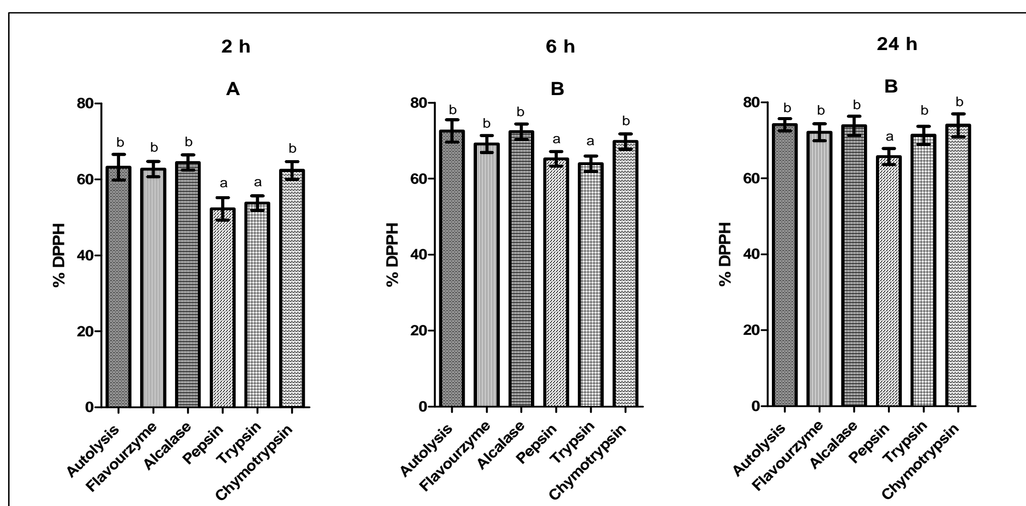


Figure 5.9 The DPPH scavenging activity of mackerel viscera hydrolysates at different hydrolysis times. Different lower case letters, within the same hydrolysis time, indicate significant ($p < 0.05$) differences between enzymes. Different upper case letters indicate significant ($p < 0.05$) differences between hydrolysis times.

All hydrolysates had similar reducing power at 2 and 6 h of hydrolysis. Significantly ($p < 0.05$) higher antioxidant activity was observed after 24 h of hydrolysis. Regardless of hydrolysis times, hydrolysates obtained with

chymotrypsin, Alcalase and the endogenous enzymes had significantly ($p < 0.05$) higher reducing power (Figure 5.10).

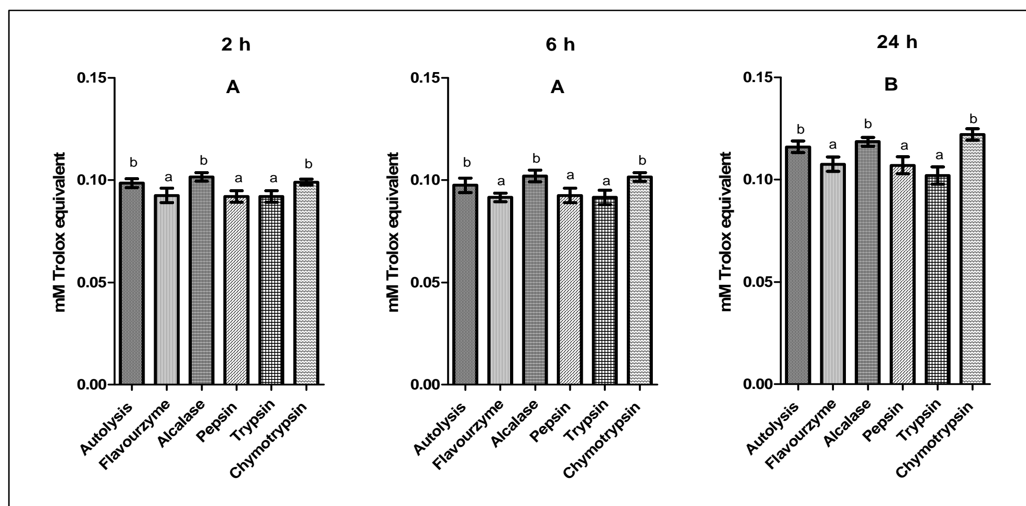


Figure 5.10 The reducing power (FRAP) of mackerel viscera hydrolysates at different hydrolysis times. Different lower case letters, within the same hydrolysis time, indicate significant ($p < 0.05$) differences between enzymes. Different upper case letters indicate significant ($p < 0.05$) differences between hydrolysis times.

It appears that protein hydrolysates from mackerel viscera could function by donating electrons to the free radicals. The reducing power of mackerel viscera protein hydrolysate was found to be dependent on the DH and the enzyme used. It was also observed that there is a positive correlation between the degree of hydrolysis and the antioxidant activity on DPPH ($R^2 = 0.99$ for Alcalase, trypsin and chymotrypsin; $R^2 = 0.97$ for autolysis and Flavourzyme & $R^2 = 0.86$ for pepsin). From these results, it could be concluded that mackerel viscera hydrolysates had high antioxidant activity and pepsin was the least efficient enzyme.

Previous investigations reported that mackerel (*Scomber austriasicus*) hydrolysates prepared by protease N showed strong *in vitro* antioxidant activity (Chuang *et al.,* 2000; Wu *et al.,* 2003). Mackerel intestinal crude enzymes were successfully used to produce peptides with antioxidant properties from Alaska pollack (*Theragra chalcogramma*) frame proteins (Je *et al.,* 2005a). Alcalase and Flavourzyme produced hydrolysates with high antioxidant activity when used to hydrolyse muscle of round scad (*Decapterus maruadsi*) and yellow stripe trevally (Thiansilakul *et al.,* 2007; Klompong *et al.,* 2007)

Other studies reported antioxidant peptides from protein hydrolysates of Alaska pollack (Kim *et al.,* 2001), capelin (Shahidi & Amarowicz, 1996; Amarowiz & Shahidi, 1997), tuna (Jao & Ko, 2002; Je *et al.,* 2007), conger eel (Ranathunga *et al.,* 2006), herring (Sathivel *et al.,* 2003), hoki (Je *et al.,* 2005b), cod (Jeon *et al.,* 1999), carp (Li *et al.,* 2006) and yellowfin sole (Jun *et al.,* 2004).

5.2.2. ACE inhibitory activity

5.2.2.1. Mackerel gelatine hydrolysates

Mackerel head and skin gelatine hydrolysates obtained after digestion with pepsin for 24 h possessed strong activity against the ACE (Figure 5.11).

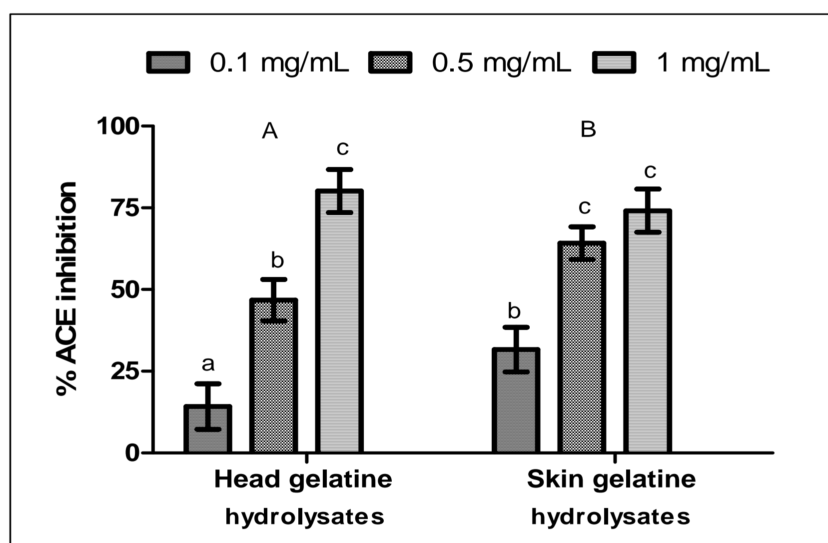


Figure 5.11 The ACE inhibitory activity of mackerel gelatine hydrolysates at different concentrations. Different lower case letters, within the same hydrolysates, indicate significant ($p < 0.05$) differences between concentrations. Different upper case letters, indicate significant ($p < 0.05$) differences between sources.

Previous studies showed that collagen and gelatine hydrolysates are possible potential sources for peptides with ACE inhibitory activity (Fahmi *et al.,* 2004; Zhao *et al.,* 2007). It was reported that ACE is strongly inhibited by peptides containing hydrophobic amino acids at the C-terminal sequence (Je *et al.,* 2004). Pepsin preferentially cleaves peptide bonds between hydrophobic amino acids. Hence, the hydrolysis of mackerel gelatine by pepsin increases the amount of hydrophobic amino acids which can act as potent ACE inhibitors (see section 5.6).

5.2.2.2. Mackerel viscera hydrolysates

Figure 5.12 shows the ACE inhibitory of mackerel viscera hydrolysates at various concentrations. Mackerel viscera hydrolysed with endogenous enzymes showed significantly ($p < 0.05$) highest ACE inhibitory activity at the highest concentration (1 mg/mL). No significant differences ($p > 0.05$) were observed between the ACE inhibitory activity, at the same protein concentration, of the hydrolysates from Flavourzyme, trypsin and chymotrypsin.

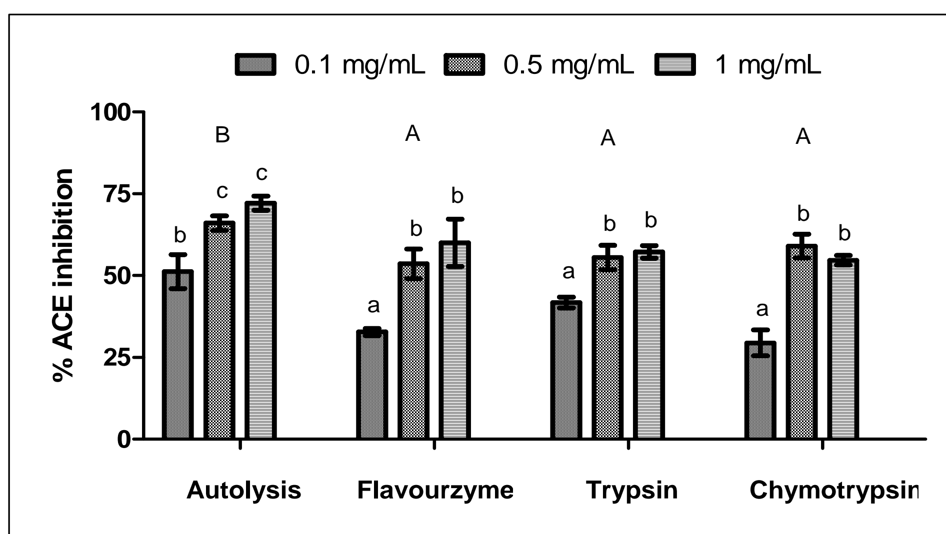


Figure 5.12 The ACE inhibitory activity of mackerel viscera hydrolysates at different concentrations. Different lower case letters, within the same enzyme, indicate significant ($p < 0.05$) differences between concentrations. Different upper case letters, indicate significant ($p < 0.05$) differences between enzymes.

The digestive tract in most fish species contains several proteolytic enzymes (Kolkovski, 2001), mainly pepsin and trypsin (Kishimura *et al.,* 2007; Pavlisko *et al.,* 1997a: Pavlisko *et al.,* 1997b; Bezerra *et al.,* 2000). Pepsin, as previously discussed, liberates hydrophobic amino acids that act as potent ACE inhibitors. Trypsin cleaves arginine and lysine. It was reported that the positive

charge in guanidine group of Arg plays a major role in inhibiting ACE (Meisel, 1998). Hence, the higher inhibition of ACE activity, observed with hydrolysates obtained from the autolysis, can be due to the combined effect of several digestive enzymes.

5.2.3. Semicarbazide-sensitive amine oxidase SSAO inhibitory activity

The SSAO inhibitory activity of hydrolysates from mackerel gelatines and viscera were assessed. Table 5.1 shows the inhibition of SSAO by different hydrolysates. Mackerel head and skin gelatine hydrolysates, obtained from pepsin after 24 h, showed about 16 % inhibition of SSAO when present in the incubation mixture at 0 min. When added to the assay mixture 2 h before adding benzylamine (substrate) the inhibition was more substantial (~ 50 %). Mackerel head and skin gelatines hydrolysates obtained after hydrolysis for 24 h with trypsin and chymotrypsin did not show any inhibition of SSAO, possibly due to the limited hydrolysis of gelatine by these enzymes. Mackerel viscera hydrolysates, obtained from autolysis and hydrolysis by Flavourzyme at 24 h, showed about 20 % inhibition of SSAO at time 0. When these hydrolysates were incubated with the enzyme before adding the substrate (benzylamine) the inhibition was stronger (52 % and 46 %, respectively). Mackerel viscera hydrolysates generated from hydrolysis by trypsin and chymotrypsin at 24 h, showed a similar trend in the inhibition of SSAO as for hydrolysates obtained from autolysis and hydrolysis by Flavourzyme. However, their inhibition of SSAO was about half of that observed with autolysis and Flavourzyme hydrolysates. No effect on SSAO activity was detected for hydrolysates obtained from pepsin and Alcalase.

Table 5.1 The SSAO inhibitory activities (%) of fish protein hydrolysates.

	Skin gelatines		Head gelatines		Viscera	
	t = 0	t = 2 h	t = 0	t = 2 h	t = 0	t = 2 h
Pepsin	$15±1.6^{bA}$	$51.3±2.2^{bB}$	$17±0.9^{bA}$	$49.8±1.9^{bB}$	$10.0±0.7^{aA}$	$9.5±1.2^{aA}$
Trypsin	$8.2±1.0^{aA}$	$8.4±1.1^{aA}$	$9.0±2.4^{aA}$	$8.3±1.4^{aA}$	$8.4±3.5^{aA}$	$23.5±8.1^{bB}$
Chymotrypsin	$8.5±0.8^{aA}$	$8.8±1.1^{aA}$	$10.7±0.9^{aA}$	$11.0±0.6^{aA}$	$8.9±1.8^{aA}$	$23.5±8.1^{bB}$
Endogenous	-	-	-	-	$18.8±3.2^{bA}$	$52.2±1.9^{cB}$
Flavourzyme	-	-	-	-	$20.8±4.5^{bA}$	$45.8±3.1^{cB}$
Alcalase	-	-	-	-	$6.3±0.6^{aA}$	$10.0±0.7^{aA}$

Values given as mean ± standard deviation. Different lower case letters in the same column indicate significant ($p < 0.05$) differences between enzymes. Different upper case letters in the same row, within the same raw material, indicate significant differences ($p < 0.05$) between the incubation times.

Hydrolysates from mackerel head and skin gelatines obtained after hydrolysis with pepsin for 24 h and hydrolysates from mackerel viscera obtained after hydrolysis with endogenous enzymes, Flavourzyme, trypsin and chymotrypsin for 24 h were found to exhibit time-dependent SSAO inhibitory activities. This result may be due to the presence of L-lysine, which is known to be an inhibitor of SSAO in a time and dose-dependent manner (Olivieri *et al.,* 2007).

The results showed that mackerel hydrolysates (either from gelatines or viscera) may act as anti-inflammatory compounds by inhibiting the SSAO. It is known that the SSAO converts primary amines into the corresponding aldehydes, generating hydrogen peroxide and ammonia. It was found that the endogenous compounds amino-acetone and methylamine are good substrates for most SSAO enzymes (Lyles & Chalmers, 1992). SSAO plays a key role in inflammation through its catalytic products, hydrogen peroxide, and reactive aldehydes. Therefore, the inhibition of SSAO activity might represent a therapeutic strategy for controlling inflammation (Lin *et al.,* 2008).

5.2.4. Platelet aggregation inhibitory activity

The platelet aggregation inhibitory activity was estimated by the ADP secretion assay. Samples were analysed at 3 different concentrations (0.6 µg/mL, 6 µg/mL & 60 µg/mL). Figure 5.13 shows the amount of ADP released (normalised) from platelets in the presence of mackerel head and skin gelatine hydrolysates obtained after hydrolysis with pepsin for 24 h. No significant differences ($p > 0.05$) were observed between the head and skin hydrolysates at

0.6 and 6 µg/mL, respectively. However, mackerel skin gelatine hydrolysates showed higher activities at 60 µg/mL. These activities were similar to that of the control inhibitors, acetyl salicylic acid and indomethacine (Figure 5.13).

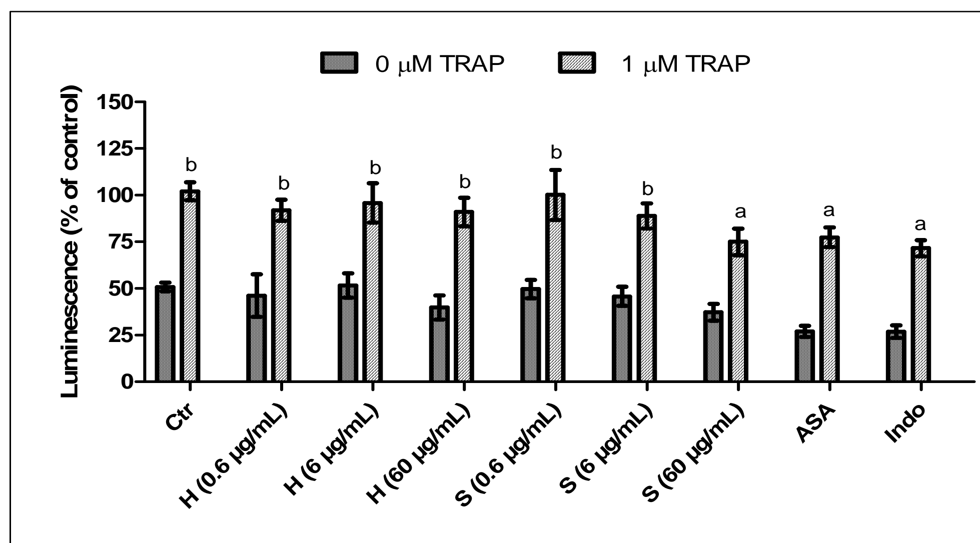


Figure 5.13 The platelet aggregation inhibitory activity of mackerel gelatine hydrolysates. Ctr: control (water), H: mackerel head gelatine hydrolysates, S: mackerel skin gelatine hydrolysates and TRAP: Thrombin receptor activating peptide. ASA (Aspirin) and Indo (Indomethacine) were used as positive controls (inhibitors). Different letters indicate significant ($p < 0.05$) differences.

Mackerel viscera hydrolysates did not show any activity when screened for inhibition of platelet aggregation (Appendix I). The inhibition of platelet aggregation by peptides has been related to the presence of tri-peptide Arg-Gly-Asp (also called RGD) which blocks the binding of fibrinogen to the activated platelets receptors (Sheu *et al.,* 1992). So far, some peptides have been identified as inhibitor for platelet aggregation. Arg-Gly-Asp sequences were previously observed in chymotryptic fragmentation of trigramin, a single chain cysteine-rich peptide (Huang *et al.,* 1987), in halysin, a cysteine-rich peptide (Huang *et al.,* 1991) as well as in triflavin, an Arg-Gly-Asp-containing, snake venom peptide (Sheu *et al.,* 1992).

The inhibition of platelet aggregation of mackerel skin gelatine hydrolysates obtained after digestion with pepsin for 24 h, suggest that this hydrolysate may contain repetitive RGD sequences.

5.2.5. Effect of FPH on microbial growth

5.2.5.1. Effect on probiotic strains

Prebiotics are usually non digestible food ingredients that stimulate the microbial growth (Lee *et al.,* 2002). Fish protein hydrolysates are usually highly digestible (Folador *et al.,* 2006). Moreover, fish protein hydrolysates are favourable substrates for bacterial growth (Dufossé *et al.,* 1997) due to the higher nitrogen content. Hence, fish protein hydrolysates can not considered as prebiotic.

In order to study the effect of fish protein hydrolysates on the growth of lactic acid bacteria and bifidobacteria, these strains were supplemented with the hydrolysates and the growth was monitored. Table 5.2 shows the enhanced growth when probiotic strains were supplemented with 100 µg of fish protein hydrolysates for 24 h compared to control (water) and reported as Log_{10} (cfu/mL).

Table 5.2 The enhanced growth when probiotic strains were supplemented with 100 µg of fish protein hydrolysates for 24 h compared to control (water).

Sample	Enzyme	*L. plantarum* Log_{10} (cfu/mL)	*B. breve* Log_{10} (cfu/mL)
Skin gelatines	Pepsin	4.89±0.02[c]	2.88±0.05[d]
Head gelatines	Pepsin	1.92±0.02[b]	1.16±0.02[c]
Viscera	Endogenous	4.04±0.02[c]	0.26±0.07[b]
Viscera	Flavourzyme	-0.01±0.03[a]	-0.25±0.03[a]
Viscera	Trypsin	2.02±0.01[b]	0.91±0.07[c]
Viscera	Chymotrypsin	4.69±0.04[c]	1.07±0.03[c]

Values given as mean ± standard deviation. Different letters in the same column indicate significant ($p < 0.05$) differences.

In general, higher growth of lactic acid bacteria, based on cfu/mL compared to control (water), than bifidobacteria was observed for all hydrolysates tested. For both bacteria, hydrolysates from mackerel skin gelatines stimulated higher growth than mackerel head gelatine hydrolysates. For mackerel viscera, hydrolysates obtained after hydrolysis with endogenous enzymes and chymotrypsin resulted in higher microbial growth. Both lactic acid bacteria and bifidobacteria did not grow compared to the control when supplemented with hydrolysates prepared with Flavourzyme. These hydrolysates may have an antibacterial activity, which may inhibit the growth of the probiotic strains.

5.2.5.2. Antimicrobial activity

No fish hydrolysats had antimicrobial activity against Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). When tested against Gram positive bacteria, hydrolysates obtained from mackerel viscera after hydrolysis with Flavourzyme, slightly inhibited *Staphylococcus aureus* (Figure 5.14A); *Lactobacillus plantarum* and *Bifidobacterium breve* were also inhibited by these hydrolysates. It is known that Gram negative bacteria are more resistant to antibacterial compounds (such as lysozyme and penicillin) than Gram positive bacteria due to the difference in the structure of their cell wall (Lehner *et al.,* 2005).

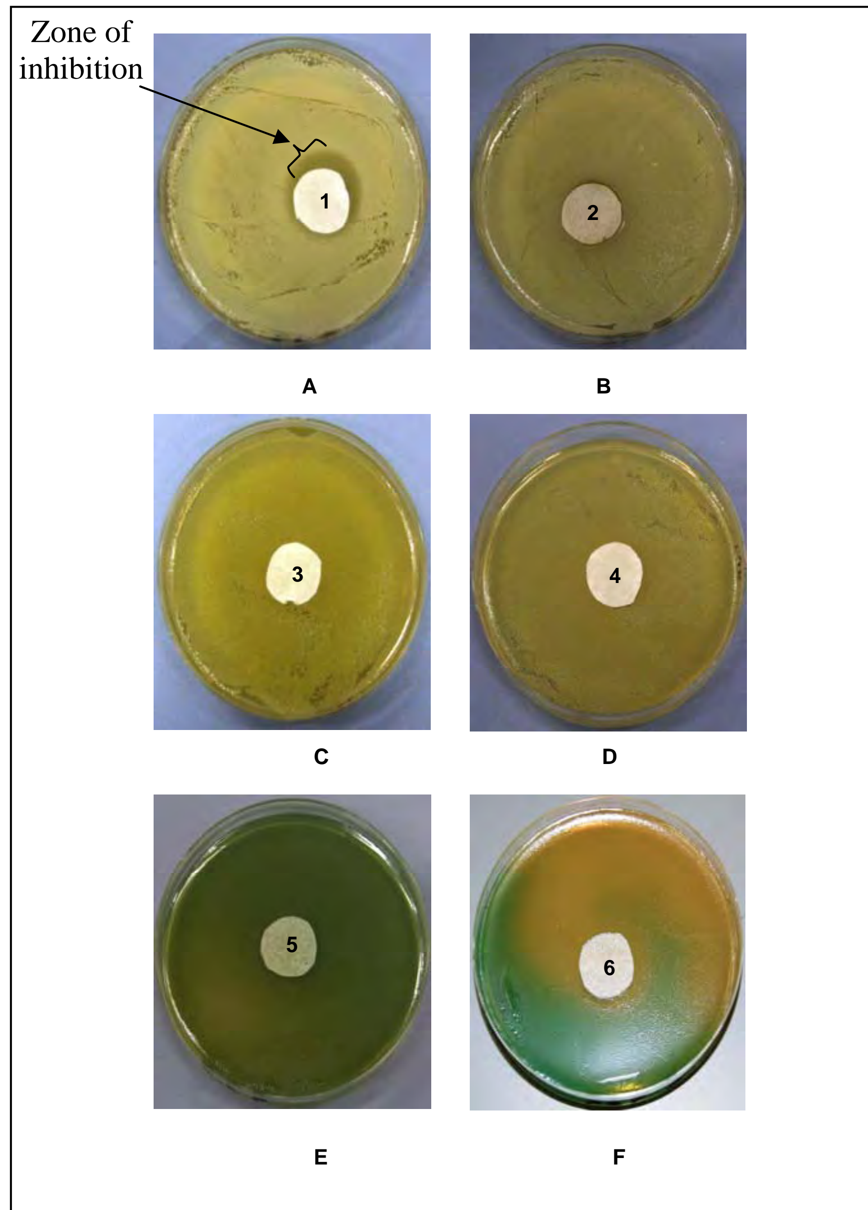


Figure 5.14 Zone of inhibition obtained in anti-bacterial disc diffusion assay. Discs 1, 3 & 5 were soaked in hydrolysates from mackerel viscera digested by Flavourzyme for 24 h and discs 2, 4 & 6 were soaked in sterile water (control). A & B assay against *S. aureus*, C & D assay against *E. coli* and D & F assay against *P. aeruginosa*.

5.3. Fractionation of bioactive FPH

To identify the compounds that are responsible for the different bioactivities, samples were fractionated using a preparative HPLC. The bioactivity level of fish protein hydrolysates are summarised in Table 5.3.

Mackerel skin gelatine hydrolysates showed higher bioactive properties than mackerel head gelatine hydrolysates. For mackerel viscera hydrolysates, endogenous enzymes generated hydrolysates with higher bioactive properties followed by hydrolysates from Flavourzyme. Due to the difficulty of controlling the autolysis, as a result of the non-specificity of the endogenous enzymes and the variability according to the season, sex, habitat and diet (Liaset *et al.,* 2000), Flavourzyme hydrolysates were preferred.

Table 5.3 Bioactivity level of fish protein hydrolysates.

Sample	Enzyme	Bioactive assays							
		Antioxidant		Bacterial growth		Inhibition of			
		DPPH	FRAP	*L. plantarum*	*B. breve*	Platelet aggregation	ACE	SSAO	Gram positive bacteria
Head gelatines	Pepsin	+++	++	++	++	+++	+++	+++	-
Skin gelatines	Pepsin	+++	++	++++	++++	++++	++	++++	-
Viscera	Endogenous	++	++	++++	+	+	++++	++++	-
Viscera	Flavourzyme	++	++	-	-	+	+++	+++	+
Viscera	Trypsin	++	++	++	++	+	+	+	-
Viscera	Chymotrypsin	++	+++	++++	++	+	+	+	-

Very low activity (-), low activity (+), average activity (++) and high activity (+++) and very high activity (++++).

Therefore, mackerel skin gelatine hydrolysates obtained from pepsin and mackerel viscera hydrolysates produced by Flavourzyme were selected for fractionation based on their bioactivity level (Table 5.3).

HPLC chromatograms of peptides, from these two hydrolysates, separated by reversed-phase HPLC column (ODS-2 C_{18}) are depicted in Figure 5.15. The majority of compounds (i.e., small peptides or amino acids) were eluted between 4 and 30 min for mackerel skin gelatine hydrolysates (Figure 5.15A) and between 2 and 18 min for mackerel viscera hydrolysates (Figure 5.15B).

The separation of peptides on ODS-2 C_{18} column was based on their polarity with no effect of their molecular mass. The peptides appearing in the beginning of the chromatogram were more hydrophilic than those separated afterwards (Wanasundara *et al.,* 2002). Factors affecting the polarity of peptides include the amino acid composition and ionisation state of the peptides (Lemieux & Amiot, 1989).

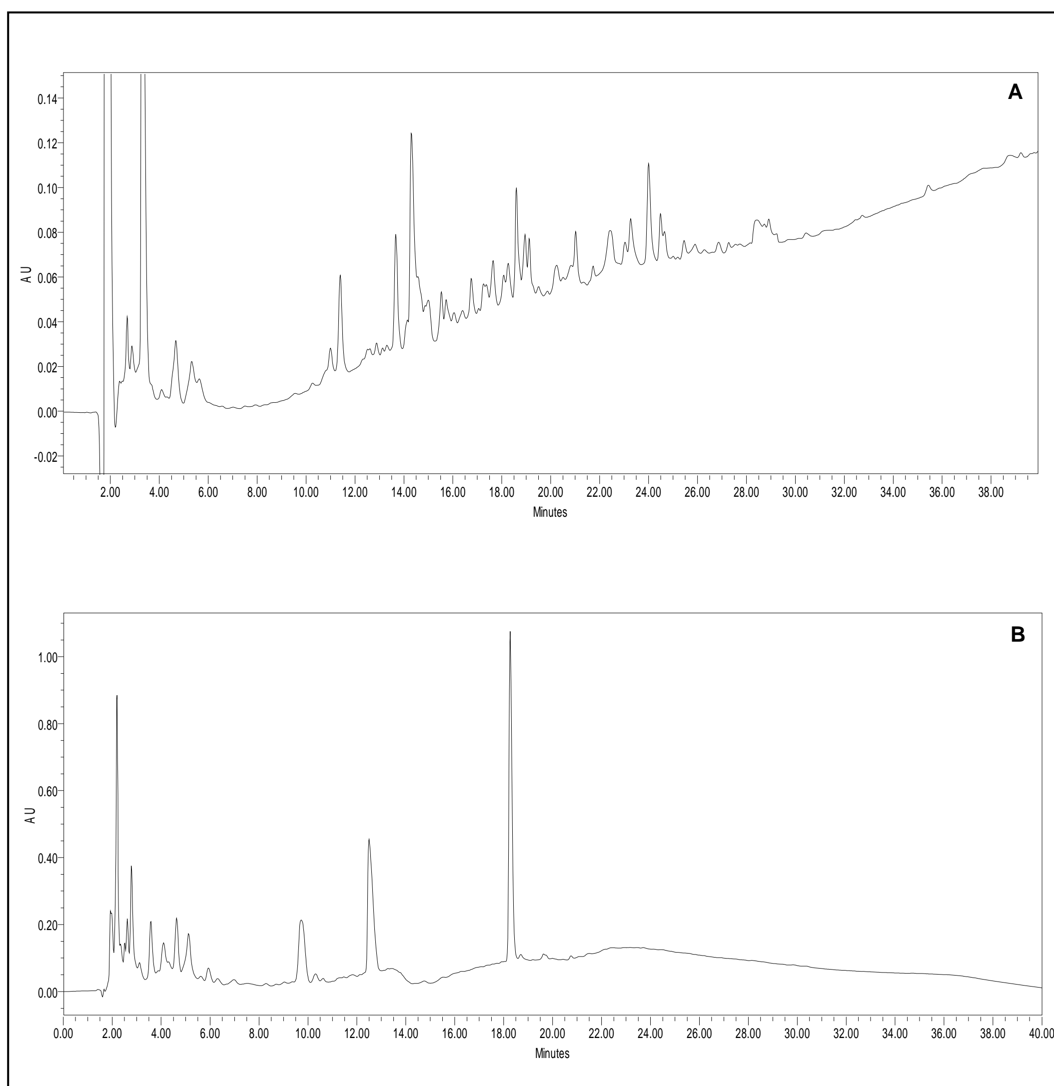


Figure 5.15 RP (C_{18}) HPLC chromatograms of mackerel hydrolysates. (A) mackerel skin gelatine hydrolysates digested by pepsin for 24 h and (B) mackerel viscera hydrolysates digested by Flavourzyme for 24 h.

Figure 5.16 shows the preparative HPLC chromatograms of mackerel skin gelatine and viscera hydrolysates.

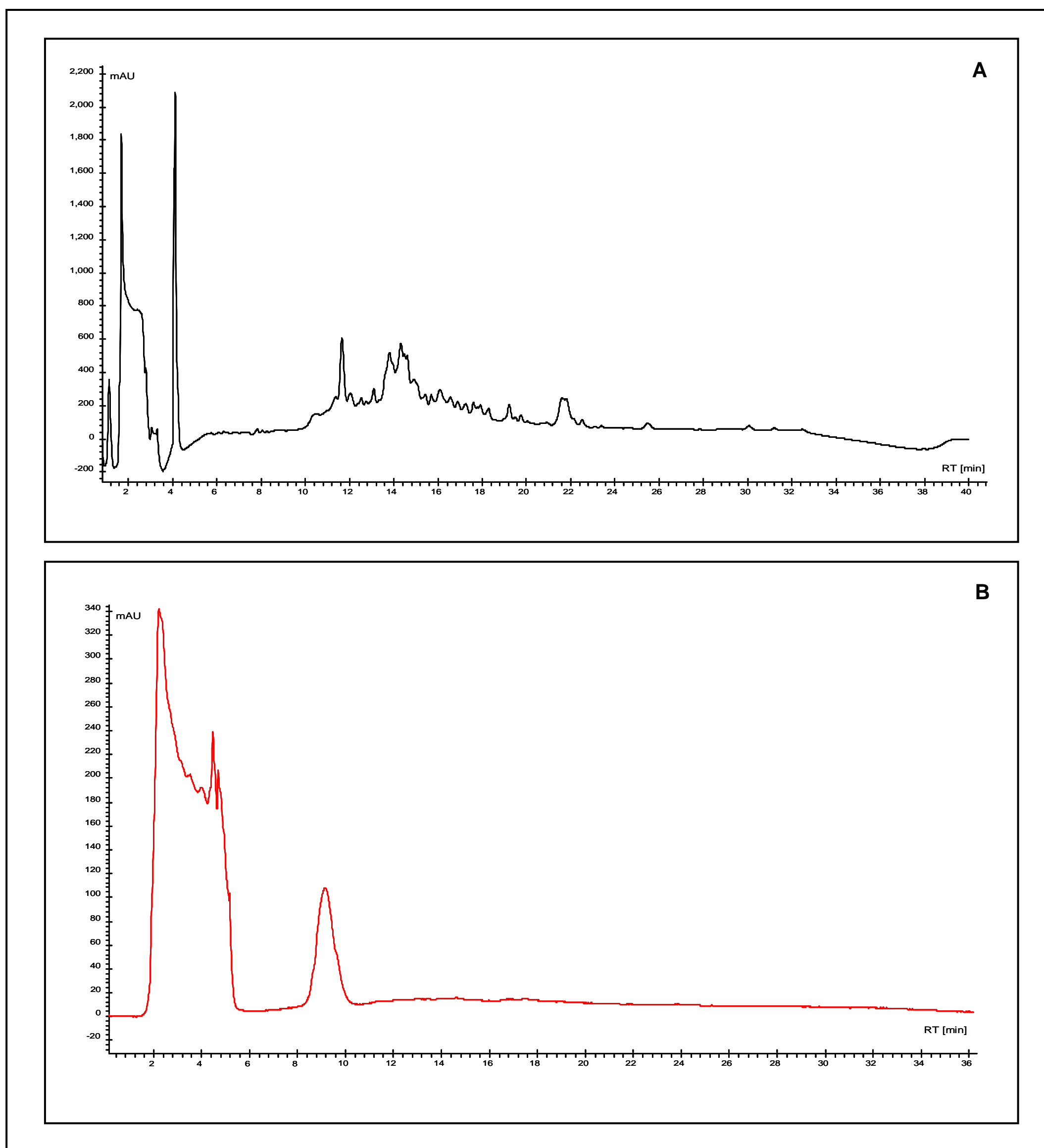


Figure 5.16 RP (C_{18}) preparative HPLC chromatograms of mackerel hydrolysates. (A) mackerel skin gelatine hydrolysates digested by pepsin for 24 hours and (B) mackerel viscera hydrolysates digested by Flavourzyme for 24 hours.

Peaks that appeared at 13 and 18 min (Figure 5.15B) were not in the preparative HPLC chromatograms of mackerel viscera hydrolysates (Figure 5.16B). This could be due to the filtration through the 3 kDa cut-off filter. These two peaks correspond to peptides that are less polar and have higher molecular size than the rest of peptides since they were eluted at the end of the analysis.

The fractions were collected in the basis of time (30 seconds). The presence of eluted peptides and their concentrations were determined by the OPA method (Figure 5.17).

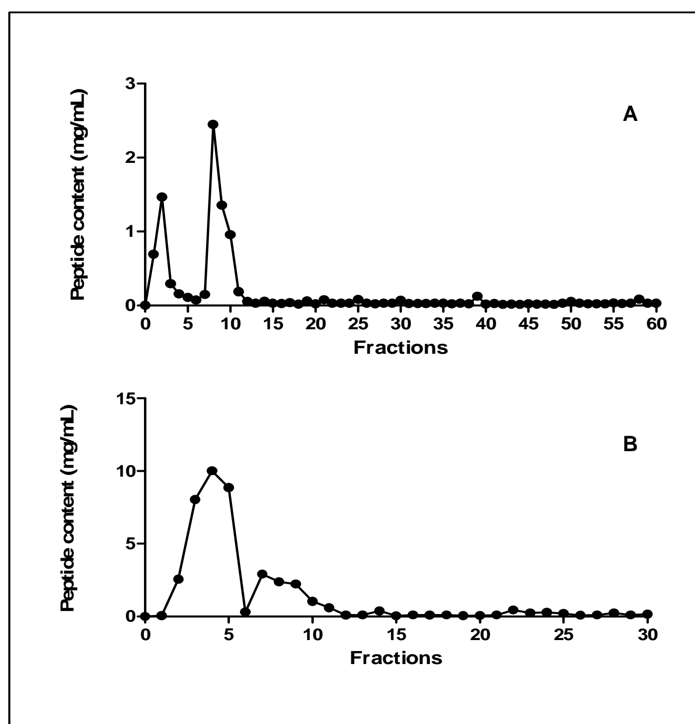


Figure 5.17 Peptide content of mackerel skin gelatines (A) and viscera (B) fractionated hydrolysates.

For mackerel skin gelatine hydrolysates, the preparative chromatography eluted 60 fractions with different peptide contents (Figure 5.17A). Two major peaks with high peptide contents (~ 1.5 and 2.5 mg/mL, respectively) were observed in the first 10 fractions. The first 10 - 12 fractions separated from mackerel viscera hydrolysates (Figure 5.17B) had high peptide contents with fractions number 3, 4 and 5 having significantly ($p < 0.05$) the highest amounts. For both mackerel skin gelatines and viscera hydrolysates, very low peptide contents were observed for samples eluted after fraction number 15.

5.4. Screening for bioactivity of fractionated FPH

5.4.1. Mackerel skin gelatine hydrolysates

Ten fractions (denoted 1 - 10) obtained from mackerel skin gelatine hydrolysates were analysed for bioactivities against several assays (Table 5.4). The DPPH scavenging activities of all fractions showed no significant ($p > 0.05$) differences. However, significantly ($p < 0.05$) higher reducing power (0.11 mM Trolox equivalent) was observed for fraction number 4 with the FRAP assay compared to the rest of fractions. The antioxidant activity observed for the crude sample (non-fractionated skin gelatine hydrolysates lower than 3000 Da) was mainly due to fraction number 4.

Table 5.4 Fractionation of mackerel skin gelatine hydrolysates and screening for bioactivities of corresponding fractions.

	Applied eluents										non fractionated hydrolysates
A (%)	94.8	93.2	91.6	90	88.4	86.8	85.2	83.6	82	80.4	
B (%)	5.2	6.8	8.4	10	11.6	13.2	14.8	16.4	18	19.6	
Fractions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
DPPH (%)	57.7[a]	60.2[a]	57.8[a]	62.7[a]	60.2[a]	58.8[a]	58.4[a]	58.3[a]	54.7[a]	52.6[a]	74.6[b]
FARP (mM Trolox)	0.07[a]	0.07[a]	0.07[a]	0.11[b]	0.07[a]	0.06[a]	0.06[a]	0.06[a]	0.06[a]	0.06[a]	0.12[b]
ACE (%)	48.4[c]	24.5[b]	18.6[b]	75.6[d]	34.2[b]	8.1[a]	20.9[b]	47.7[c]	58.1[c]	24.6[b]	78.1[d]
SSAO (%)	46.0[d]	32.4[c]	32.0[c]	17.5[b]	30.7[c]	19.6[b]	9.5[a]	21.6[b]	25.8[b]	25.0[b]	49.3[d]

A: Water & B: Acetonitrile. Both eluents (A & B) contained 0.1 % TFA. Different letters in the same row indicate significant ($p < 0.05$) differences.

The evaluation of the antihypertensive properties showed that fractions number 1, 4, 8 and 9 had higher ACE inhibitory activities compared to the rest of fractions. Fraction number 4 was significantly ($p < 0.05$) very potent against ACE (75.6 %) and showed inhibition activity slightly lower than that observed for the non fractionated hydrolysates (78.1 %). The screening for anti-inflammatory activities found that three adjacent fractions (1, 2, and 3) had significantly ($p < 0.05$) higher SSAO inhibitory activity. It was also observed that fractions 2 and 3 exhibited similar efficacy on inhibiting the SSAO (~ 32 %) and the SSAO inhibitory activity of fraction number 1 was very close to that of the non fractionated hydrolysates.

The platelet aggregation inhibitory activities of various fractions from mackerel skin gelatine hydrolysates are presented in Figure 5.18.

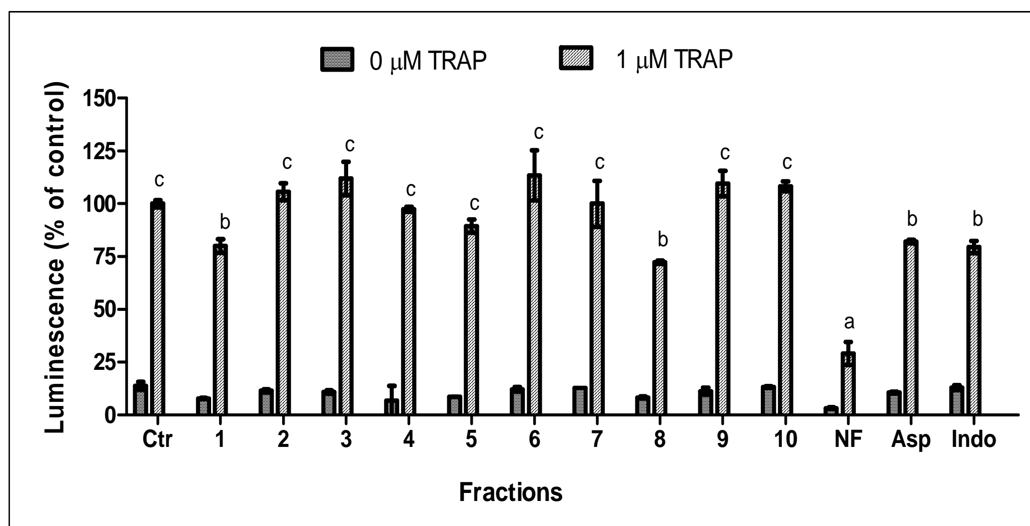


Figure 5.18 The platelet aggregation inhibitory activity of fractionated mackerel skin gelatine hydrolysates. Ctr: control (water), NF: non fractionated hydrolysates (< 3000 Da), TRAP: Thrombin receptor activating peptide. Aspirin (ASA) and Indomethacine (Indo) were used as positive controls (inhibitors). Different letters indicate significant ($p < 0.05$) differences.

It was observed that fractions 1, 5 and 8 were the most active against the platelet aggregation with activities close to that of the commercial inhibitors but lower than the non fractionated mackerel skin gelatine hydrolysates (< 3000 Da).

5.4.2. Mackerel viscera hydrolysates

Table 5.5 shows the antioxidant, antihypertensive and anti-inflammatory activities of 15 fractions (denoted 1 - 15) obtained from mackerel viscera hydrolysates.

Two adjacent fractions (3 and 4) showed higher DPPH scavenging activities (74.5 % and 69.3 %, respectively) compared to the rest of fractions. The radical scavenging activity of fraction number 3 was very similar to that observed for the non fractionated hydrolysates (78.3 %) When analysed against the FRAP assay, no significant ($p > 0.05$) differences were observed among all 15 fractions. The ACE inhibitory activity of mackerel viscera hydrolysates was mainly due to the presence of fractions 2 and 3. These two fractions showed the highest ACE inhibitory activities (59.6 % and 69.7 %, respectively) which were slightly lower than the ACE inhibitory effect of the non fractionated hydrolysates (74.5 %). Fractions 7 and 11 showed highest SSAO inhibitory activities. The SSAO inhibitory activity of fraction 7 was close to that of the non fractionated hydrolysates (41.6 % and 49.3 %, respectively).

Table 5.5 Fractionation of mackerel viscera hydrolysates and screening for the bioactivities of corresponding fractions.

	Applied eluents															non fractionated hydrolysates
A (%)	94.8	93.2	91.6	90	88.4	86.8	85.2	83.6	82	80.4	94.8	93.2	91.6	90	88.4	
B (%)	5.2	6.8	8.4	10	11.6	13.2	14.8	16.4	18	19.6	21.2	22.8	24.4	26	27.6	
Fractions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	
DPPH (%)	52.4^{a}	57.8^{a}	74.5^{c}	69.3^{b}	63.6^{b}	49.7^{a}	52.2^{a}	53.4^{a}	55.0^{a}	55.7^{a}	56.5^{a}	60.3^{b}	61.5^{b}	58.3^{a}	58.5^{a}	78.3^{c}
FARP (mM Trolox)	0.06^{a}	0.07^{a}	0.09^{a}	0.08^{a}	0.08^{a}	0.07^{a}	0.08^{a}	0.08^{a}	0.08^{a}	0.07^{a}	0.07^{a}	0.06^{a}	0.06^{a}	0.06^{a}	0.06^{a}	0.09^{a}
ACE (%)	29.8^{c}	59.6^{e}	69.7^{f}	43.6^{d}	38.0^{d}	16.1^{b}	23.2^{c}	17.1^{b}	26.5^{c}	9.3^{a}	30.7^{c}	14.6^{b}	25.5^{c}	33.0^{c}	20.8^{b}	74.5^{f}
SSAO (%)	11.0^{a}	14.2^{a}	24.8^{b}	19.6^{b}	11.8^{a}	25.1^{b}	41.6^{c}	21.3^{b}	27.6^{b}	25.3^{b}	31.6^{c}	25.6^{b}	24.0^{b}	25.5^{b}	20.4^{b}	49.3^{c}

A: Water & B: Acetonitrile. Both eluents (A & B) contained 0.1 % TFA. Different letters in the same row indicate significant ($p < 0.05$) differences.

5.5. Selection of fractions with highest bioactivity

The bioactivity levels of different fractions from mackerel skin gelatine and viscera hydrolysates are shown in Table 5.6. Two fractions from each sample were selected based on their activities to determine the amino acid sequence possibly responsible for their bioactivity.

Fractions number 1 & 4 from mackerel skin gelatine hydrolysates and fractions number 3 & 7 from mackerel viscera hydrolysates were the most bioactive ones. Hence, these fractions were chosen for sequence analysis.

Table 5.6 Bioactivity level of different fractions from mackerel skin gelatine and viscera hydrolysates.

		Fractions														
	Assays	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Skin gelatine	**SSAO**	++++	++	++	+	+++	+	-	+	+	+					
	ACE	++	+	+	++++	++	-	+	++	++	+					
	FRAP	+	+	+	+++	+	+	+	+	+	+					
	DPPH	+	+	+	+	+	+	+	+	+	+					
	Anti-platelet aggregation	+++	+	-	+	++	-	-	+++	-	-					
Viscera	**SSAO**	+	+	++	+	+	+	++++	+	+	+	++	++	+	+	+
	ACE	+	++	+++	++	+	-	+	+	+	-	+	-	+	+	+
	FRAP	+	+	++	++	++	+	++	++	++	+	+	+	+	+	+
	DPPH	+	+	++++	++	++	+	+	+	+	+	+	+	+	+	+

Very low activity (-), low activity (+), average activity (++), high activity (+++) and very high activity (++++).

A second injection into the analytical HPLC was performed (Figure 5.19) to determine the purity of these fractions. The chromatograms showed that all the fractions contain pure peptides, except fraction number 3 from mackerel viscera hydrolysates (Figure 5.19C).

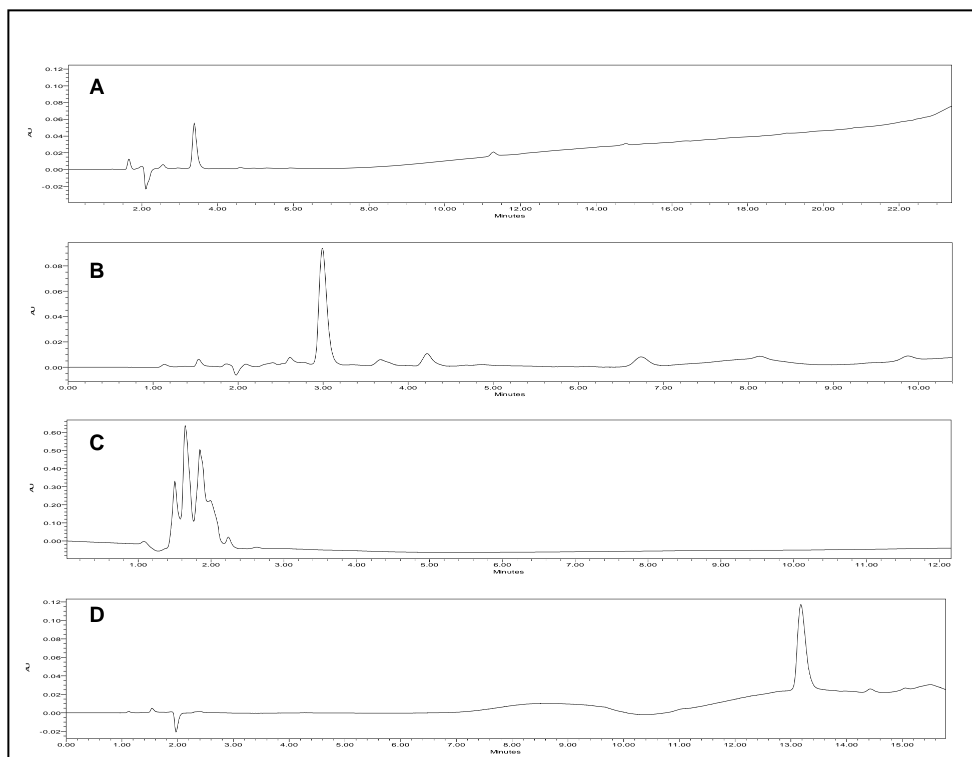


Figure 5.19 RP-HPLC C_{18} chromatograms of fractions from the bioactive hydrolysates. (A) Fraction number 1 from mackerel skin gelatine hydrolysates, (B) Fraction number 4 from mackerel skin gelatine hydrolysates, (C) Fraction number 3 from mackerel viscera hydrolysates and (D) Fraction number 7 from mackerel viscera hydrolysates.

The IC_{50} for the SSAO inhibitory activity of different bioactive fractions are presented in Figure 5.20.

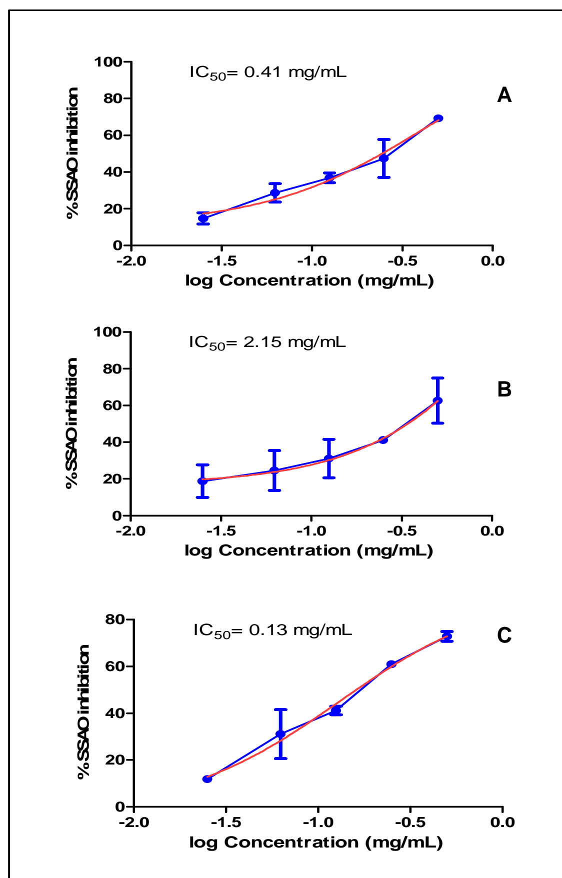


Figure 5.20 IC_{50} values for the SSAO inhibitory activity of the bioactive fractions. (A) Fraction number 1 from mackerel skin gelatine hydrolysates, (B) Fraction number 3 from mackerel viscera hydrolysates and (C) Fraction number 7 from mackerel viscera hydrolysates.

Fraction number 3 from mackerel viscera hydrolysates had the highest IC_{50} value (2.15 mg/mL), followed by fraction number 1 from mackerel skin gelatine hydrolysates with an IC_{50} value of 0.41 mg/mL. Fraction number 7 from mackerel viscera hydrolysates exhibited the lowest IC_{50} value (0.13 mg/mL). The IC_{50} value (2.15 mg/mL) of fraction number 3 from mackerel viscera hydrolysates

was half of that reported by Oliveiri *et al.* (2010) for soluble elastin (4.6 mg/mL) suggesting better inhibition. The lower IC_{50} value, observed with fraction number 7, indicates a higher inhibitory potency against SSAO. The inhibition of SSAO prevents the production of toxic aldehydes and reduces the development of inflammatory diseases (Jeon & Sayre, 2003).

The IC_{50} for the ACE inhibitory activity of different bioactive fractions are shown in Figure 5.21.

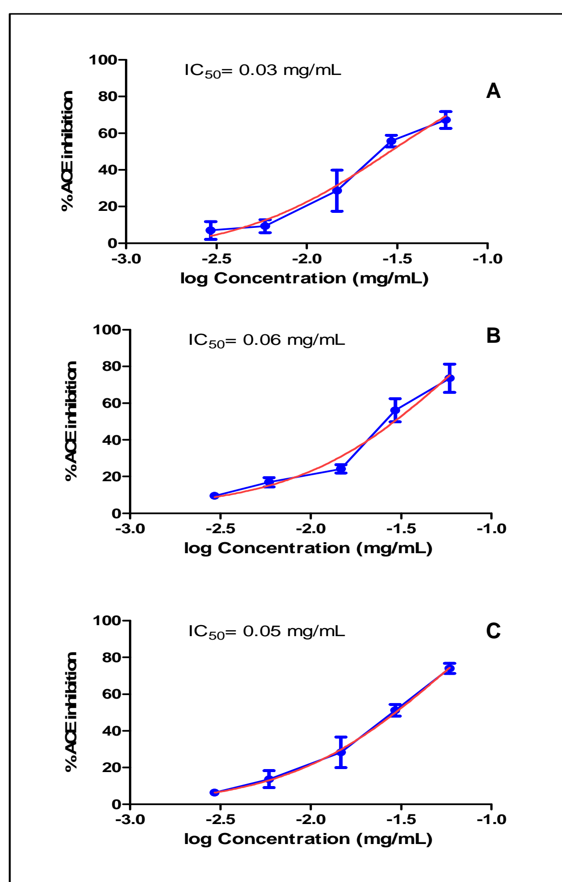


Figure 5.21 IC_{50} values for the ACE inhibitory activity of the bioactive fractions. (A) Fraction number 1 from mackerel skin gelatine hydrolysates, (B) Fraction number 4 from mackerel skin gelatine hydrolysates and (C) Fraction number 3 from mackerel viscera hydrolysates.

Fraction number 4 from mackerel skin gelatine hydrolysates and fraction number 3 from mackerel viscera hydrolysates showed similar IC_{50} against the ACE (~ 0.055 mg/mL). These IC_{50} values were 16 times lower than the IC_{50} value (0.81 mg/mL) for ACE inhibitory activities of peptides from sardinelle by-products protein hydrolysates (Bougatef *et al.,* 2008) and 4 times lower than the IC_{50} (0.2 mg/mL) for amaranth trypsin-digested glutelins (De La Rosa *et al.,* 2010). Fraction number 1 from mackerel skin gelatine hydrolysates had a lower IC_{50} (0.03 mg/mL) than fraction number 4. The calculated IC_{50} was very similar to that observed with hard clam meat hydrolysates (0.036 mg/mL) as reported by Tsai *et al.* (2008). The lower IC_{50} value obtained with fraction number 1 suggests a better antihypertensive property. The inhibition of ACE lowers the blood pressure and reduces the risk of cardiovascular and renal diseases (Odama & Bakris, 2000) (Chapter 1, section 1.2.2.3).

5.6. Peptide sequence analysis

The fragmentation of the peptide present in fraction number 1 from mackerel skin gelatine hydrolysates exhibited a signal at m/z 387, which was the molecular ion $[M+H]^+$ of Cys-His-Lys (C-H-K). The product ion spectrum of this ion is shown in Figure 5.22A. The m/z 147 was due to y_1 C-terminus sequence ion. From the screening studies, it was observed that this peptide inhibits the SSAO activity and suppresses the platelet activation.

The presence of lysine, in this tripeptide, could be responsible for the inhibition of SSAO. Olivieri *et al.* (2010) studied the kinetics of inhibition of SSAO in presence of L-lysine and they suggested an uncompetitive inhibition,

where the L-lysine binds only to the complex formed between the enzyme and the substrate or to an intermediate product.

The amino acid sequence (C-H-K) was previously identified in human, monkey, bovine and mouse genomes as a tripeptide motif of serine protease (EMBL/GenBank/DDBJ databases). Thrombin, a serine protease, is a potent agonist that activates the platelet. Thrombin cleaves the extracellular N-terminus of protease-activated receptors (PARs) exposing a new amino terminus capable of forming a ligand (Boysen *et al.,* 2002). Since this peptide (C-H-K) is a sequence motif of serine protease, it might have competed with thrombin and formed a bond with the PARs of the platelets inhibiting them from being activated.

The peptide present in fraction number 4 from mackerel skin gelatine hydrolysates showed a molecular ion $[M+H]^+$ at m/z 615 (Figure 5.22B). Several ions were identified in the spectrum as product ions. The ions at m/z 175, 193 and 307 were due to y_1, y_2 and y_3 C-terminus sequence ions, respectively. The ions at m/z 212, 309 and 423 were due to b_2, b_3 and b_4 N-terminus sequence ions, respectively. The peptide in this fraction was assigned as Pro-Asn-Pro-Asn-Ala-Cys (P-N-P-N-A-C).

The ACE inhibitory activity of peptides was linked to the presence of hydrophobic amino acids at the C-terminal end (Miyoshi *et al.,* 1991; Yokoyama *et al.,* 1993) and Pro-Asn-Pro-Asn-Ala-Cys was also composed of hydrophobic amino acid (Cys) at the C-terminal end which might be responsible for inhibiting ACE.

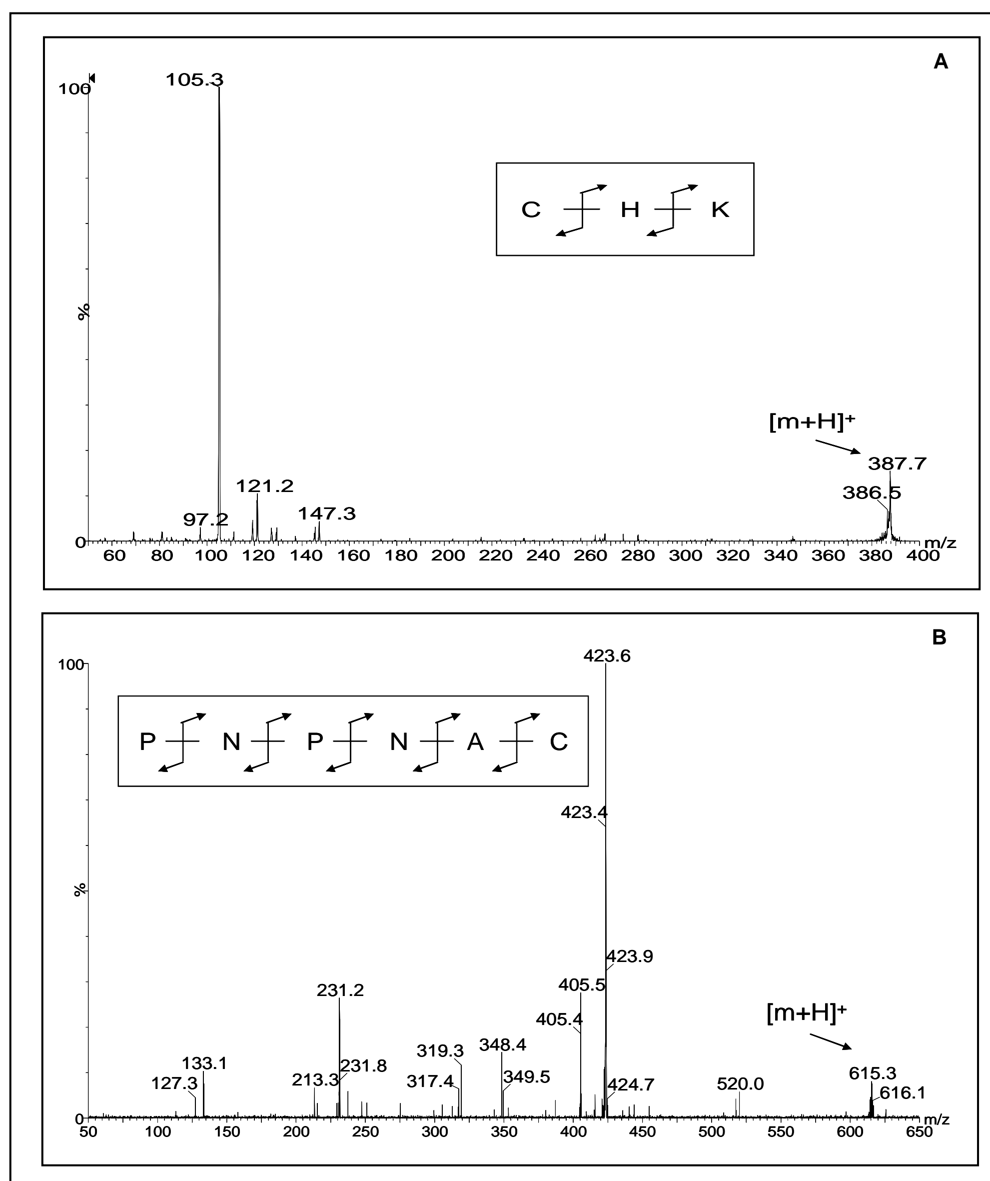


Figure 5.22 MS/MS spectra of the molecular ions present in mackerel skin gelatine fractions. (A) m/z = 387 present in fraction number 1 and (B) m/z = 615 present in fraction number 4.

No useful MS data could be obtained by the analysis of fractions number 3 and 7 from mackerel viscera hydrolysates digested with Flavourzyme. Hence, the sequence determination of this fraction was not attempted.

5.7. Screening for bioactivity of blue whiting gelatine hydrolysates

The hydrolysis of gelatines from mackerel heads and skins with pepsin for 24 h produced hydrolysates with several bioactive properties. Gelatines extracted from blue whiting heads and skins were also hydrolysed under the same conditions to obtain possible bioactive peptides. Hydrolysates from blue whiting heads and skin were screened for their radical scavenging capacity using DPPH and results are presented in Figure 5.23.

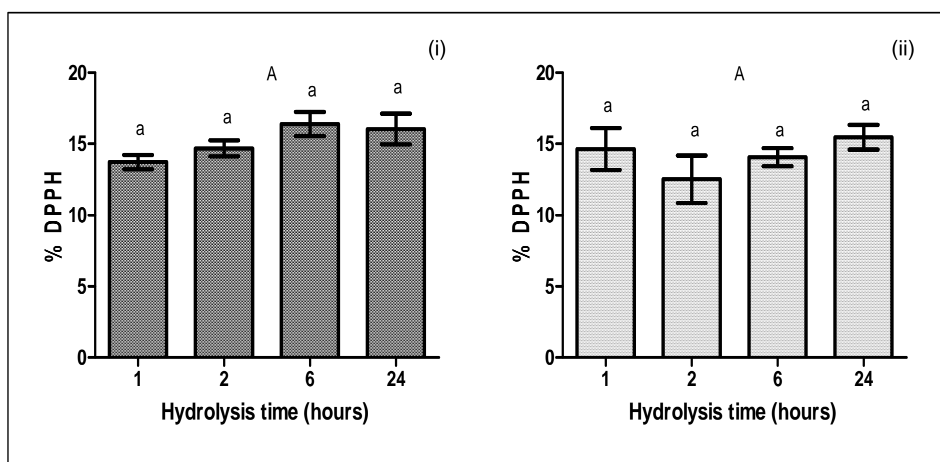


Figure 5.23 The DPPH scavenging activity of blue whiting gelatine hydrolysates prepared from heads and skins at different hydrolysis times. Gelatine hydrolysates from: (i) blue whiting heads and (ii) blue whiting skins. Different lower case letters indicate significant ($p < 0.05$) differences between hydrolysis times. Different upper case letters indicate significant ($p < 0.05$) differences between sources.

The screening for the antioxidant activity found that, unlike mackerel gelatine hydrolysates, those obtained from blue whiting did not have any relevant radical scavenging properties.

5.8. Conclusions

Enzymatic hydrolysis represents an effective way for upgrading fish waste and low value fish by-products to highly nutritious functional ingredients. The enzymatic hydrolysis of mackerel waste resulted in hydrolysates with potent bioactive properties. Pepsin and Flavourzyme (proteases from *Aspegillus oryzae*) were the most efficient enzymes for digesting gelatine and mackerel viscera, respectively.

The prepared hydrolysates had high antioxidant, anti-inflammatory and antihypertensive activity and could have potential application in the food and pharmaceutical industries.

The peptides obtained from mackerel skin gelatine after hydrolysis with pepsin inhibited the platelet aggregation and as consequence may serve as natural antithrombotic compounds.

The anti-inflammatory and the antithrombotic activities of mackerel skin gelatine hydrolysates were due to the presence of a tripeptide (Cys-His-Lys) with a molecular weight of 387 Da. The antihypertensive and antioxidant activities were possibly due to a peptide assigned as Pro-Asn-Pro-Asn-Ala-Cys with a molecular weight of 615 Da.

Chapter 6 - Fish oils

6.1. Extraction and characterisation

6.1.1. Yield of extraction

The yields of extracted oils using various procedures are presented in Figure 6.1 and Figure 6.2. The amount of extracted oil varied significantly ($p < 0.05$) depending on the method used for the extraction. For the same extraction process, no differences between yields from heads and skins were observed. However, regardless of the source (heads or skins) the solvent extraction showed the highest recovery yields while the alkaline pre-treatment resulted in lowest yields.

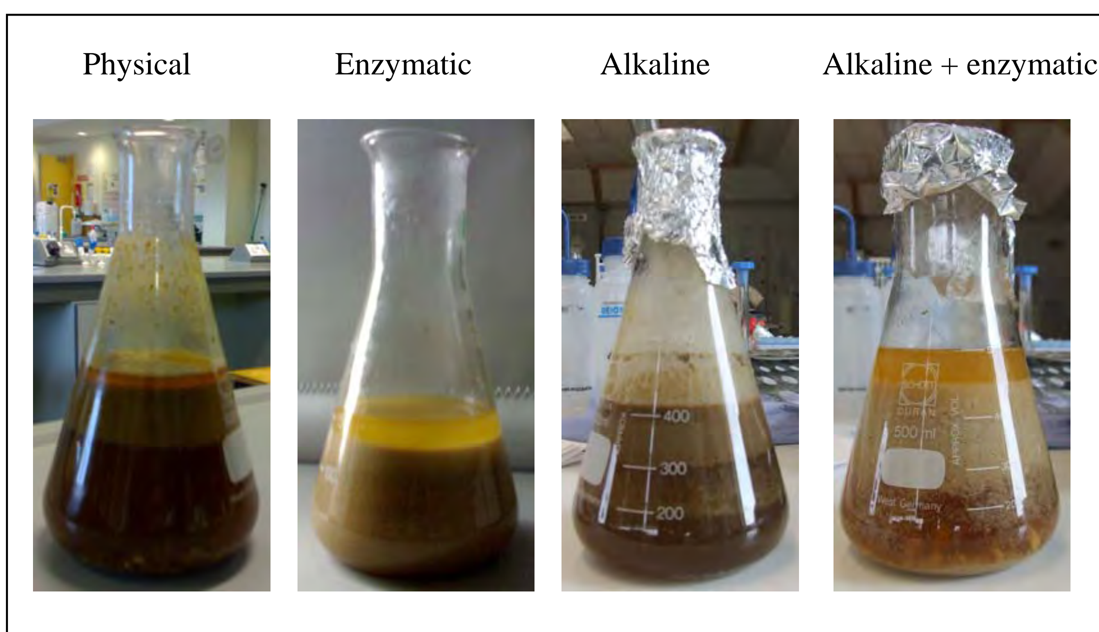


Figure 6.1 Mackerel oils extracted with different procedures.

All samples extracted with hexane had significantly ($p < 0.05$) the highest yield (> 90 %). Hexane was used by Aryee *et al.* (2009) to extract oils from salmon skins with high recovery yields (~ 60 %). The enzymatic hydrolysis yielded 54.7 % of oil from mackerel heads and 56.5 % of oil from mackerel skins. Slightly lower yields were obtained when applying the method based on heat treatment with a yield of 45.9 % and 40.5 % for mackerel heads and skins, respectively.

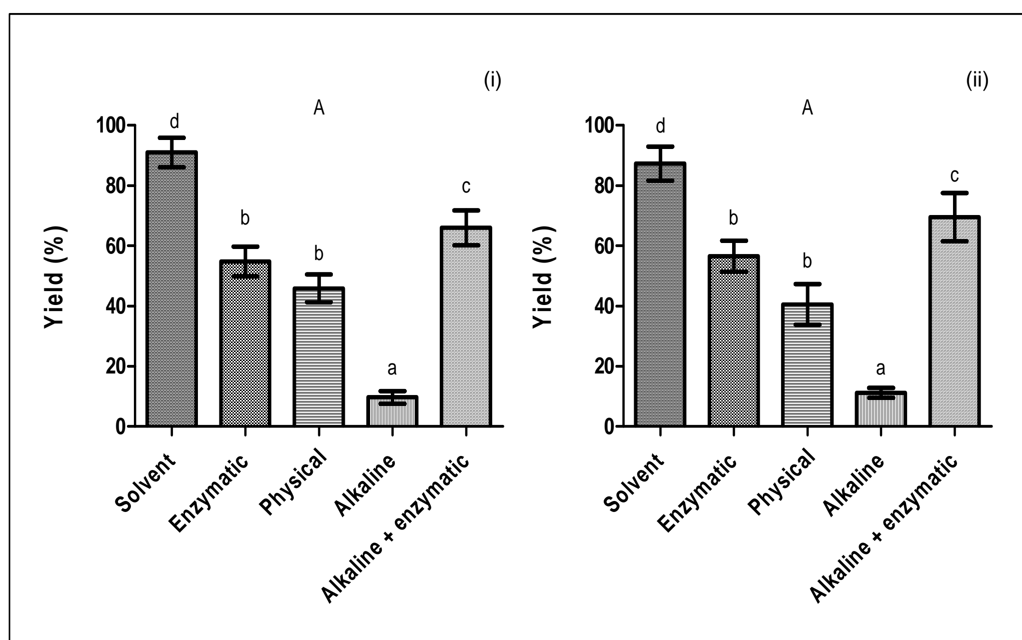


Figure 6.2 Extraction yields of mackerel head (i) and skin (ii) oils as affected by the extraction procedure. Different lower case letters indicate significant ($p < 0.05$) differences between treatments. Different upper case letters indicate significant ($p < 0.05$) differences between sources.

Oils extracted after centrifugation of the alkaline solution had very low yields, 9.7 % and 11.2 % for heads and skins, respectively. Sardine oils were collected from surimi waste-water by centrifugation yielding 34 % of oils (Toyoshima *et al.,* 2004). Higher yields were obtained from alkaline solution when a hydrolysis step was introduced. The yield increased about 6 fold and resulted in higher oil recovery (> 65 %) than those obtained with the other extraction methods (heat-treated or hydrolysed samples).

Several factors, including the time, the temperature and the solvent nature, affect the extraction yield (De Boer, 1988). In this study, the low yields obtained after the centrifugation of alkaline waste-water could be due to either insufficient contact between the sodium hydroxide and the matrix or the inability of the sodium hydroxide to break the cell membrane and release oils.

Turon *et al.* (2005) evaluated the extraction of oil from Tilapia heads using heat treatment and the yield was around 18 %. The enzymatic hydrolysis of salmon heads recovered 17.4 % of oils (Linder *et al.,* 2005). These reported yields for heat and enzymatic treatments are in agreement to those obtained from this study.

6.1.2. Physicochemical and colour analysis of mackerel oils

Results for colour and the quality measurements of oils extracted from mackerel heads and skins using several procedures are presented in Table 6.1.

Table 6.1 Colour and quality parameters of oils from mackerel.

		Colour parameters			Quality parameters		
Source	**Procedure**	**L***	**a***	**b***	**Acid value**	**Peroxide value**	**Iodine value**
Heads	**Alkaline**	43.8[b] ±1.5	3.6[c] ±0.1	13.0[c] ±0.9	1.9[a] ±0.5	8.6[a] ±0.2	125.9[b] ±1.4
	Alkaline + enzymatic	51.9[d] ±3.8	4.6[c] ±1.1	27.5[d] ±2.0	2.8[a] ±0.6	16.7[b] ±0.3	111.4[a] ±5.9
	Enzymatic	55.8[d] ±2.6	-0.2[c] ±0.2	26.6[d] ±1.7	2.7[a] ±0. 4	10.6[a] ±0.4	134.6[b] ±5.2
	Physical	26.7[a] ±1.2	-1.7[a] ±0.1	5.8[b] ±0.4	2.7[a] ±0.2	12.7[a] ±0.1	107.9[a] ±7.9
	Solvent	48.2[c] ±1.3	3.8[c] ±0.4	25.1[d] ±1.7	2.1[a] ±0.2	10.9[a] ±1.3	127.3[b] ±5.8
Skins	**Alkaline**	41.7[b] ±0.8	5.5[c] ±0.6	12.4[c] ±1.3	2.1[a] ±0. 4	10.8[a] ±0.8	130.0[b] ±8.1
	Alkaline + enzymatic	26.9[a] ±1.5	-1.5[a] ±0.1	5.4[b] ±1.0	2.5[a] ±0.2	14.1[b] ±0.4	104.0[a] ±11.4
	Enzymatic	39.8[b] ±0.7	-1.1[b] ±0.1	19.5[c] ±1.2	2.3[a] ± 0.8	11.2[a] ±0.5	112.8[a] ±6.4
	Physical	28.8[a] ±1.1	3.8[c] ±0.6	0.6[a] ±0.2	2.2[a] ±0.4	12.9[a] ±1.6	118.2[a] ±8.9
	Solvent	38.0[b] ±0.6	-0.8[b] ±0.1	14.9[c] ±0.9	2.0[a] ±0.2	9.7[a] ±1.2	114.4[a] ±3.3

Values given as mean ± standard deviation. Different letters in the same column indicate significant ($p < 0.05$) differences between treatments. Acid value was expressed as mg KOH/g; Peroxide value was expressed as meq O_2/kg and Iodine value was expressed as g I_2/100g.

The acid value has been widely used as a quality parameter for classifying oils (Baccouri *et al.,* 2007). The acid value of mackerel head and skin crude oils obtained with the different methods was relatively low (1.9 - 2.8 mg KOH/g). The acid values of all extracted oils were lower than the limit of 4.0 mg KOH/g for crude oils (*Codex Alimentarius* Commission, 2005). Slightly higher acid values were observed with the heat treated samples. The heat treated samples (either through the heating process or microwave inactivation) showed a relatively higher acid value than solvent or alkaline extracted oils. Usually oils extracted at low

temperatures have low acid values since the hydrolysis of triglyceride ester bonds is reduced compared to high temperature extraction processes (Chantachum *et al.,* 2000). The extracted oils can not be used for human consumption without being refined to reach an acid value below 0.6 mg KOH/g (*Codex Alimentarius* Commission, 2005).

The peroxide value (PV), a measurement of the hydroperoxide content, is used to assay lipid and fat oxidation (Yildiz *et al.,* 2003; Baccouri *et al.,* 2007). Oils can be classified depending on their oxidation stages as indicated by the PV. It was reported that oils with PV raging from (3 - 5), (10 - 12) and (16 - 18) are considered low, moderate and highly oxidised oils, respectively (Warner *et al.,* 1989). The peroxide values of mackerel oils (PV = 8.6 - 14.1 meq O_2/kg) were in the acceptable range for crude oils (lower than 15 meq O_2/kg) according to the *Codex Alimentarius* (*Codex Alimentarius* Commission, 2005). Higher PV was observed with oil obtained from heads after the combined alkaline and enzymatic extraction (PV = 16.7 meq O_2/kg).

When comparing the extraction procedures, high peroxide values were observed for heat treated samples, but more substantially for all mackerel head extracted samples. It is known that heat processing causes the denaturation of proteins. Myoglobin, an iron containing protein in the blood, was mainly present in mackerel heads and the heat treatment might have liberated the iron, a catalyst for lipid oxidation (Decker & Welch, 1990).

Mackerel head oils extracted after the heat treatment and the combined alkaline and enzymatic process, as well as mackerel skin oils extracted after the alkaline treatment, the combined alkaline and enzymatic process and the

enzymatic hydrolysis can not be classified as edible oils without further refining. The peroxide value for edible oils must be lower than 10 meq O_2/kg (*Codex Alimentarius* Commission, 2005).

Mackerel oils had high iodine values due to the high number of unsaturated fats. The decline in iodine value gave an indication of the oxidation of oils (Naz *et al.,* 2004). Oils extracted from mackerel heads and skins using heat treatment and the combined alkaline and enzymatic hydrolysis were the most oxidised oil as observed by the low iodine values (Table 6.1). From the results of peroxide determination, it was observed that these oils were highly oxidised. The alkaline treatment along with the effect of the enzymatic hydrolysis and the elevated temperatures resulted in breaking cell membranes, denaturating proteins and releasing oils. The liberated oils reacted with free ferrous ions, strong oxidation catalysts (Beltrán De Heradia *et al.,* 2001), leading to higher oxidation rate (Chantachum *et al.,* 2000).

The oils extracted from mackerel heads and skins in this study showed slightly lower acid value, similar iodine value and higher peroxide value than mackerel oils extracted by other researchers. Mackerel oils with acid value of 3.6 mg KOH/g, peroxide value of 1 meq O_2/kg and iodine value of 145 g I_2/100g were reported for Atlantic mackerel (*Scomber scombrus*) by Ke *et al.* (1975). The published data for oils from mackerel skins included an acid value of 4 mg KOH/g, a peroxide value of 3.3 meq O_2/kg and an iodine value of 133 g I_2/100g (Zuta *et al.,* 2003). These differences could be due to the extraction conditions and the raw material used.

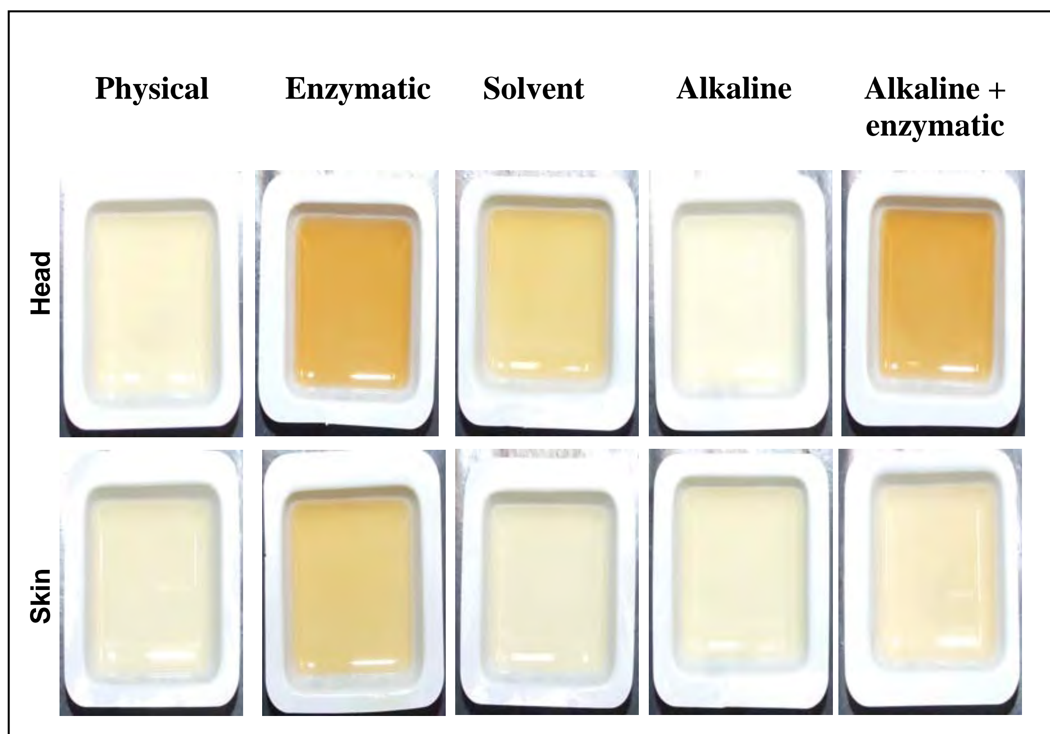


Figure 6.3 Colour of mackerel head and skin oils as affected by the extraction procedure.

It was observed that the colour of mackerel oils depended on the extraction method (Table 6.1 and Figure 6.3). The extraction procedure influenced both the lightness and redness values of mackerel oils. In general, oils from mackerel skins had lighter colour compared to oils from mackerel heads. Oils from mackerel heads extracted by solvent, after the enzymatic hydrolysis and using the combined alkaline and enzymatic hydrolysis had the highest b* values. These values reflected the intense brownish colour of these oils. Slightly lower b* values were observed for oils from mackerel skins extracted by solvent and after enzymatic hydrolysis. Several conditions lead to the darker colour of fish oils. Some oxidation products, such as aldehydes, may form Schiff bases with proteins (Crexi *et al.,* 2009) leading to the darker colour. The heat treatment at the end of the

hydrolysis process may have contributed to the oils colouration. With elevated temperatures and due to the high unsaturation, mackerel oils could undergo isomeration and conjugation of some double bonds which absorb in the blue region and reflect a more brownish colour (Crexi *et al.,* 2009). The colour of mackerel oil is very important since it affects greatly its market value and plays a major role in its acceptability (Shahidi *et al.,* 1998). For commercial applications, the dark extracted mackerel oils need to be bleached and deodourised.

6.1.3. FTIR analysis of mackerel oils

All mackerel oils showed similar FTIR spectra (Figure 6.4) with characteristic absorptions bands of aliphatic hydrocarbons assigned at 3000 - 2800 cm^{-1} (stretching), 1460 - 1377 cm^{-1} (bending) and 720 cm^{-1} (rocking) (Le Dréau *et al.,* 2009). Mackerel oils were mainly composed of triglycerides as confirmed by the presence of the functional groups of the triglyceride in the FTIR spectra. The C-H stretching appeared at 2922 cm^{-1} and 2853 cm^{-1} (Yang *et al.,* 2005; Guillén & Cobo, 1997). The peak at 1744 cm^{-1} corresponded to C=O stretching (Ismail *et al.,* 1993; Lerma-Garcia *et al.,* 2010). Peaks around 1151 cm^{-1} and 721 cm^{-1} represented the C-O stretching and C-H rocking, respectively (Ismail *et al.,* 2006). These peaks were present in all the fish oil samples analysed.

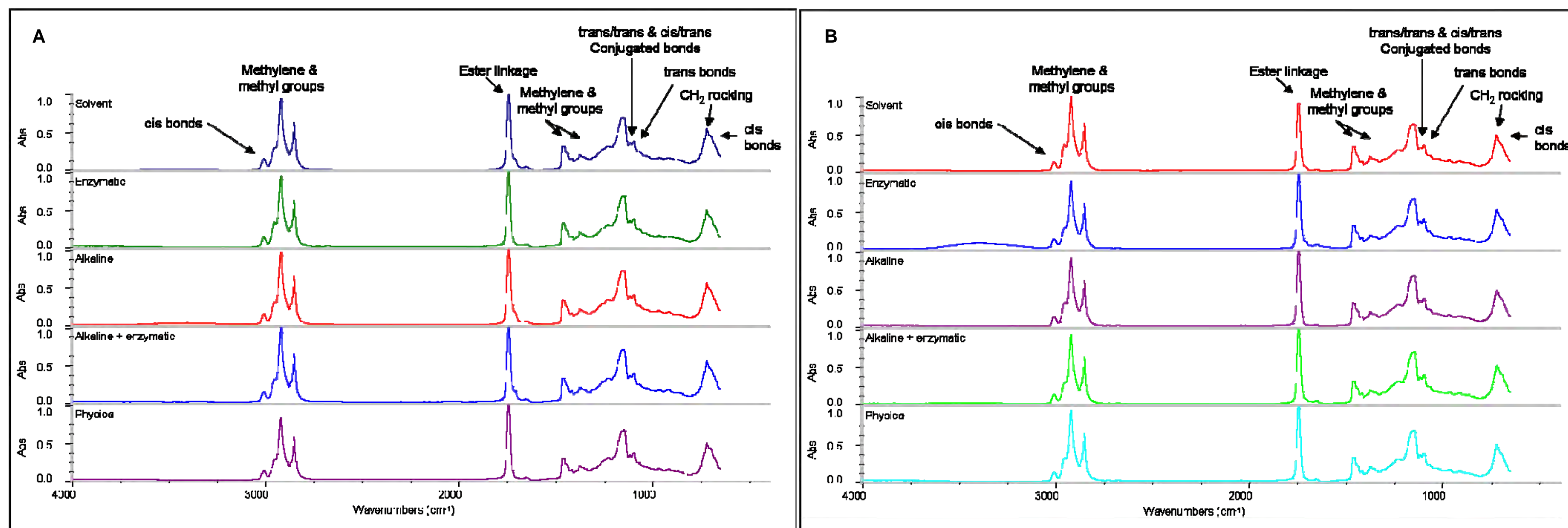


Figure 6.4 FTIR spectra of mackerel head (A) and skin (B) oils extracted using various procedures.

The FTIR analysis of organic compounds helps in the elucidation of their molecular structure (Sherazi *et al.,* 2009). In this study, the FTIR spectra indicated that the extraction procedure did not affect the characteristic of the oils since the result did not show any spectral variations or difference among the peak intensities of mackerel oils. This could be due to the constant molecular feature of triacylglycerols (Sherazi *et al.,* 2009).

6.1.4. Chromatographic characterisation of mackerel oils

6.1.4.1. Analysis of triglycerides and lipid classes

The lipid profile of the extracted mackerel oils depended on the source and the extraction procedure. For both head and skin oils, the main components were neutral lipids (FFA, MAG, DAG and TAG) as shown in Figure 6.5.

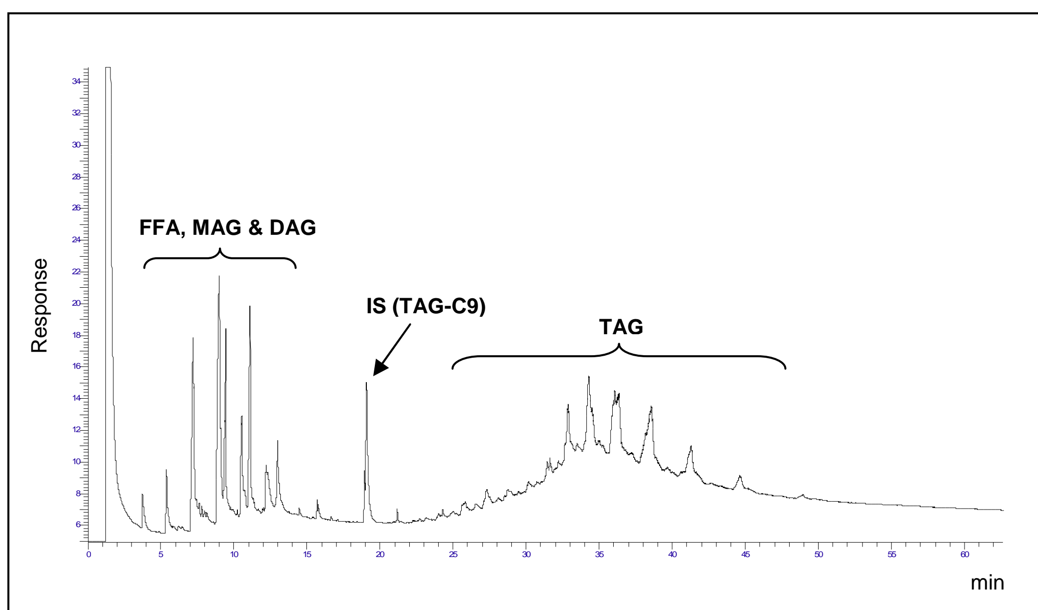


Figure 6.5 Typical gas chromatogram of lipid classes from mackerel oil. IS (TAG-C9) refers to the internal standard.

TAG was the major lipid class observed in these oils with amounts higher than 75 %. Low levels of MAG were found in all oils (0 - 3.6 %). Moderate levels of FFA and DAG were also observed, however, higher values were obtained for mackerel head oils compared to skin oils (Table 6.2).

Table 6.2 Lipid classes of mackerel head and skin oils (from GC measurements).

	Extraction	FFA	MAG	DAG	TAG
Heads	**Solvent**	8.4±0.8[b]	1.3±0.1[a]	9.3±1.0[c]	81.0±1.9[a]
	Enzymatic	7.8±0.1[b]	0.0±0.1[a]	7.4±0.3[c]	83.9±0.4[a]
	Alkaline	2.0±0.4[a]	0.0±0.1[a]	1.9±0.0[a]	96.1±0.4[b]
	Alkaline + enzymatic	3.9±0.5[a]	3.6±0.4[b]	1.5±0.4[a]	92.3±0.9[b]
	Physical	8.6±1.9[b]	1.2±0.1[a]	8.5±0.5[c]	81.7±2.5[a]
	Folch method	7.7±1.0[b]	1.1±0.1[a]	8.9±0.0[c]	77.8±1.7[a]
Skins	**Solvent**	5.4±1.2[b]	0.7±0.1[a]	5.7±0.7[b]	88.2±2.0[b]
	Enzymatic	2.7±0.1[a]	0.4±0.1[a]	4.1±0.1[b]	91.6±0.0[b]
	Alkaline	3.3±0.7[a]	0.1±0.1[a]	2.7±0.3[a]	93.9±1.0[b]
	Alkaline + enzymatic	3.7±0.1[a]	0.2±0.0[a]	4.3±0.0[b]	91.9±0.1[b]
	Physical	5.4±1.5[b]	0.6±0.1[a]	5.3±0.6[b]	88.7±2.1[b]
	Folch method	3.1±0.0[a]	0.5±0.0[a]	4.5±0.3[b]	90.5±0.4[b]

Values given as mean ± standard deviation. Different letters in the same column indicate significant ($p < 0.05$) differences between treatments.

The level of FFA gives an indication of the oxidation state of the oil. FFA are very susceptible to oxidation leading to the deterioration of the taste of the oil (Refsgaard *et al.,* 2000). The present findings were similar to those obtained for mackerel (*Scomber australasicus*) by Osako *et al.* (2006) where the TAG content

ranged from 69.0 % to 90.4 %, DAG content varied from 0.7 % to 3.6 % and FFA were in the range of 1.2 % to 10.9 %.

The phospholipids were analysed using HPLC/ELSD and a typical HPLC/ELSD chromatogram of mackerel oils shown in Figure 6.6.

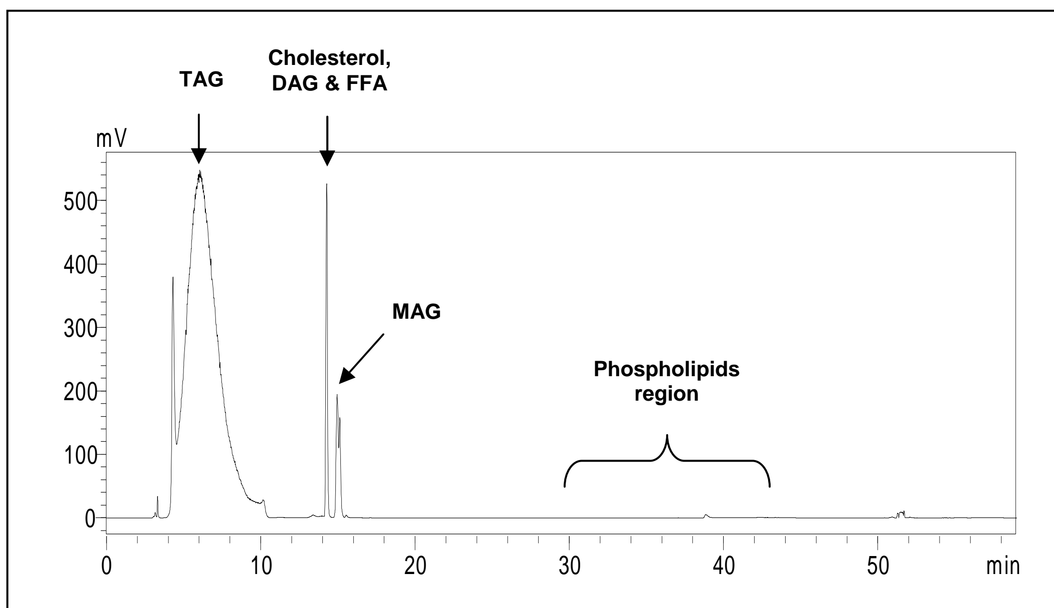


Figure 6.6 HPLC/ELSD chromatogram of mackerel oil extracted using the Folch method.

Mackerel oils did not appear to contain phospholipids as confirmed by the absence of peaks in the HPLC/ELSD chromatograms. Previous investigations showed that mackerel phospholipids were concentrated in the fillets with an amount less than 4 % of the total lipid content (Brix *et al.,* 2009).

6.1.4.2. Fatty acid composition of mackerel oils

Fatty acid methyl esters derived from mackerel fish oils were analysed by a gas chromatography system equipped with a flame ionisation detector (GC - FID). Typical GC - FID chromatograms are presented in Figure 6.7.

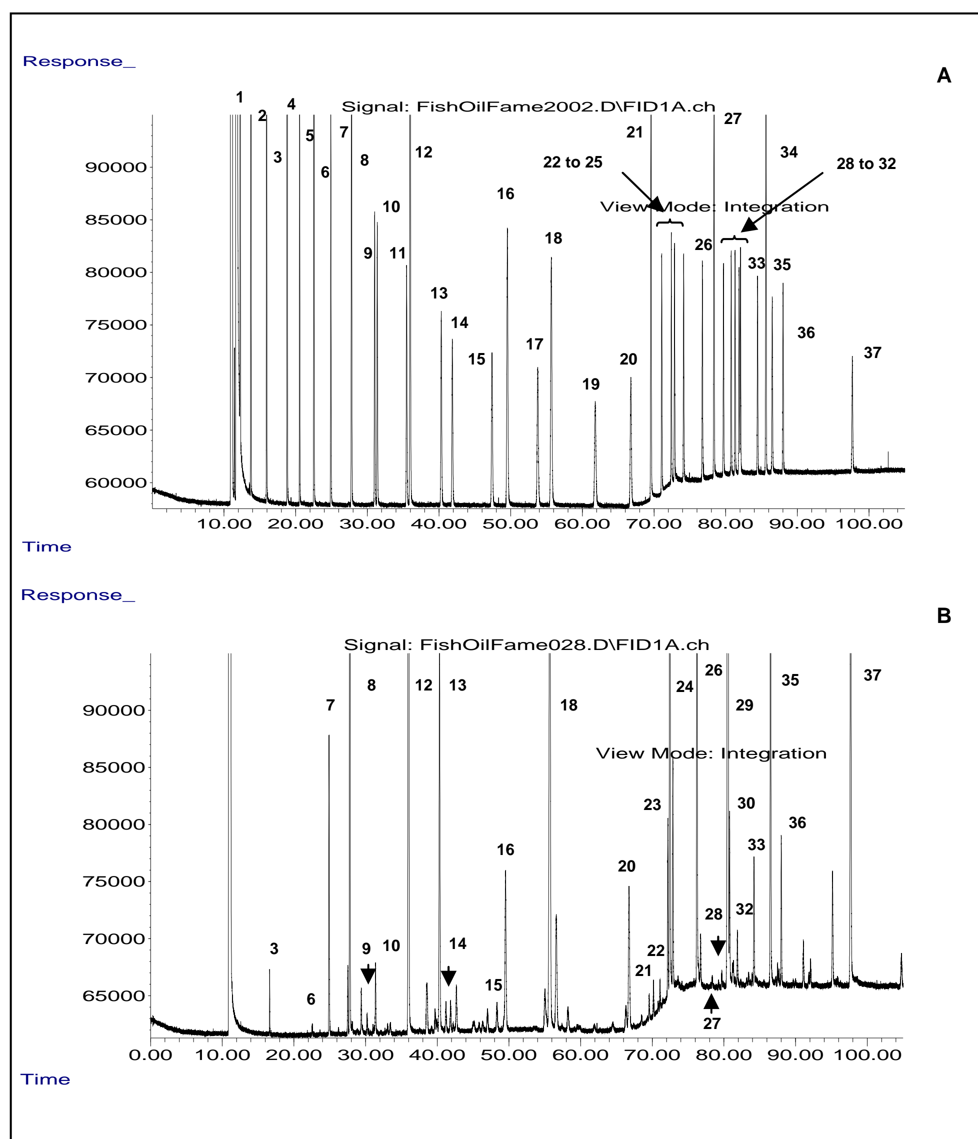


Figure 6.7 Gas chromatograms of fatty acid methyl esters (FAME). A: Supleco 37 standard and B: mackerel oil extracted from head by Folch method. FAMEs were represented by peaks: 1 - C4:0; 2 - C6:0; 3 - C8:0; 4 - C10:0; 5 - C11:0; 6 - C12:0; 7 - C13:0 (Internal standard); 8 - C14:0; 9 - C14:1; 10 - C15:0; 11 - C15:1; 12 - C16:0; 13 - C16:1; 14 - C17:0; 15 - C17:1; 16 - C18:0; 17 - C18:1n9t; 18 - C18:1n9c; 19 - C18:2n6t; 20 - C18:2n6c; 21 - C20:0; 22 - C18:3n6; 23 - C20:1; 24 - C18:3n3; 25 - C21:0; 26 - C20:2; 27 - C22:0; 28 - C20:3n6; 29 - C21:1n9; 30 - C20:3n3; 31 - C20:4n6; 32 - C23:0; 33 - C22:2; 34 - C24:0; 35 - C20:5n3 (EPA); 36 - C24:1; 37 - C22:6n3 (DHA).

A total of 27 FA were identified from the mackerel oil (Table 6.3). These included saturated FA (14:0, 15:0, 16:0, and 18:0), monounsaturated FA (16:1, 18:1n-9, 20:1, 21:1n-9 and 24:1) and polyunsaturated FA (18:2n-6, 18:3n-6, 18:3n-3, 20:2, 20:4n-6, 20:3n-6, 20:3n-3, 20:5n-3, and 22:6n-3). Results revealed that 14:0, 16:0, 18:1n-9, 20:1, 20:5n-3, 20:1n-6 and 22:6n-3 were the major FA and their contents were in the range of 8.4 to 22.1 % of the total FA. In this study, saturated fatty acids were present in lower content than monounsaturated and polyunsaturated fatty acids.

Regardless of the extraction procedure, mackerel head oils had significantly ($p < 0.05$) higher DHA content and lower ω-6 fatty acids than mackerel skin oils (Table 6.3). This was due to the difference between the distribution and amount of fat in the tissues. Oil from fish heads are usually rich in DHA which is mainly due to their presence, with high level, in the brain and eyes of the fish (Sargent, 2000).

For both heads and skins, the lowest recovery of EPA and DHA were observed using the solvent extraction (103.4 mg/g and 85.2 mg/g, respectively) and the highest with alkaline extraction (149.2 mg/g and 106.2 mg/g, respectively). PUFA contents in mackerel muscle have been reported to vary in the range of 21.2 - 51.7 % (Zuta *et al.,* 2003; Osako *et al.,* 2006). Several parameters affect the percentage of PUFA, including EPA and DHA in fish oil. Differences were associated with the diet, season and reproductive state of the fish (Arts *et al.,* 2001). However, since all the samples were extracted during the same period, the differences between the EPA and DHA recoveries can be attributed to the extraction method and the type of tissues (heads or skins).

Table 6.3 Fatty acid composition (% of total peak area) of mackerel oils extracted from heads and skins using various procedures.

	Head oils					Skin oils				
	Physical	**Alkaline**	**Alkaline & enzymatic**	**Solvent**	**Enzymatic**	**Physical**	**Alkaline**	**Alkaline & enzymatic**	**Solvent**	**Enzymatic**
C8:0	0.2±0.01	0.2±0.00	0.2±0.02	0.2±0.02	0.2±0.05	0.3±0.03	0.2±0.01	0.3±0.01	0.3±0.04	0.3±0.03
C12:0	0.1±0.01	0.1±0.01	0.1±0.02	0.1±0.01	0.1±0.04	0.1±0.02	0.1±0.03	0.1±0.01	0.1±0.03	0.1±0.00
C13:0	1.8±0.27	1.8±0.04	1.9±0.13	2±0.14	2±0.44	1.8±0.07	1.6±0.01	1.9±0.19	1.9±0.07	1.9±0.03
C14:0	8.4±0.11	8.4±0.13	8.8±0.07	9.3±0.39	9.9±1.36	9.2±0.05	9.3±0.02	9.4±0.10	10.3±1.33	9.4±0.52
C14:1	0.1±0.00	0±0.07	0.1±0.00	0±0.00	0±0.00	0±0.00	0±0.06	0±0.00	0±0.00	0.1±0.00
C15:0	0.6±0.01	0.6±0.02	0.6±0.01	0.6±0.01	0.7±0.12	0.6±0.00	0.6±0.01	0.6±0.00	0.7±0.10	0.6±0.02
C16:0	15.9±0.14[b]	15.3±0.49[c]	16.1±0.33[c]	17.4±0.3[c]	18.5±1.8[c]	14.5±0.01[b]	14.3±0.02[b]	14.6±0.41[b]	7.2±0.13[a]	14.7±0.93[b]
C16:1	5±0.01	4.9±0.18	4.7±0.12	5±0.03	5.2±0.55	4.4±0.04	4.3±0.08	4.7±0.05	5.1±0.65	4.7±0.29
C17:0	0.4±0.00	0.4±0.03	0.4±0.02	0.4±0.02	0.4±0.07	0.4±0.00	0.5±0.09	0.4±0.02	0.5±0.07	0.4±0.02
C17:1	0.3±0.01	0.3±0.01	0.3±0.02	0.3±0.02	0.3±0.05	0.3±0.01	0.3±0.01	0.3±0.01	0.3±0.07	0.3±0.02
C18:0	2.6±0.03	2.5±0.06	2.5±0.01	2.7±0.01	2.8±0.22	2.4±0.06	2.4±0.03	2.5±0.01	2.8±0.38	2.5±0.11
C18:1n9c	13.1±0.0[b]	10.9±0.2[a]	12±0.14[b]	12.4±0.0[b]	13.2±1.0[b]	9.7±0.03[a]	9.9±0.08[a]	10.6±0.2[a]	11.2±1.0[a]	10.2±0.80[a]
C18:2n6c	1.9±0.03	2.1±0.15	2.2±0.47	1.9±0.06	2±0.32	2±0.10	2.3±0.13	2.2±0.09	2.4±0.16	2.1±0.02
C20:0	0.2±0.00	0.2±0.01	0.2±0.00	0.2±0.01	0.2±0.02	0.2±0.01	0.2±0.08	0.2±0.02	0.2±0.03	0.2±0.01
C18:3n6	0.2±0.03	0.2±0.04	0.2±0.01	0.2±0.02	0.2±0.10	0.3±0.03	0.3±0.03	0.3±0.00	0.3±0.04	0.2±0.01
C20:1	9.4±0.08[b]	10±0.04[b]	10.2±0.0[b]	9.8±0.0[b]	4.8±0.84[a]	12.3±0.0[c]	12.7±0.0[c]	12.7±0.0[c]	13.8±1.41[c]	12.3±0.5[c]
C18:3n3	1.9±0.14	2.5±0.00	2.1±0.01	2±0.10	2.2±0.36	2.1±0.06	2.2±0.09	2.1±0.04	2.3±0.28	2.1±0.16
C20:2	0.5±0.06	0.6±0.03	0.5±0.02	0.5±0.01	0.6±0.09	0.6±0.02	0.5±0.04	0.5±0.05	0.6±0.17	0.6±0.01
C22:0	0±0.00	0±0.00	0±0.00	0±0.00	0.1±0.10	0±0.00	0.1±0.01	0±0.00	0±0.00	0±0.00
C20:3n6	14.9±0.1[a]	16.7±0.52[a]	16.3±0.17[a]	14.8±0.45[a]	15.5±0.27[a]	21.1±0.05[b]	21.1±0.00[b]	20.6±0.68[b]	22.1±1.48[b]	19.9±2.47[b]
C21:1n9	1.2±0.02	1.2±0.03	1.3±0.01	1.2±0.02	1.3±0.07	0±0.00	0±0.00	1.4±0.05	1.5±0.15	1.4±0.16
C20:3n3	0.2±0.24[a]	0.3±0.10[a]	0.1±0.13[a]	0±0.00[a]	0.2±0.25[a]	1.5±0.04[b]	0.3±0.11[a]	0.3±0.12[a]	0.4±0.02[a]	0.4±0.03[a]
C20:4n6	0.5±0.01	0.5±0.00	0.4±0.01	0.5±0.03	0.4±0.02	0.4±0.00	0.5±0.00	0.5±0.01	0.5±0.04	0±0.00
C22:2	1.2±0.01	1.3±0.06	1.1±0.01	1.1±0.04	1.2±0.08	1±0.00	1.1±0.02	1.1±0.03	1.1±0.15	1.1±0.03
C20:5n3 (EPA)	8±0.01	7.6±0.06	7.4±0.00	7.4±0.02	7.5±0.20	6.5±0.02	6.1±0.11	5.9±0.15	6.6±1.15	6.5±0.36
C24:1	1.2±0.03	1.3±0.05	1.3±0.02	1.3±0.07	1.3±0.02	1.4±0.00	1.4±0.01	1.2±0.09	1.3±0.08	1.3±0.18
C22:6n3 (DHA)	11.9±0.08[b]	11.9±0.55[b]	10.9±0.13[b]	10.6±0.30[b]	11.2±0.41[b]	8.7±0.04[a]	8.8±0.09[a]	7.5±0.03[a]	8.4±1.23[a]	8.5±0.05[a]
Σ ω-6	17.5±0.2	19.6±0.7	19.1±0.7	17.4±0.5	18.1±0.8	23.8±0.2	24.2±0.2	23.6±0.8	25.3±1.7	22.2±3.1
Σ ω-3	22.1±0.5	22.2±0.7	20.5±0.3	20.1±0.4	21.0±1.2	18.8±0.2	17.4±0.4	15.8±0.3	17.6±2.7	17.6±2.5
ω-3/ω-6	1.3	1.1	1.1	1.2	1.2	0.8	0.7	0.7	0.7	0.8
EPA (mg/g)	48.1±4.6	52.2±3.4	44.2±3.6	38.5±0.1	49.2±0.0	41.0±3.4	39.4±0.99	36.0±4.2	34.1±1.4	35.7±2.6
DHA (mg/g)	84.1±8.7	97.0±1.2	76.8±5.4	64.9±1.8	86.5±0.8	64.5±5.3	66.8±1.1	54.1±5.1	51.1±0.7	55.1±1.3

Values given as mean ± standard deviation. Different letters in the same row, indicate significant ($p < 0.05$) differences between treatments (shown only for C16:0, C18:1, C20:1, C20:3n6, C20:3n3 and C22:6n3).

The ratio of ω-3/ω-6 FA in mackerel head oils (1.1 - 1.3) was higher than that of mackerel skin oils (0.7 - 0.8). The difference between the ratios of ω-3/ω-6 FA indicated that mackerel head oils have better fatty acid composition than mackerel skin oils. It was proven that a higher ω3/ω6 fatty acid ratio in diet plays an important role in reducing the risk of coronary heart diseases (Kinsella *et al.*, 1990).

6.2. Antioxidant activity of mackerel oils

The *in vitro* antioxidant activity of mackerel head and skin oils extracted by various processes was evaluated using the DPPH assay (Figure 6.8). The antioxidant activity of fish oil was assayed over a range of dilutions. In general, the antioxidant activity increased by increasing the oil concentration. Oils from mackerel heads at 25 and 50 mg/mL as well as oils from mackerel skins at 50 mg/mL showed higher DPPH scavenging capacity. The source and the extraction procedure significantly ($p < 0.05$) affected the antioxidant activity of the oils. Mackerel head oils had higher antioxidant activity than mackerel skin oils. Oils extracted from mackerel heads with solvent and after the enzymatic hydrolysis showed the highest scavenging activity against DPPH (Figure 6.8).

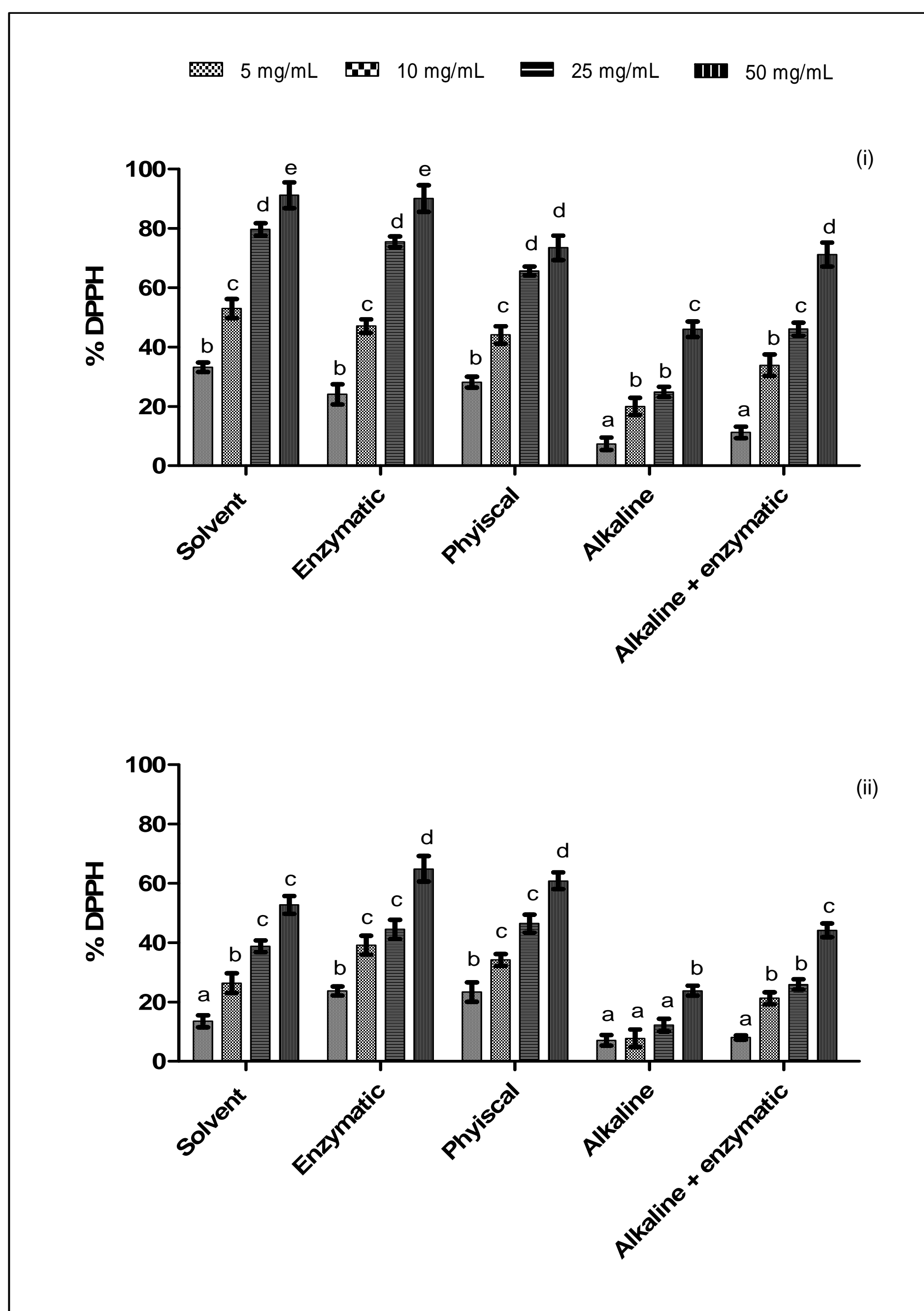


Figure 6.8 DPPH scavenging activity of mackerel head (i) and skin (ii) oils extracted using various procedures. Different lower case letters, within the same treatment indicate significant ($p < 0.05$) differences between the concentrations.

The EPA and DHA do not seem to be responsible for the antioxidant activity of these oils since the solvent extracted oils had the lowest EPA and DHA content (38.5 % and 64.9 %, respectively), but showed higher antioxidant activity than oils extracted from mackerel heads and skins using the alkaline treatment, the heat process and the combined alkaline and hydrolysis method. The HPLC/ELSD chromatograms of these two bioactive oils (from mackerel heads extracted by solvent and by enzymatic hydrolysis), showed the presence of unknown peaks (Figure 6.9).

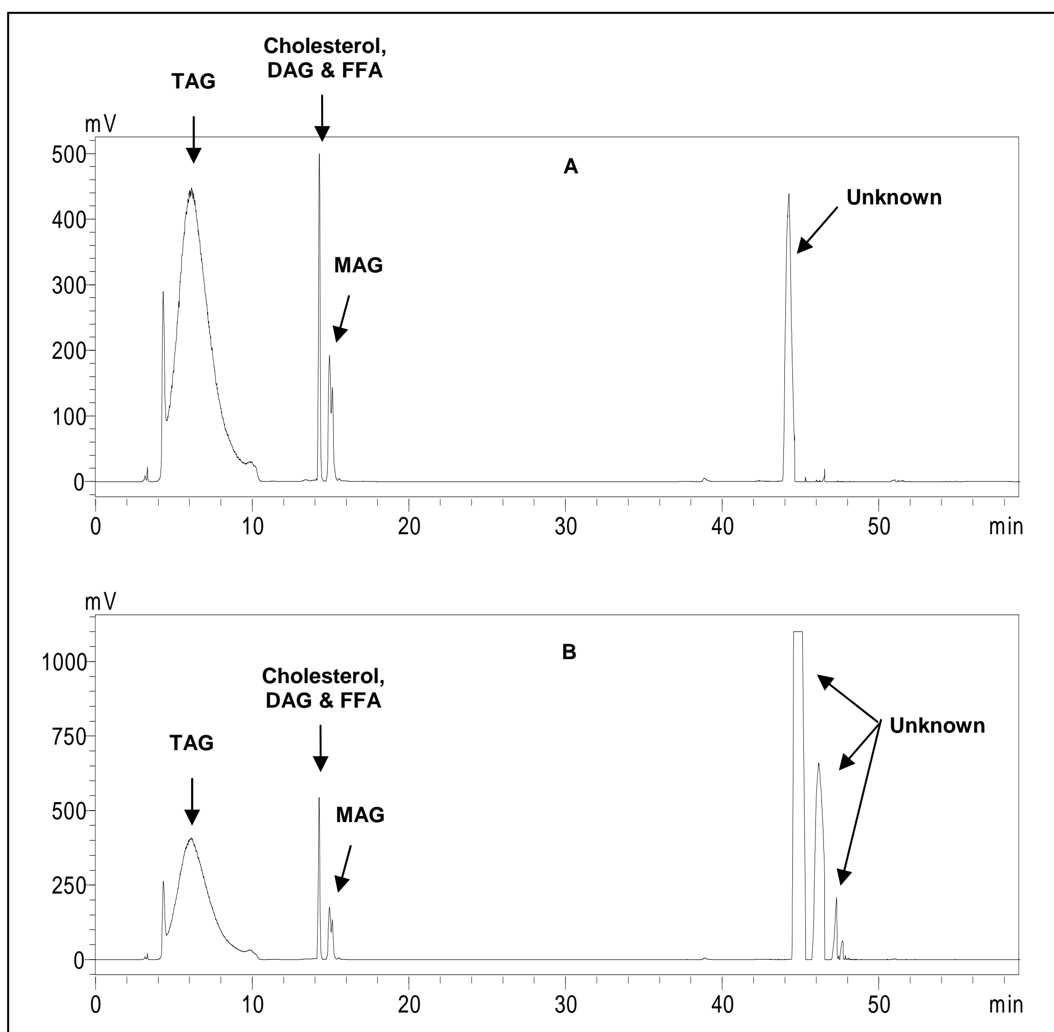


Figure 6.9 HPLC/ELSD chromatograms of mackerel head oils. (A) solvent extracted oil and (B) oil extracted after enzymatic hydrolysis.

These peaks correspond to low molecular compounds with high polarity since they were eluted at the end of the chromatograms. The DPPH scavenging activity of mackerel oils could be due to these compounds.

The polar and non polar fractions in mackerel head oils extracted after the enzymatic hydrolysis were separated by adsorption chromatography on Silica gel and visualised on TLC plates (Figure 6.10). It was noticed that the polar fraction (Figure 6.10, lane 3) contained coloured compounds.

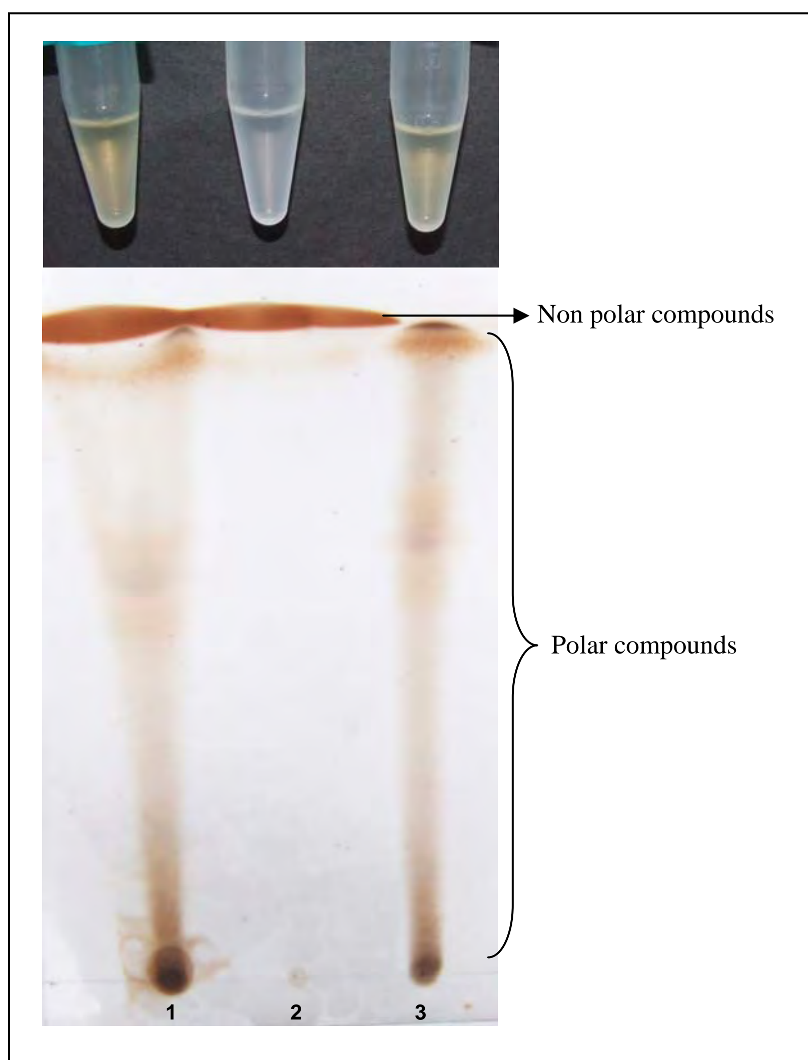


Figure 6.10 Separation of oil fraction by thin layer chromatography (TLC). 1: total fish oil sample; 2: non polar fraction and 3: polar fraction.

The two fractions along with the non-fractionated oil were analysed for their antioxidant activity. Results showed that the polar fractions exhibited higher DPPH scavenging capacity than the neutral fraction as seen in Figure 6.11.

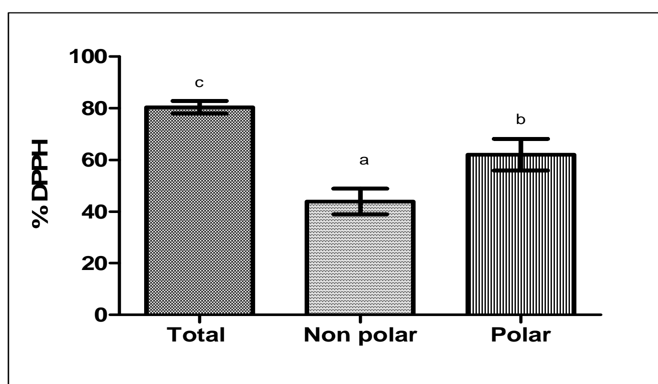


Figure 6.11 DPPH scavenging activity of fractionated mackerel head oil extracted after hydrolysis with Alcalase. Different letters indicate significant ($p < 0.05$) differences.

The higher antioxidant activity associated with the coloured fraction (polar fraction) could be due to the presence of pigments. The polyene structure of carotenoids can provide a chromophoric system responsible for the colouration of oil (Silva *et al.,* 2010). Previous studies showed that astaxanthin, a potent antioxidant carotenoid, was present in mackerel at levels of 6 - 11 mg/kg (Torrissen *et al.,* 1989). So far, the solvent and the enzymatic extractions were the main procedures to recover carotenes (Nollet, 2000). However, the alkaline conditions for the enzymatic hydrolysis may have converted astaxanthin to astacene (Goodwin, 1980) leading to the presence of more peaks in the HPLC-ELSD chromatogram. Hence, one can suggest that the radical scavenging activities of mackerel head oils could be the result of the presence of pigments (carotenoids).

6.3. Conclusions

Mackerel head and skin oils were mainly composed of TAG. The analysis of lipid classes showed that these oils did not contain the polar phospholipids. The chromatographic analysis revealed that mackerel oils had high levels of polyunsaturated long chain fatty acids. EPA and DHA were the main omega-3 fatty acid present with amounts in the range of 34 to 49 mg/g for EPA and 51 to 97 mg/g for DHA.

The oil recovered from the alkaline extraction had better physicochemical and visual qualities (low acidity, lower oxidation state and lighter colour) than oils extracted with the other procedures. However, the low extraction yield makes this process inefficient for industrial reasons. The enzymatic hydrolysis of mackerel heads liberated high amounts of oil with strong antioxidant activity and well balanced fatty acid composition. The antioxidant activity of these oils may be due to carotenoids that were recovered during the hydrolysis process.

Chapter 7 - General conclusions

- The processing of mackerel and blue whiting generated significant amount of waste (greater than 50 %). This waste consisted of heads, skins, bones and viscera and contained a high content of valuable compounds (proteins and fish oils).

- Although blue whiting waste contained high level of proteins, the low quality of these fish made them unsuitable for use.

- Mackerel waste is a potential source of gelatines, oils and protein hydrolysates.

- The best gelatines in terms of textural, rheological and functional properties were extracted from mackerel skin.

- The use of acetic acid in the pre-treatment of fish waste was recommended in order to obtain gelatines with higher functional properties. However, gelatines extracted after pre-treatment with this acid had lower visual quality (darker colour).

- The hydrolysis of mackerel skin gelatine with pepsin, for 24 h, resulted in hydrolysates with antioxidant, antihypertensive, anti-inflammatory and antithrombotic activities.

- Hydrolysates from mackerel viscera prepared with Flavourzyme, for 24 h, inhibited ACE and SSAO enzymes and showed antimicrobial and antioxidant activities.

- Chemical, physical and enzymatic methods were developed for extracting oils from mackerel waste. The enzymatic hydrolysis was found to recover higher amounts of oil with potent antioxidant activity.

An optimised protocol for recovering bioactive compounds from mackerel waste is presented in Figure 7.1.

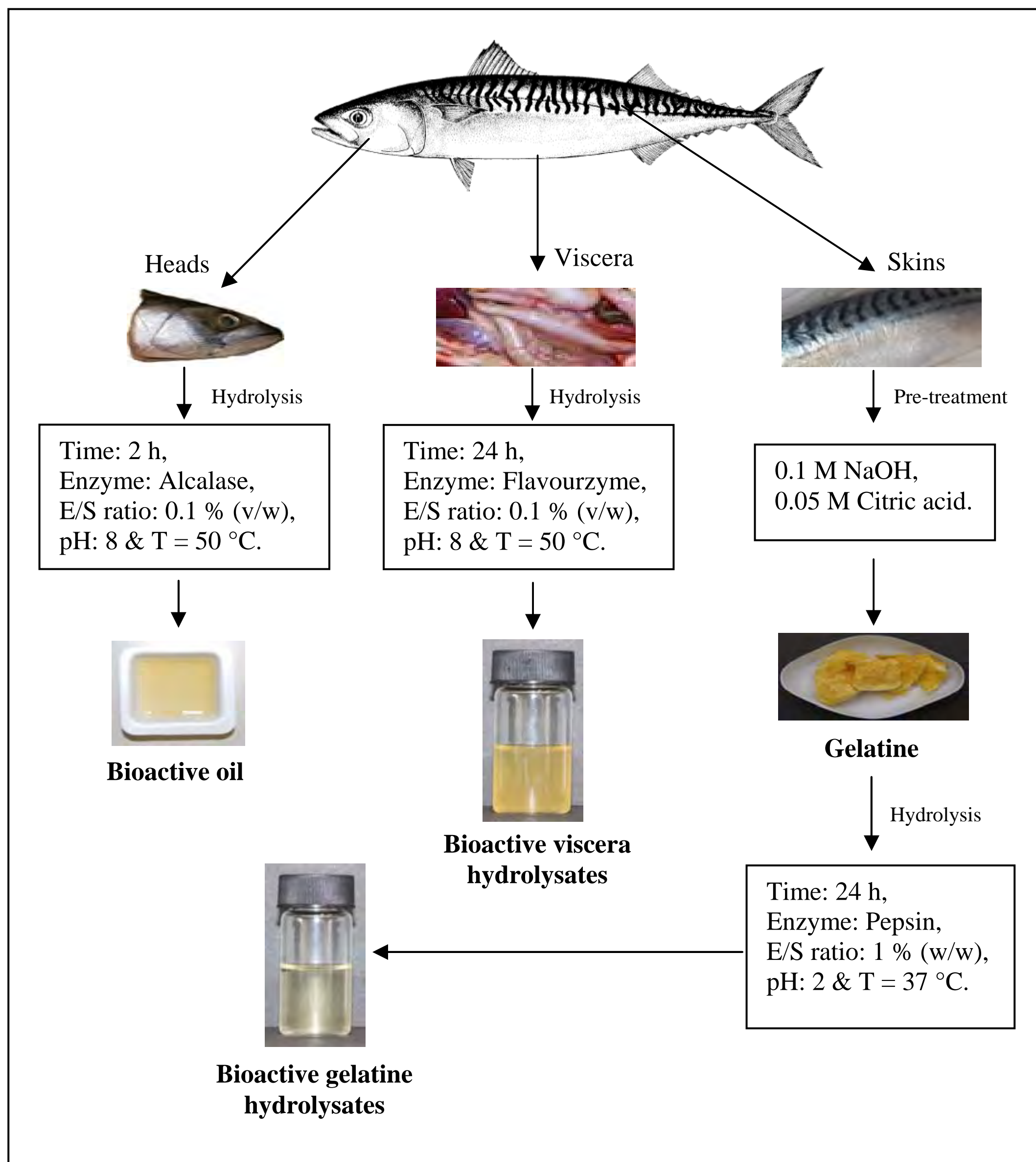


Figure 7.1 An optimised protocol for extracting bioactive compounds from mackerel waste.

Chapter 8 - References

Ababouch, L. (2005). Composition of fish. In: *FAO Fisheries and Aquaculture Department.* Rome, Italy. Available at: http://www.fao.org/fishery/topic/12318/en.

Abbaș, K.A., Mohamed, A., Jamilah, B. and Ebrahimian, M. (2008). A Review on Correlations between Fish Freshness and pH during Cold Storage. *American Journal of Biochemistry and Biotechnology.* 4, 416-421.

Adler-Nissen, J. (1986). Methods in food protein hydrolysis. In: *Enzymic hydrolysis of food proteins*, Adler-Nissen, J. (Ed.). Elsevier Applied Science Publishers, New York, USA. 116-124.

Aewsiri, T., Benjakul, S., Visessanguan, W. and Tanaka, M. (2008). Chemical compositions and functional properties of gelatin from pre-cooked tuna fin. *International Journal of Food Science and Technology*. 43, 685-693.

Agyare, K.K., Addo, K., and Xiong, L.Y. (2009). Emulsifying and foaming properties of transglutaminase-treated wheat gluten hydrolysate as influenced by pH, temperature and salt. *Food Hydrocolloids*. 23, 72-81.

Ajami, B., Bennett, J.L., Krieger, C., Tetzlaff, W. and Rossi, F.M. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature Neuroscence.* 10, 1538-1543.

Amarowicz, R. and Shahidi, F. (1997). Antioxidant activity of peptide fractions of capelin protein hydrolysates. *Food Chemistry*. 58, 355-359.

Amarowicz, R., Pegg, R.B., Rahimi-Moghaddam, P., Barl, B. and Weil, J.A. (2004). Free radical scavenging capacity and antioxidant activity of selected plant species from Canadian prairies. *Food Chemistry.* 84, 551-562.

AOAC (2000). Association of Official Analytical Chemist. In: *Official Methods of Analysis of AOAC International,* Horwitz, W. (Ed.). AOAC International, Maryland-Gaithersburg, USA.

Archer, M.H., Dillion, V.M., Campbell-Platt, G. and Owens, J.D. (1996). Effect of diacetyl on growth rate of Salmonella tyohimurium determination form detection time measured in a micro-well plate photometer. *Food Control.* 7, 63-67.

Ariyoshi, Y. (1993). Angiotensin-converting enzyme inhibitors derived from food proteins. *Trends in Food Science and Technology*. 4, 139-144.

Arnesen, J.A. and Gildberg, A. (2002). Preparation and characterisation of gelatine from the skin of harp seal (*Phoca groendlandica*). *Bioresource Technology.* 82, 191-194.

Arnesen, J.A. and Gildberg, A. (2006). Extraction of muscle proteins and gelatine from cod head. *Process Biochemistry.* 41, 697-700.

Arnesen, J.A. and Gildberg, A. (2007). Extraction and characterisation of gelatine from Atlantic salmon (*Salmo salar*) skin. *Bioresource Technology.* 98, 53-57.

Arts, M.J.T.J., Haenen, G.R.M.M., Voss, H. P. and Bast, A. (2001). Masking of antioxidant activity by the interaction of flavonoids with proteins. *Food and Chemical Toxicology.* 39, 787-791.

Aryee, A.N.A. and Simpson, B.K. (2009). Comparative studies on the yield and quality of solvent-extracted oil from salmon skin. *Journal of Food Engineering*. 92, 353-358.

Aryee, A.N.A., Van Der Voort, F.R. and Simpson, B.K. (2009). FTIR determination of free fatty acids in fish oils intended for biodiesel production. *Process Biochemistry*. 44, 401-405.

Aspmo, S.I., Horn, S.J. and Eijsink, V.G.H. (2005). Enzymatic hydrolysis of Atlantic cod (*Gadus morhua L.*) viscera. *Process Biochemistry*. 40, 1957-1966.

Aubourg, S.P., Rodríguez, A. and Gallardo, J.M. (2005). Rancidity development during frozen storage of mackerel (*Scomber scombrus*): effect of catching season and commercial presentation. *European Journal of Lipid Science and Technology*. 107, 316-323.

Baccouri, B., Ben Temime, S., Taamalli, W., Daoud, D., M'Sallem, M. and Zarrouk, M. (2007). Analytical characteristics of virgin olive oils from two new varieties obtained by controlled crossing on Meski variety. *Journal of Food Lipids.* 14, 19-34.

Badii, F. and Howell, K.N. (2006). Fish gelatin: Structure, gelling properties and interaction with egg albumen proteins. *Food Hydrocolloids*. 20, 630-640.

Baek, H.H. and Cadwallader, K. R. (1995). Enzymatic hydrolysis of crayfish processing by-products. *Journal of Food Science*. 60, 929-934.

Baily, A.J. and Light. N.D. (1989). Genes, Biosynthesis and Degradation of Collagen in *Connective tissue in meat and meat products*. Elsevier Applied Science, London, UK.

Balian, G. and Bowes, J.H. (1977). The structure and properties of collagen. In: *The science and technology of gelatin*, Ward, A.G. and Courts, A. (Eds.). Academic Press, London, UK. 1-30.

Bandekar, J., (1992). Amide modes and protein conformation *Biochimestry and Biophysic Acta*. 1120, 123-143.

Bautista-Banos, S., Hernandez-Lauzardo, A.N., Velazquez-del Valle, M.G., Hernandez-Lopez, M., Barka, E.A. and Bosquez-Molina, E. (2006). Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. *Crop Protection.* 25, 108-118.

Bech-Larsen, T. and Scholderer, J. (2007). Functional Foods in Europe: Consumer Research, Market Experiences and Regulatory Aspects. *Trends in Food Science and Technology*. 18, 231-234.

Beltrán De Heredia, J., Torregrosa, J., Dominguez, J.R. and Peres, J.A. (2001). Kinetic model for phenolic compound oxidation by Fenton's reagent. *Chemosphere*. 45, 85-90.

Benjakul, S. and Morrissey, M. (1997). Protein hydrolysates from Pacific whiting solid wastes. *Journal of Agricultural and Food Chemistry.* 45, 3423–3430.

Benjwal, S., Verma, S., Röhm, K.H. and Gursky, O. (2006). Monitoring protein aggregation during thermal unfolding in circular dichroism experiments. *Protein Science*. 15, 635-639.

Benkouider, C. (2004). Functional foods: A global overview. *International Food Ingredients.* 5, 66-68.

Benzie, I.F.F. and Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry.* 239, 70-76.

Berdeaux, O., Voinot, E., Juaneda, P. and Sebedio, J.L. (1998). Simple method of preparation of ethyl trans-10, cis-12- and cis-9, trans-11-octadecadienoates from methyl linoleate. *Journal of American Oil Chemist's Society.* 75, 1749-1755.

Bezerra, R.S., Dos Santos, J.F., Lino, M.A.S., Vieira, V.L. and Carvalho, L.B. (2000). Characterization of stomach and pyloric caeca proteinases of tambaqui *(Colossoma macropomum). Journal of Food Biochemistry*. 24, 188-199.

Bhaskar, N. and Mahendrakar, N.S. (2008). Protein hydrolysate from visceral waste proteins of Catla (*Catla catla*): Optimization of hydrolysis conditions for a commercial neutral protease. *Bioresource Technology*. 99, 4105-4111.

Biesalski, H.K., Dragsted, L.O., Elmadfa, I., Grossklaus, R., Müller, M., Schrenk, D., Walter, P. and Weber, P. (2009). Bioactive compounds: Definition and assessment of activity. *Nutrution.* 25, 1202-1205.

BIM (2010). The Irish Seafood National Programme 2007-2013. Available at: http://www.bim.ie/templates/reports.asp?node_id=384.

Bini, G. (1969). Atlante dei pesci delle coste Italiane. Mondo Sommerso Publisher, Rome, Italy.

Binsi, P.K., Shamasundar, B.A., Dileep, A.O., Badii, F. and Howell, N.K. (2009). Rheological and functional properties of gelatin from the skin of Bigeye snapper (*Priacanthus hamrur*) fish: Influence of gelatin on the gel-forming ability of fish mince. *Food Hydrocolloids*. 23, 132-145.

Bligh, E.G. and Dyer, W.J. (1959). A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology.* 37, 911-917.

Bomford, R. (1989). Aluminium salts: perspectives in their use as adjuvants. In: *Immunological Adjuvants and Vaccines*, Gregoriadis, G., Allison, A.C. and Poste, G. (Eds.). Plenum Press, New York, USA.

Boom, R.M. (2007). Emulsions: principles and preparation. In: *Food materials science: principles and practice*, Aguilera, J.M. and Lilford, P.J. (Eds.). Springer, New York, USA. 305-339.

Boomsma, F., De Kam, P.J., Tjeerdsma, G., Van Den Meiracker, A.H. and Van Veldhuisen, D.J. (2000). Plasma semicarbazide-sensitive amine oxidase (SSAO) is an independent prognostic marker for mortality in chronic heart failure. *European Heart Journal.* 21, 1859-1863.

Boran, G., Karacam, H. and Boran, M. 2006. Changes in the quality of fish oils due to storage temperature and time. *Food Chemistry*. 98: 693-698.

Bougatef, A., Nedjar-Arroume, N., Ravallec-Plé, R., Leroy, Y., Guillochon, D., Barkia, A. and 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, 350-356.

Bourne, M.C. (2002). Food Texture and Viscosity: Concept and Measurement. Academic Press, San Diego, USA.

Bovet, P., Faeh, D., Madeleine, G., Viswanathan, B. and Paccaud, F. (2007). Decrease in blood triglycerides associated with the consumption of eggs of hens fed with food supplemented with fish oil. *Nutrition, Metabolism and Cardiovascular Diseases*. 17, 280-287.

Boyle, E.C., Bishop, J.L., Grassl, G.A. and Finlay, B.B. (2007). Salmonella: from pathogenesis to therapeutics. *Journal of Bacteriology*.189, 1489-1495.

Boysen, R.I., Jong, A.J.O. and Hearn, M.T.W. (2002). Thermodynamic Assessment of the Stability of Thrombin Receptor Antagonistic Peptides in Hydrophobic Environments. *Biophysical Journal*. 82**,** 2279-2292.

Brennan, M.H. and Gormley, T.R. (1999). The Quality of Under-Utilised Deep-Water Fish Species. Project Report - 4560, Teagasc, Dublin, Ireland.

Bridgman, J.C., Chik, W. and Heddle, W. (2006). Wolf in Wolf's Clothing-A Case of Prosthetic Aortic Valve Thrombosis Presenting as an Acute Coronary Syndrome, Heart. *Lung and Circulation*. 15, 334-336.

BSI. (1975). Methods for sampling and testing gelatin (physical and chemical methods), British Standard Institution (No. 757), London, UK.

Brix, O., Apablaza, P., Baker, A., Taxt, T. and Grüner, R. (2009). Chemical shift based MR imaging and gas chromatography for quantification and localization of fat in Atlantic mackerel. *Journal of Experimental Marine Biology and Ecology*. 376, 68-75

Brodsky-Doyle, B., Bendit, E.G. and Blout, E.R. (1975). Infrared Spectroscopy of Collagen and Collagen-Like Polypeptides. *Biopolymers*. 14, 937-957.

Bunning, P., Holmquist, B. and Riordan, J. F. (1983). Substrate specificity and kineticcharacteristics of angiotensin converting enzyme. *Biochemistry.* 221, 110-116.

Bykov, V.P. (1983). Marine fishes: chemical composition and processing properties. Amerind Publishing Co., New Delhi, India.

Bykowski, P. and Dutkiewicz, D. (1996). Freshwater fish processing and equipment in small plants. *FAO Fisheries Circulars*. C905, Rome, Italy.

Caessens, P.W.J.R., Gruppen, H., Slangen, C.J., Visser, S. and Voragen, A.G.J. (1999). Functionality of β-casein peptides: Importance of amphipathicity for emulsion-stabilizing properties. *Journal of Agricultural and Food Chemistry.* 47, 1856-1862.

Carocci, F. (2008). Fishing areas for statistical purposes. In: *CWP handbook of fishery statistical standards, Section H*. Food and Agriculture Organisation of the United Nations (FAO), Fisheries and Aquaculture Department, Rome. Available at: www.fao.org/fishery/cwp/handbook/H.

Castillo-Yanez, F.J., Pacheco-Aguilar, R., Garcia-Carreno, F.L. and Toro, M.A.N. (2005). Isolation and characterization of trypsin from pyloric caeca of Monterey sardine Sardinops sagax caerulea. *Comparative Biochemistry and Physiology*. 140B, 91-98.

Chantachum, S., Benjakul, S. and Sriwirat, N. (2000). Separation and quality of fish oil from precooked and non-precooked tuna heads. *Food Chemistry.* 69, 289-294.

Cheftel, J.C., Cuq, J.L. and Lorient, D. (1985). In: *Food chemistry* (2nd edition), Fennema, O.R. (Ed.). Marcel Dekker, Inc., New York. USA. 245-369.

Cheow, C.S., Norizah, M.S., Kyaw, Z.Y. and Howell, N.K. (2007). Preparation and characterisation of gelatins from the skins of sin croaker (*Johnius dussumieri*) and shortfin scad (*Decapterus macrosoma*). *Food Chemistry.* 101, 386-391.

Chittur, KK. (1998). FTIR/ATR for protein adsorption to biomaterial surfaces. *Biomaterials*. 19, 357-369.

Cho, S.M., Gu, Y.S. and Kim, S.B. (2005). Extraction optimization and physical properties of yellowfin tuna (*Thunnus albacares*) skin gelatine compared to mammalian gelatins. *Food Hydrocolloids.* 19, 221-229.

Chopra, I. (2007). The increasing use of silver-based products as antimicrobial agents: A useful development or a concern. *Journal of antimicrobial Chemotherapy.* 59, 587-590.

Chuang, W.U., Pan, B.S. and Tsai, J.S. (2000). Inhibition of lipoxygenase and blood thinning effects of mackerel protein hydrolysates. *Journal of Food Biochemistry*. 24, 333-343.

Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. (1983). Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *Journal of Dairy Science.* 66, 1219-1227.

Codex Alimentarius Commission (2005). CODEX Standard for named vegetable oils, CODEX Stan 210, fats, oils and related products. Food and Agriculture Organisation of the United Nations, Rome, Italy.

Cohen, D.M., Inada, T., Iwamoto, T. and Scialabba, N. (1990). FAO species catalogue. Vol. 10. Gadiform fishes of the world (*Order Gadiformes*). An annotated and illustrated catalogue of cods, hakes, grenadiers and other gadiform fishes known to date. *FAO Fisheries Synopsis*.10 (125).

Cole, C.G.B. (2000). Gelatin. Encyclopedia of Food Science and Technology (2nd edition), Francis, J.F. (Ed.). John Wiley & Sons, New York, USA.

Colin, P. L. (1992). Reproduction of the Nassau grouper, *Epinephelus striatus* (*Pisces: Serranidae*) and its relationship to environmental conditions. *Environmental Biology of Fish*. 34, 357-377.

Collette, B.B. and Nauen, C.E. (1983). FAO species catalogue. Vol. 2. Scombrids of the world. An annotated and illustrated catalogue of tunas, mackerels, bonitos and related species known to date. *FAO Fisheries Synopsis.* 125(2).

Comas, D.I., Wagner, J.R. and Tomas, M.C. (2006). Creaming stability of oil in water (O/W) emulsions: Influence of pH on soybean protein-lecithin interaction. *Food Hydrocolloids*. 20, 990-996.

Commission of the European Communities (2003). Proposal for a regulation of the European Parliament and of the Council on nutrition and health claims made on foods, COM/2003/0424. Commission of the European Communities, Brussels.

Connor, W.E. (2000). Importance of n-3 fatty acids in health and disease. *American Journal of Clinical Nutrition*. 71 suppl, 171S-175S.

Council Directive (1999). Council Directive 1999/31/EC of 26 April 1999 on the landfill of waste. *Official Journal of the European Communities*. L 182, 16/07/1999.

Crexi, V.T., Grunennvaldt, F.L., Soares, L.A.D.S. and Pinto, L.A.A. (2009). Deodorisation process variables for croaker (M. furnieri) oil. *Food Chemistry.* 114, 396-401.

Cruz, C., Barbosa, C. and Saraiva, A. (2007). Distribution of larval anisakids in blue whiting off Portuguese fish market. *Helminthologia*. 44, 21-24.

Cuisset, T., Frere, C., Quilici, J., Morange, P.E., Nait-Saidi, L., Carvajal, J., Lehmann, A., Lambert, M., Bonnet, J.L. and Alessi, M.C. (2006). Benefit of a 600-mg loading dose of clopidogrel on platelet reactivity and clinical outcomes in patients with non-ST-segment elevation acute coronary syndrome undergoing coronary stenting. *Journal of the American College of Cardiology.* 48, 1339-1345.

Cushman, D.W. and Cheung, H.S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical Pharmacology*. 20, 1637-1648.

Dai, C.A., Chen, Y.F. and Ming-Wei Liu, M.W. (2006). Thermal properties measurements of renatured gelatin using conventional and temperature modulated differential scanning calorimetry. *Journal of Applied Polymer Science*. 99, 1795-1801.

Damodaran, S. (1996). Amino acids, peptides and proteins. In: *Food chemistry*, Fennema, O.R. (Ed.). Marcel Dekker, Inc., New York, USA. 321-429.

Darby, J.N. and Creighton, T.E.C. (1993). Protein structure. Oxford University Press, Oxford, UK.

Davarnejad, R., Kassim, K.M., Zainal, A. and Sata, A.S. (2008). Extraction of fish oil by fractionation through supercritical carbon dioxide. *Journal of Chemical Engineering Data*. 53, 2128-2132.

De La Rosa, A.P.B., A. Montoya, B., Martinez-Cuevas, P., Hernandez-Ledesma, B., Leon-Galvan, M.F., De Leon-Rodriguez, A. and Gonzalez, C. (2010). Tryptic amaranth glutelin digests induce endothelial nitric oxide production through inhibition of ACE: Antihypertensive role of amaranth peptides. *Nitric Oxide*. 23, 106-111.

De Lurdes, M., Dapkevicius, E., IBatista, I., Nout, M. J. R., Rombouts, F.M. and Houben, J.H. (1998). Lipid and protein changes during the ensilage of blue whiting (*Micromesistius poutassou Risso*) by acid and biological methods. *Food Chemistry*. 63, 97-102.

De Boer, J. (1988). Chlorobiphenyls in bound and non-bound lipids of fishes: comparison of different extraction methods. *Chemosphere*. 17, 1803-1810.

Decker, E.A. and Welch, B. (1990). The role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*. 38, 674-677.

Devienne, K.F. and Raddi, M.S.G. (2002). Screening for antimicrobial activity of natural products using a microplate photometer. *Brazilian Journal of Microbiology*. 33, 166-168.

Di Giuseppe, E., Funiciello, F., Corbi, F., Ranalli, G. And Mojoli, G. (2009). Gelatins as rock analogs: A systematic study of their rheological and physical properties. *Tectonophysics*. 473, 391-403.

Djabourov, M. (1988). Architecture of gelatin gels. *Contemporary Physics*. 29, 273-297.

Djabourov, M., Lechaire, J. P. and Gaill, F. (1993). Structure and rheology of gelatin and collagen gels. *Biorheology*. 30, 191-205.

Djukic, M., Mildner, A., Schmidt, H., Czesnik, D., Bruck, W., Priller, J., Nau, R. and Prinz, M. (2006). Circulating monocytes engraft in the brain, differentiate into microglia and contribute to the pathology following meningitis in mice. *Brain*. 129, 2394-2403.

Dobson, G. (2008). Analysis of fatty acids in functional foods with emphasis on ω3 fatty acids and conjugated linoleic acid. In: *Methods of Analysis for Functional Foods and Nutraceuticals*. CRC Press, Boca Raton, USA. 85-145.

Dufossé, L., De La Broisse, D. and Guerard, F. (1997). Fish protein hydrolysates as nitrogen sources for microbial growth and metabolite production. *Recent Research and Development in Microbiology*. 1, 365-381.

Dyerberg, J. and Bang, H.O. (1979). Hemostatic function and platelet polyunsaturated fatty acids in eskimos. *Lancet.* 2, 433-435.

Eastoe, J.E. and Eastoe, B. (1952). A method for the determination of total nitrogen in proteins. *The British Gelatine and Glue Research Association Research Report, Series B 5*. 1-17.

Eastoe, J.E. and Leach, A.A. (1958). A survey of recent work on the amino acid composition of vertebrate collagen and gelatine. In: *Recent Advances in Gelatin and Glue Research*, Stainsby, G. (Ed.). Pergamon Press, London, UK.

Elias, R.J. Bridgewater. J.M. Vachet, R.W. Waraho, T. Mcclements, D.J. and Decker, E.A. (2006). Antioxidant Mechanisms of Enzymatic Hydrolysates of *α*-Lactoglobulin in Food Lipid Dispersions. *Journal of Agricultural and Food Chemistry.* 54, 9565-9572.

Enrique-Tarancón, G., Marti, L., Morin, N., Lizcano, J.M., Unzeta, M., Sevilla, L., Camps, M., Palacín, M., Testar, X., Carpéné, C. and Zorzano, A. (1998). Role of semicarbazide-sensitive amine oxidase on glucose transport and GLUT4 recruitment to the cell surface in adipose cells. *Journal of Biological Chemistry*. 273, 8025-8032.

Enzyme Nomenclature, NC-IUBMB. (1992). Academic Press, New-York, USA.

Exerowa, D. and Kruglyakov, P. (1998). Foam and Foam Films: Theory, Experiment, Application (Studies in Interface Science). Elsevier, Amsterdam, Belgium.

Eysturskard, J., Haug, I.J., Elharfaoui, N., Djabourov, M. and Draget, K.I. (2009). Structural and mechanical properties of fish gelatin as a function of extraction conditions. *Food Hydrocolloids* 23, 1702-1711.

Fahmi, A., Morimura, S., Guo, H. C., Shigematsu, T., Kida, K. and Uemura, Y. (2004). Production of angiotensin I converting enzyme inhibitory peptides from sea bream scales. *Process Biochemistry*. 39, 1195-1200.

FAO (2010). Fisheries and Aquaculture Department, Data and Statistics Unit. Fishstat Plus: Universal software for fishery statistical time series. *Global Production Statistics.* 1950-2010.

FAO FishFinder (2010). The Species Identification and Data Programme, Species distribution maps In: *FAO Fisheries and Aquaculture Department*. Rome, Italy. Available at: http://www.fao.org/fishery/fishfinder/maps/en. (Accessed on 09/02/2010)

Fabian, H. and Mantele, W. (2002). Infrared Spectroscopy of Proteins. In: *Handbook of Vibrational Spectroscopy*, Chalmers, J.M. and Griffiths, P.R. (Eds.). Wiley, Chichester, UK. 3399-3425.

Farmer, T. (2003). Fish and seafood utilization. In: *FAO Fisheries and Aquaculture Department*. Rome, Italy. Available at: http://www.fao.org/Fishery /topic/424/en.

Fernandez, E., Chatenoud, L., La Vecchia, C., Negri, E. and ceschi, S. (1999). Fish consumption and cancer risk. *American Journal of Clinical Nutrition*. 70, 85-90.

Fernandez, Q.A. and Macarulla, M.T. (1997). Composition and functional properties of protein isolates obtained from commercial legumes grown in northern Spain. *Plant Foods for Human Nutrition.* 51, 331-342.

Ferrer, I., Lizcano, J.M., Hernandez, M. and Unzeta, M. (2002). Overexpression of semicarbazide sensitive amine oxidase in the cerebral blood vessels in patients

with Alzheimer's disease and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *Neuroscience Letters*. 321, 21-24.

Ferry, J.D. (1980). Viscoelastic Properties of Polymers. (3rd edition), Wiley, NewYork, USA.

Flugel, A., Bradl, M., Kreutzberg, G.W. and Graeber, M.B. (2001). Transformation of donor-derived bone marrow precursors into host microglia during autoimmune CNS inflammation and during the retrograde response to axotomy. *Journal of Neuroscience Research.* 66, 74-82.

Foegeding, E.A., Laniner, T.C. and Hultin, H.O. (1996). Collagen. In: *Food chemistry* (3rd edition), Fennema, O.R. (Ed.). Marcel Dekker, Inc., New York, USA. 902-906.

Foegeding, E.A., Luck, P.J. and Davis, J.P. (2006). Factors determining the physical properties of protein foams. *Food Hydrocolloids.* 20, 284-292.

Folador, J.F., Karr-Lilienthal, L.K., Parsons, C.M., Bauer, L.L., Utterback, P.L., Schasteen, C.S., Bechtel, P.J., Fahey, G.C.J. (2006). Fish meals, fish components, and fish protein hydrolysates as potential ingredients in pet foods. *Journal of Animal Science*. 84, 2752-2765.

Folch, J., Lees, M. and Stanley, G.H.S. (1957). A simple method for the isolation and purification of lipids from animal tissues. *Journal of Biological Chemistry.* 226, 497-509.

Fontecha, J., Diaz, V., Fraga, M.J. and Juarez, M. (1998). Triglyceride analysis by GC in assessment of authenticity of goat milk fat. *Journal of the American Oil Chemists' Society.* 75, 1893-1896.

Frankel, E.N. and Meyer, A.S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants - review. *Journal of the Science of Food and Agriculture*. 80, 1925-1941.

Friedewald, V.E., Bates, E.R., Granger, C.B., Yusuf, S. and Roberts, W.C. (2007). The Editor's Roundtable: arterial thrombosis and acute coronary syndromes. *American Journal of Cardiology*. 100, 974-980.

Friess, W. and Lee, G. (1996). Basic thermoanalytical studies of insoluble collagen matrices. *Biomaterials.* 17, 2289-2294.

FSAI (2008). *Compendium of Food law in Ireland*. Food Safety Authority of Ireland, Dublin, Ireland.

Fukami, H., Holmsen, H., Kowalska, M. and Niewiarowski, S. (2001). Platelet secretion. In: *Haemostasis and Thrombosis: Basic Principles and Clinical Practice* (4th edition), Colman, RW., Hirsh, J., Marder, V.J., Clowes, A.W. and James N. George, J.N. (Eds.). Lippincott Williams and Wilkins, Philadelphia, USA. 561-574.

Gbogouri, G. A., Linder, M., Fanni, J. and Parmentier, M. (2004). Influence of hydrolysis degree on the functional properties of salmon by-products hydrolysates. *Journal of Food Science*. 69, 615-622.

Geelen, A., Brouwer, I.A., Schouten, E.G., Maan, A.C., Katan, M.B. and Zock, P.L. (2005). Effects of n-3 fatty acids from fish on premature ventricular complexes and heart rate in humans. *American Journal of Clinical Nutrition*. 81, 416-20.

German, J.B. and Phillips, L. (1994). Protein interactions in foams: protein–gas phase interactions. In: *Protein Functionality in Food Systems,* Hettiarachchy, N.S. and Ziegler, G.R. (Eds.). *IFT Basic Symposium Series 9*.

Gerrard, J.A. (2006). Protein cross-linking in foods. In: *Food biochemistry and food processing*, Hui, Y.H. and Nip. W.K. (Eds.). Wiley-Blackwell, Oxford, UK. 223-240.

Gildberg, A. (1993). Enzymatic processing of marine raw materials. *Process Biochemistry*. 28, 1-15.

Gildberg, A., Arnesen, J.A. and Carlehog, M. (2002). Utilisation of cod backbone by biochemical fractionation. *Process Biochemistry*. 38, 475-480.

Gilmartin, L and Jervis, L. (2002). Production of cod (*Gadus morhua*) muscle hydrolysates: influence of combinations of commercial enzyme preparations on hydrolysate peptide size range. *Journal of Agricultural and Food Chemistry.* 50, 5417-5423.

Gilsenan, P.M. and Ross-Murphy, S.B. (2000). Rheological characterisation of gelatins from mammalian and marine sources. *Food Hydrocolloids.* 14, 191-196.

Giménez, B., Turnay, J., Lizarbe, M.A., Montero, P. and Gómez-Guillén, M.C. (2005). Use of lactic acid for extraction of fish skin gelatine. *Food Hydrocolloids.* 19, 941-950.

Giugliano, R.P., Wiviott, S.D., Stone, P.H., Simon, D.I., Schweiger, M.J., Bouchard, A., Leesar, M.A., Goulder, M.A., Deitcher, S.R., McCabe, C.H. and Braunwald, E. (2007). Recombinant nematode anticoagulant protein c2 in patients with non–ST-segment elevation acute coronary syndrome: the ANTHEM–TIMI-32 Trial. *Journal of the American College of Cardiology.* 49, 2398-2407.

Glaser, L.A., Paulson, A.T., Speers, R.A., Yada, R.Y. and Rousseau, D. (2007). Foaming behavior of mixed bovine serum albumin-protamine system. *Food Hydrocolloids.* 21, 495-506.

GME (2008). Gelatin Manufacturers of Europe. Available at: http://www.gelatine.org/en /gelatine/overview/127.htm.

Gómez-Guillén, M.C. and Montero, P. (2001). Extraction of gelatine from megrim (*Lepidorhombus boscii*) skins with several organic acids. *Journal of Food Science*. 66, 213-216.

Gómez-Guillén, M.C., Giménez, B. and Montero, P. (2005). Extraction of gelatin from fish skins by high pressure treatment. *Food Hydrocolloids*. 19, 923-928.

Gómez-Guillén, M.C., Ihl, M., Bifani, V., Silva, A. and Montero, P. (2007). Edible films made from tuna-fish gelatin with antioxidant extracts of two different murta ecotypes leaves (*Ugni molinae Turcz*). *Food Hydrocolloids*. 21, 1133-1143.

Gómez-Guillén, M.C., Turnay, J., Fernandez-Diaz, M.D., Ulmo, N., Lizarbe, M.A. and Montero, P. (2002). Structural and physical properties of gelatin extracted from different marine species: a comparative study. *Food Hydrocolloids*. 16, 25-34.

Goodwin, T.W. (1980). Nature and properties. In: *The biochemistry of carotenoids* (2nd edition.). Chapman and Hall, London, UK. 1-32.

Gornall, A.G., Bardawill, C.J. and David, M.M. (1949). Determination of serum proteins by means of the biuret reaction. *The Journal of Biological Chemistry*. 177, 751-766.

Goto, N., Kato, H., Maeyama, J.I., Eto, K. and Yoshihara, S. (1993). Studies on the toxicities of aluminium hydroxide and calcium phosphate as immunological adjuvants for vaccines. *Vaccine.* 11, 914-918.

Goto, S., Sakai, H., Goto, M., Ono, M., Ikeda, Y., Handa, S. and Ruggeri, Z.M. (1999). Enhanced shear-induced platelet aggregation in acute myocardial infarction. *Circulation*. 99, 608-613.

Goulas, A.E. and Kontominas, M.G. (2007). Combined effect of light salting, modified atmosphere packaging and oregano essential oil on the shelf-life of sea bream (*Sparus aurata*): Biochemical and sensory attributes. *Food Chemistry*. 100, 287-296.

Grant, M.E. (2007). From collagen chemistry towards cell therapy-a personal journey. *International Journal of Experimental Pathology*. 88, 203-214

Grossman, S. and Bergman, M. (1992). Process for the production of gelatin from fish skins. US Patent 5,093,474.

Guérard, F., Guimas, L. and Binet, A. (2002). Production of tuna waste hydrolysates by a commercial neutral protease preparation. *Journal of Molecular Catalysis B: Enzymatic.* 19-20, 489-498.

Guérard, F. and Maria-Teresa Sumaya-Martinez, M.T. (2003). Antioxidant effects of protein hydrolysates in the reaction with glucose. *Journal of the American Oil Chemists' Society*. 80, 467-470.

Guillen, M.D. and Cabo, N. (1997). Characterization of edible oils and lard by Fourier transform infrared spectroscopy. Relationships between composition and frequency of concrete bands in the fingerprint region. *Journal of the American Oil Chemists' Society.* 74, 1281-1286.

Gundmundsson, H. and Hafsteinsson, H. (1997). Gelatin from cod skins as affected by chemical treatments. *Journal of Food Science.* 52, 37-39.

Hamazaki, K., Itomura, M., Sawazaki, S. and Hamazaki, T. (2006). Fish oil reduces tooth loss mainly through its anti-inflammatory effects? *Medical Hypotheses*. 67, 868-870.

Haris, P.I. and Chapman, D. (1994). Analysis of Polypeptide and Protein Structures Using Fourier Transform Infrared Spectroscopy. In: *Microscopy, Optical Spectroscopy and Macroscopic Techniques (Methods in Molecular Biology)*, Jones, C., Mulloy, B. and Thomas, A.H. (Eds.). The Humana Press, Totowa, NJ. USA. 183-202.

Harris W.S. (2005). Extending the cardiovascular benefits of omega-3 fatty acids. *Current Atherosclerosis Report*. 7, 375-380.

Haug, I.J., Draget, K.I. and Smidsrød, O. (2004). Physical and rheological properties of fish gelatin compared to mammalian gelatine. *Food Hydrocolloids.* 18, 203-213.

Hayashi, T. and Mukamel, S. (2008). Two-dimensional vibrational lineshapes of amide III, II, I and A bands in a helical peptide. *Journal of Molecular Liquids*. 141, 149-154.

He, H., Chen, X., Sun, C., Zhang, Y. and Gao, P. (2006). Preparation and functional evaluation of oligopeptide-enriched hydrolysate from shrimp (*Acetes chinensis*) treated with crude protease from *Bacillus sp*. SM98011. *Bioresource Technology*. 97, 385-390.

He, H.L., Chen, X.L., Sun, C.Y., Zhang, Y.Z. and Zhou, B.C. (2007). High throughput and rapid screening of marine protein hydrolysates enriched in peptides with angiotensin-I-convering enzyme inhibitory activity by capillary electrophoresis. *Bioresource Technology.* 98, 3499-3505.

He, K., Song, Y., Daviglus, M.L., Liu, K., Van Horn, L., Dyer, A.R. and Greenland, P. (2004). Accumulated evidence on fish consumption and coronary heart disease mortality: a meta-analysis of cohort studies. *Circulation*. 109, 2705-2711.

Hermansson, A.M. (2007). Structuring water by gelation. In: *Food materials science*, Aguilera, J.M. and Lilford, P.J. (Eds.). Springer, New York, USA.

Hevia, P., Whiteker, J. R. and Olcott, H. S. (1976). Solubilization of a fish protein concentrate with proteolytic enzymes. *Journal of Agricultural and Food Chemistry.* 24, 383-386.

Higdon, J.V. and Frei, B. (2003). Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Critical Reviews in Food Science and Nutrition.* 43, 89-143.

Hirata, H., Saeki, H., Nonaka, M., Kawasaki, K., Ooizumi, T. and Motoe, K. (1993). Recovery of fish oil from the manufacturing process of highly nutritional fish meat for foodstuffs from sardine. *Nippon Suisan Gakkaishi*. 59, 111-116.

Holt, A., Sharman, D.F., Baker, G.B. and Palcic, M.M. (1997). A continuous spectrophotometric assay for monoamine oxidase and related enzymes in tissue homogenates. *Analytical Biochemistry*. 244, 384-392.

Horton, R.H., Moran, A.L., Ochs, S.R., Rawn, D.J. and Scrimgeour, G.K. (1993). Principle of Biochemistry. Prentice-Hall International, Inc., Englewood Cliffs, NJ, USA.

Hou, C.W., Chen, H.J. and Yaw-Huei, L. (2003). Antioxidant peptides with angiotensin converting enzyme inhibitory activities and applications for angiotensin-I-converting enzyme purification. *Journal of Agricultural and Food Chemistry.* 51, 1706-1709.

Houen, G. (1999). Mammalian Cu-containing amine oxidases (CAOs): new methods of analysis, structural relationships, and possible functions. *Acta Pathologica, Microbiologica et Immunologica Scandinavica*. 96, 1-46.

Huang, D., Ou, B. and Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*. 53, 1841-1856.

Huang, T.F., Chao-Zong, L., Chaoho, O. and Che-Ming, T. (1991). Halysin, an antiplatelet Arg-Gly-Asp-containing snake venom peptide, as fibrinogen receptor antagonist. *Biochemical Pharmacology*. 42, 1209-1219.

Huang, T.F., Wu Y.G. and Ouyang, C. (1987). Characterization of a potent platelet aggregation inhibitor from Agkistrodon rhodostoma snake venom. *Biochimica et Biophysica Acta (BBA)*. 925, 248-257.

Huebner, J., Wehling, R.L. and Hutkins, R.W. (2007). Functional activity of commercial prebiotics. *International Dairy Journal*. 17, 770-775.

Hung, S.C. and Zayas, J.F. (1991). Emulsifying capacity and emulsion stability of milk proteins and corn germ protein flour. *Journal of Food Science*. 56, 1216-1223.

Huss, H.H. (1988). Fresh fish - quality and quality changes: a training manual. Food and Agriculture Organization of the United Nations (FAO). Rome, Italy.

Huss, H.H. (1995). Quality and quality changes in fresh fish. Food and Agriculture Organisation of the United Nations (FAO). Rome, Italy. Available at: http://www.fao.org/docrep/v7180e/V7180E00.HTM#Contents.

ICES (2006). Report of the Working Group on the Assessment of Mackerel, Horse Mackerel, Sardine and Anchovy (WGMHSA). ICES CM 2006/ACFM:08.

Ismail, A.A., Van de Voort, F.R., Emo, G. And Sedman, J. (1993). Rapid quantitative determination of free fatty acid in fats and oils by Fourier Transform Infrared Spectrocopy. *Journal of American Oil Chemists Society*. 70, 335-341.

Ismail, A.A., Cocciardi, R., Alvarez, P. and Sedman, J. (2006). Infrared and Raman Spectroscopy in Food Science**.** In: *Handbook of Food Science, Technology and Engineering*, Hui**,** Y. H.**,** (Ed.)**.** CRC Press**,** Boca Raton**,** USA**.** 44.1**-** 44.19.

ISO (2002). Milk fat-Preparation of fatty acid methylesters. International Standard method ISO-IDF, No. 15884, 182. International Organisation for Standardisation (Geneva, Switzerland) and International Dairy Federation (Brussels, Belgium).

IUPAC (1987). Determination of polar compounds in frying fats (Standard Method 2.507). In: *Standard Methods for the Analysis of Oils, Fats and Derivatives* (7th edition). International Union of Pure and Applied Chemistry, Blackwell, Oxford, UK.

Jao, C.L. and Ko, W.C. (2002). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolyzates from tuna cooking juice. *Fisheries Science.* 68**,** 430-435.

Je, J.Y., Park, P.J. and Kim, S.K. (2005a). Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Food Research International.* 38, 45-50.

Je, Y., Kim, S.Y. and Kim, S.K. (2005b). Preparation and antioxidative activity of hoki frame protein hydrolysate using ultrafiltration membranes. *European Food Research and Technology*. 221,157-162.

Je, J.Y., Park, P.J., Kwon, J.Y. and Kim, S.K. (2004). A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (*Theragra*

chalcogramma) frame protein hydrolysate. *Journal of Agricultural and Food Chemistry.* 52, 7842-7845.

Je, J.Y., Qian, Z.J., Byun, H.G. and Kim, S.K. (2007). Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry.* 42, 840-846.

Jeon, Y.J., Byun, H.G. and Kim, S.K. (1999). Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. *Process Biochemistry*. 35, 471-478.

Jeon, H.B. and Sayre, L.M. (2003). Highly potent propargylamine and allylamine inhibitors of bovine plasma amine oxidase. *Biochemical and Biophysical Research Communications.* 304, 788-794.

Jespersen, C., Christiansen, K. and Hummelmose, B. (2000). Cleaner production assessment in fish processing. Prepared by COWI consulting engineers and planners AS. Paris: United Nations Environment Programme and Danish EPA.

Jobling, M., Larsen, A.V., Andreassen, B., Sigholt, T. and Olsen, R.L. (2002). Influence of a dietary shift on temporal changes in fat deposition and fatty acid composition of Atlantic salmon post-smolt during the early phase of seawater rearing. *Aquaculture Research*. 33, 875-889.

Johns, P. and Courts, A. (1977). Relationship between collagen and gelatine. In: *The Science and Technology of Gelatin*, Ward, A.G. and Courts, A. (Eds.). Academic Press, London. UK. 138-177.

Johnston-Banks, F.A. (1990). Gelatin. In: *Food gels*, Harris, P. (Ed.). Elsevier Applied Science Publishers, London, UK. 233-289.

Jones, R.N. (1977). Uses of gelatine in edible products. In: *The science and technology of gelatine,* Ward, A. and Courts, A. (Eds.). Academic Press Inc., London, UK. 366-394.

Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. (2006). Skin gelatin from bigeye snapper and brownstripe red snapper: Chemical compositions and effect of microbial transglutaminase on gel properties. *Food Hydrocolloids*. 20, 1216-1222.

Jun, S.Y., Park, P.J., Jung, W.K. and Kim, S.K. (2004). Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *European Food Research and Technology*. 219, 20-26.

Jung , W.K., Mendis, E., Je, J.Y., Park, P.J., Son, B.W., Kim., H.C., Choi, Y.K. and 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, 26-32.

Karádi, I., Mészáros, Z., Csányi, A., Szombathy, T., Hosszúfalusi, N., Romics, L. and Magyar, K. (2002). Serum semicarbazide-sensitive amine oxidase (SSAO) activity is an independent marker of carotid atherosclerosis. *Clinica Chimica Acta*. 323, 139-146.

Kasapis, S., Norton, I.T. and Ubbink, J.B. (2009). Modern Biopolymer Science: Bridging the Divide between Fundamental Treatise and Industrial Application. Academic Press, London, UK.

Ke, P.J., Ackman, R.G. and Linke, B.A. (1975). Autoxidation of polyun- saturated fatty compounds in mackerel oil: Formation of 2,4,7decatrienals. *Journal of American Oil Chemists Society*. 52, 349-353.

Keil, B. (1992). Specificity of proteolysis. Springer, NewYork, USA.

Kemp, W. (1987). Organic Spectroscopy (2nd edition). Macmillan Education Ltd., Hampshire, UK.

Kerschensteiner, M., Meinl, E. and Hohlfeld, R. (2008) Neuro-immune crosstalk in CNS diseases. *Neuroscience*. 158, 1122-1132.

Kielty, C.M., Hopkinson, I. and Grant. M.E. (1993). Molecular, genetic, and medical aspects. In: Connective Tissue and its Heritable Disorders, Royce, P.M. and Steinmann, B. (Eds.). Wiley-Liss, New York. USA. 103-147.

Kim, J.S. and Park, J.W. (2004). Characterization of acid-soluble collagen from Pacific whiting surimi processing byproducts. *Journal of Food Science*. 69, C637-C642.

Kim, S.K. and Mendis, E. (2006). Bioactive compounds from marine processing byproducts - A review. *Food Research International.* 39, 383-393.

Kim, S.K., Kim, Y.T., Byun, H.G., Nam, K.S., Joo, D.S. and Shahidi, F. (2001). Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Allaska Pollack skin. *Journal of Agricultural and Food Chemistry.* 49, 1984-1989.

Kim, S.K., Mendis, E. and Shahidi, F. (2008). Marine fisheries by-products as potential nutraceuticals: an overview. In: Marine nutraceuticals and functional foods, Barrow, C. and Shahidi, F. (Eds.). CRC / Taylor & Francis, Boca Raton, USA. 1-22.

Kimura, S. and Ohno, Y. (1987). Fish type I collagen: Tissue specific existence of two molecular forms, (α1)2α2 and α1α2α3 in Alaska pollack. *Comparative Biochemistry and Physiology.* 88B, 409-413.

Kimura, S., Zhu, X., Matsui, R., Shijoh, M. and Takamizawa, S. (1988). Characterisation of fish muscle type I collagen. *Journal of Food Science.* 23, 1315-1316.

Kinsella, J.E. (1976). Functional properties of proteins in foods: A Survey, *CRC Critical Review in Food Science and Nutrition.* 7, 219-280.

Kinsella, J.E. (1981). Functional properties of proteins: Possible relationships between structure and function in foams. *Food Chemistry*. 7, 273-288.

Kinsella, J.E. (1982). Relationship between structure and functional properties of food proteins. In: *Food proteins*, Fox, P.F. and Cowden, J.J. (Eds.). Applied Science Publisher, London, UK. 51-103.

Kinsella, J.E. and Phillips, L. (1989). Structure–function relationships in food proteins, film and foaming behavior. In: *Food proteins*, Kinsella, J.E. and Soucie, W. (Eds.). *The American Oil Chemistry Society*. Champaign, USA. 52-77.

Kinsella, J.E., Lokesh, B. and Stone, R.A. (1990). Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: possible mechanisms. *American Journal of Clinical Nutrition.* 52, 1-28.

Kishimura, H., Tokuda, Y., Klomklao, S., Benjakul, S. and Ando, S. (2006). Enzymatic characteristics of trypsin from pyloric ceca of spotted mackerel (*Scomber Australasicus*). *Journal of Food Biochemistry*. 30, 466-477.

Kishimura, H., Tokuda, Y., Yabe, M., Klomklao, S., Benjakul, S. and Ando, S. (2007). Trypsins from the pyloric ceca of jacopever (Sebastes schlegelii) and elkhorn sculpin (Alcichthys alcicornis): Isolation and characterization. *Food Chemistry*. 100, 1490-1495.

Kitson, F.G., Larsen, B.S. and McEwen, C.N. (1996). What is GC/MS?. In: *Gas chromatography and mass spectrometry: a practical guide*, Kitson, F.G., Larsen, B.S. and McEwen, C.N. (Eds.). Academic Press, San Diego, USA. 3-23.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. (2005). Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). *Food Chemistry*. 89, 363-372.

Kivirikko, K. I. (1993). Collagens and their abnormalities in a wide spectrum of diseases. *Annals of Medicine*. 25, 113-126.

Klompong, V., Benjakul, S., Kantachote, D. and 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, 1317-1327.

Koike, H., Ito, K., Miyamoto, M. and Nishino, H. (1980). Effects of long-term blockade of angiotensin-converting enzyme with captopril (SQ 14, 225) on hemodynamics and circulating blood volume in SHR. *Hypertension*. 2, 229-303.

Kolkovski, S. (2001). Digestive enzymes in fish larvae and juveniles-implications and applications to formulated diets. *Aquaculture.* 200, 181-201.

Kolodziejska, I., Skierka, E., Sadowska, M., Kolodziejski, W. and Niecikowska, C. (2008). Effect of extracting time and temperature on yield of gelatin from different fish offal. *Food Chemistry*. 107, 700-706.

Kotch F.W., Guzei I.A. and Raines R.T. (2008). Stabilization of the collagen triple helix by O-methylation of hydroxyproline residues. *Journal of the American Chemical Society*. 130, 2952-2953.

Kotilainen, L., Rajalahti, R., Ragasa, C., & Pehu, E. (2006). Health enhancing foods:Opportunities for strengthening the sector in developing countries. Agriculture and Rural Development Discussion Paper 30.

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

Kristinsson, H.G. and Rasco, B.A. (2000b). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition.* 40, 43-81.

Kuraishi, C., Nakagoshi, H., Tanno, H. and Tanaka, H. (2000). Application of transglutaminase for food processing. *Hydrocolloids*. 2, 281-285.

Kurkijärvi, R., Adams, D.H., Leino, R., Möttönen, T., Jalkanen, S. and Salmi, M. (1998). Circulating form of human vascular adhesion protein-1 (VAP-1): increased serum levels in inflammatory liver diseases. *Journal of Immunology*. 161, 1549-1557.

Ladeby, R., Wirenfeldt, M., Garcia-Ovejero, D., Fenger, C., Dissing-Olesen, L., Dalmau, I. and Finsen, B. (2005). Microglial cell population dynamics in the injured adult central nervous system. *Brain Research Reviews.* 48, 196-206.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227, 680-685.

Lara, J.J., Economou, M., Wallace, A.M., Rumley, A., Lowe, G., Slater, C., Caslake, M., Sattar, N and Lean, M. E.J. (2007). Benefits of salmon eating on traditional and novel vascular risk factors in young, non-obese healthy subjects. *Atherosclerosis*. 193, 213-221.

Leclerc, P.L., Gauthier, S.F. Bachelard, H. Santure, M. and Roy, D. (2002). Antihypertensiveactivity of casein-enriched milk fermented by *Lactobacillus helveticus*. *International Dairy Journal*. 12, 995-1004.

Le Dréau, Y., Dupuy, N., Artaud, J., Ollivier, D. and Kister, J. (2009). Infrared study of aging of edible oils by oxidative spectroscopic index and MCR–ALS chemometric method, *Talanta.* 77, 1748-1756.

Lee, H.W., Park, Y.S., Jung, J.S. and Shin, W.S. (2002). Chitosan oligosaccharides, dp 2-8, have prebiotic effect on the *Bifidobacterium bifidium* and *Lactobacillus sp*. *Anaerobe*. 8, 319-324.

Lee, K.H., Ryu, H.S. and Rhee, K.C. (2003). Protein solubility characteristics of commercial soy protein products. *Journal of the American Oil Chemists' Society*. 80, 85-90.

Lehner, A., Loy, A., Behr, T., Gaenge, H., Ludwig, W., Wagner, M. and Schleifer, K.H. (2005). Oligonucleotide microarray for identification of *Enterococcus* species. *FEMS Microbiology Letters*. 246, 133-142.

Lemieux, L. and Amiot, J. (1989). Application of RP-HPLC to the separation of peptides from phosphorylated and dephosphorylated casein hydrolysates. *Journal of Chromatography*. 473, 189-206.

Lerma-Garcia, M.J., Ramis-Ramos, G., Herrero-Martinez, J.M. and Simo-Alfonso, E.F. (2010). Authentication of extra virgin olive oils by Fourier transform infrared spectroscopy. *Food Chemistry*. 118, 78-83.

Leu, S.N., Jhaveri, P.A. and Karakoltsidis S.M. (1981). Constantinides, Atlantic mackerel (*Scomber scombrus*): seasonal variation in proximate composition and distribution of chemical nutrients. *Journal of Food Science.* 46, 1635-1638.

Li, L., Wang, J., Cui, C. and Jiang, Y. (2006). Artificial Neural Network for Production of Antioxidant. Peptides Derived from Bighead Carp Muscles with Alcalase. *Food Technology and Biotechnology*. 44, 441-448.

Liaset, B. Lied, E. and Espe, M. (2000). Enzymatic hydrolysis of by-products from the fish-filleting industry; chemical characterization and nutritional evaluation. *Journal of the Science of Food and Agriculture*. 80, 581-589.

Liaset, B., Julshamn, K. and Espe, M. (2003). Chemical composition and theoretical nutritional evaluation of the produced fractions from enzymic hydrolysis of salmon frames with Protamex(TM). *Process Biochemistry*. 38, 1747-1759.

Liceaga-Gesualdo, A.M. and Li-Chan, E.C.Y. (1999). Functional properties of fish protein hydrolysate. *Journal of Food Science*. 64, 1000-1004.

Lima Dos Santos, C., James, D. and Teutscher, F. (1981). Guidelines for chilled fish storage experiments. *FAO Fisheries Technical Papers* (no. 210).

Lin, L. and Li, B.F. (2006). Radical scavenging properties of protein hydrolysates from Jumbo flying squid (*Dosidicus eschrichitii Steenstrup*) skin gelatin. *Journal of the Science of Food and Agriculture*. 86, 2290-2295.

Lin, S.Y., Wang, C.C., Lu, Y.L., Wu, W.C. and Hou, W.C. (2008). Antioxidant, anti-semicarbazide-sensitive amine oxidase, and anti-hypertensive activities of geraniin isolated from *Phyllanthus urinaria*. *Food and Chemical Toxicology*. 46, 2485-2492.

Linder, M., Fanni, J. and Parmentier, M. (2005). Proteolytic extraction of. salmon oil and PUFA concentration by lipases. *Marine Biotechnology*. 15, 70-76

Lindner, H. and Helliger, W. (2001). Age-dependent deamidation of asparagine residues in proteins. *Experimental Gerontology*. 36, 1551-1563.

Ljima, N. (2003). Purification and characterization of three isoforms of chrysophsin, a novel antimicrobial peptide in the gills of the red sea bream, *Chrysophrys major*. *European Journal of Biochemistry*. 270, 675-686.

Lopez-García, B., Veynat, A., Perez-Paya, E., Gonzalez-Candelas, L. and Marcos, J.F. (2003). Comparison of the activity and antifungal hexapeptides and the fungicides thiabendazole and imazalil against postharvest fungal pathogens. *International Journal of Food Microbiology*. 89, 163-170.

Lucas, P.W., Prinz, J.F., Agrawal, K.R. and Bruce, I.C. (2002). Food physics and oral physiology. *Food Quality and Preference.* 13, 203-213.

Lyles, G.A. (1996). Mammalian plasma and tissue-bound semicarbazide-sensitive amine oxidases: biochemical, pharmacological and toxicological aspects. *International Journal of Biochemistry and Cell Biology*. 28, 259-274.

Lyles, G.A. and Chalmers, J. (1992). The metabolism of aminoacetone to methylglyoxal by semicarbazide-sensitive amine oxidase in human umbilical artery. *Biochemical Pharmacololgy.* 43, 1409-1414.

Maguire, P.B., Moran, N., Cagney, G. and Fitzgerald, D.J. (2004). Application of Proteomics to the Study of Platelet Regulatory Mechanisms. *Trends in Cardiovascular Medicine*.14, 207-220.

Mäkinen-Aakula, M. (2006). Trends in functional foods dairy market. *Proceedings of the third functional food net meeting,* Liverpool, UK.

Martin, K., Meade, G., Moran, N., Shields, D.C. and Kenny, D. (2003). A palmitylated peptide derived from the glycoprotein Ib beta cytoplasmic tail inhibits platelet activation. *Journal of Thrombosis and Haemostasis*.1, 2643-2652.

Martinez, I., Bathen, T., Standal, I.B., Halvosen, J., Aursand, M., Gribbestad, I.S. and Axleson, D.E. (2005). Bioactive Compounds in Cod (*Gadus morhua*) Products and Suitability of ^{1}H NMR Metabolite Profiling for Classification of the Products Using Multivariate Data Analyses. *Journal of Agricultural and Food Chemistry.* 53, 6889-6895.

Maruyama, S., Miyoshi, S. and Tanaka, H. (1989). Angiotensin I-converting enzyme inhibitors derived from *Ficus carica*. *Agricultural and Biological Chemistry*. 53, 2763-2767.

Mbarki, R., Ben Miloud, N., Selmi, S., Dhib, S. and Sadok, S. (2009). Effect of vacuum packaging and low-dose irradiation on the microbial, chemical and sensory characteristics of chub mackerel (*Scomber japonicus*). *Food Microbiology*. 26, 821-826.

Mbatia, B., Adlercreutz, P., Mulaa, F. and Mattiasson, B. (2010). Enzymatic enrichment of omega-3 polyunsaturated fatty acids in Nile perch (*Lates niloticus*) viscera oil. *European Journal of Lipid Science and Technology*.112, 977-984.

McGill, A.S. and Moffat, C.F. (1992). A study of the composition of fish liver and body oil triglycerides. *Lipids.* 27, 360-370.

McNair, H.M. and Miller, J.M. (1998). Basic Gas Chromatography (1st edition). Wiley-Interscience, New York, USA.

Meier, S., Mjøs, S.A., Joensen, H. and Grahl-Nielsen, O. (2006). Validation of a one-step extraction/methylation method for determination of fatty acids and cholesterol in marine tissues. *Journal of Chromatography A*. 1104, 291-298.

Meisel, H. (1998). Overview on milk protein-derived peptides. *International Dairy Journal.* 8, 363-373.

Meisel, H. and Schlimme, E. (1994). Inhibitors of angitensin converting enzyme derived from bovine casein (*casokinins*). In: β-casomorphins and related peptides: Recent development, Brantl, V. and Teschemacher, H. (Eds.). Weinheim, VCH: New York, USA. 27-33.

Metin, S., Erkan, N., Varlik, C. and Aran, N. (2001). Extension of shelf-life of chub mackerel (*Scomber japonicus Houttuyn* 1780) treated with lactic acid. *European Food Research and Technology*. 213, 174-177.

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

Mendis, E., Rajapakse, N., Byun, H. G. and Kim, S. K. (2005b). Investigation of jumbo squid (*Dosidicus gigas*) skin gelatine peptides for their in vitro antioxidant effects. *Life Sciences*. 77, 2166-2178.

Milewski, S. (2001). Protein structure and physicochemical properties. In: *Chemical and Functional Properties of Food Proteins*, Sikorski, Z.E (Ed.). Technomic Publishing Comp. Inc., Lancaster, USA. 35-56.

Mishra, V.K., Temelli, F. and Ooraikul, B. (1993). Extraction and purification of w-3 fatty acids with an emphasis on supercritical fluid extraction - A review. *Food Research International*. 26, 217-226.

Miwa, N., Yokoyama, K., Wakabayashi, H. and Nio, N. (2010). Effect of deamidation by protein-glutaminase on physicochemical and functional properties of skim milk. *International Dairy Journal*. 20, 393-399.

Miyoshi, S., Ishikawa, H., Kaneko, T., Fukui, F., Tanaka, H. and Maruyama, S. (1991). Structures and activity of angiotensin-converting enzyme inhibitors in an a-zein hydrolysate. *Agricultural and Biological Chemistry*. 55, 1313-1318.

Mizuno, S., Nishimura, S., Matsuura, K., Gotou, T. and Yamamoto, N. (2004). Release of short and proline-rich antihypertensive peptides from casein hydrolysate with an *Aspergillus oryzae* protease. *Journal of Dairy Science*. 87, 3183-3188.

Moffat, C.F., McGill, A.S., Hardy, R. and Anderson, R. S. (1993). The production of fish oils enriched in polyunsaturated fatty acids containing triglycerides. *Journal of the American Oil Chemists' Society.* 70, 133-138.

Montagna, F., Ferronato, G. and Martinelli, F. (2000). Patologia orale orientata per problemi diagnosi differenziale e terapia. Associazione Nazionale Dentisti Italiani, Promoass, Roma, Italy.

Montero, P. and Gómez-Guillén, M.C. (2000) Extracting conditions for megrim (*Lepidorhombus boscii*) skin collagen affect functional properties of the resulting gelatine. *Journal of Food Science.* 65, 434-438.

Montero, P., Jimenez-Colmenero, F. and Borderias, J. (1991). Effect of pH and the presence of NaCl on some hydration properties of collagenous material from trout (*Salmo irideus Gibb*) muscle and skin. *Journal the Science of Food and Agriculture*. 54, 137-146.

Montero, P., Gómez-Guillén, M.C. and Borderias, A.J. (1999). Functional characterisation of muscle and skin collagenous material from hake (*Merluccius merluccius L.*). *Food Chemistry.* 65, 55-59.

Morimura, S., Nagata, H., Uemura, Y., Fahmi, A., Shigematsu, T. and Kida, K. (2002). Development of an effective process for utilization of collagen from livestock and fish waste. *Process Biochemistry*. 37, 1403-1412.

Motoki, M. and Kumazawa, Y. (2000). Recent trends in transglutaminase technology for food processing. *Food Science Technology and Research*. 6, 151-160.

Mukundan, M.K., Gopakumar, K. and Nair, M.R. (1985). Purification of a lipase from the hepatopancreas of oil sardine (*Sardinella longiceps Linnaeus*) and its characteristics and properties. *Journal of the Science of Food and Agriculture*. 36, 191-203.

Mullaly, M.M., O'Callaghan, D.M., Fitzgerald, R.J., Donnelly, W.J. and Dalton, J.P. (1995). Zymogen activation in pancreatic endoproteolytic preparations and influence on some whey protein characteristics. *Journal of Food Science.* 60, 227-233.

Muyonga, J.H., Cole, C.G.B. and Duodu, K.G. (2004). Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*). *Food Chemistry*. 86, 325-332.

Nabrzyski, M. (2007). Functional role of some minerals in foods. In: *Mineral Components in Foods*, Szefer, P., Jerome, P. and Nriagu, O. (Eds.). CRC Press/Taylor & Francis, Boca Raton, USA. 123-161.

Nagai, T. and Suzuki, N. (2000). Isolation of collagen from fish waste material-skin, bone and fins. *Food Chemistry.* 68, 277-281.

Nagai, T. Araki, Y. and Suzuki. N. (2002). Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chemistry.* 78, 173-177.

Nawar, W.W. (1985). Lipids. In: *Food chemistry Food chemistry* (2nd edition), Fennema, O.R. (Ed.). Marcel Dekker Inc., New York USA. 139-244.

Nayak, R., Khan, S.A., Watson, R.H. and Cernigila, C.E. (2002). Influence of growth media on Vancomycin resistence Enterococcus isolates and correlation with resistance gene determinants. *FEMS Microbiological Letters*. 214, 159-173.

Naz, S., Sheikh, H., Saddiqi, R. and. Sayeed, S.A (2004). Oxidative stability of olive, corn and soybean oil under different conditions. *Food Chemistry*. 88, 253-259.

Neklyudov, A.D., Berdutina, A.V., Ivankin, A.N., Mitaleva, S.I. and Evstaf'eva, E.A. (2003). Collagen Fractions Obtained by Water-Salt Extraction from Raw Materials of Animal Origin. *Applied Biochemistry and Microbiology*. 39, 426-430.

Nielsen, P.M., Petersen, D. and Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science.* 66, 642-646.

Nollet, L.M. (2000). Food analysis by HPLC (2nd edition). Marcel Dekker, Inc., New York, USA.

Norland, R.E. (1990). Fish gelatin, technical aspects. In: *Advances in fisheries and technology and biotechnology for increased profitability*, Voight, M.N. and Botta, J.K. (Eds.). Technomic Publishing Co, Lancaster, USA. 325-332.

Ockerman, H.W. and Hansen, C.L. (1988). Animal by-product processing. Ellis Horwood Ltd., Chichester, England.

Odama, U. and Bakris, G.L. (2000). Target organ damage in hypertension. *Journal of Clinical Hypertension*. 2, 312-318.

Oehlenschläger, J., Karl, H., Mitchell, M., Fagan, J. and Gormley, R. (2008). Evaluation of quality parameters of canned and frozen blue whiting (*Micromesistius poutassou*). *Journal of FisheriesSciences.com*. 2, 722-732

Ogawa, M., Moody, M.W., Portier, R.J., Bell, J., Schexnayder, M. and Losso, J.N. (2003). Biochemical properties of black drum and sheepshead seabream skin collagen. *Journal of Agricultural and Food Chemistry*. 51, 8088-8092.

Oktay, M., Gülçin, İ. and Küfrevioğlu, Ö.İ. (2003). Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. *Lebensmittel Wissenchaft und Technologie*. 36, 263-271.

Olivieri, A., O'Sullivan, J., Fortuny, LR., Vives, IL. and Tipton, KF. (2010). Interaction of L-lysine and soluble elastin with the semicarbazide-sensitive amine oxidase in the context of its vascular-adhesion and tissue maturation functions. *Biochimica et Biophysica Acta (BBA)***.** 1804, 941-947.

Olivieri, A., Tipton, K. and O'Sullivan, J. (2007). L-Lysine as a recognition molecule for the VAP-1 function of SSAO. *Journal of Neural Transmission.* 114, 747-749.

Ondetti, M.A., Rubin, B. and Cushman, D.W. (1977). Design of specific inhibitors of angiotensinconverting enzyme: New class of orally active antihypertensive agents. *Science*. 196, 441-444.

Onodenalore, A.C. and Shahidi, F. (1996). Protein dispersions and hydrolysates from shark (*Isurus oxyrinchus*). *Journal of Aquatic Food Product Technology.* 5, 43-59.

Osako, K., Saito, H., Hossain, M.A., Kuwahara, K. and Okamoto, A. 2006. Docosahexaenoic Acid Levels in the Lipids of Spotted Mackerel *Scomber australasicus*. *Lipids.* 41, 713-720

Ostrowska, E., Dunshea, F.R., Muralitharan, M. and Cross, R.F. (2000). Comparison of silver-ion high-performance liquid chromatographic quantification of free and methylated conjugated linoleic acids. *Lipids.* 35, 1147-1153.

O'Sullivan, J., Unzeta, M., Healy, J., O'Sullivan, M.I., Davey, G. and Tipton, K.F. (2004). Semicarbazide-sensitive amine oxidases: enzymes with quite a lot to do. *Neurotoxicology.* 25, 303-315.

Ou, B., Hampsch-Woodill, M. and Prior, R. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry.* 49, 4619-4626.

Pacheco-Aguilar, R., Mazorra-Manzano, M.A. and Ramírez-Suárez, J.C. (2008). Functional properties of fish protein hydrolysates from Pacific whiting (*Merluccius productus*) muscle produced by a commercial protease. *Food Chemistry*. 109, 782-789.

Parisi, G., Franci, O. and Poli, B.M. (2002). Application of multivariate analysis to sensorial and instrumental parameters of freshness in refrigerated sea bass (*Dicentrarchus labrax*) during shelf life. *Aquaculture.* 214, 153-167.

Patat, S.A., Carnegie, R.B., Kingsbury, C., Gross, P.S., Chapman, R. and Schey, K.L. (2004). Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. *European Journal of Biochemsitry.* 271, 4825-4833.

Pavlisko, A., Rial, A. and Coppes, Z. (1997b). Characterization of trypsin purified from the pyloric caeca of the Southwest Atlantic white croaker *Micropogonias furmieri* (*Scianidae*). *Journal of Food Biochemistry.* 21, 383-400.

Pavlisko, A., Rial, A., De Vecchi, S. and Coppes, Z. (1997a). Properties of pepsin and trypsin isolated from the digestive tract of *Parona Szgnata* "*Palometa*". *Journal of Food Biochemistry*. 21, 289-308.

Pavlisko, A. and Coppes, Z. (1999). Digestion of myofibrillar and sarcoplasmic fractions from menhaden muscle by the action of menhaden (*Brevoortia spp.)* and white croaker (*Micropogonias furnieri*) trypsins. *Journal of Food Biochemistry*. 23, 547-558.

Pavlisko, A., De Vecchi, S. and Coppes, Z. (1999). Enzymatic digestion of muscle from menhaden (*Brevoortza spp.*) at different pHs. *Journal of Food Biochemistry*. 23, 451-467.

Payne K.J. and Veis, A. (1988). Fourier-transform IR spectroscopy of collagen and gelatin solutions: Deconvolution of the amide I band for conformational studies. *Biopolymers.* 27, 1749-1760.

Pearce, K.N. and Kinsella, J.E. (1978). Emulsifying properties of proteins. Evaluation of a turbidimetric method. *Journal of Agricultural and Food Chemistry.* 26, 716-721.

Pena-Ramos, E.A. and Xiong, Y.L. (2001). Antioxydant activity of whey protein hydrolysates in a liposomal system. *Journal of Dairy Science*. 84, 2577-2583.

Pérez-Serradilla, J.A., Japón-Luján, R. and. Luque de Castro, M.D. (2007). Simultaneous microwave-assisted solid-liquid extraction of polar and nonpolar compounds from alperujo. *Analytica Chimica Acta*. 602, 82-88.

Perioli, L., Pagano, C., Mazzitelli, S., Rossi, C. and Nastruzzi, C. (2008). Rheological and functional characterization of new antiinflammatory delivery

systems designed for buccal administration. *International Journal of Pharmaceutics*. 356, 19-28.

Petersen, H.J., Keane, C., Jenkinson, H.F., Vickerman, M.M., Jesionowski, A., Waterhouse, J.C., Cox, D. and Kerrigan, S.W. (2010). Human Platelets Recognize a Novel Surface Protein, PadA, on Streptococcus gordonii through a Unique Interaction Involving Fibrinogen Receptor GPIIbIIIa. *Infection and Immunity*. 78, 413-422.

Pfeiffer, N. (2003). Disposal and re-utilisation of fish and fish processing waste (including aquaculture wastes). Nautilus Consultants Ireland Ltd., Galway, Ireland. Edited by Marine Institute (Ref: DK/01/003).

Phillips L.G., Whitehead D.M. and Kinsella J.E. (1994). Structure-function properties of food proteins. Academic Press, San Diego, USA.

Phillips, M.C. (1981). Protein conformation at liquid interfaces and its role in stabilizing emulsions and foams. *Food Technology.* 35, 50-57.

Piazza, G., Seddighzadeh, A. and Goldhaber, S.Z. (2008). Heart failure in patients with deep vein thrombosis. *American Journal of Cardiology.* 101, 1056-1059.

Picot, L., Bordenave, S., Didelot, S., Fruitier-Arnaudin, I., Sannier, F., Thorkelsson, G., Berge, J.P., Guerard, F., Chabeaud, A. and Piot, J.M. (2006). Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. *Process Biochemistry*. 41, 1217-1222.

Pierce G.J., Dyson J., Kelly E., Eggleton J.D., Whomersley P., Young I. A.G., Santos M.B., Wang, J. and Spencer, N.J. (2002). Results of a short study on by-catches and discards in pelagic fisheries in Scotland (UK). *Aquatic Living Resources.* 15, 327-334.

Pihlajaniemi, T. and Rehn, M. (1995). Two new collagen subgroups: membrane-associated collages and type XV and XVIII. *Progress in Nucleic Acid Research and Molecular Biology*. 50, 225-262.
Poppe, J. (1992). Gelatin. In: *Thickening and gelling agents for food*, Imeson, A. (Ed.). Blackie Academic and Professional, Glasgow, UK. 98-123.

Prioult, G., Pecquet, S. and Fliss, I. (2005). Allergenicity of acidic peptides from bovine β-lactoglobulin is reduced by hydrolysis with *Bifidobacterium lactis* NCC362 enzymes. *International Dairy Journal*. 15, 439-448.

Prockop, D.J. and Kivirikko, K.I. (1995). Collagens. Molecular biology, diseases, and potentials for therapy. *Annual Review of Biochemistry*. 64, 403-434.

Qian, Z.J., Je, J.Y. and Kim, S.K. (2007). Antihypertensive effect of angiotensin I converting enzyme-inhibitory peptide from hydrolysates of Bigeye tuna dark muscle, Thunnus obesus. *Journal of Agricultural and Food Chemistry.* 55, 8398-8403.

Quaglia, G.B. and Orban, E. (1990). Influence of enzymatic hydrolysis on structure and emulsifying properties of sardine (*Sardina pilchardus*) protein hydrolysate. *Journal of Food Science.* 55, 1571-1573.

Rahali, V., Chobert, J.M., Haertle, T. and Gueguen, J. (2000). Emulsification of chemical and enzymatic hydrolysates of β-lactoglobulin: characterization of the peptides adsorbed at the interface. *Nahrung*. 44, 89-95.

Ranathunga, S., Rajapakse, N. and Kim, S.K. (2006). Purification and characterization of antioxidative peptide derived from muscle of conger eel (*Conger myriaster*). *European Food Research and Technology*. 222, 310-315.

Rao, M.A. (1999). Rheology of Fluids and Semisolid Foods: Principles and Applications. Springer , New York, USA.

Rdzanek, A., Pietrasik, A., Kochman, J., Wilczynska, J. and Opolski, G. (2007). Acute coronary syndrome caused by left main coronary artery plaque rupture and thrombosis - Resolution after pharmacological treatment. *International Journal of Cardiology*. 117, e92-e94.

Reed, G.L., Fitzgerald, M.L. and Polgár, J. (2000). Molecular mechanisms of platelet exocytosis: insights into the "secrete" life of thrombocytes. *Blood*. 96, 3334-3342.

Refsgaard, H., Tsai, L. and Stadtman, E. (2000). Modifications of proteins by polyunsaturated fatty acid peroxidation products. *Proceedings of the National Academy of Sciences of the United States of America*. 97, 611-616.

Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. *Official Journal of the European Union*. L 12, 3-18.

Regulation (EC) No.1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. *Official Journal of the European Union*.L273**.** 1-95.

Richards, R.C., O'Neil, D.B., Thibault, P. and Ewart, K.V. (2001). Histone H1: an antimicrobial protein of Atlantic salmon (*Salmo salar*). *Biochemistry and Biophysics Research Communications.* 284, 549-555.

Rigby, B.J. (1968). Amino acid composition and thermal stability of the skin collagen of the Antarctic ice-fish. *Nature.* 219,166-167.

Roach, J.A.G., Yurawecz, M.P., Kramer, J.K.G., Mossoba, M.M., Eulitz, K. and Ku, Y. (2000). Gas chromatography-high resolution selected-ion mass spectrometric identification of trace 21:0 and 20:2 fatty acids eluting with conjugated linoleic acid isomers. *Lipids.* 35, 797-802.

Roberfroid, M.B. (2000). Concepts and strategy of functional food science: the European perspective. *American Journal of Clinical Nutrition.* 71(suppl):1660S-1664S.

Rodriguez-Alcala, L.M., Harte, F. Fontecha, J. (2009). Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization. *Innovative Food Science and Emerging Technologies*. 10, 32-36.

Roynette, C.E., Calder, P.C., Dupertuis, Y.M. and Pichard, C. (2004). n-3 polyunsaturated fatty acids and colon cancer prevention. *Clinical. Nutrition*. 23, 139-151.

Ruggeri, Z.M. (2003). Von Willebrand factor, platelets and endothelial cell interactions. *Journal of Thrombosis and Haemostasis*. 1, 1335-1342.

Rustad, T. (2003). Utilisation of marine by-products. *Electronic Journal of Environmental, Agricultural and Food Chemistry.* 2, 458-463.

Saito, Y., Wanezaki, K., Kawato, A. and Imayasu, S. (1994). Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. *Bioscience, Biotechnology, and Biochemistry*. 58, 1767-1771.

Sajjadi, S. (2007). Formation of fine emulsions by emulsification at high viscosity or low interfacial tension; A comparative study. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 299, 73-78.

Samaranayaka, A.G.P. and Li-Chan, E.C.Y. (2008). Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (*Merluccius productus*). *Food Chemistry*. 107, 768-776.

Sargent, J.R. (2000). Functions and metabolism of lipids in marine organism: an overview. In: *Marine lipids: Proceedings of the Symposium held in Brest,* Baudimant, G., Guezennec, J., Philippe, R. and Samain, J.F. (Eds.). *Actes de colloques - IFREMER.* 27, 181-182.

Sathe, S.K and Venkatachalam, M. (2007) Fractionation and biochemical characterization of moth bean (*Vigna aconitifolia L.)* proteins. *LWT - Food Science and Technology*. 40, 600-610.

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

Sathivel., S., Smiley, S., Prinyawiwatkul, W. and Bechtel., P.J. (2005). Functional and nutritional properties of red salmon (*Oncorhynchus nerka*) enzymatic hydrolysates. *Journal of Food Science*. 70, C401-C406.

Schägger, H. (2006). Tricine-SDS-PAGE. *Nature Protocols*. 1, 16-22.

Schrieber, R. and Gareis, H. (2007). Gelatine Handbook: Theory and industrial practice. Wiley-VCH GmbH & Co, Weinhem, Germany.

Schuhardt, U. and Lopes, O.C. (1988). Tetramethylguanidine catalyzed transesterification of fats and oils: a new method for rapid determination of their composition. *Journal of American Oil Chemist's Society.* 65, 1940-1941.

Selvakumar, D., Dey, S. and Das, D. (1999). Production and bioassay of bialaphos biosynthesized by *Streptomyces hygroscopicus* NRRL B-16256. *BioProcess Engineering***.** 20, 459-462.

Shukla, P.J. (2005). Fish & Fisheries. Rastogi Publications, Meerut, India.

Sentandreu, M.A. and Toldrá, F. (2006). A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. *Food Chemistry*. 97, 546-554.

Shahidi, F. (1994). Seafood processing by-products. In: *Seafoods chemistry, processing, technology and quality*, Shahidi, F. and Botta, J.R. (Eds.). Blackie Academic and Professional, London UK. 320-334.

Shahidi, F. and Amarowicz, R. (1996). Antioxidant activity of protein hydrolysates from aquatic species. *Journal of American Oil Chemists Society*. 73, 1197-1199.

Shahidi, F. and Kamil, J.Y.V.A. (2001). Enzymes from fish and aquatic invertebrates and their application in the food industry. *Trends in Food Science and Technology.* 12, 435-464.

Shahidi, F., Han, X.Q. and Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). *Food Chemistry.* 53, 285-293.

Shahidi, F., Metusalach, and Brown, J.A. (1998). Carotenoid pigments in seafoods and aquaculture. *Critical Reviews in Food Science and Nutrition*. 38, 1-67.

Shalaby, S.M., Zakora, M. and Otte, J. (2006). Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. *Journal of Dairy Research*. 73, 178-186.

Sherazi, S.T.H., Kandhro, A., Mahesar, S.A., Bhanger, M.I., Talpur, M.Y. and Arain, S. (2009). Application of transmission FT-IR spectroscopy for the trans fat determination in the industrially processed edible oils. *Food Chemistry.* 114, 323-327.

Sheu, J.R., Lin, C.H., Chung, J.L., Teng, C.M. and Huang, T.F. (1992). Triflavin, an Arg-Gly-Asp-containing antiplatelet peptide inhibits cell-substratum adhesion and melanoma cell-induced lung colonization. *Japanese Journal of Cancer Research*. 83, 885-893.

Shimizu, M., Sawashita, N., Morimatsu, F., Ichikawa, J., Taguchi, Y., Ijiri, Y. and Yamamoto, J. (2008). Antithrombotic papain-hydrolyzed peptides isolated from pork meat. *Thrombosis Research*. 123, 753-757.

Shukla, P.J. (2005). Fish & Fisheries. Rastogi Publications, Meerut, India.

Sigholt, T., Erikson, U., Rustad, T., Johansen, S., Nordtvedt, T. and Seland, A. (1997). Handling stress and storage temperature affect meat quality of farm-raised Atlantic salmon (*Salmo salar*). *Journal of Food Science.* 62, 898-905.

Sikorski, Z.E. (2001). Functional Properties of Proteins in Food Systems. In: *Chemical and Functional Properties of Food Proteins.* Sikorski, Z.E. (Ed.). Technomic Publishing Comp. Inc., Lancaster, USA. 113-136.

Sikorski, Z.E. and Naczk, M. (1981). Modification of technological properties of fish protein concentrate. *Critical Reviews in Food Science and Nutrition.* 14, 201-230.

Silva, L., Garcia, B. and Paiva-Martins, F. (2010). Oxidative stability of olive oil and its polyphenolic compounds after boiling vegetable process. *LWT - Food Science and Technology*. 43, 1336-1344.

Simpson, B.K. and Haard, N.F. (1999). Marine enzymes (2nd edition). In: *Encyclopedia of food science and technology*, Francis, F.J. (Ed.). Wiley, New York, USA. 495-528.

Simpson, B.K., Nayeri, G., Yaylayan, V. and Ashie, I. N. A. (1998). Enzymatic hydrolysis of shrimp meat. *Food Chemistry*. 61, 131-138.

Šližytė, R., Daukšas, E., Falch, E., Storrø, I. and Rustad., T. (2005). Characteristics of protein fractions generated from hydrolysed cod (*Gadus morhua*) by-products. *Process Biochemistry.* 40, 2021-2033.

Smith, D.J., Salmi, M., Bono, P., Hellman, J., Leu, T. and Jalkanen, S. (1998). Cloning of vascular adhesion protein 1 reveals a novel multifunctional adhesion molecule. *Journal of Experimental Medicine.* 188**,** 17-27.

Sørensen, B.K., Højrup, P., Østergard, E., Jørgensen, C.S., Enghild, J., Ryder, L.R. and Houen, G. (2002). Silver staining of proteins on electroblotting membranes and intensification of silver staining of proteins separated by polyacrylamide gel electrophoresis. *Analytical Biochemistry*. 304, 33-41.

Steffe, J.F. (1996). Rheological methods in food process engineering (2nd edition). Freeman Press, East Lansing, USA.

Stephens, G., O'Luanaigh, N., Reilly, D., Harriott, P., Walker, B., Fitzgerald, D. and Moran, N. (1998). A sequence within the cytoplasmic tail of GpIIb independently activates platelet aggregation and thromboxane synthesis. *Journal of Biological Chemistry*. 273, 20317-20322.

Stevens, P.V. (1992). Trace bioorganic constituents of gelatins - A review. *Food Australia***.** 44, 320-324.

Stolyhwo, A., Kolodziejska, I. and Sikorski, Z.E. (2006). Long chain polyunsaturated fatty acids in smoked Atlantic mackerel and Baltic sprats. *Food Chemistry*. 94, 589-595.

Stuart, B. (2004). Infrared Spectroscopy: Fundamentals and Applications. John Wiley & Sons, Ltd., Chichester, UK.

Stuart K. and Panitch A. (2009). Characterization of gels composed of blends of collagen I, collagen III, and chondroitin sulfate. *Biomacromolecules*. 10, 25-31

Sun, B., Tandon, N.N., Yamamoto, N., Yoshitake, M. and Kambayashi, J. (2001). Luminometric assay of platelet activation in 96-well microplate. *Biotechniques*. 31, 1174-1181.

Susi, H., Ard, J.S. and Carroll, R.J. (1971). Hydration and Denaturation of Collagen as Observed by Infrared Spectroscopy. *Journal of the American Leather Chemists Association*. 66, 508-519.

Susi, H. and Byler, M.D. (1988). Fourier Deconvolution of the Amide I Raman of Proteins as Related to Conformation. *Applied Spectroscopy*. 42, 819-825.

Sztuka, K. and Kołodziejska, I. (2008). Effect of transglutaminase and EDC on biodegradation of fish gelatin and gelatine-chitosan films. *European Food Research and Technology*. 226, 1127-1133.

Tadros, T.F. (2009). Emulsion Science and Technology: A General Introduction. In: *Emulsion Science and Technology*, Tadros, T.F. (Ed.). Wiley-VCH, Weinheim, Germany. 1-56.

Taskaya, L., Chen, Y.C. and Jaczynski, J. (2009). Functional properties of proteins recovered from whole gutted silver carp (*Hypophthalmichthys molitrix*)

by isoelectric solubilization/precipitation. *LWT – Food Science and Technology*. 42, 1082-1089.

Taton, T.A. (2001). Boning Up on Biology. *Nature*. 412, 491-492.

Te Nijenhuis, K. (1997). Thermoreversible networks: viscoelastic properties and structure of gels. *Advances Polymer Science*.130, 1-267.

Terry, P.D., Rohan, T.E. and Wolk, A. (2003). Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. *American Journal of Clinical Nutrition*. 77, 532-543.

Thiansilakul, Y., Benjakul, S. and Shahidi, F. (2007). Antioxidative activity of protein hydrolysate from round scad muscle using Alcalase and Flavourzyme. *Journal of Food Biochemistry*. 31, 266-287.

Tolleson, T.R., Newby, L.K., Harrington, R.A., Bhapkar, M.V., Verheugt, F.W.A., Berger, P.B., Moliterno, D.J., White, H.D., Ohman, E.M., Van de Werf, F., Topol, E.J. and Califf, R.M. (2003). Frequency of stent thrombosis after acute coronary syndromes (from the SYMPHONY and 2nd SYMPHONY trials). *American Journal of Cardiology***.** 92, 330-333.

Toppe, J., Albrektsen, S., Hope, B., Aksnes, A. (2007). Chemical composition, mineral content and amino acid and lipid profiles in bones from various fish species. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 146, 395-401.

Torrissen, O.J. (1989). Pigmentation of salmonids: interactions of astaxanthin and canthaxanthin on pigment deposition in rainbow trout, (*Salmo gairdneri R*.). *Aquaculture*. 79, 363-374.

Tosh, S.M., Marangoni A.G., Hallet F.R. and Britt I.J. (2003). Aging dynamics in gelatin gel microstructure. *Food Hydrocolloid*. 17, 503-513.

Toyoshima, K., Noguchi, R., Hosokawa, M., Fukunaga, K., Nishiyama, T., Takahashi, R. and Miyashita, K. (2004). Separation of Sardine Oil without Heating from Surimi Waste and Its Effect on Lipid Metabolism in Rats. *Journal of Agriculture and Food Chemistry*. 52, 2372-2375.

Tsai, J.S., Chen, J.J. and Pan, B.S. (2008). ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (*Meretrix lusoria*). *Process Biochemistry.* 47, 743-747.

Turon, F., Rwabwogo, B., Baréa, B., Pina, M. and Graille, J. (2005). Fatty acid composition of oil extracted from Nile perch (*Lates niloticus*) head. *Journal of Food Composition and Analysis.* 18, 717-722.

Tyler-Cross, R. and Schirch, V. (1991). Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. *Journal of Biological Chemistry*. 266, 22549-22556.

Tzikas, Z., Amvrosiadis, I. and Soultos, N. (2007). Sp. Georgakis, Seasonal variation in the chemical composition and microbiological condition of Mediterranean horse mackerel (Trachurus mediterraneus) muscle from the North Aegean Sea (Greece). *Food Control*. 18, 251-257.

Uauy, R. and Valenzuela, A. (2000). Marine oils: the health benefits of n-3 fatty acids. *Nutrition.* 16, 680-684.

Usydus, Z., Szlinder-Richert, J. and Adamczyk, M. (2009). Protein quality and amino acid profiles of fish products available in Poland. *Food Chemistry*. 112, 139-145.

Van Der Rest, M. and Garrone, R. (1991). Collagen family of proteins. *Journal of the Federation of American Societies for Experimental Biology*. 5, 2814-2823.

Van Trijp, H. (2007). Consumer understanding and nutritional communication, *International developments in science and health claims,* ILSI international symposium on functional foods in Europe. Malta.

Van Vliet, T. (1999). Factors determining small-deformation behaviour of gels. In: *Food emulsions and foams; interfaces, interactions and stability*, Dickinson, E. and Rodriguez Patino, J.M. (Eds). Royal Society of Chemistry, Cambridge, UK.

Vattem, D.A. and Shetty, K. (2006). Biochemical Markers for Antioxidant Functionality. In: *Food Biotechnology*, Shetty, K., Paliyath, G., Pometto, A. and Levin, R.E. (Eds.). CRC Press, Boca Raton, USA.

Veis, A. (1965). The macromolecular chemistry of gelatine. Academic Press, New York, USA.

Venugopal, V. (2008). Marine products for healthcare: functional and bioactive nutraceutical compounds from the ocean CRC Press/Taylor & Francis, Boca Raton, USA.

Venugopal, V. and Shahidi, F. (1995). Value-added products from underutilized fish species. *Critical Reviews in Food Science and Nutrition.* 35, 431-453.

Vermeirssen, V., Van Camp, J. and Verstraete, W. (2002). Optimisation and validation of an Angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *Journal of Biochemical and Biophysical Methods*. 51, 75-87.

Vidacek, S., De Las Heras, C. and Tejada, M. (2009). Quality of Fish Muscle Infested with *Anisakis simplex*. *Food Science and Technology International.* 15, 283-290.

Vojdani, F. (1996). Solubility. In: *Methods of testing protein functionality* (1st edition), Hall, G.M. (Ed.). St Edmundsbury Press, Bury St. Edmunds, UK. 11-60.

Wanasundara, P.K.J.P.D., Amarowicz, R.A., Pegg, R.B. and Shand, P.J. (2002). Preparation and characterization of hydrolyzed proteins from defibrinated bovine plasma. *Journal of Food Science*. 67, 623-630.

Wangtueai, S. and Noomhorm, A. (2009). Processing optimization and characterization of gelatin from lizardfish (*Saurida spp.*) scales. *LWT - Food Science and Technology*. 42, 825-834.

Ward, A.G. and Courts, A. (1977). The science and technology of gelatine. Academic Press, New York, USA.

Warner, K., Frankel, E.N. and Mounts, T.L. (1989). Flavor and oxidative stability of soybean, sunflower and low erucic acid rapeseed oils. *Journal of the American Oil Chemists' Society*. 66, 558-564.

Wellner, N., Belton, P.S. and Tatham, A.S. (1996). Fourier transform IR spectroscopic study of hydration induced structure changes in the solid state of *ω*-gliadins. *Biochemical Journal.* 319, 741-747.

WHO (2002). *Food safety and foodborne illness*. Geneva, Switzerland: World Health Organization Fact sheet 237.

Wilde, P.J. and Clark, D.C. (1996). Foam formation and stability. In: *Methods of Testing Protein Functionality* (1st edition), Hall, G.M. (Ed.). St Edmundsbury Press, Bury St. Edmunds, UK. 110-152.

Williamson, M.P. and Asakura, T. (1997). Protein chemical shifts. In: *Protein NMR Techniques (Methods in Molecular Biology)*, Reid, D.G. (Ed.). Humana Press, Totowa, USA. 53-59.

Wong, D.W.S. (1989). Mechanism and theory in food chemistry. Van Nostrand Reinhold, New York, USA.

Woufle, D., Yang, J. and Brass, L. (2001). ADP and platelets: the end of the beginning. *Journal of Clinical Investigation*. 107, 1503-1505.

Wu, H.C., Chen, H.M. and 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, 949-957.

Wu, J., Aluko, R.E. and Muir, A.D. (2002). Improved method for direct high-performance liquid chromatography assay of angiotensin-converting enzyme-catalysed reactions. *Journal of Chromatography A.* 950, 125-130.

Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.V., Gebhardt, S.E. and Prior, R.L. (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *Journal of Agricultural and Food Chemistry.* 52, 4026-4037.

Xiu-Qin, L., Chao, J., Yan-Yan, S., Min-Li, Y. and Xiao-Gang, C. (2009). Analysis of synthetic antioxidants and preservatives in edible vegetable oil by HPLC/TOF-MS. *Food Chemistry*. 113, 692-700.

Yakimets, I., Wellner, N., Smith, A.C., Wilson, R.H., Farhat, I. and Mitchell, J. (2005). Mechanical properties with respect to water content of gelatin films in glassy state. *Polymer.* 46, 12577-12585.

Yamamoto, N. and Takano, T. (1999). Antihypertensive peptides derived from milk proteins. *Die Nahrung*. 43, 159-164.

Yang, H., Irudayaraj, J. and Paradkar, M.M. (2005). Discriminant analysis of edible oils and fats by FTIR, FT-NIR and FT-Raman spectroscopy. *Food Chemistry.* 93, 25-32.

Yang, W. J., Griffiths, P.R., Byler, M.D. and Susi, H. (1985). Protein Conformation by Infrared Spectroscopy: Resolution Enhancement by Fourier Self Deconvolution. *Applied Spectroscopy*. 39, 282-287

Yen, G., and Wu, J. (1999). Antioxidant and radical scavenging properties of extract from Ganoderma tsugae. *Food Chemistry*. 65, 375-379.

Yildiz, G., Wehling, R.L. and Cuppett, S.L. (2003). Comparison of four analytical methods for the determination of peroxide value in oxidized soybean oils. *Journal of the American Oil Chemists' Society.* 80, 103-107.

Yokoyama, K., Chiba, H. and Yoshikawa, M. (1993). Peptide inhibitors for angiotensin I converting enzyme from a thermolysin digest of dried bonito. *Bioscience Biotechnology and Biochemistry*. 56, 1541-1545.

Yoshie-Stark, Y., Tsukamoto, M., Futagawa, K., Kubota, M. and Ogushi, M. (2009). Bioactivities of surimi from southern blue whiting prepared by different ways. *Food Chemistry*. 113, 47-52.

Zayas, J.F. (1997). Solubility of proteins. In: *Functionality of Proteins in Food*. Springer-Verlag. Berlin, Germany. 6-22.

Zhang, S., Wang, Y., Herring, J.L. and Oh, J.H. (2007). Characterization of edible film fabricated with channel catfish (*Ictalurus punctatus*) gelatin extract using selected pretreatment methods. *Journal of Food Science*. 72, C498-C503.

Zhao, Y., Li, B., Liu, Z., Dong, S., Zhao, X. and Zeng, M. (2007). Antihypertensive effect and purification of an ACE inhibitory peptide from sea cucumber gelatin hydrolysate. *Process Biochemistry*. 42, 1586-1591.

Zhou, P. and Regenstein, J.M. (2005). Effects of alkaline and acid pretreatments on Alaska pollock skin gelatin extraction. *Journal of Food Science*. 70, 392-396.

Zulueta, A., Esteve, M.J. and Frigola, A. (2009). ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry*. 114, 310-316.

Zuta, C.P., Simpson, B.K., Chan, H.M. and Philips, L. (2003). Concentrating PUFA from mackerel processing waste. *Journal of American Oil Chemist's Society*. 80, 933-936.

Internet

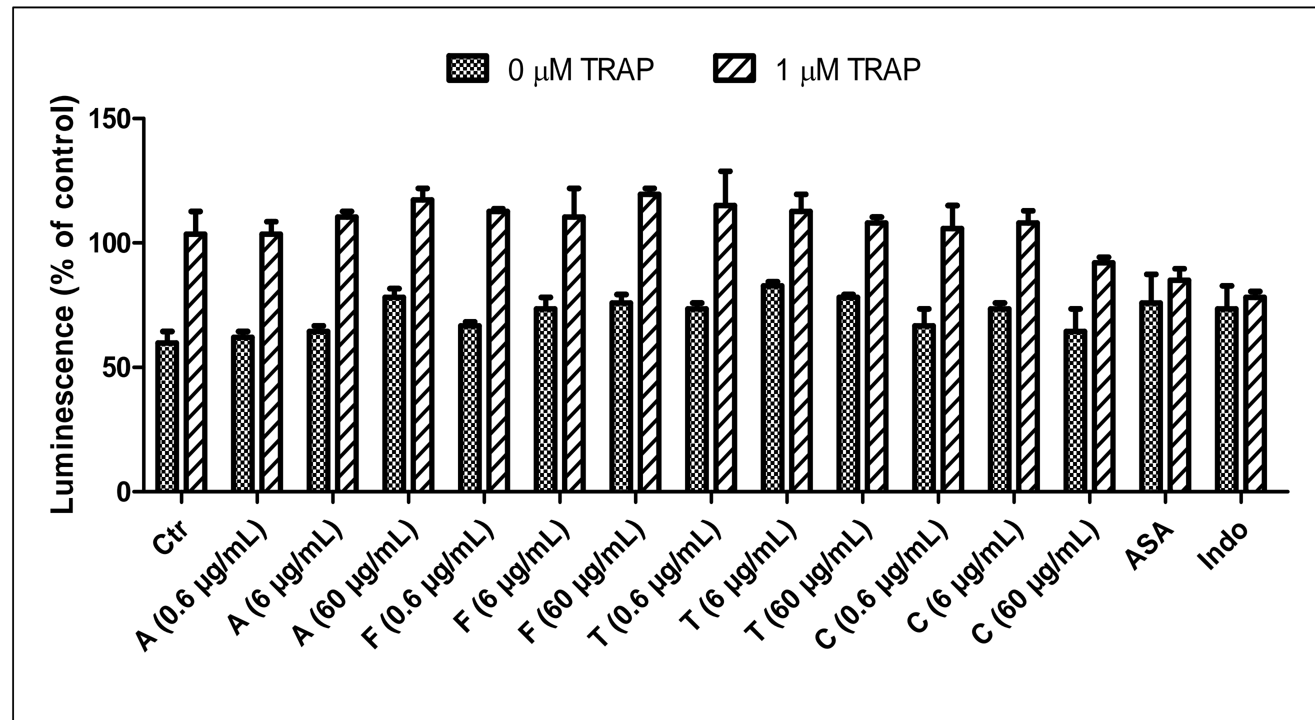
Braunschweig Enzyme Database (BRENDA) (http://www.brenda-enzymes.org/).

EMBL/GenBank/DDBJ databases (http://www.expasy.ch/enzyme/).

Nomenclature Committee of the International Union of Biochemistry and

Molecular Biology (NC-IUBMB) (http://www.chem.qmul.ac.uk/iubmb/enzyme/).

Appendices



Appendix I. The platelet aggregation inhibitory activity of mackerel viscera hydrolysates. Ctr: control (water), A: autolysis, F: Flavourzyme, T: trypsin, C: chymotrypsin, TRAP: Thrombin receptor activating peptide. Aspirin (ASA) and Indomethacine (Indo) were used as positive controls (inhibitors).

List of publications

Zied Khiari, Daniel Rico, Ana Belen Martin-Diana, Catherine Barry-Ryan. (2011). The extraction of gelatine from Mackerel (*Scomber scombrus*) heads with the use of different organic acids. *Journal of FisheriesSciences.com*. 5, 52-63.

Comparison between gelatines extracted from Mackerel and Blue Whiting heads using different organic acids. (2009). Zied Khiari, Daniel Rico, Ana Belen Martin-Diana and Catherine Barry-Ryan. In: *Total Food, Sustainability of the Agri-Food Chain*. Waldron, K.W., Moates, G.K. and Faulds, C.B. (Eds.). 323, 138 - 141.

Bioactive properties of hydrolysates from Mackerel viscera. (2009). Zied Khiari, Daniel Rico, Ana Belen Martin-Diana and Catherine Barry-Ryan. In: *Total Food, Sustainability of the Agri-Food Chain*. Waldron, K.W., Moates, G.K. and Faulds, C.B. (Eds.). 323, 142 - 146.

Conferences presentations

Functional properties of gelatines extracted from Mackerel and Blue Whiting bones by different pre- treatments. Zied Khiari, Daniel Rico, Ana Belen Martin-Diana and Catherine Barry-Ryan. 3rd Joint Trans-Atlantic Fisheries Technology Conference, Copenhagen, Denmark. September 15-18, 2009.

Comparison between gelatines extracted from Mackerel and Blue Whiting heads using different organic acids. Zied Khiari, Daniel Rico, Ana Belen Martin-Diana and Catherine Barry-Ryan. Total Food 2009 - Sustainability of the Agri-Food Chain, Norwich BioScience Institutes, Norwich, UK. April 22-24, 2009.

Bioactive properties of hydrolysates from Mackerel viscera. Zied Khiari, Daniel Rico, Ana Belen Martin-Diana and Catherine Barry-Ryan. Total Food 2009 - Sustainability of the Agri-Food Chain, Norwich BioScience Institutes, Norwich, UK. April 22-24, 2009.

Functional properties of gelatines extracted from head and skin of Mackerel using different organic acids. Zied Khiari, Daniel Rico, Ana Belen Martin-Diana and Catherine Barry-Ryan. 38th Annual Research Conference Food, Nutrition and Consumer Sciences. UCC, Cork, Ireland. September 4, 2008.

Antioxidant Properties of Mackerel's Gelatine Hydrolysates Extracted from Head and Skin Using Different Organic Acids. Zied Khiari, Daniel Rico, Ana Belen Martin-Diana and Catherine Barry-Ryan. 1st International Congress of Seafood Technology, Ege University. Cesme, Turkey. May 18-21, 2008.