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UNDERUTILIZED FISH SPECIES AND FISH PROCESSING BY-PRODUCTS UPGRADING STRATEGIES

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Tese de Candidatura ao grau de Doutor em Ciência Animal, Especialidade em Tecnologia dos Produtos Animais submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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A verdadeira viagem de descoberta não consiste em buscar novas paisagens,
mas sim num novo olhar

Marcel Proust

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Resumo

Os produtos da pesca e aquicultura apresentam um grande potencial, dada a ampla diversidade de espécies, a larga gama de aplicações e o valor nutricional. Nomeadamente, os benefícios para a saúde de uma dieta rica em peixe têm sido reconhecidos. Vários componentes benéficos têm sido identificados, mormente, vitaminas, minerais, proteínas facilmente digeríveis e ácidos gordos polinsaturados ω3 (ω3-PUFA). Adicionalmente, a preocupação crescente dos consumidores com a segurança alimentar e a saúde tem levado a uma maior procura destes produtos. Todavia, a exploração dos recursos para além da capacidade de regeneração destes e as alterações ambientais têm impedido a pesca de acompanhar a evolução da procura, assim favorecendo o crescimento da produção de peixe de aquicultura. No entanto, esta é apenas uma resposta possível aos problemas. Na verdade, existem grandes quantidades de peixe sem valor comercial, tratadas como desperdício ou usadas na produção de farinha de peixe. Assim sendo, há recursos insuficientemente explorados, como peixe capturado e rejeitado ou sub-produtos da indústria de processamento de pescado. Tipicamente, estes recursos não apresentam valor comercial por diversas razões, como um sabor desagradável, uma textura pouco firme, demasiadas espinhas, excessiva gordura ou, ainda, o pequeno tamanho dos adultos. Contudo, estes são recursos valiosos, contendo, tal e qual os produtos comerciais, proteínas, vitaminas, minerais e ω3-PUFA de alto valor nutricional, contanto que sejam asseguradas condições adequadas de manuseamento e armazenagem.

Donde, a valorização de espécies sub-utilizadas e sub-produtos da indústria de processamento de pescado é um problema fundamental do sector, o qual, para ser resolvido, requer estratégias tecnológicas inovadoras e apropriadas. Uma resposta essencial pode ser encontrada na área dos produtos reestruturados. Esta tecnologia oferece a possibilidade de modificar a composição e textura de um produto. O processamento permite a remoção de componentes indesejáveis e a adição de outros vantajosos, nomeadamente, componentes que aumentem o tempo de prateleira do produto, o enriqueçam nutricionalmente —em particular, aditivos benéficos para a saúde, como a fibra dietética (FD)— ou modifiquem as suas propriedades no sentido de maior aceitação pelos consumidores. Existem diferentes vias de preparação de produtos reestruturados, envolvendo diferentes produtos intermédios. Entre estes, o peixe picado não lavado e o surimi (peixe picado, lavado e refinado) são os mais importantes. Estes produtos intermédios podem ser sujeitos a duas estratégias possíveis de valorização: a incorporação de aditivos e/ou a aplicação de tecnologias de processamento alternativas.

Neste trabalho foram experimentados dois grupos de aditivos: FD (de maça, Vcl; de polpa de ervilha, Swe; de casca de ervilha, Exa; oligofrutose de raiz de chicória, Fbls; inulina de raiz de chicória, Fib; carragenato de algas, Carr; e glucomanano de 'konjac', Kjc) e a enzima de origem microbiana, transglutaminase (MTGase). Cada um destes aditivos afecta de maneira diferente a estrutura e propriedades do produto, podendo interagir com outros aditivos, os ingredientes do produto e, obviamente, com o tipo de músculo do peixe. Relativamente às novas tecnologias de processamento, a alta pressão hidrostática (APH) e a irradiação UV (IUV) foram usadas como processos de gelificação alternativos à tradicional gelificação térmica. Como a incorporação de aditivos, estas tecnologias permitem melhorar e transformar em diferentes sentidos a estrutura e propriedades dos produtos.

Como matérias-primas representativas, foram usadas aparas (músculo aderente às espinhas e pele) de pescada (*Merluccius capensis*), robalo (*Dicentrarchus labrax*) e dourada (*Sparus aurata*), bem como filetes de sarda (*Scomber scombrus*) e cavala (*Scomber japonicus*). Estas duas espécies (particularmente a sarda) são espécies de peixe sub-utilizadas e um recurso rico em proteína desperdiçado em Portugal.

Assim sendo, este trabalho abarca dez estudos diferentes relativos a estratégias de valorização para estes sub-produtos e espécies sub-utilizadas e visa dois objectivos, que embora diferentes, são indissociáveis: por um lado, a compreensão dos fenómenos bioquímicos subjacentes às propriedades dos produtos e ao efeito de aditivos e tecnologias de processamento e, por outro, a utilização deste conhecimento na selecção dos melhores aditivos e tecnologias e na optimização dos seus parâmetros, sempre no intuito de produzir alimentos inovadores, atraentes e viáveis do ponto de vista comercial. No tocante a estes, destaca-se a preparação e estudo da estabilidade durante a armazenagem de um produto semelhante a um tradicional alimento cárnico e de um outro produto de conceito inovador e com valor acrescentado.

Relativamente ao primeiro dos objectivos enunciados, um conhecimento mais profundo dos efeitos devidos às matérias-primas, aos aditivos e às tecnologias de processamento foi alcançado.

Em particular, provou-se que a material-prima é um factor crucial. Com efeito, a qualidade textural superior dos produtos gelificados termicamente de peixe fresco em relação à observada nos produtos de peixe congelado foi atribuída à desnaturação proteica e agregação, decorrentes da congelação e armazenagem em congelado. Acresce a isto, que diferentes níveis de desnaturação proteica e agregação conduziram a diferentes modos de actuação da MTGase. Especificamente, no caso da matéria-prima ser peixe congelado, a MTGase catalisou ligações cruzadas entre os poucos resíduos de Gln e Lys expostos à superfície das proteínas, assim ligando um pequeno número de

moléculas e não indo além de um efeito de curto alcance, expresso texturalmente por um considerável endurecimento. No caso de ser peixe fresco, a MTGase pôde, dado o maior desdobramento das proteínas, catalisar ligações cruzadas entre praticamente todos os resíduos de Gln e Lys, unindo um grande número de moléculas e conseguindo o estabelecimento de uma vasta estrutura reticular, expressa texturalmente por uma maior deformabilidade dos produtos.

Relativamente aos efeitos bioquímicos dos aditivos, para além da MTGase, foi observada para cada FD uma forma diferente de interagir com a matriz proteica, levando, por conseguinte, a uma diferente qualidade textural e geral dos produtos.

No que diz respeito às tecnologias alternativas de gelificação, ao passo que a IUV pareceu favorecer a degradação enzimática das proteínas, sem que se observasse algum benefício na acção da MTGase ou para a proteínas miofibrilares, a APH mostrou-se mais promissora, ao favorecer uma forma diferente de agregação proteica, caracterizada por interacções proteicas com baixos níveis de desnaturação e, portanto, contrastante com as grandes alterações conformacionais observadas na gelificação térmica.

A informação obtida permitiu algumas escolhas no tocante à incorporação de aditivos ou à aplicação de tecnologias alternativas de gelificação na preparação e optimização de novos alimentos capazes de valorizar espécies sub-utilizadas e sub-produtos.

Quanto à selecção de aditivos, a MTGase (de 0,1 até 0,5 %, p/p) apresentou um efeito positivo nos diferentes produtos testados, preparados a partir de diversas matérias-primas e sujeitos a diferentes condições de processamento, especialmente ao nível textural. Entre as FD, Swe, Fib, Carr e Kjc foram as mais vantajosas, pois aumentaram o teor em FD do alimento sem causar efeitos negativos na textura (ou, mesmo, nalguns casos, com melhoria textural do produto). Mais ainda, enquanto Swe (4 %, p/p) e Carr (1 %, p/p) foram particularmente eficientes no endurecimento de produtos preparados a partir de diversas matérias-primas, Fib (2 ou 4 %, p/p) foi mais eficaz no aumento da deformabilidade dos produtos. Porém, há uma nota de cautela nestes resultados: os benefícios da adição de FD podem depender da qualidade da matéria-prima, uma vez que, no caso da FD Swe, a substituição da proteína por FD só logrou ser vantajosa para proteína de baixa qualidade.

As tecnologias de gelificação experimentadas deram diferentes resultados. Enquanto a IUV não apresentou vantagem alguma, a aplicação de APH (200 MPa, 15 min e um único ciclo de compressão) melhorou e modificou (em sentidos potencialmente úteis) os produtos gelificados face ao observado com produtos gelificados termicamente. APH pode vir a ser utilizada na valorização de produtos preparados de matérias-primas de menor qualidade, como as aparas de pescada congelada.

Estes resultados mostraram ser possível a obtenção de produtos reestruturados de peixe com qualidade a partir de matérias-primas de relativamente baixa qualidade. Assim, estes resultados encontraram aplicação no desenvolvimento de novos produtos com potencial comercial. Nomeadamente, uma salsicha tipo Frankfurt de peixe, saudável e aceitável do ponto de vista sensorial (comparável às salsichas comerciais de porco) com mês e meio de estabilidade de armazenagem em frio (2 ºC) foi produzida através da combinação da FD Swe (aditivo que conferiu dureza), da Fib (aditivo que conferiu a sensação cremosa típica da gordura), do baixo nível lipídico e dos nutrientes do peixe. Adicionalmente, foi produzido um aperitivo pronto a servir, incorporando FD e ω3-PUFA, sem perda significativa de qualidade ao longo de dois meses de armazenagem (2 ou 10 ºC).

Por conseguinte, este trabalho cumpriu com os seus objectivos de testar diferentes estratégias de valorização de espécies sub-utilizadas e sub-produtos da indústria e provar a viabilidade de algumas destas estratégias.

Abstract

Fish products present a great potential, since they offer a wide diversity of species, a broad range of applications and are nutritionally invaluable for human health. The health benefits of a diet rich in fish have been extensively recognized. Various beneficial components have been identified, namely, vitamins, minerals, readily digested proteins and ω3 polyunsaturated fatty acids (ω3-PUFA). The growing concern of consumers with food safety and health has led to a higher demand for seafood products. However, as a result of overexploitation and environmental changes, world captures have not been able to keep pace with this evolution, thus leading to an increase of aquaculture production. But this may be only one possible solution to these problems. There are large quantities of fish without commercial value which are treated as waste or used to produce fish meal. Hence, there are underexploited resources, fish by-catch or by-products from the fish processing industry, which are deemed unmarketable for various reasons, such as unpleasant taste, too soft texture, too many bones, too much fat or small size of the adults. These are valuable resources from a nutritional point of view, containing proteins, vitamins, minerals and ω3-PUFA as good as those contained in the commercial products, provided that adequate handling and storage conditions are ensured.

The upgrading of underutilized fish species and by-products of the fish processing industry is a fundamental difficulty for the sector, which requires adequate and innovative technological strategies to be overcome. A fundamental answer may be found in the restructured fish products' field. This technology offers the possibility to modify the composition and texture of a product. The required processing enables the removal of undesirable components and the addition of others to a product, which may bring about various advantages. Namely, components that lengthen the storage period of a product, nutritionally enrich it —particularly with health promoting additives, such as dietary fibre (DF)— or modify any of its properties in a way that improves its acceptability in the market. There are many different ways of preparing restructured fish products, namely, involving different intermediate materials. Among these, unwashed fish mince and surimi (thoroughly washed and refined fish mince) are the most meaningful. Both of these intermediate materials can be subjected to two main upgrading strategies: the incorporation of additives and/or the application of alternative processing technologies.

In this work two main groups of additives were tested: DFs (apple fibre, Vcl; inner pea fibre, Swe; outer pea fibre Exa; chicory root oligofructose, Fbls; inulin, Fib; carrageenan, Carr; and konjac glucomannan, Kjc) and the microbial enzyme transglutaminase (MTGase). Each of these additives affects the product's structure and properties in different ways and may interact with each other as well as with the products' ingredients

and, of course, the type of fish muscle. Regarding new processing technologies, high hydrostatic pressure (HHP) and UV irradiation (UVI) were used as alternative gelation processes to the traditional thermal gelation. As the additives incorporation, these technologies enable to improve and transform in different ways the structure and properties of the products.

As representative raw materials, hake (*Merluccius capensis*), sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) trimmings (muscle joined to the bones and skin) and Atlantic (*Scomber scombrus*) and chub mackerel (*Scomber japonicus*) were used. The latter are underutilized fish species (particularly Atlantic mackerel) and a largely wasted animal protein resource in Portugal.

This work encompassed ten different studies concerning upgrading strategies for these by-products and underutilized fish species and aimed to address two different albeit interconnected objectives: to understand the biochemical phenomena underlying fish products properties and the impact of additives and processing techniques on them and to use this knowledge for selecting the best additives and techniques as well as finding their optimal parameters in order to prepare innovative, attractive and commercially viable food products. Concerning these, it was planned to prepare and to study the storage stability of a fish product mimicking a traditional meat food as well as a new value-added food concept.

Regarding the first objective, significant breakthroughs were achieved that helped to better understand the effects due to the raw materials, the various additives and the processing technology choices.

With respect to raw materials, they proved to be a major factor. The superior and different textural quality of heat-induced gel products prepared from fresh fish (sea bass) with respect to those from frozen fish (hake) was traced back to the protein denaturation and aggregation due to freezing and frozen storage. The different levels of protein denaturation and aggregation led to different MTGase action modes. For frozen fish, MTGase catalyzed the cross-linking between the small number of Gln and Lys residues exposed at the surface of proteins, thus linking a small number of molecules and exhibiting a short distance effect, which was texturally expressed by a substantial hardening effect. For fresh fish, MTGase, due to a greater unfolding of proteins, catalyzed the cross-linking between almost all Gln and Lys residues of the proteins, thereby uniting a large number of molecules and succeeding in the establishment of a vast network structure, which was texturally expressed by a greater deformability of the products.

Concerning the biochemical effects of the additives, besides MTGase, it was found that each DF has its own specific way for interacting with the protein matrix, thus favouring different outcomes for the textural, colour and general quality of the fish products.

Regarding alternative gelation technologies, whereas UVI apparently stimulated protein degradation by enzymes without any positive biochemical action upon MTGase or myofibrillar proteins, HHP seemed to promote a kind of protein aggregation characterized by side-to-side interactions of proteins with reduced denaturation instead of large conformational changes as with thermal gelation.

From all these data, some relevant choices could be done regarding additives incorporation or the utilization of alternative technologies for the preparation and optimization of new food products, capable of upgrading underutilized fish products and by-products of fish processing.

Concerning the selection of additives, MTGase (from 0.1 up to 0.5 %, w/w) had a positive effect on the different tested products prepared from various raw materials and subjected to diverse processing conditions, especially at the textural level. Swe, Fib, Carr and Kjc were the most advantageous from tested DFs, since they augment DF content in food without any major deleterious effect on texture (or, sometimes, with some textural improvement for the product). While Swe (4 %, w/w) and Carr (1 %, w/w) were particularly efficient in the hardening of various fish products prepared from different raw materials, Fib (2 or 4 %, w/w) was more efficient in the enhancement of the products' deformability. But, there is an important caution note in these results: the beneficial effects of DF addition may depend on the quality of the raw material, since, for Swe, it was found that replacing protein by DF was only beneficial for protein of poor quality.

The alternative gelation technologies yielded different results. Whereas, UVI showed no advantage, HHP treatment (200 MPa, 15 min and one single compression cycle) led to the improvement and transformation (in potentially useful ways) of fish gel products with respect to heat-induced gels. HHP may be able to upgrade products prepared from low quality raw materials, such as frozen hake trimmings.

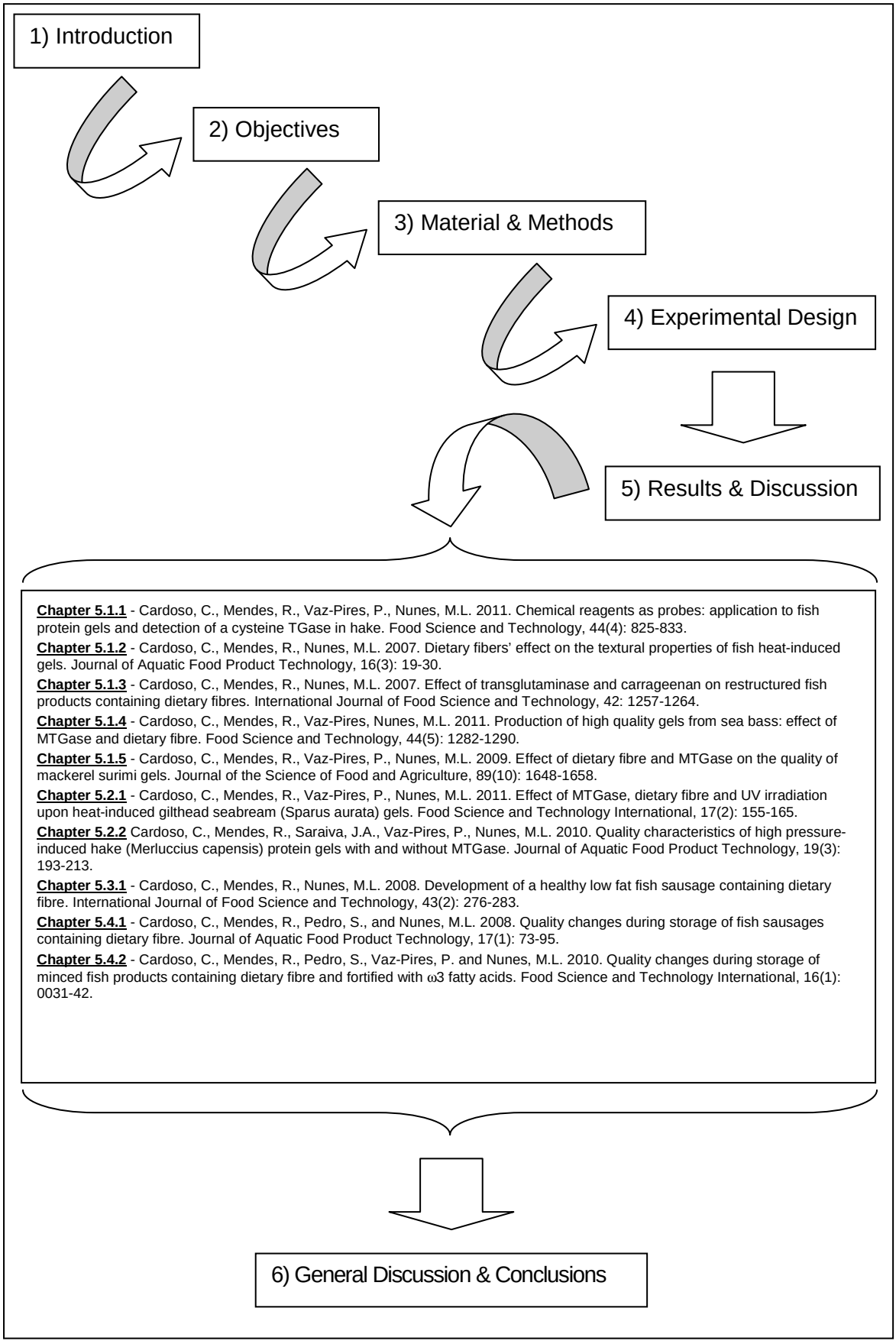
These results showed that it is possible to attain good quality restructured fish products from low quality (albeit acceptable) raw materials. Thus, these results were applied to the development of new fish products with commercial potential. A sensory acceptable and healthy Frankfurter fish sausage (comparable to commercial pork sausages) with 1.5 month storage stability (at 2 ºC) was produced through the combination of Swe (hardening additive), Fib (mimicking fat from a sensory point of view), low fat level and fish nutrients. Additionally, a ready-to-eat fish appetizer with no significant quality loss over two months of storage (at 2 or 10 ºC) was produced with incorporation of DF and ω3-PUFA, thereby addressing health concerns.

This work was able to test different technological strategies suitable for the upgrading of underutilized fish species and fish processing by-products and to prove the viability of some of them.

Structure of the Thesis

This work is composed by six parts and it yielded ten published papers (see scheme below):

- The 1st part is a general introduction to the main scientific and technical problems of this work. It encompasses a description of the fisheries sector situation and points to the need of upgrading some raw materials through the production of restructured fish products. Moreover, some important concepts are explained. Afterwards, the biochemical phenomena underlying the preparation processes of these products are presented. Finally, the main upgrading strategies are introduced and discussed, regarding their characteristics, effects and potential for the fish processing sector;
- In the 2nd part, the main objectives of this work are presented;
- In the 3rd part, all material and methods used in the work are thoroughly described;
- In the 4th part, the experimental design of the whole work and the integration of the different sets of experiments in it are clearly outlined;
- The 5th part is composed by ten chapters corresponding to ten already published papers. In this part, results are fully presented through tables and figures and the discussion is focused on each of the main experimental sets;
- Finally, a general discussion and the conclusions of this work form the 6th part. In this part, a global view of the results is given and the discussion seeks to find the common ground of the various experiments and their main conclusions. The connection between the different experimental sets is emphasized, thereby expounding the usefulness of the results of the initial experimental work for the design and particular choices of the later experiments.



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Appendix

List of Abbreviations

A	Actin
A_1	Area of first compression (TPA)
A_2	Area of second compression (TPA)
a*	CIELAB colour parameter
ACT	Actinin
ADP	Adenosine diphosphate
AHA	American Heart Association
ALM	Amidated low methoxyl pectin
Arg	Arginine
Asn	Asparagine
ATP	Adenosine triphosphate
ATPase	Enzyme that catalyses the decomposition of ATP into ADP+P
a_w	Water activity
b*	CIELAB colour parameter
BPP	Beef plasma protein
BSA	Bovine serum albumine
C-Pt	C-protein
Ca^{2+}	Calcium ion
$Ca(OH)_2$	Calcium hydroxide
Car	Carbonic anhydrase
Carr	Carrageenan (algal dietary fibre)
Cl^-	Chloride ion
CLO	Cod liver oil
CO_2	Carbon dioxide
Cys	Cysteine
D	Desmin
DF	Dietary fibre
DHA	Docosahexaenoic acid
D.M.	Dry matter
DRM	Dynamic rheological measurements
DTT	Dithiothreitol
E_1	Elastic modulus
E_e	Elastic modulus at equilibrium
EC	Enzyme commission number

EDTA	Ethylenediamine tetraacetic acid
EPA	Eicosapentaenoic acid
η_1	Viscous modulus
EW	Egg white
Exa	Outer pea dietary fibre
F_0	Force registered at the onset of relaxation
F_1	Force registered after one min of relaxation
FA	Formaldehyde
FAO	Food and Agriculture Organisation
Fbls	Chicory root oligofructose (dietary fibre)
Fib	Chicory root inulin (dietary fibre)
FT	Folding Test
FW	Final Weight (purge loss)
G’	Storage modulus
Gal	β-galactosidase
Gln	Glutamine
Glu	Glutamic dehydrogenase
Gly	Glycine
GRAS	Generally recognized as safe
GS	Gel strength
H	Moisture
H-bond	Hydrogen bond
HHP	High hydrostatic pressure
HMW	High molecular weight protein bands
HPLC	High performance liquid chromatography
HSD	Honestly significant difference
IW	Initial weight (purge loss)
KGM	Konjac glucomannan (dietary fibre)
Kjc	Glucomannan (konjac dietary fibre)
L_1	Original compression distance (TPA)
L_2	Detected height of the product in the second compression (TPA)
L*	Luminosity/CIELAB colour parameter
Lac	α-lactalbumin
Leu	Leucine
Lys	Lysine

M-Pt	M-protein
MA	Malonaldehyde
MAC	α2-macroglobulin
Max.	Maximum
MHC	Myosin heavy chain
Min.	Minimum
MLC1	Myosin light chain 1
MLC2	Myosin light chain 2
MLC3	Myosin light chain 3
MTGase	Microbial transglutaminase
MW	Molecular weight
Myo	Myosin
Na^+	Sodium ion
NaOH	Sodium hydroxide
NEM	N-ethylmaleimide
NH_3	Ammonia
ntwk.	Network
O_2	Oxygen
ω3-PUFA	Omega3 polyunsaturated fatty acids
ω6 PUFA	Omega6 polyunsaturated fatty acids
Ova	Ovalbumin
P	Free phosphate ion
PAGE	Polyacrylamide gel electrophoresis
Pho	Phosphorylase B
PL	Purge loss
Pro	Proline
Pv's	Parvalbumins
R^2	Coefficient of determination
RDI	Recommended daily intake
SDS	Sodium dodecylsulphate
SEM	Scanning electron microscopy
Ser	Serine
SH	Sulfhydryl group
σ	Decaying stress
σ_0	Initial stress
σ_e	Stress at equilibrium

STI	Soybean trypsin inhibitor
Swe	Inner pea dietary fibre
SWOT	Strengths, weaknesses, opportunities and threats
t	Time
τ_i	Relaxation time
TBARS	Thiobarbituric acid reactive species
TCA	Trichloroacetic acid
TGase	Transglutaminase
Thr	Threonine
TM	Tropomyosin
TMAO	Trimethylamine oxide
TNC	Troponin-C
TNI	Troponin-I
TNT	Troponin-T
TPA	Texture profile analysis
Tra	Transferrin
Tris-HCl	Tris(hydroxymethyl)-aminomethane
Trp	Tryptophan
Tyr	Tyrosine
U	Urea
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UVI	Ultraviolet irradiation
Val	Valine
Vcl	Apple dietary fibre
VO	Vegetable oil
WBC	Water binding capacity
W_f	Final weight
WHC	Water holding capacity
W_i	Initial weight
WPC	Whey protein concentrate
W_s	Sample weight
w/v	Weight per volume
w/w	Weight per weight
Y_T	Relaxation

List of Units

ºC	degree Celsius
ºC.min^{-1}	degree Celsius per minute
cm	centimeter
cm^{-1}	number of waves per centimeter
cm^3/(m^2.day.bar)	cubic centimetre per square meter, day and bar
×g	multiple of the gravitational acceleration
g	gram
g/day	gram per day
g/kg	gram per kilogram
g/l or g.l^{-1}	gram per liter
g/person	gram per person
h	hour
Hz	Hertz
kcal	kilocalorie
kcal/mol	kilocalorie per mole
kDa	kiloDalton
kg	kilogram
kN	kiloNewton
kPa	kiloPascal
kPa.s	kiloPascal times second
kV	kiloVolt
Log (CFU)/g	log colony forming units per gram
M	molar (mol per litre)
mA	miliAmpere
mg	miligram
mg/ml	milligram per milimeter
min	minute
ml	mililitre
µm	micrometer
mm	milimeter
mm/min	milimeter per minute
mm/s	milimeter per second
µmol	micromol

mM	milimolar (milimol per litre)
MPa	megaPascal
MPa/min	megaPascal per minute
$\mu W/cm^2$	microWatt per square centimeter
mW/cm^2	miliWatt per square centimeter
N	Newton
N.mm	Newton times milimeter
nm	nanometer
Pa	Pascal
rpm	revolutions per minute
s	second
U	unit of MTGase activity
$U.g^{-1}$	unit of MTGase activity per gram of MTGase product
V	Volt
W	Watt

1 - Introduction

1.1 - General Introduction

Seafood products present a great potential, since they offer a wide diversity of species, a broad range of applications —from fish meal to caviar— and large amounts of different nutrients, invaluable for human health. The health benefits of a diet rich in fish have been extensively recognized in the last decade. Among various nutritionally beneficial components, such as vitamins, minerals and readily digested proteins (Borderías et al., 2005), ω3 polyunsaturated fatty acids (ω3-PUFA), mainly eicosapentaenoic acid or EPA (20:5ω3) and docosahexaenoic acid or DHA (22:6ω3), are associated with decreased morbidity and mortality from various diseases. Several epidemiological studies (Simopoulos, 2002) have found a reduced risk of coronary heart disease, hypertension, stroke and consequent further effects due to the beneficial effects of these fatty acids on lipids, namely cholesterol, blood pressure and eicosanoid levels as well as on the coagulation rate. Indeed, the American Heart Association (AHA) recommends a daily intake (RDI) of 500 mg EPA+DHA (Kris-Etherton et al., 2002).

Accordingly, the increasing concern of consumers with food safety and health has led to a growing demand for seafood products. However, as a result of overexploitation and environmental changes, world captures have not been able to keep pace with this evolution, thus leading to an increase of aquaculture production (Table 1.1) (SOFIA, 2009). In fact, world marine captures may be approaching the catch ceiling for commercial resources. There are widespread concerns about collapsing stocks, over-capacity in fishing fleets, unsustainable fishing practices, fish discards, wasting of fish processing by-products and all the consequent environmental degradation.

Table 1.1 – Worldwide evolution of seafood production (from: SOFIA, 2009).

Production (million tonnes)	2000	2001	2002	2003	2004	2005	2006	2010*
Sea waters fishing	86.8	84.2	84.5	81.5	85.7	84.5	81.9	83.3
Inland waters fishing	8.8	8.9	8.7	9.0	8.9	9.7	10.1	10.9
Total fishing	95.6	93.1	93.2	90.5	94.6	94.2	92.0	94.2
Aquaculture	35.5	37.9	40.4	42.7	45.9	48.5	51.7	57.7
World Total	131.1	131.0	133.6	133.2	140.5	142.7	143.6	151.9

* Estimate based on data published by FAO 2004 (FAO, 2004).

Fish products are also a very important component of the Portuguese diet. In fact, recent studies (Cardoso et al., 2010) based on the latest statistically available data found fish and other seafood consumption in Portugal as one the highest at the European level. The amount of effectively consumed seafood (considering the edible portion) surpassed

the 520 g/(person.week), ahead of Spain with 440 g/(person.week) and much higher than, for instance, the UK, with 140 g/(person.week). The five most consumed fishery products in Portugal are cod, hake, sardine, horse mackerel and tuna. The estimated effective consumptions are 112, 49, 49, 45 and 33 g/(person.week), respectively (Cardoso et al., 2010a). These five fish species accounted for more than half of total seafood consumption (Figure 1.1) and represented a huge pressure on these fish resources, either caught in Portuguese waters (as sardine and horse mackerel) or elsewhere (as cod, for instance, in Norwegian waters, or hake, in Southern African waters). Besides the high *per capita* consumption values (consumer perspective) and the environmental impact, the importance of fish in Portugal arises from the significant role of the fisheries and fish industry in the Portuguese economic life and from the social impact of these activities. Namely, the still numerous coastal communities, which are hard hit (and would be even more in the future) by dwindling fish resources and the employees of the fish processing industry which could see their jobs at risk.

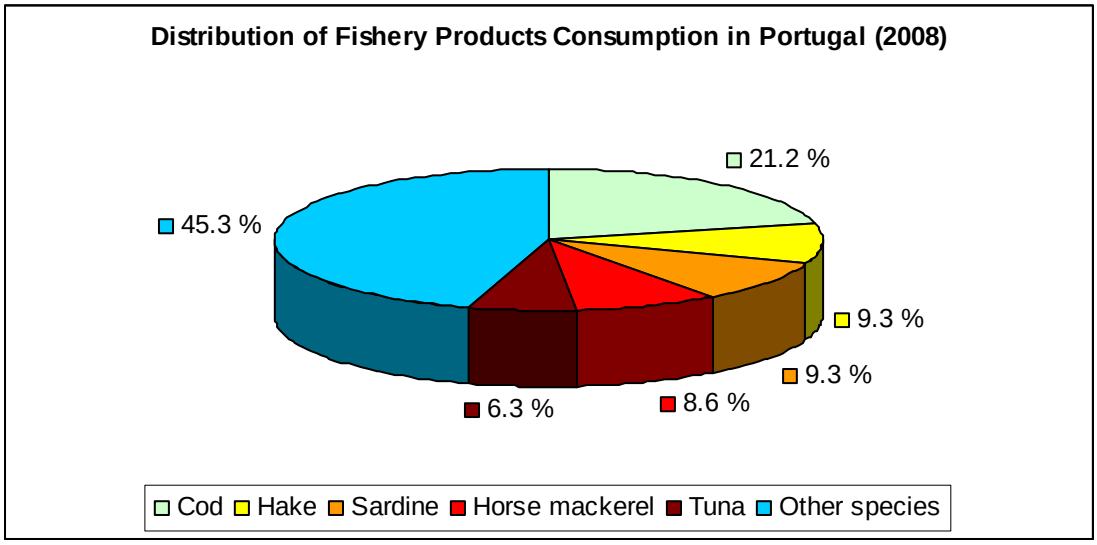

Figure 1.1 - Distribution of the consumption of fishery products in Portugal (year 2008) (from: Cardoso et al., 2010a).

The present state of the Portuguese fisheries and fish industry sectors results from a combination of multiple factors. Firstly, various fish resources are overexploited and, as such, their fishing is limited by the European and Portuguese authorities. This entails that a large portion of the consumer demand must be met with imports (which may lead to overexploitation of foreign resources). Effectively, in 2007, Portugal imported 422,000 tonnes of fish products worth almost 1,400 million euros, exported 144,000 tonnes worth 518 million euros and, accordingly, exports' value only accounted for 37.1 % of the imports' expenditure (INE, 2008). The reliance in foreign resources is a factor of economic dependence on other countries, posing some serious risks to companies, especially if

foreign governments bar access to their fishing grounds and raise other obstacles, regardless of these actions being economically motivated (promotion of their own fisheries sector) or drawn by political reasons.

However, there are large amounts of fish without commercial value, which are treated as waste or used to produce fish meal. There are underexploited resources, fish by-catch or by-products from the fish processing industry, which are deemed unmarketable for various reasons, such as unpleasant taste, too soft texture, too many bones, too much fat content or small size of the adults (which may render uneconomical some usual processing steps, like filleting). The waste of these resources is not merely an environmental liability and an economical mismanagement of scarce raw materials. In fact, these are precious resources from a nutritional point of view, containing proteins, vitamins, minerals and ω3-PUFA as good as those contained in the commercial resources, provided that adequate handling and storage conditions are ensured. These conditions must prevent any microbiological risk or loss of quality, in order to upgrade such resources to human food quality standards, required by the Portuguese and European legal framework and enforced by the hygiene and health authorities. Recent studies have identified a number of bioactive compounds from remaining fish muscle proteins, fish oil, fish bone, internal organs, and, even, shellfish and crustacean shells (Je et al., 2005; Jeon and Kim, 2002; Kim et al., 2001).

These facts pose a technological challenge to the fish sector. Upgrading strategies involving technological advancement in the formulation and/or processing of new fish products may be a very advantageous option, since the sensory attributes of such processed products can be directed to consumer preferences, thereby circumventing some of the previously mentioned marketability problems. Particularly, combining fish minces with new food ingredients has been used as a way of upgrading low-value species and by-products generated by the fish processing industry (Sánchez *et al.*, 2004).

1.2 - Underutilized Fish Resources

There are two main classes of underutilized fish resources: the by-products resulting from the fish processing industry (from such operations as heading, filleting, washing, etc.) and, as already mentioned, underutilized fish species (Figure 1.2). All these resources may benefit from a deeper knowledge of the biochemical phenomena determining sensory properties and storage stability of the products as well from new technologies addressing the shortcomings usually ascribed to these resources.

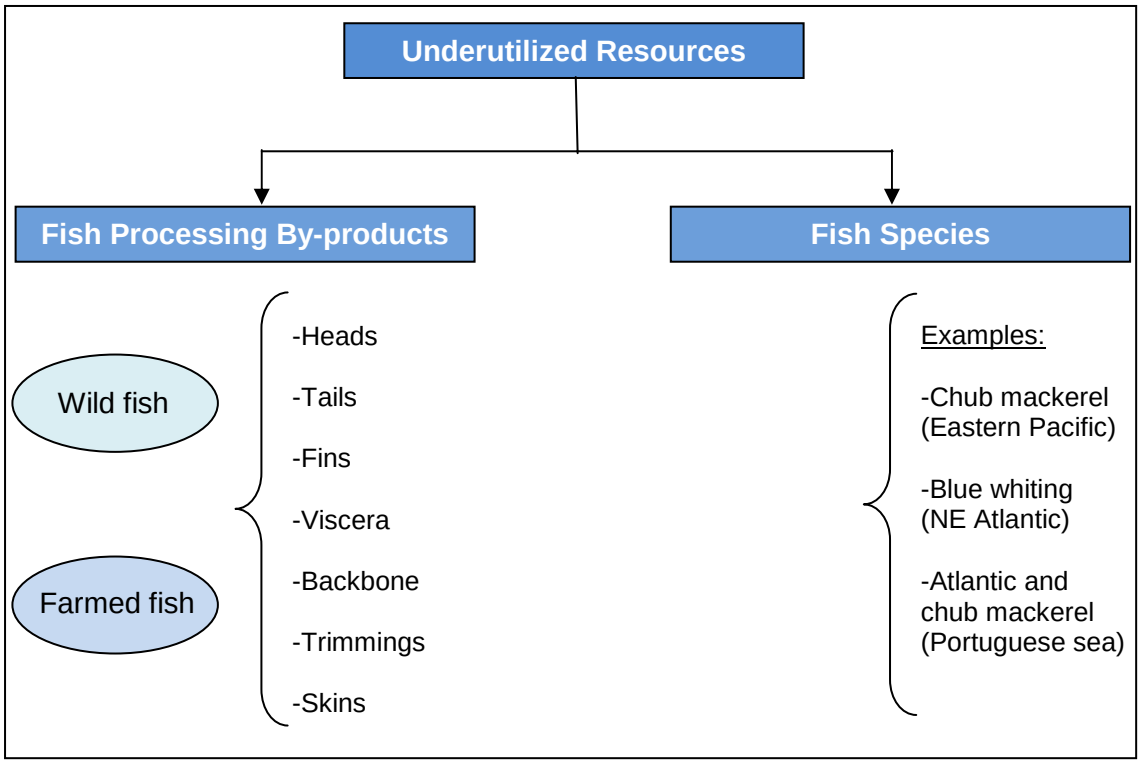

Figure 1.2 – General overview of the underutilized resources.

1.2.1 - Fish Processing By-products

The fish processing industry with the purpose of producing value-added convenient products such as frozen fillets or loins generates a large amount of by-products (Shahidi, 2007). This trend to produce convenient seafood encompasses not only wild fish as raw material, but also farmed fish. The market for whole fish of various species produced by the aquaculture sector, such as sea bass (*Dicentrarchus labrax*), is showing signs of saturation, at least, in South European countries (Cardoso et al., 2010a). There is also a new and growing problem of by-products that are generated by the processing of farmed fish, for instance, with the purpose of preparing ready-to-cook sea bass fillets. These by-products are potentially an undervalued resource, whose importance will certainly increase in the future. The conversion to powdered fish meal as with other fish by-products (Guerard et al., 2002), represents a loss of valuable protein for food.

Generated by-products include heads, tails, fins, viscera, and backbones, as well as trimmings (i.e., muscle joined to the skin and bones) (Figure 1.3). Whereas trimmings may be a valuable food source, the other by-products raise more problems, as a result of higher microbiological loads and/or higher levels of autolytic enzymes (for instance, the viscera). The trimmings contain a high level of nutritionally valuable components (for instance, proteins with a balanced amino acid profile or ω3-PUFA), which may represent a valuable food resource, safely obtainable, provided that care is taken with storage and handling of these by-products. Of course that size (for instance, small flesh portions in the fins), appearance (defective fillets or blood spots), high bone density and technological difficulty of separation (trimmings) pose some problems for the utilization of these by-products for food purposes.

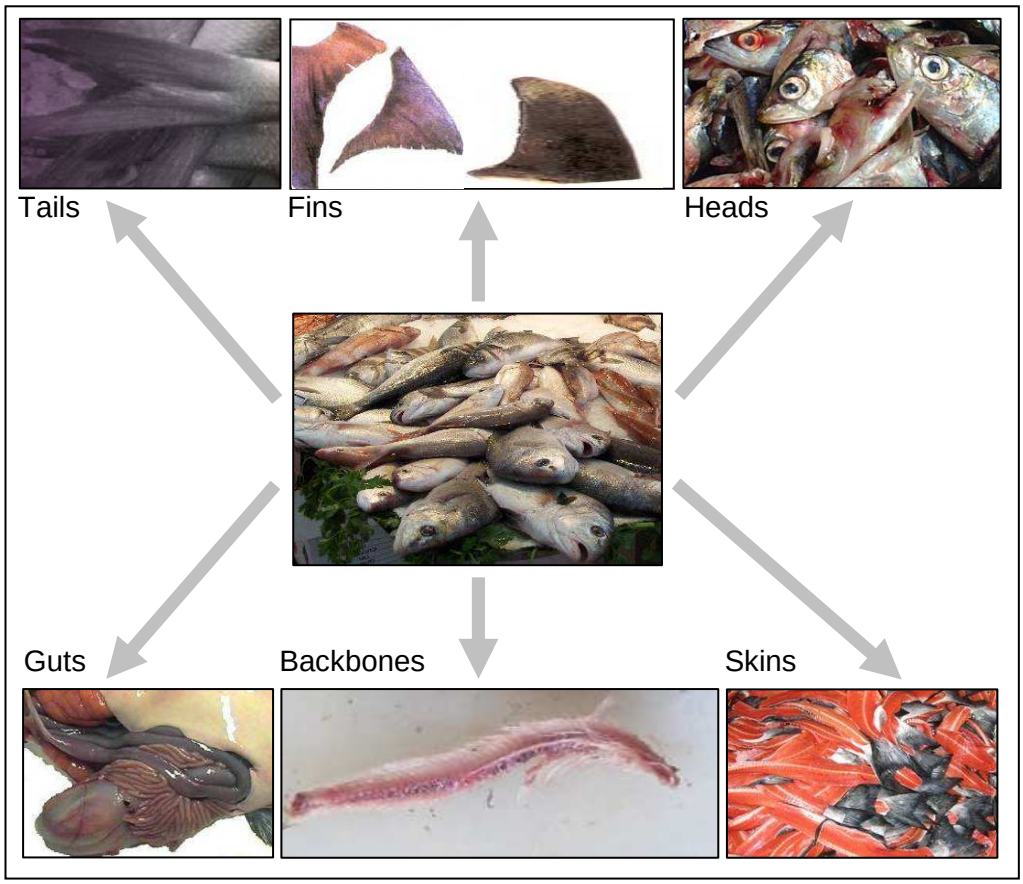

Figure1.3 – By-products generated during fish processing (pictures from: IPIMAR).

Considerable amounts of protein-rich by-products from fish processing plants are discarded (Figure 1.4) without any attempt to carry out recovery or are sold for fish meal or fish oil production (Mackie, 1983; Wasswa et al., 2007). For instance, the Nile perch (*Lates niloticus*) is an important international example of a species which is processed into chilled fish fillet for export along Lake Victoria, thereby generating large quantities of by-products (annual solid waste of 36000 tonnes and 1838000 m^3 of waste water)

(Gumisiriza et al., 2009), that remain unutilized. This is simultaneously an environmental and a resource loss serious problem. Many more could be mentioned.

Figure 1.4 – Sawdust, trimmings and other by-products generated during hake processing (picture from: IPIMAR).

Different utilizations of these by-products may be devised, like the production of novel and value-added products for nutraceutical, pharmaceutical and fine chemical industries, such as immune-enhancer biopeptides, ω3-PUFA dietary supplements or squalene for skin care (Shahidi, 2007) (Figure 1.5). Another solution is the production of food, such as extraction of cheeks and tongue from cod (Batista, 2007), utilization of fish stomachs, maws and liver (Batista, 2007) and production of surimi (see below) from the flesh joined to fish frames. For this purpose, different technological processes may be followed in order to recover protein from fish by-products, namely, mechanical separation from frames, base extraction or hydrolysis (Shahidi, 2007). While hydrolysis of fish proteins by endogenous enzymes may lead to some quality deterioration, such processes may be intentionally carried out to produce fish sauce and silage (Dapkevicius, 2002).

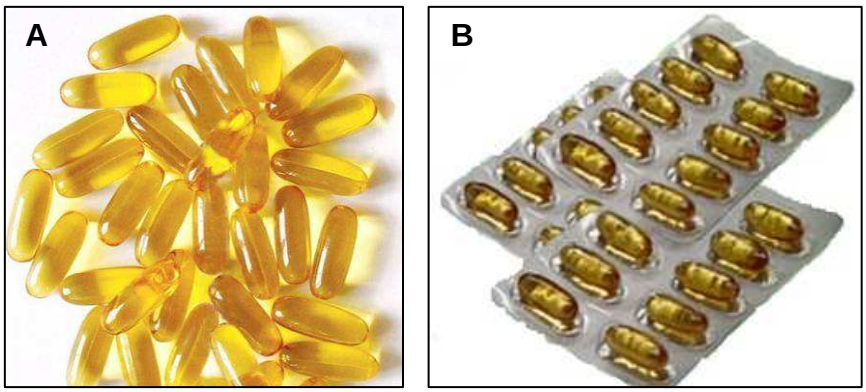

Figure 1.5 – Nutraceuticals by-products from fish processing (A: ω3-PUFA dietary supplements and B: squalene for skin care; pictures from: IPIMAR).

There are also chemical processing methods for protein recovery, such as chemical extraction of a fish protein concentrate, chemical hydrolysis, surimi production (see 1.3.2), production of fish protein isolates by the pH-shift method and other processes (Kristinsson et al., 2007).

However, in this work, the main focus will be on the mechanical separation of by-products, particularly, separation of skin and bones from trimmings and the utilization of the remaining flesh. Regarding this subject, is worth mentioning that as a result of filleting up to 50 % of the whole fish may be commonly discarded as waste (Windsor and Barlow, 1981). The remaining flesh attached to the filleting by-product (for instance, a frame) of fish, such as cod and haddock, can make up to 60 % of the by-product's weight (Ravichander and Keay, 1976). The main advantage of mechanical separation techniques lies in the preservation of the nutritional value and organoleptic characteristics of fish flesh in the attained products (Taylor et al., 2007). The application and further development of the mechanical recovery of fish flesh is desirable because it provides maximum yields at a reasonable processing cost and produces a product (minced fish) that offers scope for further improvement (see below). As long as there is a demand for such products, the mechanical separation of fish flesh from filleting by-products offers a profitable alternative to the production of fish meal or fish silage (Taylor et al., 2007). The production of minced fish requires specific equipment, which, in most cases, is based on the physical screening of flesh from non-flesh components through a perforated filter. Such flesh-bone separators must be able to recover as much of the remaining flesh from the by-product as possible, without destroying the flesh structure. One of the most common devices is the belt and drum system of the Baader equipment (used in this work, see 3.2.2.1 and Figure 1.6).

The production of fish mince from by-products also presents some drawbacks (a SWOT summary is shown in Table 1.2). For demersal species, it can accelerate the degradation of lipids, proteins, and also results in a product that is often aesthetically unacceptable (Taylor et al., 2007). Regarding this latter issue, a thorough rinsing of the by-products is required in order to remove any contamination from coloured, non-flesh components such as blood or some skin fragments. Minced fish offers a great control over product flavouring, texture, appearance, and storage properties (for instance, addition of antioxidants). Moreover, in subsequent processing, a range of textural variations can be induced by mechanical mixing, often in the presence of salt and other additives, and addition of flavour or other desirable additives is more effectively accomplished (Ravichander and Keay, 1976).

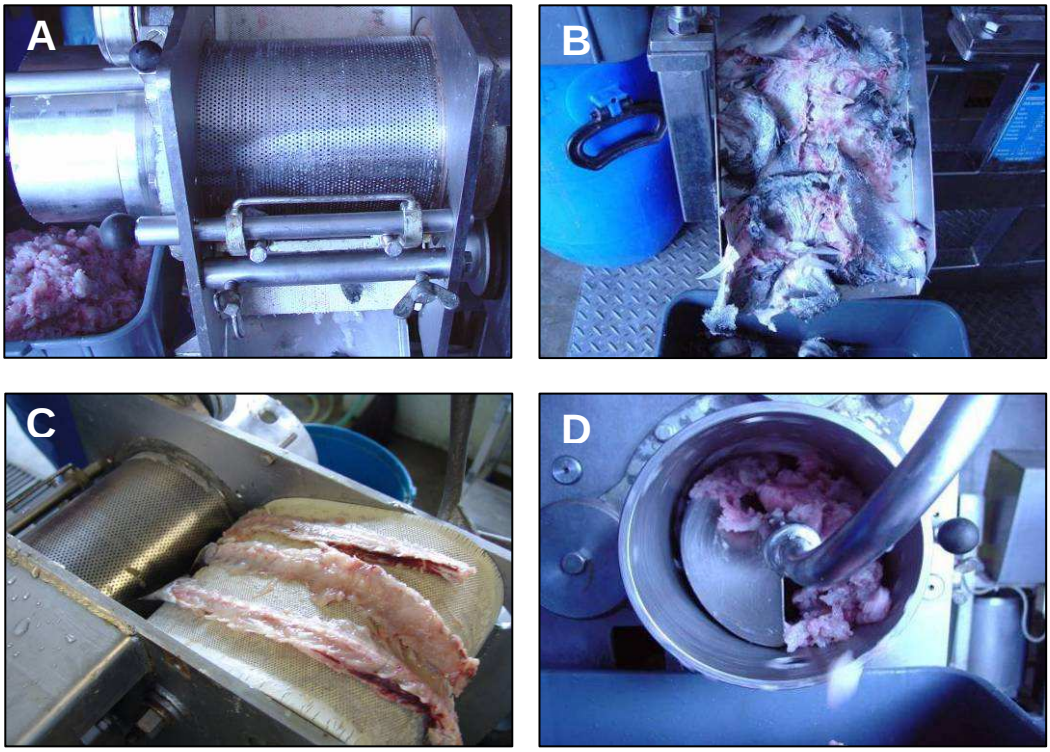

Figure 1.6 – Baader flesh-bone separator. A – General view; B – Discarded material; C – Feeding area; and D – Perforated drum and mince production (pictures from: IPIMAR).

Table 1.2 – A SWOT (strengths, weaknesses, opportunities and threats) analysis of fish mincing (from: Seafish, 2005).

Strengths	Weaknesses
-Flexibility in the type and scale of the process -Utilization of a wide range of raw material -Wide range of products can be prepared -Mince can be frozen for use by a third party or used directly to make products in-house -Mincing can be established within existing premises	-Risk of accelerated degradation of nutrients -Large-scale producers face high capital and operational costs -Legislative requirements establish very high standards of hygiene and operation -Lack of identification for all grades of mince
Opportunities	**Threats**
-Development of new markets/new food products (greater control over product characteristics) -Existence of markets for leftover bone, such as gelatine production	-Supplies of commercial blocks from other countries may dominate the world markets -Without the development of new markets, an excess of mince blocks may flood the market place, driving prices down

1.2.2 - Underutilized Fish Species

The declining stocks of traditionally commercial species promote the search for alternative species, still underutilized. Fish technologists and fish trade have turned their attention to some other sources of raw material (Flick et al., 1992; Shahidi and Venugopal, 1997), such as menhaden (Flick et al., 1992) or Mediterranean mackerel (Tzikas et al., 2009). According to some estimates, world fish consumption could be more than doubled if these resources were brought to the human food chain (Grantham, 1981). Such

estimates are based on various assumptions, are old (though fishing did not increase much in the last decades, overexploitation of some species has worsened) and have been dismissed as flawed (for instance, underreporting of effective captures) by some critics (Jacquet, 2004). Nevertheless, fish underutilization is a very important phenomenon with various aspects: species untargeted by fishing, discarded species, or fish used for other purposes than human consumption. It has been reported (SOFIA, 2009) that around one-fifth (year 2007) of the stock groups monitored by FAO were underexploited (2 %) or moderately exploited (18 %) and could perhaps produce more. For instance, some limited possibilities for expansion are offered by a few stocks of chub mackerel (*Scomber japonicus*), which are moderately exploited in the Eastern Pacific. In 2006, about 23 percent (33.6 million tonnes) of estimated world fish production was used for non-food products, in particular the manufacture of fishmeal and oil, the remaining 77 percent (110 million tonnes) was used for direct human consumption (SOFIA, 2009) (Figure 1.7). Large amounts of underutilized fish species are caught as by-catch during commercial fishing and then thrown back to the ocean (Venugopal and Shahidi, 1998), they may be not included in the final numbers of fish production, but are recognized as exploited resources by FAO. Some sources (Infofish, 1995) have estimated that commercial fisheries globally produce 27 million tonnes of discards each year. Shrimp trawl fisheries, particularly for tropical species, were found to generate more discards than any other type of fishery and accounted for one-third of global amount. As a result, some fish species, while still remaining underutilized, may also be overexploited.

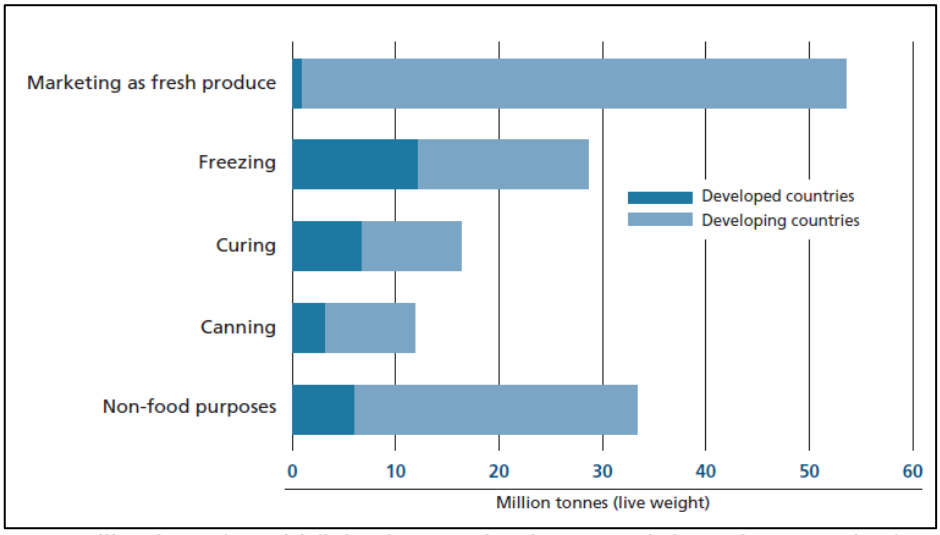

Figure 1.7 – Utilization of world fisheries production. Breakdown by quantity for the year 2006 (from: SOFIA, 2009).

There are reasons for not targeting some species, discarding them or using them for non-food ends, since many of these fish resources present rapid spoilage, seasonal variations in catch and composition, size or morphology features that render them less

attractive to industry and/or consumer. The rapid spoilage is a serious cause of resource underutilization, since the bulk of the world fish catch (about 30 %) is made up of relatively small-size pelagics (Hansen, 1996). In fact, due to the high fat content, most of these species deteriorate rapidly, thus presenting a major impediment to their effective utilization. Their small size and fragility causes them to break easily and disintegrate as a result of rough handling. For these reasons, species such as herring, mackerel or sardine are partially used for fish meal manufacture.

Different approaches to solve these problems have been proposed, namely, the recovery of flesh by mechanical deboning (small fish) and the development of value-added processed products, such as surimi and surimi-based products, sausages, fermented products, protein concentrates and hydrolysates, extruded products, and biotechnological possibilities (Venugopal and Shahidi, 1995). Some of these products (such as surimi) could be prepared on board and frozen stored. For instance, potentially underutilized sardine has been successfully tested as an alternative to Alaska pollock (*Theragra chalcogramma*) for production of fish cake products (Hirasawa, 1984).

One important example of such an untapped resource is the blue whiting (*Micromesistius poutassou*) (Figure 1.8), a gadoid abundant in the Northeastern Atlantic (Dagbjartsson, 1975; FAO inform, 2010). This species is considered a highly productive deep-sea species and to support larger fisheries. Being its organoleptic features not appealing to consumers and presenting a rapid deterioration process, efforts have been made to employ it in the manufacture of a large number of restructured products from fish mince (Borderías et al., 1997; Pérez-Mateos et al., 1997). Different gelation technologies (by pressure and heat) were tested and some progress regarding optimization of the operational parameters has been achieved (Pérez-Mateos et al., 1997).

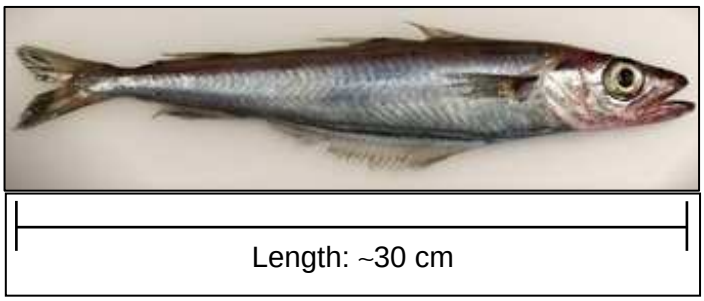

Figure 1.8 – External appearance of the blue whiting (pictures from: IPIMAR).

Other examples from Portuguese waters may be mentioned, Atlantic (*Scomber scombrus*) and chub mackerel (*Scomber japonicus*) (Figure 1.9). These are two abundant fish species in European waters, being sometimes caught together off the Iberian coast (ICES, 2006). Unfortunately, they are underutilized fish species (particularly Atlantic mackerel) and a largely wasted animal protein resource. Although chub mackerel is used

as a raw material by the fish canning industry, leaner fish are not used. Hence, a large portion of the captured mackerel is discarded by fishermen, since there is scarce demand for these fish in the market, making them low commercial value species (Cabral et al., 2003). In fact, it has been reported that this fishery targets bigger fish, thus, discarding marketable albeit smaller fish (ICES, 2006), a problem already mentioned above. A solution may be found in the production of good quality surimi or fish pastes, which are used in the manufacture of processed seafood products, whose sensory attributes can be controlled and directed to consumer preferences.

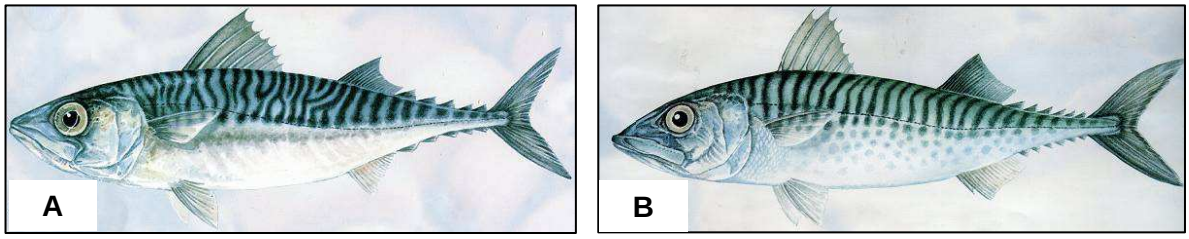

Figure 1.9 – External appearance of Atlantic (A) and chub mackerel (B) (pictures from: IPIMAR).

Nevertheless, is a well known fact that process development for underutilized species demands a thorough knowledge of each species with respect to their biology, proximate composition, catching technique, maximum sustainable yields, product development (including restructured products, such as surimi and surimi-derived products), marketing, and industry response (Harte et al., 2008; Jhaveri and Constantinides, 1982).

1.3 - Restructured Fish Products

Using minced and/or chopped fish muscle as raw materials and subjecting them to different technological processes, with or without ingredients admixture, new products with a different appearance and texture may be prepared. These new products, given their differences with respect to minimally processed fish products, may be termed restructured fish products (Borderías et al., 2005). This class of products encompasses a large range of products, from fish fingers specially targeted for children (covered with breadcrumbs, frozen, and to be used as fried products) to a new generation of fish products called analogues or substitutes, most of them mimicking traditional seafood products or, even, other high value products. These have not only become popular in Japan and other countries of the Asia-Pacific area, but have also gained market share in North America and, more recently, in Europe. The vast majority of these products are prepared from surimi (see 1.3.2), which is thoroughly washed and refined fish mince.

One major reason for restructuring fish muscle is related to the limited supply of high quality fishery products (see 1.1 and 1.2). Underutilized fish resources, such as fish processing by-products and underutilized fish species (species that have not traditionally

been commercialized either very much or at all), must be used as a precious source of animal protein and other nutrients. However, these resources are usually underutilized as the result of unattractive sensory characteristics or very fast spoilage processes. These problems make the restructured fish products an advisable outlet for these raw materials. One of the chief advantages of restructured products is that the composition of the end product can be modified by reformulation of the original product once this has been chopped and/or minced. Some constituents can be eliminated (as in the surimi manufacturing process) and new components can be added. These components can fulfil different roles, they can improve or transform sensory characteristics (taste, hardness, succulence, colour, etc.), enrich the nutritional content of the final product, even acting from a nutraceutical standpoint, and extend shelf life. For some products, at least, it is possible to distinguish these components as ingredients or additives. Ingredients are substances, whose incorporation is indispensable in order to achieve a certain product, for instance, breadcrumbs for a fish finger. On the other hand, additives are incorporated with the sole purpose of modifying the properties of the product without changing its identity (for instance, dietary fibre incorporated for nutraceutical reasons in crab meat analogues from surimi).

It is possible to consider different categories of restructured fish products on the basis of the type of raw material treatment or of the intermediate material produced during the preparation process (Figure 1.10). There are products prepared directly from minced fish, others from surimi and others from fish protein isolates or recovered protein (see 1.2.1). One particular case is the mixture of fish mince (or recovered fish protein) with high amounts of fat, requiring incorporation of emulsifier proteins and termed emulsion fish products. In this introduction, special emphasis will be put on those products which were prepared and tested in this work.

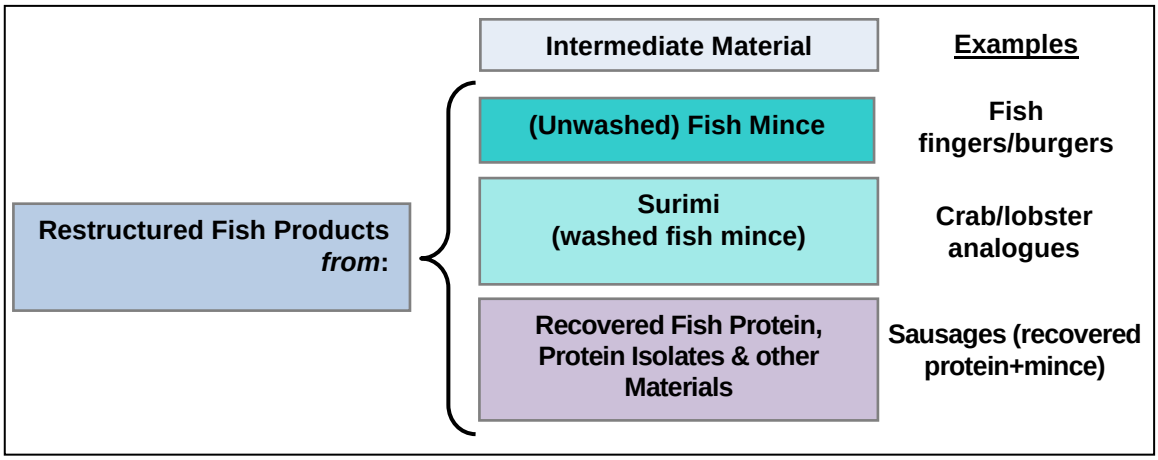

Figure 1.10 – Categories and examples of restructured fish products.

1.3.1 - Products based on Minced Fish

Fish mince can be defined as deboned and unwashed fish flesh. Minced fish contains all the proteins and biomolecules of the fish muscle, this means, besides structural proteins, all enzymes. Fish mince —especially if prepared from industry by-products and underutilized fish species— can be an inexpensive intermediate material for manufacturing various seafood products (Kim and Park, 2007). As a result of a low level of food processing (no washing steps), it offers nutritional advantages (containing water-soluble vitamins, minerals, and lipids) as well as functional advantages (exhibiting meat-like texture) compared to the other intermediate materials (Lanier, 1994; Magnusdottir, 1995). Additionally, higher yields in fish mince (unwashed) are especially important as many fishery resources are overexploited and there is a shift toward higher utilization of harvest. But, the conversion of fish mince to seafood final products with functional properties (from an organoleptic or health point of view) requires the addition of enzyme inhibitors or rapid cooking in order to minimize quality deterioration by enzymatic activity.

Diverse final products have been prepared from fish mince. From simple fish fingers or burgers (Figure 1.11) to other products involving the transformation of fish mince in a way that mimics muscle fibres, resulting in fillet analogues (Moreno et al., 2008; Moreno, 2009).

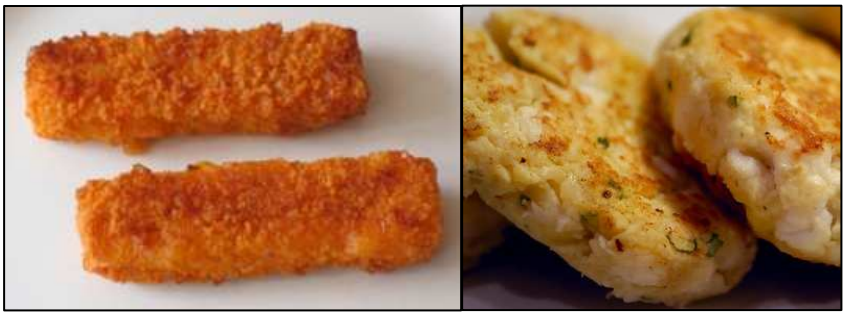

Figure 1.11 – Examples of restructured products prepared from fish mince (pictures from: IPIMAR).

For all these products, salt addition is fundamental, since it is required for the extraction and solubilisation of the muscle fibre (myofibrillar) proteins (Kuraishi et al., 1997). This also enables the gelling phenomenon (see 1.4), which is the basis for the manufacture of a large number of restructured products from fish mince, the gel usually being induced by heat treatment (Borderías et al., 1997). Usually a decrease in salt level to below 2.0 % (w/w) has a negative effect on the mechanical properties. Some authors (Gómez-Guillén et al., 1997a) proposed a minimum level of 1.5 % (w/w) salt. Nevertheless, low-salt restructured fish products with microbial transglutaminase as binding agent were obtained using mechanically deboned fish meat from filleting by-products of silver carp (*Hypophthalmichthys molitrix*) (Téllez-Luis et al., 2002). These authors demonstrated the

feasibility of attaining low-salt (1.0 %, w/w) restructured fish products with improved mechanical properties from silver carp mince. Several restructured fish products have been developed following different techniques and using various additives: vacuum tumbling processing of trimmed salmonid fish with subsequent canning and retorting or tumbling catfish using egg white as binder and several other processing techniques (Yetim and Ockermann, 1995; Zimmerman et al., 1996). Another important example of the utilization of fish mince in the preparation of restructured fish food is given by striped mullet (*Mugil cephalus*) (Ramírez et al., 2007a). This is an abundant species in Mexican waters. However, it is mainly captured to commercialize its roe and the flesh has low commercial value. Incorporation of additives (amidated low methoxyl pectin) was required for achieving products with adequate mechanical properties (Ramírez et al., 2007a). In this case, it was also possible to lower salt content (to 1.0 %, w/w) through transglutaminase or whey protein concentrate addition (Ramírez et al., 2007b). The combination of dietary fibre and minced fat fish has been reported (Sánchez-Alonso et al., 2007b). It was found that the addition of grape antioxidant dietary fibre to minced horse mackerel (*Trachurus trachurus*) muscle had a delaying effect on lipid oxidation. Though this is not an underutilized resource, it proves the feasibility of combining additives and unwashed (and, basically, untreated) raw materials with high fat levels, thereby mitigating their rancidity problems.

From all examples given above (summarized in Table 1.3), it can be concluded that there is a vast field of applications for minced fish as an intermediate material for the preparation of a wide range of food products and that many of the shortcomings (associated to a low degree of processing) of the minced fish can be overcome through new processing and additive solutions.

1.3.2 - Products based on Surimi

The utilization of unwashed minced fish shows some important problems. It must be mentioned that most intermediate materials used for manufacturing various seafood products are frozen to keep quality good for a relatively long period (Kim and Park, 2007). However, frozen stability of fish mince is poor, particularly for cold water species like Alaska pollock, due to their higher content of active enzyme systems, such as trimethylamine oxidase (Jahncke et al., 1992). The presence of these enzyme systems may have a deleterious effect on the mechanical properties of the final products, since some enzymes are proteases (Ayensa et al., 2002). For instance, heat-induced gelation at temperatures between 50 and 70 ºC results in maximum myofibrillar protein degradation in various fish species, namely, in arrowtooth flounder (*Atheresthes stomias*) (Uresti et al., 2006). Myosin, the myofibrillar protein responsible for functional and mechanical

properties, is subjected to proteolytic degradation leading to the loss of textural quality (Ramírez et al., 2002). This is mainly due to metalloproteases which selectively cleave the myosin molecule into heavy meromyosin and light meromyosin (Konno and Fukazawa, 1993).

Table 1.3 – Brief literature review on the subject of restructured fish products prepared from fish mince.

Raw material	Final product	Most significant findings	Reference
Hake muscle	Fish fillet analogue	-Additives, such sodium alginate (0.5 %, w/w), calcium chloride (0.1 %, w/w), and microbial transglutaminase (1.0 %, w/w), suitable for the preparation of products with the appearance of fresh fish	Moreno et al., 2008
Filleting by-products of silver carp	Heat-induced gel product	-Feasibility of attaining acceptable low-salt (1.0 %, w/w) products from poor quality raw materials through incorporation of microbial transglutaminase (0.3 %, w/w)	Téllez-Luis et al., 2002
Salmonid fish	Heat-induced gel product	-Preparation of a good quality ready-to-eat product through an innovative process involving vacuum tumbling, marinade solution penetration, canning, and retorting at high temperature and pressure	Zimmerman et al., 1996
Striped mullet muscle	Heat-induced gel product	-Feasibility of attaining acceptable low-salt (1.0 %, w/w) products through addition of microbial transglutaminase (0.3 %, w/w)	Ramírez et al., 2007b
Horse mackerel muscle	Frozen mince mixed with dietary fibre	-Incorporation of grape antioxidant dietary fibre (up to 4 %, w/w) delayed lipid oxidation during the first 3 months of frozen storage	Sánchez-Alonso et al., 2007b

The surimi processing method was developed in Japan (on the basis of traditional ancient processes, in fact, surimi is a word originally coined in Japan, meaning minced muscle) to overcome these problems, namely, to extend frozen storage shelf life (Yoon et al., 1991). In this processing method (Figure 1.12), minced flesh is repeatedly washed using chilled water (<10 ºC) (or using saline solutions containing sodium bicarbonate or sodium chloride) which removes unnecessary components that promote protein denaturation, particularly, during frozen storage (Park and Lin, 2005).

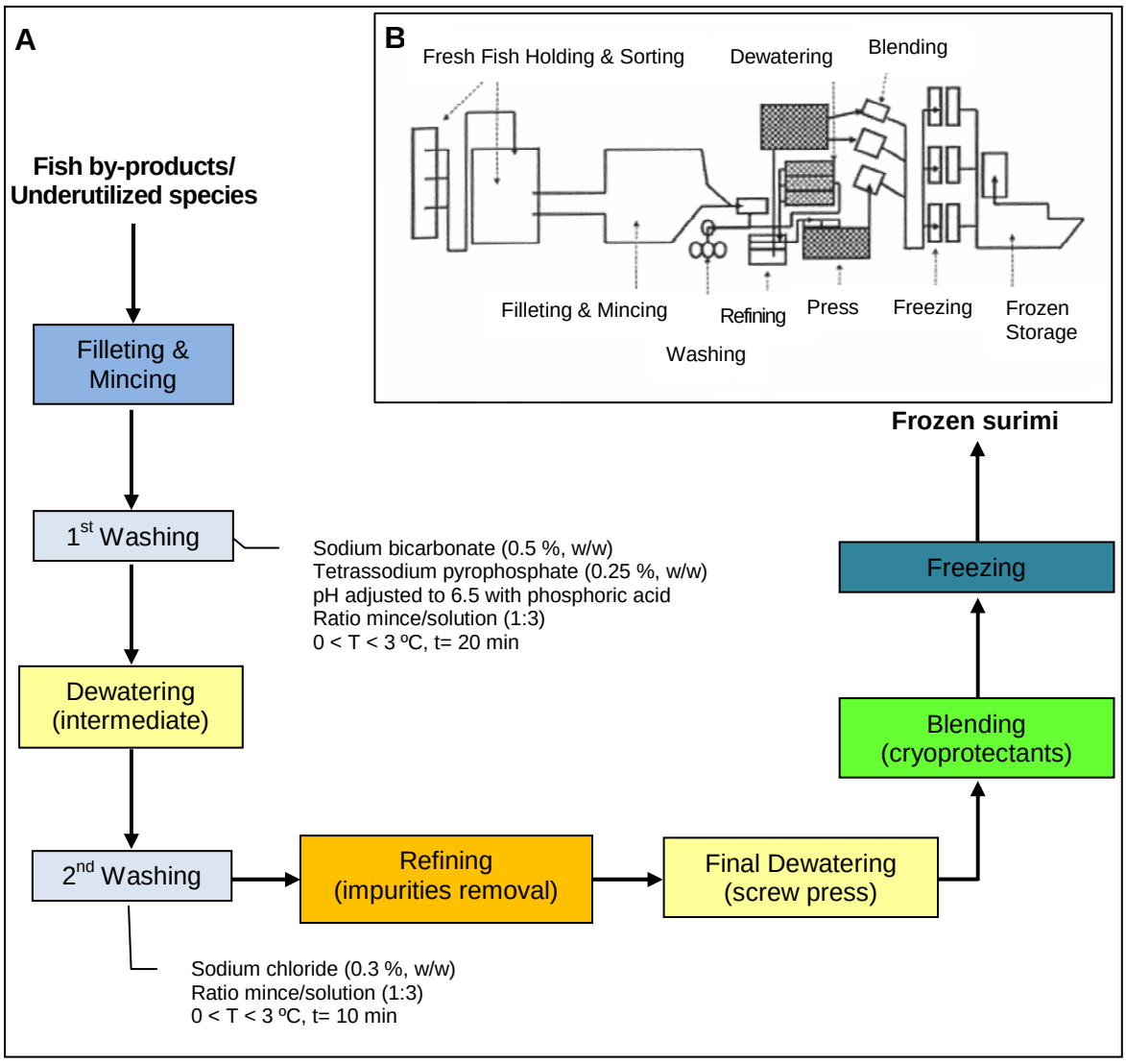

Figure 1.12 – Main steps of a typical process (A) and flow chart (B, adapted from: Draves, 2003) of surimi manufacturing.

Before 1960, surimi was manufactured and used within a few days as a refrigerated raw material, because freezing induced protein denaturation. With the discovery of cryoprotectants, the surimi industry was able to tap into previously unexploitable resources (Park and Lin, 2005). A group of Japanese scientists (Nishiya et al., 1960) found that the addition of low molecular weight carbohydrates, such as sucrose and sorbitol, to the dewatered myofibrillar proteins prior to freezing prevented freeze denaturation of proteins in Alaska pollock muscle. Since then the surimi industry had a great expansion, not only in Japan, but also in the USA (more modestly in Europe) (Kim and Park, 2007). In general, it is considered that surimi has excellent gelling and water-binding properties (Lanier, 1986; Lee, 1984). The high concentration of myofibrillar protein in surimi (Figure 1.13) enables the product to gel upon heating to produce a chewy and elastic texture (Bertak and Karahadian, 1995).

Figure 1.13 – External appearance of frozen surimi blocks (picture from: IPIMAR).

Due to its peculiar characteristics, surimi is used as an intermediate material for producing seafood products of various types and flavours, such as lobster or crab meat analogues (Babbit and Reppond, 1987). In Portugal, the crabsticks are probably the best known surimi-based products (Figure 1.14). Worldwide, crabsticks have also been one of the most prevalent surimi-based fabricated seafood products in the marketplace (Hollingworth et al., 1991). Surimi has found other applications beyond shellfish analogues. Fish nuggets (Heng and Eong, 2005), fish patties and Frankfurter sausages — produced from red hake (*Urophycis chuss*)— (Buck and Fafard, 1985) can be mentioned (Table 1.4).

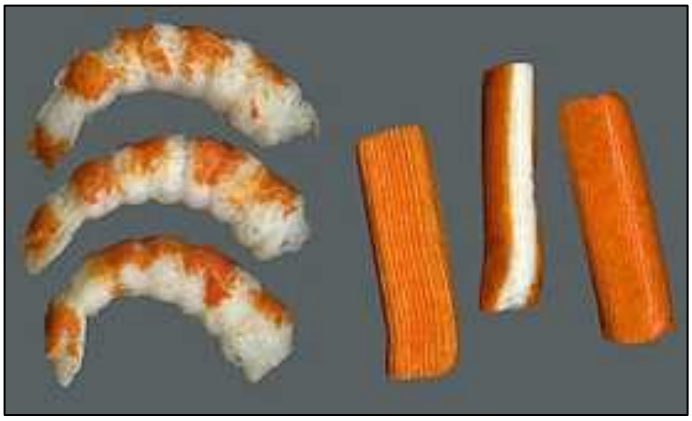

Figure 1.14 – Examples of restructured products prepared from surimi: shrimp and crab meat (crabsticks) analogues (picture from: IPIMAR).

As previously mentioned for unwashed fish mince, surimi has also been produced from underutilized fish resources. Regarding small pelagic fish (*Sardinops pilchardusts*) underutilized by the industry, some works (Bentis et al., 2005) have shown the feasibility of preparing final products with adequate textural quality (hardness and elasticity) and high nutritional value (ω3-PUFA). This has been part of a wider effort to exploit some

underutilized fish species, given the limited access to the Alaska Pollock sources (Chen et al., 1997), the main raw material for the production of surimi (Guenneugues and Morrissey, 2005). But, despite serious attempts to use underutilized small pelagic species, such as mackerel, the control of the factors influencing the large-scale processing of such raw materials has remained difficult (Chen et al., 1997). Namely, factors such high fat content or the pigment and trimethylamine oxide in dark-fleshed raw material. Different and more adequate washing procedures were required in order to prepare high quality surimi from such raw material (Shimizu et al., 1992). For instance, improvements in the colour of surimi with a higher number of washing cycles (Kim et al., 1996) or as a result of different washing times and water quantities (Chen et al., 1997) have been reported. Another example of the utilization of a low-cost species for the preparation of surimi is the silver carp (Luo et al., 2008). This is a freshwater species, which, in China, represents a large proportion of the aquaculture production, but with a very limited distribution sphere and storage period, thus justifying the investigation of a possible surimi outlet for the production surplus.

Surimi has also some drawbacks: low recovery yields, loss of some nutritionally valuable components, and the production of large quantities of wastewater, which are a serious environmental problem. That is, surimi manufacturing not only does not use by-products (such as trimmings) from the fish processing industry, but also generates itself various by-products (Kim and Park, 2007), that require application or treatment. Unwashed fish mince may be an interesting option, especially for industry by-products, provided that a deeper knowledge of the various biochemical processes is achieved and new solutions are found to avoid or compensate some negative effects of the enzyme systems, thus improving mechanical properties.

1.3.3 - Other restructured fish products

There are restructured fish products prepared from intermediate materials other than fish mince or surimi. These intermediate materials range from coarsely chopped fish flesh (lowest degree of processing) (Borderías et al., 2005) to fish protein concentrates or isolates produced by chemical techniques and involving separation of various components and a very high degree of processing (Kristinsson et al., 2007). Intermediate solutions were also proposed (Pires et al., 2009), involving the mixture of unwashed fish mince with proteins recovered by the pH-shift method (regarding this method, see Figure 1.15). These authors produced a sensory acceptable Frankfurter sausage with 20 % addition of recovered protein from Cape hake (*Merluccius capensis*) by-products, instead of hake mince (Figure 1.16). Another interesting study combined Alaska pollock surimi with isolated carp proteins (carp proteins were also recovered with pH-shift and isoelectric

precipitation) and demonstrated that properties, namely whiteness, of restructured fish products based on recovered proteins can be similar to those of surimi-based seafood, provided that appropriate additives are used (Taskaya et al., 2010) (Table 1.5).

Table 1.4 – Brief literature review on the subject of restructured fish products based on surimi.

Raw material	Final product	Most significant findings	Reference
Alaska pollock	Crab meat analogue	-Shore-based produced surimi can be used to produce an excellent (gel strength>70 N.mm) crab meat analogue	Babbit and Reppond, 1987
Small pelagic fish species	Fish nuggets	-Low value pelagic fish can be utilized in the production of fish nuggets with promising results	Heng and Eong, 2005
Red hake	Frankfurter sausage	-Frankfurter analogue competed well in consumer tests with a chicken Frankfurter -Frankfurter analogue proved to have lower levels of certain microorganisms	Buck and Fafard, 1985
Small pelagic fish	Heat-induced gel product	-Protein loss during the fabrication process was relatively small (6.9 % on a dry weight basis) -Hardness and elasticity of the products optimized regarding sorbitol as well as various salts: sodium, calcium, and ammonium chlorides	Bentis et al., 2005
Silver carp	Heat-induced gel product	-Setting temperature and protein concentration major factors affecting the gel strength -Combination of 90 % surimi protein and 10 % soy protein isolate (SPI) yielded a higher breaking force than surimi alone -Breaking force and distance of silver carp surimi gels decreased when the protein ratio of SPI (in a range of 10-40 % addition) was increased in the total protein (for 1 h setting at 30 or 40 ºC and 30 min heating at 85 ºC)	Luo et al., 2008

For this work, there is a specific category of restructured fish products deserving greater attention, the emulsion fish products. These are restructured products prepared from diverse intermediate materials (unwashed fish mince, surimi or fish protein isolates), but with a common characteristic, a high fat content, requiring the addition of emulsifier proteins. The addition of other ingredients, as is the case with Frankfurter sausages (Pires et al., 2009), dilutes the concentration of myofibrillar protein, thereby limiting the role of gel biochemical phenomena for the products' final properties (see 1.4).

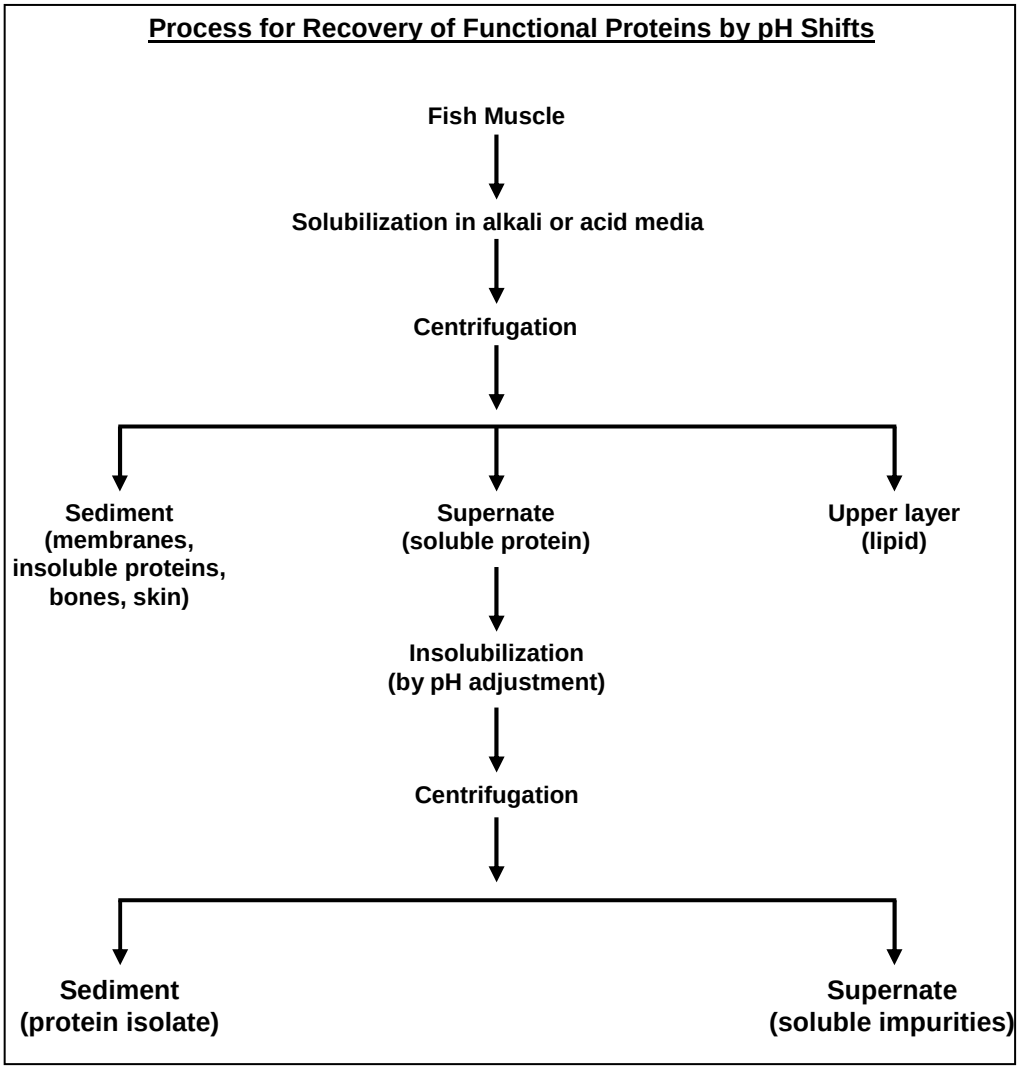

Figure 1.15 – Scheme of the pH-shift process (from: Hultin et al., 2005).

In these products, a good emulsion is fundamental for an adequate texture. Another work (Cavestany et al., 1994) reported that incorporation of sardine (*Sardina pilchardus*) surimi (up to 20 %) did not produce any important alteration in the rheological characteristics of meat products (Bologna sausages), but that fat level was an important factor. Indeed, when fat content was reduced, there was a significant decline both in the binding properties and in the shear and penetration forces of the products. As in the previous example, most fish sausages neither totally replace pork meat (Morris, 1988) nor mimic the common Frankfurter sausage (Chuapoehuk et al., 2001, López-Caballero et al., 2005). For instance, sausages from catfish (*Clarias macrocephalus* and *Clarias gariepinus*) meat and surimi were subjected to sensory analysis and the highest acceptability was found for a 10 % pork fat sausage (Chuapoehuk et al., 2001). It has been reported that one sausage formulation with similar quantities of surimi and pork meat could maintain the hardness of commercial sausage and, furthermore, without adversely affecting its flavour, acceptability and consumer preference (Murphy et al., 2004). The

production of a Frankfurter sausage with total replacement of pork meat and the minimization of pork fat (and of fat level altogether) were an untapped field of research that, accordingly, received special attention in this work.

Figure 1.16 – Encasing, twisting and tying of fish Frankfurter sausages (pictures from: IPIMAR).

Table 1.5 – Brief literature review on the subject of other restructured fish products.

Raw material	Final product	Most significant findings	Reference
Hake mince + recovered protein from hake by-products	Frankfurter fish sausages	-Acceptable Frankfurter sausage regarding texture, colour and sensory evaluation with 20 % addition of recovered protein -Fish sausages showed lighter colour and softer texture than pork sausages -Enrichment in ω3-PUFA did not affect flavour and overall acceptability	Pires et al., 2009
Alaska pollock surimi + isolated carp proteins	Heat-induced gel product	-Whiteness of restructured fish products based on recovered proteins can be similar to that of surimi-based seafood, provided that $0.2 \leq TiO_2 \leq 0.5$ % (w/w) is added -TiO_2 did not affect texture or viscoelasticity	Taskaya et al., 2010
Sardine surimi + pork meat	Bologna sausages	-Incorporation of sardine surimi (up to 20 %) did not change the fat and water binding properties as well as the rheological characteristics of the products -Fat level was an important factor: fat reduction reduced both the binding properties and the shear and penetration forces of the products	Cavestany et al., 1994
Catfish meat + surimi	Sausage product	-Sensory analysis showed that fish sausage prepared from fish meat and surimi (ratio 40:60) with 10 % pork fat had highest acceptability -No negative effects from ω3-PUFA addition	Chuapoehuk et al., 2001
Whiting surimi + pork meat	Sausage product	-Partial replacing pork meat with fish proteins: a combination of 25.3 % (w/w) surimi, 22.2 % (w/w) fat, and 25.3 % (w/w) pork ensured hardness & sensory evaluation similar to commercial control	Murphy et al., 2004

1.4 - Gel Proteins and Gel Mechanisms

Regarding restructured fish products, especially those containing high concentration of myofibrillar proteins (such as surimi), the biochemistry of gelation plays a major role in their properties. The various experiments carried out in this work involved the preparation of model gel products (from unwashed fish mince or surimi) or of products whose quality was much influenced by the gelation phenomena (including a low fat Frankfurter sausage). Therefore, it is important a good understanding of fish proteins and gel mechanisms.

First and foremost, it must be well understood what is meant by a food gel. This is a food with viscoelastic properties, i.e., a food that displays a combination of viscous and elastic characteristics if subjected to a deformation (Meyers and Chawla, 1998). Most gelling carbohydrates and gelatin form hydrogels (water confined in a polymer matrix) when their concentrated solutions are cooled, however they melt upon heating (i.e., are thermo-reversible) (Lanier et al., 2005). On the other hand, fish myofibrillar proteins (see 1.4.1) like the muscle proteins of other animal species, as well as egg white, wheat gluten, and milk β-lactoglobulin, form a thermo-irreversible gel upon heating (Figure 1.17) that does not melt with further temperature change (Lanier et al., 2005). These fish proteins enable the preparation of products of high textural quality and potential. In particular, surimi is known to produce gels of very high gel strength and deformability, quite unique among protein-rich foods. The excellent heat-induced gelation properties of surimi make it useful as a food ingredient (Lanier et al., 2005).

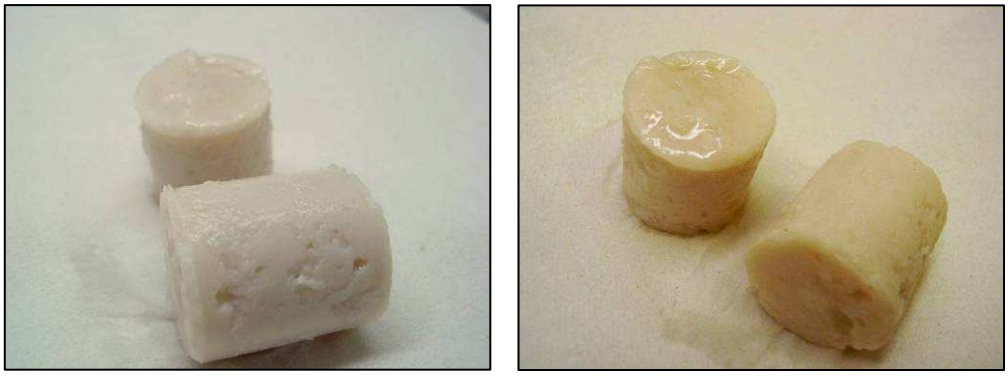

Figure 1.17 – Thermo-irreversible fish gels (pictures from: IPIMAR).

In order to recognize how these properties of fish gels are brought about, it is necessary not only to know the various classes of fish proteins and their characteristics, but also to better understand their chemical interactions. This, in turn, will enable to comprehend the different phenomena that occur during heat-induced gelation. Such phenomena can be viewed along the sequential process of manufacture of the gels as the operations and the thermal process advance. There is a first phase, known as setting,

followed by a critical phase of intermediate temperatures and, finally, a heating step at higher temperature that completes the gelation. There are other gels (which vie to reach or, even, surpass the textural quality of traditional surimi-based products) that are not heat-induced, but produced through different technical means, such as high hydrostatic pressure.

1.4.1 - Fish Proteins

There are three main classes of fish proteins: myofibrillar, stroma, and sarcoplasmic. Each group will be analysed below and fulfils different roles in the living fish as well as in the preparation of a good quality gel product.

Myofibrillar proteins are the main proteins in the muscle fibres, which contain a very large number of myofibrils (Figure 1.18). The myofibrils are constructed of end-on-end contractile units called sarcomeres, which contain three types of filaments —thick, thin, and connecting— arranged in such a way that give a striated appearance to the muscle under the microscope (Lanier et al., 2005).

The predominant protein in the sarcomere (in the thick filament) is myosin (55-60 % of total myofibrillar proteins). Myosin is a relatively large protein, with a molecular weight of 470 kDa (Bechtel, 1986) and it has both fibrous (long, extended shape) and globular (spherical shaped) properties. Each myosin molecule is composed of two ~220 kDa heavy amino acid chains and two pairs of light chains (<22 kDa) (Lowey and Risby, 1971). The heavy chains interact to form two different domains: a pair of globular “heads” and an elongated domain, “rod”. The amount and characteristics of myosin make this protein the most important for the formation of various chemical interactions underlying the gelation phenomena (see 1.4.2). Actin (comprising 15 to 30 % of the myofibrillar protein) is a globular protein with a molecular weight of 43 kDa (Lanier et al., 2005). This globular conformation reduces the exposure of some amino acid residues susceptible to cross-linking reactions (Ramírez-Suárez et al., 2005). Therefore, this protein has a less important role in the development of a gel texture. Other small fractions of proteins associated with either myosin or actin are tropomyosin, troponin complexes, actinins, M-proteins, and C-proteins (Asghar and Pearson, 1980). Whereas tropomyosin is a light chain protein (30-40 kDa), M- and C-proteins are heavier. All these fractions play important roles in the structural integrity of the sarcomere. During processing of surimi into a final product, the disassembly of the sarcomeres, which is an important requirement to attain an even protein distribution in the heat-induced gel, may require their selective solubilisation or degradation (Lanier et al., 2005).

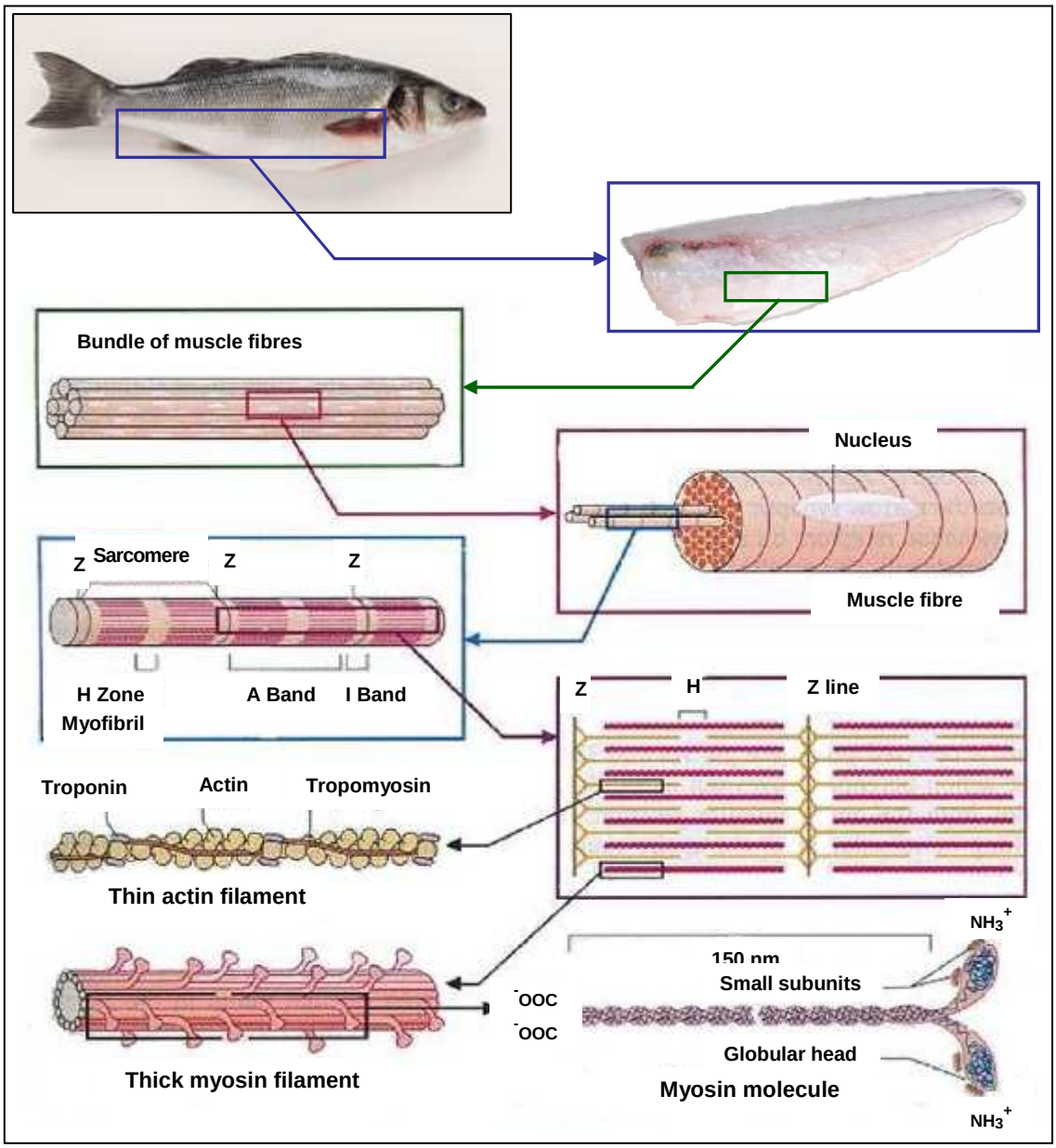

Figure 1.18 – Diagram depicting successively greater detail in fish muscle microstructure (adapted from: Macdonald et al., 1990 and Koolman and Röhm, 1996).

Stroma proteins are those proteins that make up the connective tissue, primarily collagen (Lanier et al., 2005). They are almost totally insoluble in water or saline solutions. The washing operations carried out in the manufacturing of surimi do not remove collagen. This may be troublesome, since it is thought that collagen interferes with the gelation of the myofibrillar proteins (Lanier et al., 2005). If present in high concentrations, such as in meats from terrestrial animals, it can accumulate in unsightly pockets in a heat-induced gelled meat product. But, fish have only a small percentage of stroma proteins, especially, if compared to their myofibrillar protein content (Table 1.6). Hence, the presence of collagen in either fish mince or surimi has probably a negligible effect on the gelling ability of these intermediate materials. This helps to explain the unique gel

properties of fish mince regardless of the utilization of washing procedures. On the other hand, it may soften the texture of traditional meat products (such as sausages), where partial or total substitution of fish flesh is tested (a challenge to one of the objectives of this work, see part 2).

Table 1.6 – Comparison of protein composition of fish and terrestrial animal meats.*

Animal species	(% of total proteins)		
	Sarcoplasmic	Myofibrillar	Stroma
Cod	21	76	3
Carp	23-25	70-72	5
Flatfish	18-24	73-79	3
Beef	16-28	39-68	16-28

* Based in data published by Lanier et al., 2005.

The sarcoplasmic proteins are water soluble (soluble at low ionic strength) proteins (Lanier et al., 2005). Most sarcoplasmic proteins, in contrast to the largely fibrillar or rod-like conformation of myosin, are globular in tertiary structure. Of course, being water soluble, sarcoplasmic proteins are largely removed by the conventional washing procedures involved in the preparation of surimi. This removal of these proteins is the main difference between two of the main intermediate materials discussed above (see 1.3.1 and 1.3.2), unwashed fish mince and surimi. It was originally thought that sarcoplasmic proteins, if not removed, would dilute the concentration of the better-gelling myofibrillar proteins and adversely affect gelation. It was also thought that sarcoplasmic proteins, if present in high concentration when the myofibrillar proteins were heated during product preparation, would unfold (denature) and attach to the myofibrillar proteins, thus blocking potential sites of protein-protein interaction between myofibrillar proteins and also leading to a reduction in the gelation quality (Okada, 1962). Some researchers have reported that sarcoplasmic proteins do not interfere with the gelation of myofibrillar proteins (Morioka and Shimizu, 1993). This means that unwashed minced fish can show a gelling ability similar to that of surimi. This idea determined many of the experimental options of this work and was largely confirmed (see 5.1.1, 5.1.4 and part 6). It is important to mention that sarcoplasmic proteins comprise several types of proteins, namely, enzymes and heme proteins (myoglobin and hemoglobin). Some enzymes, such as certain heat-stable proteases, certainly have a negative effect on the gelation of myofibrillar proteins due to their ability to cleave and thereby weaken the protein structures, as previously mentioned (Ayensa et al., 2002; Konno and Fukazawa, 1993; Ramírez et al., 2002; Uresti et al., 2006). On the other hand, other sarcoplasmic enzymes, such as transglutaminase (TGase), promote protein cross-linking, resulting in stronger textural properties. Protease and TGase activities in the sarcoplasmic fraction depend on

the fish species and protease activity can be reduced by proper heat processing or the addition of food-grade protease inhibitors (Lanier et al., 2005). Among the most problematic enzymes, a distinction can be made between the trimethylamine oxide (TMAO) demethylase and the proteolytic enzymes. The former is an enzyme especially prevalent in gadoid (cod-like) species, such as whiting or pollock, degrading TMAO to formaldehyde (FA) and dimethylamine during frozen storage. FA has a strong denaturing effect upon proteins, thus negatively affecting gelling properties, provided that this enzyme system is active and present at sufficient concentration (Lanier et al., 2005). Thorough removal prior to deboning of some organ tissues, such as the kidney or the liver, can reduce the impact of TMAO demethylase. On the other hand, proteolytic enzymes attack the myofibrillar proteins most actively during the cooking step, when the temperature is between 50 and 70 ºC (Uresti et al., 2006). Such enzymes disintegrate the protein network formed by the gelation of myofibrillar proteins, resulting in a mushy, rather than firm gel texture. In many species, the origin of these heat-stable proteases appears to be gut tissues (particularly, kidney and stomach). Rapid gutting and thorough cleaning and rinsing of the fish before and during processing (Figure 1.19), especially when fish are feeding prior to harvest, will reduce proteolytic activity in the meat and hamper phenomena of enzyme migration from the gut tissue into the muscle.

Figure 1.19 – Good hygiene conditions and practices during fish processing (pictures from: IPIMAR).

Regarding heme proteins, they are iron-containing proteins of the blood and red muscle cells (Lanier et al., 2005). It must be stressed that denaturation of the heme proteins, before or during processing, can result in their binding to myofibrillar proteins and cause discolouration. Besides, ferric ion is a known catalyst of lipid oxidation, which also affects gelation quality and appearance (Min and Boff, 2002). A possible solution is the physical separation of the light and dark muscles in fish prior to processing. Avoidance of the dark muscle (if possible) may be desirable to improve the gelling ability and appearance of the products. Dark muscle is more predominant in pelagic fish such as

sardine and mackerel and this has limited the use of these species for the production of surimi (Lanier et al., 2005). Apparently, the most straightforward approach to obtaining a good quality surimi is to use species that are mainly composed of light muscle. But, some strategies (mainly additives incorporation) may offer an alternative and this was also a challenge for this work (see production of mackerel surimi in 5.1.5).

As expounded above, these various fish proteins (Table 1.7) interact in different ways and such interaction is of paramount importance for the gelation phenomena. A deeper understanding of these interactions can only be accomplished by a better knowledge of the chemical bonds and reactions between proteins.

Table 1.7 – Characterization of the main fish protein classes.

Fish proteins	Characterization	Reference
Myofibrillar	-Are the main proteins in the muscle fibres -Myosin is the predominant protein (55-60 % of total myofibrillar protein) & fundamental for gelation -Myosin has a long fibrous portion and a globular head	Lanier et al., 2005
	-Myosin has a molecular weight of 470 kDa	Bechtel, 1986
	-Myosin is composed of two ~220 kDa heavy amino acid chains and two pairs of light chains (<22 kDa)	Lowey and Risby, 1971
	-Actin (15-30 % of myofibrillar protein) is a protein with a globular conformation & a molecular weight of 43 kDa	Lanier et al., 2005
	-Actin's conformation reduces residues exposure & cross-linking, thus actin has a minor role in gelation phenomena	Ramírez-Suárez et al., 2005
Stroma	-Make up the connective tissue (primarily collagen) -Are insoluble in water/saline solutions -Are < 5 % of total protein (minor effect on gelling ability)	Lanier et al., 2005
Sarcoplasmic	-Enzymes and heme proteins are the dominant -Are water soluble, being removed in surimi preparation -Present mostly a globular conformation	Lanier et al., 2005
	-As a whole, may not interfere with myofibrillar proteins' gelation, thus favouring the use of unwashed fish mince	Morioka and Shimizu, 1993
	-Some enzymes (heat-stable proteases) have a negative effect on gelation of myofibrillar proteins	Ayensa et al., 1992 (& others)
	-Other enzymes, such as TGase show a positive effect -Denaturation of heme (iron-containing) proteins can result in their binding to myofibrillar proteins & discolouration	Lanier et al., 2005
	-The ferric ion promotes lipid oxidation, thus affecting gelation quality and appearance	Min and Boff, 2002

1.4.2 - Chemical Interactions

Fish myofibrillar proteins play a major role in gelation and are the largest protein group in fish muscle (Table 1.6). These proteins have highly reactive surfaces once the protein is unfolded. During heating of fish minces (washed or not), proteins unfold, exposing their reactive surfaces to the neighbouring protein molecules, which then interact to form

intermolecular bonds. When enough bonding occurs, a three-dimensional network is formed, resulting in a gel (Lanier et al., 2005). Two main bond categories may be formed between proteins, non-covalent and covalent bonds. The former are divided into three main types: hydrogen bonds, ionic linkages and hydrophobic interactions. These bonds require different energies in order to be broken: covalent (~50-100 kcal/mol), ionic (~50-60 kcal/mol), hydrogen (~5 kcal/mol) and hydrophobic (~0.5 kcal/mol) (Atkins, 1990) (Figure 1.20). The relative importance of each bond type does not depend exclusively on the individual bond strength. All bonds are important for the gelation phenomena: hydrogen bonds after cooking (during cooling), ionic linkages disruption is a requisite for gelation, and hydrophobic and covalent bonds seem to be fundamental for the formation of a thermo-irreversible gel at high temperature (Gilleland et al., 1997).

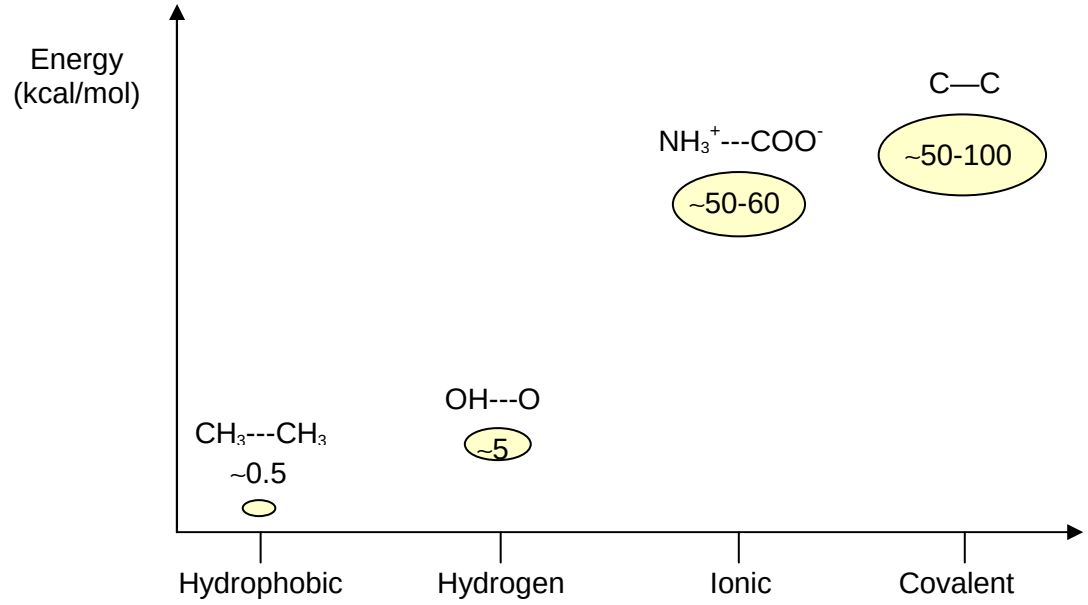

Figure 1.20 – Required energy for rupture and examples of chemical bonds between proteins.

Covalent bonds are rigid chemical bonds formed by the sharing of electrons between proteins and their rupture requires high amounts of energy (Atkins, 1990). There are significant differences of required energy between different atom pairs. Whereas one mol of S-S bridges requires about 60 kcal to be broken, one mol of C-N and of C-C require >70 kcal and >80 kcal, respectively (Atkins, 1990). In contrast to the other types of protein bonds previously discussed, covalent bonds are largely temperature insensitive once formed. For this reason, the formation of these bonds, in addition to hydrophobic interactions, is thought to be a primary mechanism for the formation of a thermo-irreversible gel (Gilleland et al., 1997). Among covalent bonds, two types deserve special attention: disulphide and ε-amino-(γ-glutamyl)-lysine bonds. Disulphide bonding (S-S) is a great contributor for protein gelation as a result of cooking (Lanier et al., 2005). This

bonding is formed by the oxidation of two cysteine residues of neighbouring protein chains, provided that they have reactive sulfhydryl (-SH) groups (Figure 1.21). As a result of these biochemical phenomena, it has been reported that surimi gels are strengthened by the addition of oxidants, which accelerate oxidation of the sulfhydryl groups (Nishimura et al., 1994). An important requirement for a successful gelation is the conversion of intramolecular S-S bonds (within a protein) to intermolecular S-S bonds through disulphide interchange, thereby leading to cross-linking of proteins. Some additives can achieve this. It was found that cystine (a dimeric amino acid formed by the oxidation of two cysteine amino acids) addition to pollock surimi pastes prior to heating induced the formation of stronger gels (Kim, 1987).

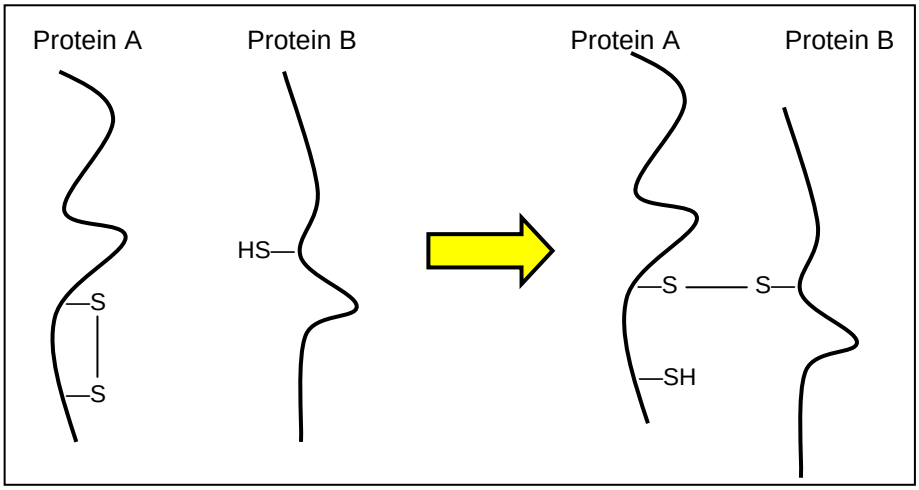

Figure 1.21 – Conversion of intramolecular S-S bonds to intermolecular S-S bonds (adapted from: Lanier et al., 2005).

On the other hand, the ε-amino-(γ-glutamyl)-lysine bonds (between glutamine and lysine residues of myosin heavy chains) are catalyzed by an enzyme, TGase (Kumazawa et al., 1993; Ramírez-Suárez et al., 2001), belonging to the group of sarcoplasmic proteins in the fish mince (see 1.4.1) and being calcium (Ca^{2+}) dependent (Saeki, 1996) (Figure 1.22). These covalent bonds make also a large contribution for protein cross-linking, thereby creating a denser bond network, i.e., a more tightly knit web, between proteins in gel products. The lesser role of actin in gelation is ascribed to its resistance to cross-linking, which, precisely, may be due to a lower exposure of the susceptible amino acid residues (Gln, Lys) in its globular conformation (Ramírez-Suárez et al., 2005). Actin is reported to have a synergistic role in the gelation of myofibrillar proteins (Ishioroshi et al., 1980), being suggested an optimal 15:1 weight ratio of myosin to actin for maximum rigidity.

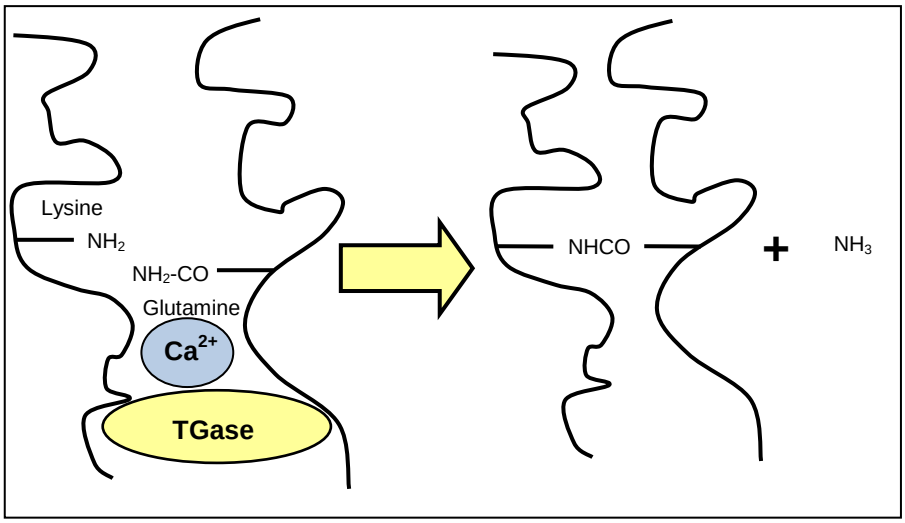

Figure 1.22– Formation of ε-amino-(γ-glutamyl)-lysine bonds by TGase (adapted from: Park, 2005a).

Within non-covalent bonds, hydrogen bonds are weaker dipole bonds (for instance, between a hydrogen atom covalently bonded to an oxygen and another oxygen atom in the vicinity, Figure 1.20) that, mainly due to their great numbers rather than individual bond strength (Atkins, 1990), can be important in the stabilization of bound water within the gel and add gel strength during cooling of surimi-based seafoods. Conversely, during heating, a large number of hydrogen bonds that maintain the folded protein structure are broken between the carbonyl and amide groups in the peptide backbone. This, in turn, allows the peptide backbone to become extensively hydrated, which, in turn, reduces the mobility of the neighbouring water molecules. This hydration of the exposed peptide backbone plays a major role in the water holding capacity of the gel that is subsequently formed by protein-protein interaction (Lanier et al., 2005). Hydrogen bonds between proteins are more numerous when the gel is colder, which explains firmer gels at lower temperature (Howe et al., 1994). Those between amino acids also stabilize the secondary structure of individual protein molecules in water. The α-helix of native and partially denatured proteins and the β structure that forms on heating and cooling are both stabilized by hydrogen bonds (Bouraoui et al., 1997) (Figure 1.23).

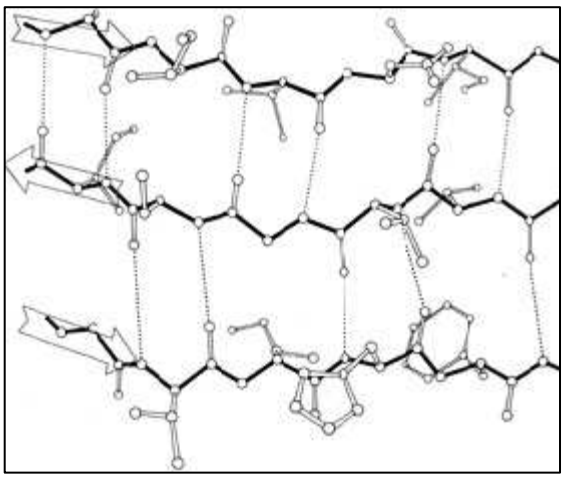

Figure 1.23 – Example of a β structure (antiparallel) stabilized by hydrogen bonds (dotted lines).

Ionic linkages are the attraction between oppositely charged sites on the protein surface. At a pH nearly neutral (as occurs in most fish raw and intermediate materials, as well as final products), the carboxyl groups (COO^-) of glutamic and aspartic acid are negatively charged, while the amino groups (NH_2^+) of lysine and arginine are positively charged (Lanier et al., 2005). The ionic attraction between these groups leads to associations of myofibrillar protein, which are insoluble in water. The ionic interactions are thought to be the most important forces involved in the assembly of myosin thick filaments (Miroshinichenko et al., 2000). Therefore, the addition of sodium chloride interferes with this electrostatic interaction, bringing about the disassembly of the thick filaments and better dispersion of myosin (Figure 1.24). For this reason, for minced fish to gel, salt must be added to break ionic linkages, assist in the dispersion of the proteins, and develop an elastic structure (Niwa, 1992). The salt ions (Na^+ and Cl^-) selectively bind to the oppositely charged groups exposed on the protein surface. However, salt addition must be followed by a sufficient degree of grinding to enhance protein solubilisation and dispersion.

Contrastingly to hydrogen bonds, hydrophobic bonds are strengthened by rising temperature (Lanier et al., 2005). The formation of intra- and intermolecular hydrophobic interactions is due to the thermodynamic response of protein surfaces exposed to the water in which they are dispersed or solubilised. The interior of the folded protein chain has a greater density of hydrophobic residues. Conversely, the residues on the surface of the folded protein are mostly hydrophilic. The folded protein achieves through this arrangement thermodynamic equilibrium in water. Protein unfolding (commonly as a response to heating) exposes the hydrophobic core to water. Water molecules near these exposed hydrophobic groups become ordered into hydrogen-bonded clathrates (Lanier et al., 2005). Such ordering decreases the mobility of the water molecules, thus decreasing the entropy.

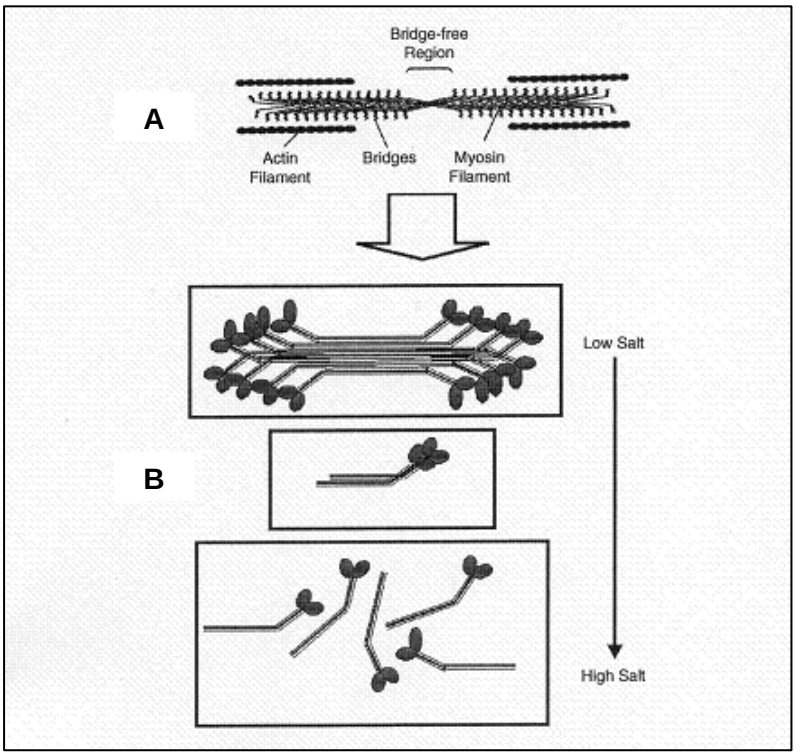

Figure 1.24 – Arrangement between thin (actin) and thick (myosin) filaments in the muscle (A, from: Yu, 2002) and disassembly of thick filaments as a result of salt addition (B, from: Cohen, 1998).

Therefore, for thermodynamic reasons (entropy increase) the hydrophobic portions of the protein associate closely with each other (Figure 1.25). This results in an effective binding of the proteins, thus leading to protein association and (under the proper conditions) the formation of a gel network.

All these different chemical interactions between proteins (Table 1.8) play a role during the process of preparation of a gelled product from unwashed fish mince or surimi (see 1.4.3., 1.4.4., and 1.4.5.) and have an important impact in the textural quality of the product.

1.4.3 - Setting Phenomenon - *Suwari*

In the process of preparation of a restructured product from an intermediate material, such as unwashed fish mince or surimi, different processing steps must be followed, which lead to different phenomena (Figure 1.26). After mixing of the ingredients, the attained batters must undergo a first phase, the so-called “setting” step.

Fish minces and specially surimi can form a less hard, but very deformable gel when thoroughly mixed with salt and held at low temperatures (0 to 40 ºC, depending on the species, namely, on its provenience from cold or warm waters) without cooking. These gels resulting from this process are referred to by the Japanese term “suwari” (Moreno et al., 2009). These gels after cooking at higher temperatures lead to the formation of

stronger gels than those cooked without this low-temperature phase (Lanier et al., 1982) (Figure 1.26). This phase, characterized by gelation and textural strengthening of the salted batter at low temperatures, is precisely the setting step. Effectively, setting is often utilized in the preparation of surimi-based seafood (Lanier, 1986).

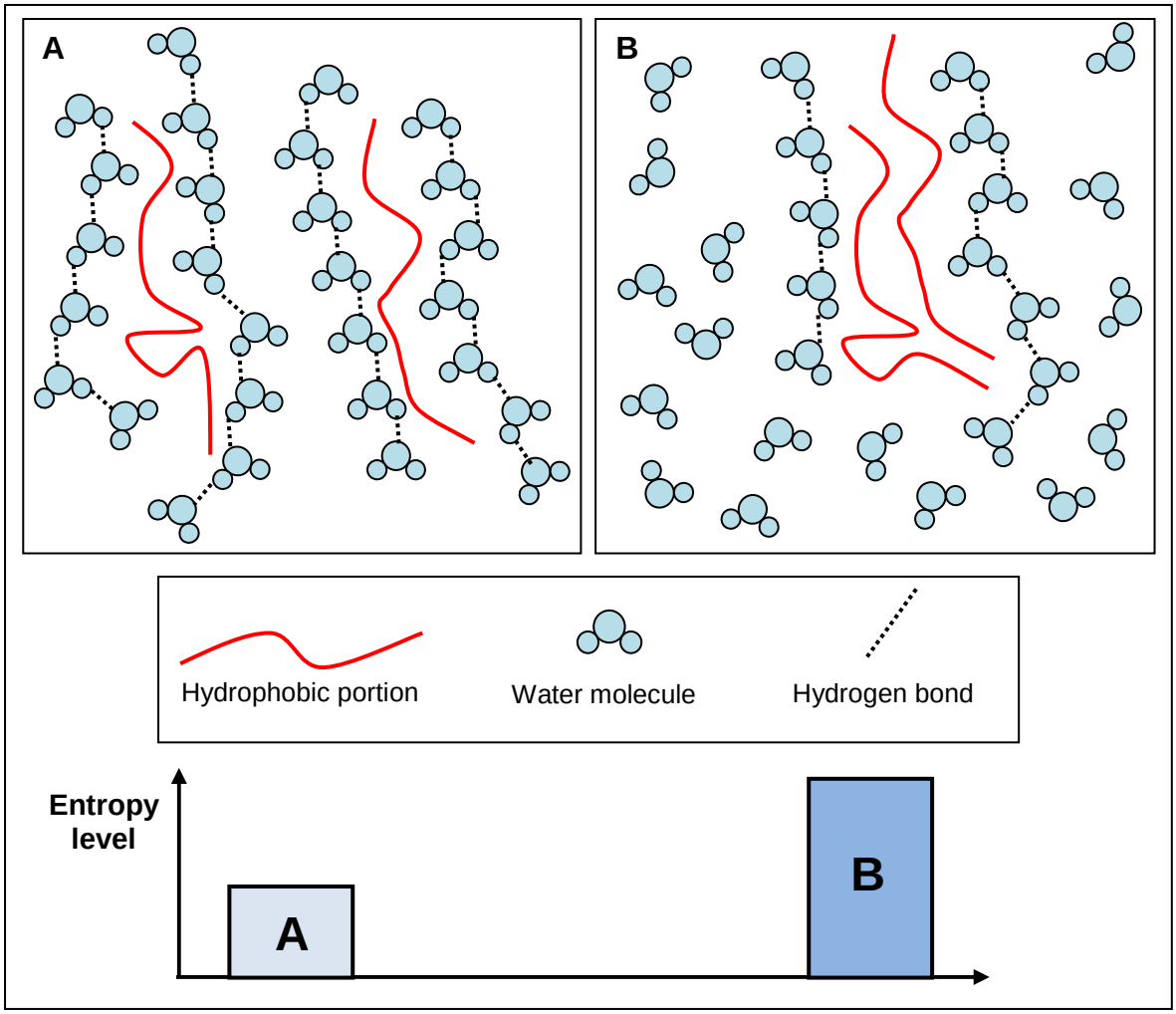

Figure 1.25 – Association of the hydrophobic portions of the proteins and obtained entropy increase: A, low entropy with water molecules ordered near the exposed hydrophobic portions; B, high entropy with higher water mobility (adapted from: Lanier et al., 2005).

This unique phenomenon is thought to result from the enzymatically catalyzed formation of non-disulphide covalent bonds between myofibrillar proteins. As previously seen (1.4.2), these bonds are due to TGase, a protein-glutamine γ-glutamyltransferase (EC 2.3.2.13) present in (endogenous to) the fish muscle. This cross-linking results in the formation of myosin polymers, with a simultaneous decrease in myosin heavy chain monomers (Esturk et al., 2004). The content of ε-amino-(γ-glutamyl)-lysine dipeptide cross-links generally correlates with the level of gel strength (Imai et al., 1996).

Table 1.8 – Main chemical interactions between proteins.

Chemical interactions	Characteristics and main effects	Reference
Hydrogen bonds	-Weak dipole bonds -Importance due to their great numbers -Stabilize the bound water within the gel -Add gel strength during cooling	Lanier et al., 2005
	-More numerous for colder gel, thus contributing to higher firmness at lower temperature	Howe et al., 1994
	-Stabilize the α-helix and β-sheet of proteins	Bouraoui et al., 1997
Ionic bonds	-Attraction of oppositely charged sites on the protein	Lanier et al., 2005
	-Importance in the assembly of myosin thick filaments	Miroshinichenko et al., 2000
	-Must be disrupted for minced fish to gel	Niwa, 1992
Hydrophobic bonds	-Interaction between hydrophobic areas -Increased by rising temperature (protein unfolding as a response to heating exposes hydrophobic areas) -Entropy increase favours association of hydrophobic portions of different proteins and contributes to formation of a gel network	Lanier et al., 2005
Covalent bonds	-Formed by the sharing of electrons belonging to atoms of different proteins	Lanier et al., 2005
	-Their rupture requires high energy amounts	Atkins, 1990
	-Largely temperature insensitive once formed, decisively contributing for the formation of thermo-irreversible gels	Gilleland et al., 1997
	-Two main studied types in fish proteins: S-S and ε-amino-(γ-glutamyl)-lysine bonds	Lanier et al., 2005

Nevertheless, it has been reported that the rate at which the cross-linking reaction proceeds seems also to be an important factor in the ultimate strength attained (Lee et al., 1997). Covalent linkages are not the only protein-protein interactions that stabilize the suwari gel. Several experimental findings suggest that intermolecular hydrophobic interactions contribute to the low-temperature setting reaction as well. An increase in protein surface hydrophobicity can be measured during setting (Niwa, 1992; Wicker et al., 1986). Involvement of hydrophobic interactions in setting was also observed in the Raman spectroscopy of suwari gels, resulting in the decreased intensity of a band near 2930 cm^{-1}, assigned to C-H stretching vibrations (Bouraoui et al., 1997). The requirement of salt addition also supports a role of hydrophobic interactions in setting, because salt acts upon water molecules in a way that favours the hydrophobic interactions between proteins (Wicker et al., 1989).

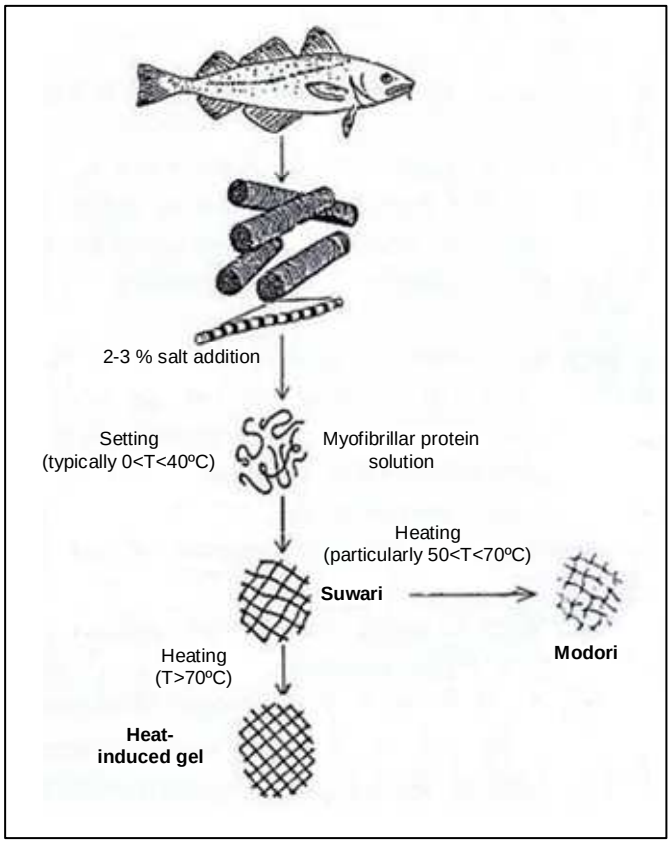

Figure 1.26 – Formation process of a gel from myofibrillar proteins (adapted from: Suzuki, 1981).

The optimum setting temperature depends on the used species (Figure 1.27), mainly because the myosin (the substrate of the reaction) has a different thermal stability for different fish species (Araki and Seki, 1993; Joseph et al., 1994; Niwa et al., 1993).

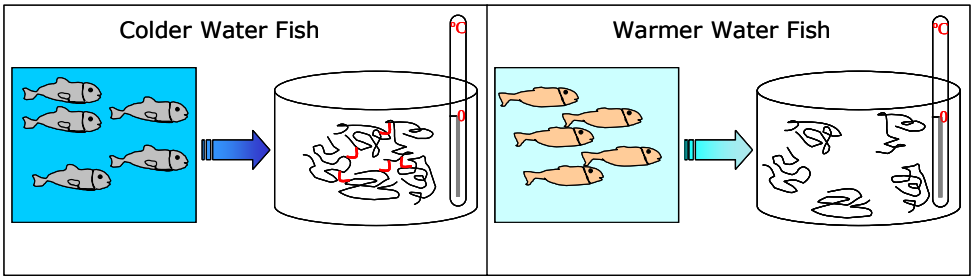

Figure 1.27 – Dependence of the optimum setting temperature on the fish species (for instance, at near 0 ºC, only myosin of colder water fish species initiates the cross-linking process).

Generally, fish that live in colder waters have the least stable proteins compared to fish living in warmer waters. Yet fish from cold, but very deep waters, may have more stable proteins than fish in the same waters nearer the surface, as a protective mechanism to the higher pressure of their habitat (Lanier et al., 2005). It has been shown that Alaska pollock surimi displays maximum gel strength when preincubated at 25 ºC for two to three hours (Kamath et al., 1992). Other authors (Lee and Park, 1998) reported that pollock gels

achieved highest gel strength when set at 5 ºC. Optimum setting temperature for warm water species was quite different, namely, between 25 and 40 ºC for threadfin bream, bigeye snapper, and lizardfish (Esturk et al., 2004). It was found that the strongest gels for Atlantic croaker (a temperate water species) were attained with 40 ºC setting (Kim, 1987). No setting was observed for this species below 25 ºC. Setting of tropical species is typically optimal at higher temperatures (Benjakul at al., 2002; Klesk et al., 2000; Morales et al., 2001). However, simple temperature dependence is not supported by the scientific literature data. For instance, small pelagic species from temperate (or moderately cold) waters, such as sardine, seem to show an adequate setting at higher temperature (35 ºC) for 1 h (Mendes et al., 1997). The setting of threadfin bream, a major resource for minced fish production in Southeast Asia, could be induced at both 25 and 40 ºC, but setting at 25 ºC took longer (4 h) than at 40 ºC (2 h) (Yongsawatdigul et al., 2002). Nonetheless, in this same study, it was shown that partial unfolding of the myofibrillar proteins seemed to be enough to induce TGase cross-linking of reactive amino acids at 25 ºC. A summary of the main aspects of the setting phenomenon is shown in Table 1.9.

Table 1.9 – Main aspects of the setting phenomenon.

Raw material	Main aspects	Reference
Fish (in general)	-Minced fish/surimi form a soft and very deformable gel (after salt mixing and at 0 < T < 40 ºC) - *suwari*	Moreno et al., 2009
	-After cooking, the previously set gels form stronger gels	Lanier et al., 1982
	-Setting promotes cross-linking, resulting in the formation of myosin polymers	Esturk et al., 2004
Colder water fish:	-Proteins are more temperature-sensitive, thus favouring setting at lower temperatures	Lanier et al., 2005
Alaska pollock	-Surimi has been reported to display maximum gel strength if set at 25 ºC for 2/3 h	Kamath et al., 1992
	-Highest gel strength was achieved after set at 5 ºC	Lee and Park, 1998
Warmer water fish:	-Setting of tropical species is optimal at higher temperatures	Benjakul et al., 2002
Threadfin bream & other species	-Showed higher optimal setting temperatures, 25 < T < 40 ºC	Esturk et al., 2004
Temperate water fish	-Small pelagic species also show an adequate setting at higher temperatures, 25 < T < 40 ºC	Mendes et al., 1997

1.4.4 - Proteases and Network Destruction - *Modori*

In the traditional process for the preparation of gelled fish products, cooking is required as the step subsequent to setting, in order to attain tougher gels. Typically, this requires heating up to temperatures of 90 ºC (see 1.4.5). This may be troublesome if the heating

rate is low and the set fish minces are subjected to temperatures between 40/50 and 70 ºC for too much time (An et al., 1996).

This is because temperature can activate endogenous enzymes that naturally occur in fish muscle. Fish muscles from various species show similar temperature effects, a structure-setting reaction below 40 ºC (see 1.4.3) and a structure-disintegrating reaction at higher temperatures, particularly between 50 and 70 ºC, which is known by the Japanese term “modori” (An et al., 1996) (Figure 1.26). This phenomenon has been associated with the presence and activity of endogenous serine and cysteine muscle proteases (An et al., 1996; Ramos-Martínez et al., 1999). Other explanations have been put forward for the occurrence of modori. An excessive formation of hydrophobic interactions between proteins may lead to a water loss and an asymmetric and heterogeneous network, with special relevance for the role of myosin heavy chain (Shimizu et al., 1983). Another hypothesis is the action of non-enzymatic proteins (between 44 and 50 kDa) with a strong reducing impact on the water binding capacity (Iwata et al., 1977). Nevertheless, the action of proteolytic enzymes seems to be the main driving force of the phenomenon (Kinoshita et al., 1990).

Modori is a problem for many species, for instance, it has been reported that thermal gel degradation of sardine surimi gels occurred with incubation at 50 and 60 ºC (Alvarez et al., 1999). The underlying proteolytic activity causes the rapid and severe degradation of myofibrillar proteins, particularly myosin (Wasson et al., 1992). This proteolytic activity has a detrimental effect on the final textural quality, lowering the gel strength (Boye and Lanier, 1988; Morrissey et al., 1993). Cysteine endoproteases have the most serious effects on texture because of their thermostability and ability to cleave internal peptide bonds, producing two shorter peptide chains (Figure 1.28).

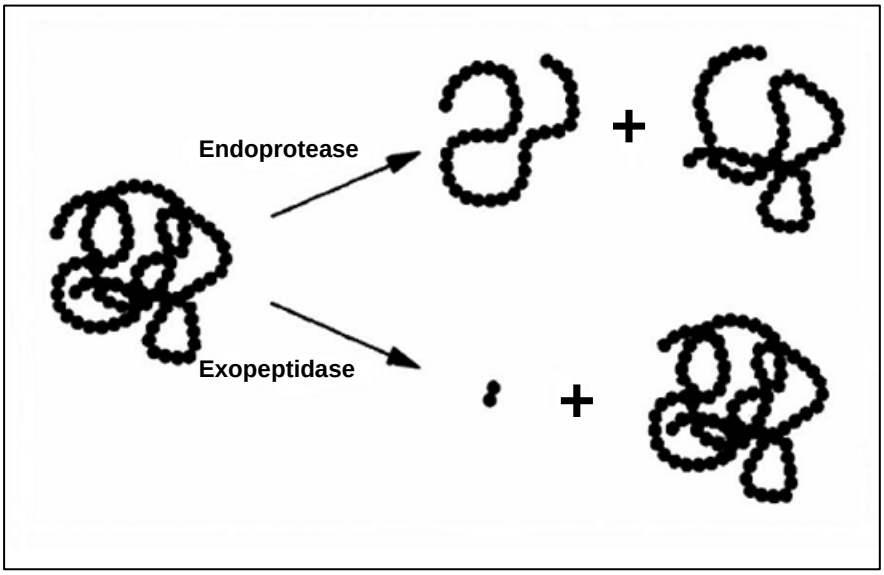

Figure 1.28 – Effects from endoproteases and exopeptidases on protein structure (from: An et al., 1996).

Exopeptidases can only cleave terminal peptide bonds (Kirschke and Barrett, 1987). The most active proteases in fish muscle differ from species to species. They are generally categorized into two major groups: cathepsins (Seymour et al., 1994; Toyohara et al., 1993) and heat-stable alkaline proteases (Makinodan et al., 1984; Wasson et al., 1992).

In many species, the origin of heat-stable proteases appears to be the gut tissues. Therefore, as previously mentioned, rapid evisceration and thorough cleaning of the fish before processing can prevent the occurrence of modori. Another solution can be the minimization of time held in the temperature zone of enzyme activation while processing the intermediate materials to final products (Lanier et al., 2005). The enzymes are inactivated by heating at 80 ºC or higher temperature. Rapid cooking, such as ohmic (Park et al., 1998) or microwave heating (Green and Babitt, 1990), eliminates the problem (Yongsawatdigul et al., 1995). Another proposed alternative is the addition of blood plasma protein and some other naturally derived proteins, which are able to inhibit the proteolytic activity (Hamann et al., 1990). Some authors (Taguchi et al., 1983) have also reported modori inhibition by alcohols, such as n-butyl-, n-amyl-, and n-hexyl-alcohols. Finally, alternative gelation technologies not relying in heating to induce gelation may prevent the phenomenon of modori (see 1.4.6). A summary of the main aspects of the modori phenomenon is shown in Table 1.10.

Table 1.10 – Main aspects of the modori phenomenon.

Raw material	Main aspects	Reference
Fish (in general)	-Minced fish/surimi subjected to 40/50 < T < 70 ºC for too much time can lose textural quality – *modori*	An et al., 1996
	-Moderate heating activates endogenous enzymes, namely serine and cysteine muscle proteases	An et al., 1996 & Ramos-Martínez et al., 1999
	-Other possible explanation of *modori* is an excessive formation of hydrophobic bonds leading to water loss and an asymmetric and heterogeneous network	Shimizu et al., 1983
Sardine	-Surimi gels lose textural quality with incubation at 50 < T < 60 ºC	Alvarez et al., 1999
Fish (many species)	-Rapid gutting and thorough cleansing of the fish before processing can prevent *modori*	Lanier et al., 2005
Fish (in general)	-Rapid cooking technologies (ohmic or microwave heating) may also prevent *modori*	Yongsawatdigul et al., 1995
Fish (in general)	-Addition of blood plasma protein and other proteins inhibits proteolytic activity and, as a result, *modori*	Hamann et al., 1990
Fish (in general)	-*Modori* inhibition by some alcohols (for instance, n-butyl-alcohol) is another possibility	Taguchi et al., 1983

1.4.5 - Heat-Induced Gelation

In order to impart more rigidity and mechanical resistance to the set (suwari) gels, thereby attaining definitive gels, they have to undergo a thermal treatment, known as cooking (Lanier et al., 2005) (Figure 1.26). This treatment ensures that proteins (mainly myofibrillar proteins) unfold and expose important chemical groups, which play a fundamental role on the gelation (see 1.4.2).

Thermal treatment also involves risks, particularly in the 50-70 ºC range (see 1.4.4), being very important to quickly reach a temperature of 80 ºC or higher. The rate of denaturation of the proteins at any time and temperature also differs between species, according to its protein stability. Protein denaturation and aggregation, induced by heating under the correct conditions, drives the gelation of unwashed minces or surimi. Denaturation of the proteins before the gel network is established will initiate interactions and the formation of bonds between proteins (premature protein aggregation) that will prevent adequate gelation later (Lanier et al., 2005).

Heating above 80 ºC generates a higher firmness level, as the gel evolves to its final characteristic structure. The rigidity of the very deformable network (previously formed during the setting) is increased as well as the aggregation phenomena go on (Roussel and Cheftel, 1990; Sano et al., 1990), until the formation of the definitive gel. Whereas the myosin long rod region has a dominant role in the cross-linking interactions occurring at low temperature, at higher temperature, the globular portion of the myosin heads is more important for the outcome (Taguchi et al., 1987; Sano et al., 1990).

It has been shown that the oxidation of sulfhydryl groups in solutions of carp myofibrillar proteins is enhanced by temperatures up to 80 ºC, which highlights the importance of disulphide bridges in the development of the final gel structure (Itoh et al., 1980). The myosin molecule presents more than 40 residues containing sulfhydryl groups, in their majority in the globular head region of the molecule (Lowey et al., 1969; Gazith et al., 1970).

Other studies have demonstrated that the large majority of the protein-protein interactions established above 50 ºC are associated to the globular myosin heads, through the formation of disulphide bridges (Taguchi et al., 1987) or hydrophobic interactions (Sano et al., 1990). According to some authors (Lanier et al., 1982), both types of interaction are simultaneously formed. A summary of the main aspects of heat-induced gelation is shown in Table 1.11.

Table 1.11 – Main aspects of heat-induced gelation.

Raw material	Main aspects	Reference
Fish (in general)	-Suwari gels must be cooked (heated > 70 ºC) in order to gain more rigidity and mechanical resistance -Rapid heating is very important (T> 80 ºC quickly attained) in order to minimize modori -Heating causes proteins to unfold, expose important chemical groups, and undergo cross-linking	Lanier et al., 2005
	-T> 80 ºC, globular portions of myosin play an important part in the gelation phenomenon	Taguchi et al., 1987 Sano et al., 1990
Carp	-Disulphide bridges important for the development of the final gel structure	Itoh et al., 1980
Fish (in general)	-Disulphide bridges and hydrophobic bonds between globular myosin heads are the most important protein interactions at higher temperatures	Taguchi et al., 1987 Sano et al., 1990 Lanier et al., 1982

1.4.6 - Alternative Gelation Technologies

The protein denaturation (modori) problems found in the production of heat-induced gels have promoted the research of alternative gelation technologies, such as high hydrostatic pressure (HHP) or ultraviolet irradiation (UVI). Although some alternative thermal treatments, such as ohmic heating (Park et al., 1998) (Figure 1.29), may minimize the permanence in the critical temperature range of 50-70 ºC, other technologies with no heat transfer (be it by conventional means, electric current or field or microwave irradiation) whatsoever, may offer different biochemical paths to gelation and to different and more interesting properties.

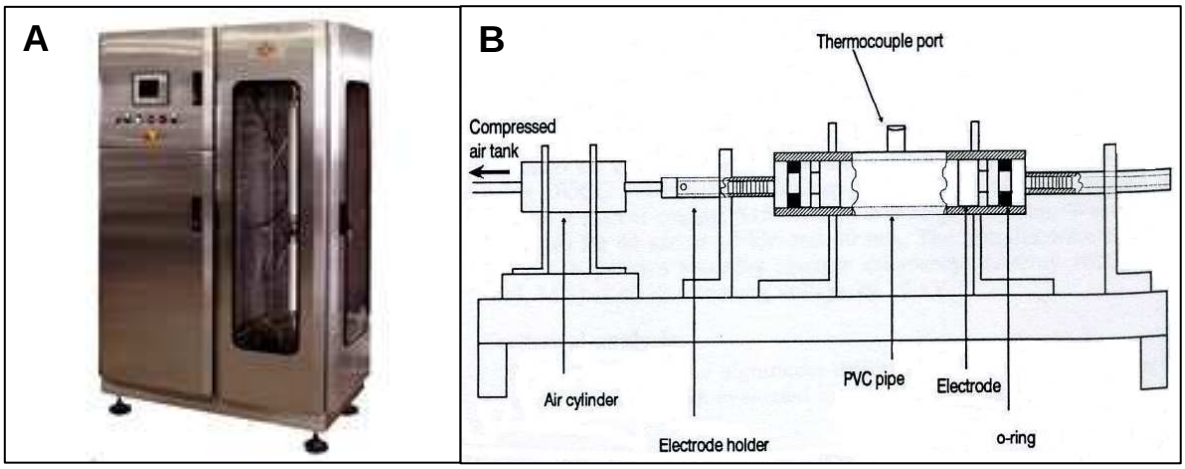

Figure 1.29 – Ohmic heating (A: example of an equipment and B: diagram presenting the operational principle) (from: Park et al., 1998).

Regarding these alternative gelation technologies, HHP has been applied to some fish products (Rastogi et al., 2007) and can be used to produce restructured gelled fish

products (Jiménez-Colmenero, 2002). HHP may achieve some degree of gelation at low temperatures, even below 10 ºC (Fernández-Martín et al., 1998). For pressure-induced gelation, a biochemical mechanism, different from that described above (see 1.4.5) for heat-induced gelation, has been proposed (Uresti et al., 2006). It seems that HHP application to fish mince at low temperature promotes a kind of protein aggregation characterized by side-to-side interactions of proteins with reduced denaturation instead of large conformational changes as with thermal gelation. Nevertheless, as in heat-induced gels, pressure-induced gelation has been ascribed (for instance, in HHP gels from Alaska pollock surimi) to increased cross-linkage of the myosin heavy chain (Shoji et al., 1990). The difference may lie in the different action mode of pressure processing, with maintenance of the original or slightly changed conformations over vast stretches of the structure of myosin and other myofibrillar proteins. Such gelation process involved in pressure-induced gel may explain the formation of different mechanical properties as reported by various investigators. This positive effect has been likened to the favorable protein aggregation induced at low temperature by microbial TGase (Uresti et al., 2006). Concerning UVI, its effect on protein structures is not well understood, there is evidence that it can promote polymerization of proteins (Ishizaki et al., 1994) and, thereby, change protein functionality. The main effects on fish protein gelation of these alternative technologies are shown in Table 1.12.

Table 1.12 – Main effects on fish protein gelation of alternative gelation technologies.

Technology	Effects	Reference
Ohmic heating	-Minimizes the permanence in the critical temperature range (50 <T< 70 ºC) and, as a result, the modori phenomenon	Park et al., 1998
HHP	-Can effectively induce gelation of fish proteins	Jiménez-Colmenero et al., 2002
HHP	-Some degree of gelation at low temperatures (for instance, T< 10 ºC)	Fernández-Martín et al., 1998
HHP	-May promote side-to-side protein interactions without large conformational changes as with thermal gelation	Uresti et al., 2006
HHP	-Gelation has been ascribed to cross-linking of the myosin heavy chain	Shoji et al., 1990
UV irradiation	-Can promote protein polymerization	Ishizaki et al., 1994

1.5 – Improvement and Transformation of Functional Properties

Although restructured fish products have been commercially successful, especially in the Far East, new challenges (convenience, health, etc.) are emerging in the food industry. These challenges are simultaneously opportunities for such processed products

as those previously described (see 1.3), because restructured products by their very nature can be more easily transformed into tailor-made products, able to please widely diverse market segments. For this change to succeed, a deep knowledge of the gelation phenomena and fish proteins (see 1.4) is an important tool. A knowledge that must always be taken into account in order to open new venues for traditional products or devise new strategies capable of bringing about new generations of restructured fish products. These strategies must improve and transform the functional properties of these food products.

Functional properties of food products can be considered accordingly to two main points of view, functionality as the whole set of sensory properties, namely texture, or under the point of view of health. This latter perspective presumes the incorporation of bioactive components into foods, that is, components that have a physiological effect on the human organism or, even, a health benefit. These incorporated components and the new foods thus produced are usually called nutraceuticals (Borderías et al., 2005). Under this perspective, the stress has been shifting into the identification of the potential of foods as promoters of physical and mental health. This notion of functionality is one of the main driving forces behind the development of new food products (Jiménez-Colmenero, 2004). In the last decades, several groups of functional compound have been identified (Goldberg, 1994): dietary fibres, amino acids, peptides, ω3-PUFA, vitamins and many more.

In this work, functionality will be always considered under both the health and sensory perspectives. For this last perspective, the three major functional properties of restructured fish products are colour, flavour, and texture (Park, 2005a). Controlling colour and flavour is relatively easy because of largely linear responses. Regarding texture, control is more difficult (Park, 2005a). The addition of ingredients and/or the application of alternative processing technologies affect the textural properties of the product mostly in a nonlinear fashion. Consequently, there is a wide range of textural properties (Figure 1.30).

A relationship has been defined between the physical parameters and sensory characteristics of texture (Lanier, 1986). When the human mouth is able to perceive the relatively high stiffness compared to the cohesiveness of the product, a “brittle” sensation is produced in the mouth. For brittle foods, the food structure strongly resists deformation, but upon subjection to sufficient force will collapse before appreciable deformation of the food has occurred. A low value of the stiffness/cohesiveness ratio points to a “rubbery” material. The overall magnitude of the two textural variables locates the textural description on a continuum, moving from a perception of “mushy” (neither stiff nor rubbery) up and rightward to one of “toughness” (both stiff and rubbery) (Lanier et al., 1985).

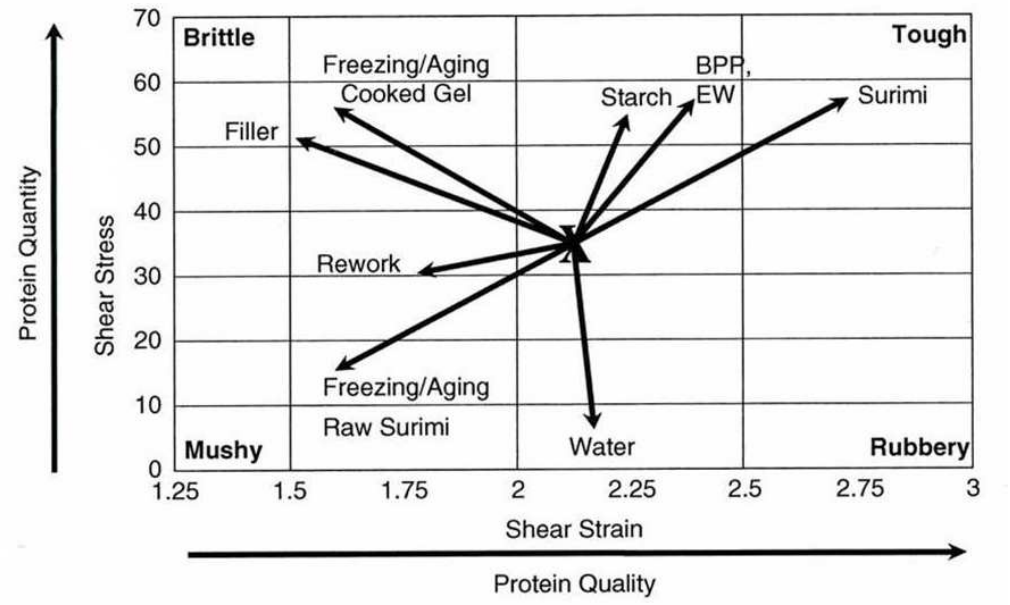

Figure 1.30 – Texture map demonstrating effects of ingredients and some processing operations on gel texture (from: Park, 2005a). BPP, beef plasma protein; EW, egg white; and Filler, comprises wheat gluten, soy protein isolate, and whey protein concentrate.

The stiffness may be associated to protein concentration in the food (regardless of its quality, for instance, its level of denaturation) and the cohesiveness to protein quality. For every restructured fish product, a relative ideal balance must be aimed at between textural stiffness and cohesiveness, in order to produce tailor-made products.

The incorporation of additives (see 1.6) and the application of alternative processing technologies (see 1.7) may offer the best solutions for the problem of reaching a right balance of textural parameters (Borderías et al., 2005; Park, 2005b) (Figure 1.31). The ideal is to achieve functionality in both senses, that is, improving sensory properties and imparting health benefits to the product. Some additives, such as dietary fibre (DF), may fulfil both objectives.

1.6 – Incorporation of Additives

The incorporation of additives, as the functional compounds previously mentioned (see 1.5), into restructured fish products is a main strategy of improvement and transformation of their functional properties.

One important point regarding additives is that their incorporation is not always advantageous. For an equal moisture level, whenever an additive is incorporated there is a protein dilution effect (the additive replaces the protein). Hence, whereas, if fish protein is of poor quality, replacing protein by an additive may have a beneficial effect, if protein presents good quality, additive substitution may have a negative net effect. That is, the advantages accrued by the additive are smaller than the costs associated to a lower

concentration of good quality protein in the product. It has been reported a negative effect on the textural quality of the product when the minced muscle protein is of high quality (Pérez-Mateos and Montero, 2000).

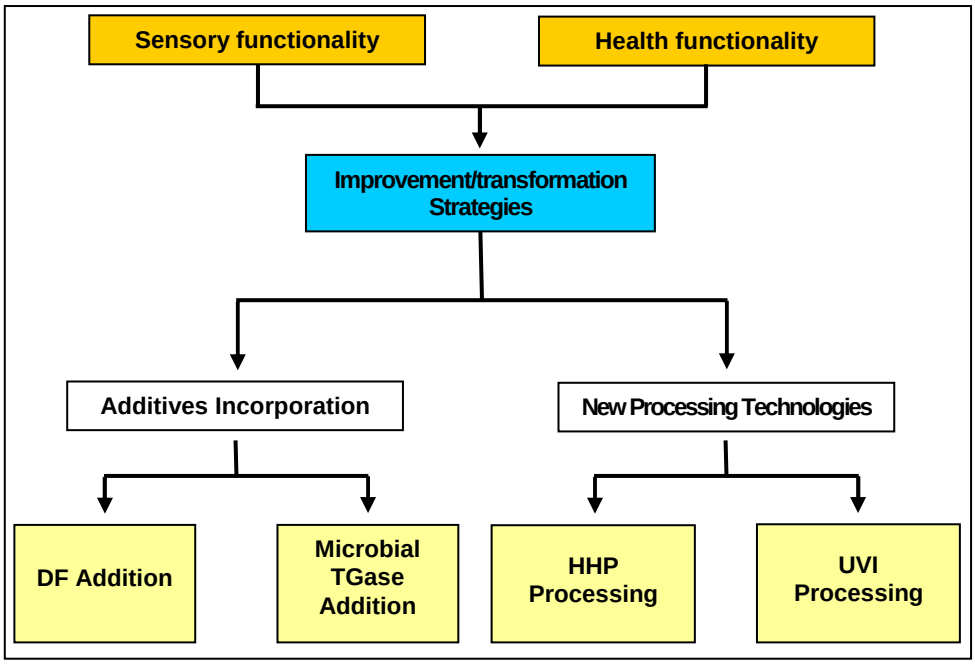

Figure 1.31 – Strategies for the improvement and modification of functional properties.

Additives are incorporated into unwashed fish mince for different reasons than into surimi. Japanese producers of surimi-based products use additives in high quality surimi in order to prepare tailor-made products, such as to adapt products to the western consumer taste (Gómez-Guillén, 1994). For the unwashed fish mince, the additives are incorporated with the purpose of improving gelation, as a means of making up for the lower gelling ability of unwashed fish mince with respect to surimi.

In this work, the incorporation of additives as a way of improving products, which otherwise would probably be of poor textural quality, is of great importance, since this work is about developing strategies to upgrade underutilized fish species and fish processing by-products. Among the various possible additives, dietary fibre (Borderías et al., 2005) and microbial transglutaminase (Téllez-Luís et al., 2002) were the chosen additives for the various experiments because they have been used in different areas of the food industry with the purpose of improving functional properties (Park, 2005a).

1.6.1 - Dietary Fibre

Dietary fibre (DF) is a very promising additive, since it combines sensory functionality with health benefits, being a full-fledged functional additive. Its application to fish products is a relatively recent development (Borderías et al., 2005). There is a substantially

untapped field of research, which may convey important findings and advances for the production of functional restructured products from fish, particularly, from underutilized fish species and fish processing by-products.

1.6.1.1 – Definition, Sources and Health Effects

DF, unlike other food components, is not attacked by the enzymes of the stomach and small intestine and, as such, it reaches the colon undegraded. DF has been defined as that fraction of the edible part of plants (from roots, seeds, fruits, etc.) or their extracts (and also synthetic analogues) that is resistant to the digestion and absorption in the small intestine, usually with partial or total fermentation in the large intestine (Prosky, 2001). The acronym DF includes polyssacharides, oligossacharides, lignin and other associated substances (Figure 1.32). Carrageenans and alginates from algae are also comprised in this definition.

More recently the definition of DF has been made more inclusive, comprising not only non-edible parts of vegetables, but also fibre of animal origin, such as chitosans, which are derived from the chitin contained in the exoskeletons of crustaceans and whose molecular structure is similar to that of plant cellulose (Borderías et al., 2005), and of microbial origin such as xanthan gum or curdlan. As previously emphasized, DFs possess two kinds of properties: technological functionality and physiological functionality. Properties differ widely between them, depending on the type of fibre.

The components of available DF include cellulose, hemicellulose, pectins, hydrocolloids (some gums are known as hydrocolloids because of the colloidal nature of these gums if dissolved or dispersed in water) (Park, 2005a), lignin and oligosaccharides such as the inulins.

With respect to water solubility these components are typically divided into two major categories: soluble (pectic substances, namely from fruits, inulins from chicory root or fruits and diverse hydrocolloids) and insoluble (cellulose, hemicellulose and lignin from grains and other vegetable material) (Figure 1.33). Much more detailed information regarding DF (especially that used in this work) can be found in the Appendix.

DF produces beneficial effects on bowel transit time (Feldheim and Wisker, 2000), affects glucose and lipid metabolism, reduces the risk of colourectal cancer (Faivre and Bonithon-Kopp, 1999), stimulates bacterial metabolic activity, detoxifies the colon luminal contents and helps maintain the equilibrium of the colon ecosystem and integrity of intestinal mucosa by acting as a prebiotic (Cherbut et al., 1995; FAO/WHO, 1997; Schweizer and Edwards, 1992).

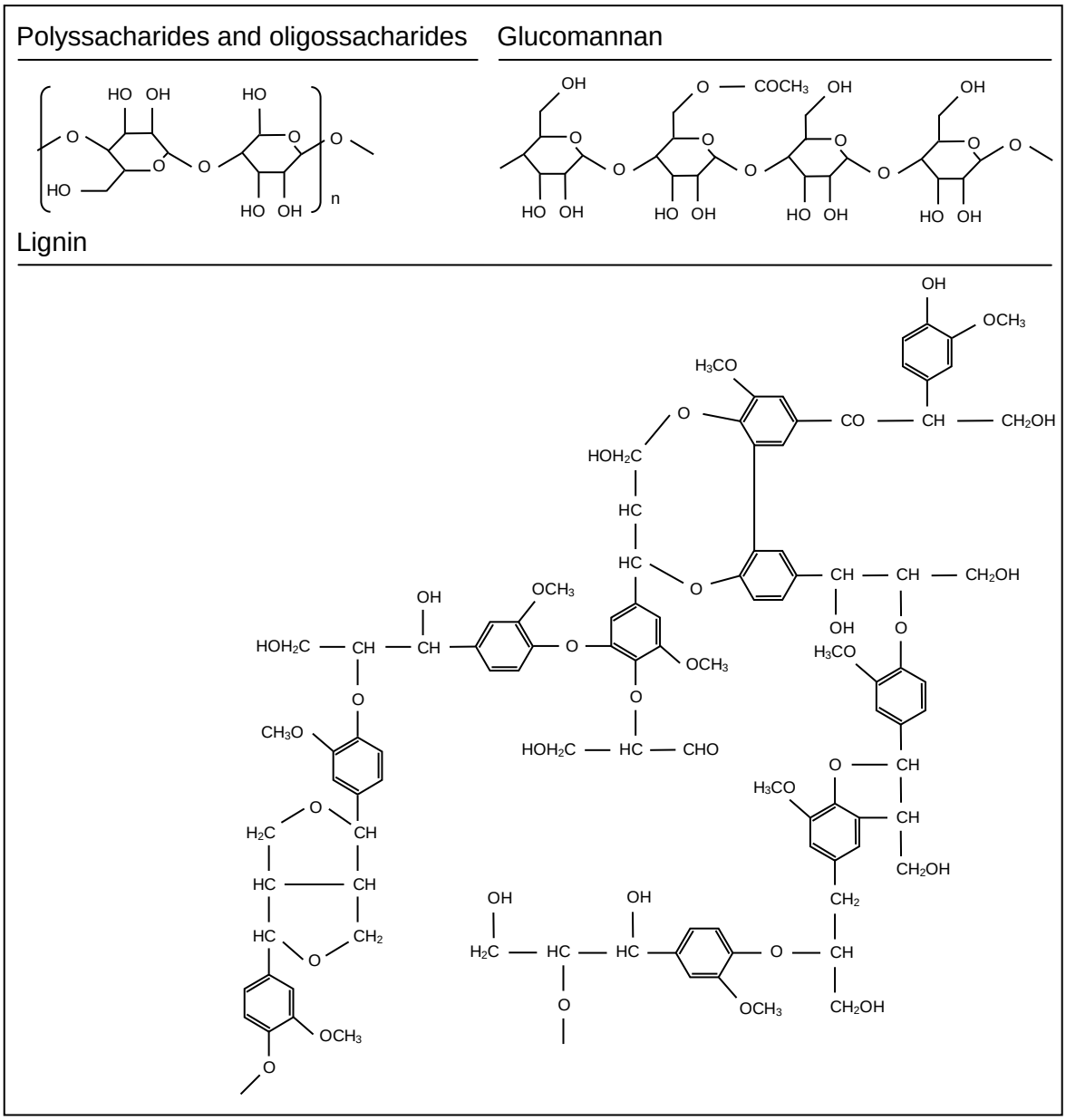

Figure 1.32 – Examples of food components most commonly defined as DF.

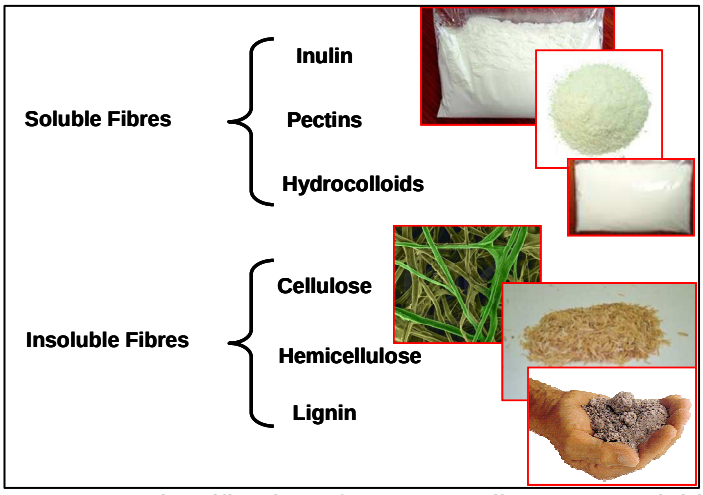

Figure 1.33 – Classification of DFs regarding water solubility.

According to the American Dietetic Association (Marlett et al., 2002), the current advisable fibre intake for adult individuals ranges from 25 to 30 g/day and the insoluble/soluble ratio should be 3:1. For Europe, a daily consumption of 20 g/person has been recommended (Borderías et al., 2005).

1.6.1.2 - Physicochemical Properties

The so-called technological functionality of DFs results from various possible positive effects upon the physicochemical properties of foods (Table 1.13). DFs may have beneficial effects upon the water holding capacity (WHC), fat binding capacity, viscosity, gel-forming ability and general textural features (Borderías et al., 2005). One of the most important properties from a technological standpoint is the ability to bind water. Soluble fibres, such as pectin and gums, possess a higher WHC than cellulosic fibres. However, when in the form of powder, cellulosic DF (such as that found in grain husks) binds several times its weight in water. This property has been related to the length and thickness of the fibre particle (Blenford, 1992). DF from algae, depending on their type, can bind up to 20 times their own volume in dry matter. Glucomannan from konjac tuber is extremely hygroscopic and also presents a very high water binding capacity (Fernández-Martín et al., 2009). Different factors may affect this property, such as the pH of the medium generally influences the WHC of a given DF. The capacity of a fibre to bind fat depends more on the porosity of the DF than on molecular affinity (Nelson, 2001). For this reason, in order to prevent fat uptake, it is advisable to hydrate the DF previously to its application, so that the water fills the pores and prevents the entry of fat. For instance, this is useful as a means of avoiding excessive absorption of frying fat when DF is used in batters. Moreover, viscosity, i.e., the formation of highly viscous solutions, is another property of some DFs, particularly, pectins, gums and polysacharides extracted from algae (Borderías et al., 2005). Plant-derived gums are generally the substances most widely used as thickeners. But, the viscosity of insoluble and some soluble DFs, such as inulins, is extremely low (Gallaher and Schneeman, 2003). The gel-forming ability of DF is another useful property with many possible applications. Many soluble fibres form gels, for instance, ι- and k-carrageenan (from algae), pectins, and glucomannan of konjac. The capacity to form a gel and the characteristics of the gel will depend on a number of factors, including concentration, temperature, presence of specific ions and pH. There is also the possibility of synergistic gel-forming effects (Pérez-Mateos et al., 2001). Finally, the utilization of DF can assist the restructuring of products based on fish and meat muscle (Nelson, 2001). In most meat products and some fishery products, the use of these DFs can help in achieving the right texture, namely, aiding in the restructuring of previously ground muscle (see 1.6.1.3).

Table 1.13 – Effects of DFs on the physicochemical properties of foods.

DF	Effects	Reference
Soluble (for instance, pectins)	-Enhancement of WHC due to the ability of these DFs to bind water	Borderías et al., 2005
Cellulose powder	-Can bind several times its weight in water, thus also enhancing WHC	Blenford, 1992
All	-Can bind fat, which may be prevented by previous hydration of the DF	Borderías et al., 2005
Pectins, gums & algae DFs	-Form highly viscous solutions, thus acting as thickeners	Borderías et al., 2005
Pectins & carrageenans	-Form gels, thereby raising the possibility of synergistic gel-forming effects with food proteins (such as myofibrillar fish proteins)	Pérez-Mateos et al., 2001
All	-Can help to change the texture of foods in different directions (firmness, elasticity, etc.)	Nelson, 2001

1.6.1.3 - Applications

The several physicochemical properties presented by DFs have led to their application in meat and fish products. This application has produced different effects and has contributed for the development of various functional restructured fish products (Nelson, 2001). Fish as such could be considered a functional food in that it is an important source of nutraceutical components such as ω3-PUFA. Nevertheless, fish would be even more complete as a food if it contained DF. The importance of the addition of DF to restructured fish products is reinforced by the fact that Western diet shows a clear deficit of DF (Borderías et al., 2005).

Many of the DFs used in fish products are soluble and are obtained from algae or seeds, selected for their functional characteristics, such as high WHC, emulsifying capacity, thickening or gel-forming ability. Concerning WHC, hydrocolloids have been incorporated in gels prepared from hake (*Merluccius australis*) sawdust to develop analogue products with high WHC (Borderías et al., 1996). The role of DF is so much more important where the used raw material is of poor quality. Carrageenans are also considered powerful enhancers of the water binding properties. Some studies (DaPonte et al., 1985) have shown this and that k-carrageenan has higher WHC than ι-carrageenan and prevented syneresis in fish gels during freezing/thawing. On the other hand, the modification of the emulsifying capacity by the addition of specific DFs is important for the sausage and fish processing industries (Borderías et al., 2005). The incorporation of DF to certain restructured fish products can help to improve viscosity, a step that may be necessary for the performance of some processes or the achievement of particular textures. Concerning the enhancement of the gelling ability of fish proteins, the main used additives have been carrageenans (Borderías et al., 1996; Gómez-Guillén et al., 1996). Other DFs have been proposed, such as garrofin, guar and xanthan gums (Montero et al.,

2000; Pérez-Mateos et al., 2001). As well as these hydrocolloids, glucomannan of konjac has been tested in surimi-based products, fish burgers and a “cooked ham” of fish (Figure 1.34).

Figure 1.34 – Example of a product (fish “cooked ham”) incorporating glucomannan with the purpose of improving its gel ability (pictures from: IPIMAR).

This DF has not been used in many fish products. The main work (Park, 1996) with this DF involved the production of temperature-tolerant gels from Alaska pollock and Pacific whiting (*Merluccius productus*). This author found out that glucomannan induces thermostable gelling in a mildly alkaline medium, so that this DF not only binds water but imparts elasticity to the products. In fact, DFs may produce structural changes in the protein matrix. There is much experimental work on the interaction between protein and DFs in aqueous systems, but there are not so many papers addressing this interaction in fish muscle (Borderías et al., 1996, Gómez-Guillén and Montero, 1996; Montero et al., 2000; Pérez-Mateos et al., 2001). In microscopy studies conducted on blue whiting muscle gels, it was found that different types of DFs behaved differently (Montero et al., 2000). Whereas thickeners (garrofin, guar and xanthan gum, or carboxymethylcellulose) formed a filamentous mesh, carragenates and alginates (which enhance gelation) coated or lined the cavities with a continuous structure. The addition of vegetable DFs with a high insoluble proportion has been the subject of much less studies (Ang and Miller, 1991). Cellulose poses some problems. The water entrapped by cellulose fibre is not as tightly bound as in biopolymers that strengthen surimi gels (Yoon and Lee, 1990). The lack of tightly bound water and mass expansion during heating may explain why this DF does not improve gel strength (Yoon and Lee, 1990). Nonetheless, the addition of 1 to 2 % (w/w) cellulose powder significantly improved the frozen storage quality of surimi gels by keeping them from becoming brittle. Recently, it has been reported (Sánchez-Alonso et al., 2007a) the successful incorporation of 3 % (w/w) wheat DF into hake (*Merluccius merluccius*) and horse mackerel restructured products. This DF, mainly composed by

cellulose and hemicellulose, increased the WHC and the final products were favourably rated by a sensory panel, only some loss of rigidity and cohesiveness was observed. Concerning the addition of fruit DFs to fish products, there are hardly any references in the literature (Borderías et al., 2005). These DFs would be very useful from both a technological and a nutritional standpoint. In this case, a recent paper (Sánchez-Alonso et al., 2008) has also brought some advancement to this research field: it was observed that the addition of white grape DF (>50 %, w/w of dry matter is insoluble DF) to a horse mackerel restructured product delayed lipid oxidation during frozen storage. This was due to high polyphenol levels in the DF product and shows the potential technological advantages that can be found with DFs from some fruits. Regarding chitosan, it has been found that addition of this DF can enhance rheological properties in surimi with poor gel forming capacity (Table 1.14), depending on the type and concentration of chitosan and on the system to which it is added (Benjakul et al., 2003). Its influence on the gelling of restructured fish products seems to result from its influence upon the enzymatic activity of endogenous TGase (Benjakul et al., 2003; Kataoka et al., 1998). But, no such effect was found on TGase of microbial origin (Benjakul et al., 2003), an important additive to restructured fish products (see 1.6.2).

Table 1.14 – Applications of DF to restructured seafood products.

DF	Effects	Reference
Hydrocolloids	-Incorporated into gels prepared from hake sawdust enhanced WHC	Borderías et al., 1996
ι-carrageenan	-Promising effects on the rheological properties of giant squid (*Dosidicus gigas*) gel products	Gómez-Guillén et al., 1996
Guar and xanthan gums	-Formed a filamentous mesh in blue whiting mince gels, thereby influencing texture as thickening agents	Montero et al., 2000
Carragenates & alginates	-Coated the cavities of the protein network with a continuous structure in the blue whiting gels, which may be related to active gel promoting action	Montero et al., 2000
Glucomannan of konjac	-Induced thermostable gelling of restructured Alaska pollock and Pacific whiting products, thereby binding water and imparting elasticity to the products	Park, 1996
from wheat (cellulose & hemicellulose)	-Successfully incorporated (up to 3 %, w/w) into restructured hake and horse mackerel products, with WHC improvement and only a small loss of textural quality	Sánchez-Alonso et al., 2007a
from white grape	-Delayed lipid oxidation during frozen storage of a restructured horse mackerel product	Sánchez-Alonso et al., 2008
Chitosan	-Improved rheological properties of surimi with poor gelling ability	Benjakul et al., 2003

From the wealth of examples given (Table 1.14), the addition of DF to the various types of restructured fish products is of great interest from a technological point of view (especially when the raw material is of poor quality, leading to final products with textural

flaws) as well as from a physiological point of view, since DF incorporation would complement the nutritional advantages of eating fish products.

1.6.2 - Microbial Transglutaminase

The important role of TGase in the gelation biochemistry was already addressed and explained (see 1.4.1 and 1.4.2). This endogenous fish enzyme has some drawbacks that limit its contribution for protein cross-linking and a favourable effect on gelation. First, it can be removed during surimi preparation if washing is too extensive, since it is water soluble (Nowsad et al., 1994). For the endogenous TGase to be active and induce setting (see 1.4.3), Ca^{2+} ions must be present at sufficient levels (Lee and Park, 1998; Saeki, 1996). Other drawback is the high variability of TGase activity, which depends on a combination of factors (Lanier et al., 2005). Different fish species may present different TGase contents, possibly as a result of habitat, feed, and other factors. For instance, there are some tropical species, such as tilapia (*Oreochromis niloticus*), which exhibit high TGase activity (Worratao and Yongsawatdigul, 2003). Also, it has been shown that in some species the water-soluble fraction of muscle also contains components that inhibit TGase activity (Wan et al., 1994).

Therefore, given these drawbacks, the utilization of a low-cost TGase from a microbial source (MTGase) as an additive may be advantageous, offering an important tool for the upgrading of the gelling ability of restructured fish products (Ando et al., 1989).

1.6.2.1 - Characterization

Two decades ago, a group of Japanese scientists isolated MTGase from the culture supernatant of a microorganism, *Streptoverticillium* sp. s-8112 (Ando et al., 1989; Nonaka et al., 1989), now termed *Streptoverticillium mobaraense* (Yan et al., 2005). The primary structure and some important features of MTGase were determined (Kanaji et al., 1993) and it was found that it consists of 331 amino acid residues with a molecular weight of 37,863 Da (Figure 1.35).

The sequence of the enzyme was found different from other TGases (Kanaji et al., 1993). The enzyme contains a sole Cys residue, which is essential for its catalytic activity. This importance was shown by treatment with N-ethylmaleimide, because this chemical substance dicarboxyethylated the sole Cys residue, thereby causing loss of the MTGase catalytic activity (Kanaji et al., 1993).

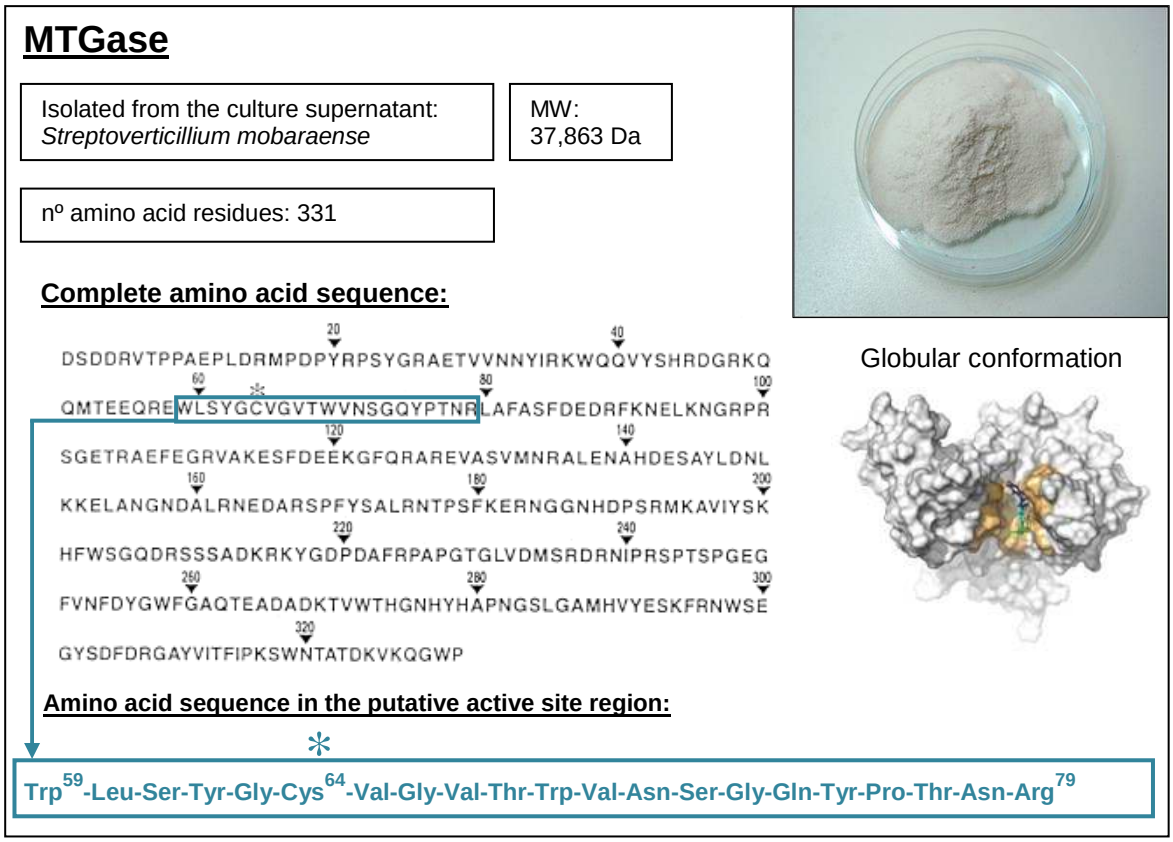

Figure 1.35 – Summary of the main MTGase characteristics. In the complete sequence all amino acids are denoted by the one letter codes; in the sequence of the active site all amino acids are denoted by the three letter codes;∗, indicates the cysteine residue essential for the catalytic activity (adapted from: Kanaji et al., 1993 and Motoki and Seguro, 1998).

MTGase is not calcium sensitive, meaning that neither chelating agents nor Ca^{2+} salts have any marked effect on its activity (Lanier et al., 2005). Moreover, being an additive incorporated in the surimi-based products after the preparation of surimi (involving various washing steps) is not removed by processing. In contrast to DF, it is a functional additive from a technological point of view only (improves gelation and various textural parameters), since no physiological or health effect is known.

1.6.2.2 - Applications

MTGase has been used in food processing (Table 1.15) for the production of a wide variety of surimi-derived products from different raw materials (Park, 2005a). Whereas some reports have indicated that the benefits from MTGase addition are mainly noticeable in products from lower-quality surimi, there is also a considerable increase in the strength of gels made from high-quality Alaska pollock surimi (Lanier et al., 2005). Thus, the range of applications of MTGase has widened to all restructured fish products. MTGase has gained acceptance in the food industry for promoting protein cross-linking (Téllez-Luis et al., 2002). In Japan, it is widely used to strengthen surimi gels and many other protein

foods (Asagami et al., 1995). Regarding restructured fish products from minced fish, it has effectively improved, in various circumstances, their textural properties (Téllez-Luis et al., 2002; Ramírez et al., 2000). Gel strength, hardness, and cohesiveness are increased by the addition of MTGase. Concerning WHC, the action of MTGase is more controversial, given the different effects reported by several studies. While some authors (Ramírez et al., 2007b) have found a favourable action of MTGase upon WHC or upon extracted water, others have reported no significant effect of MTGase upon the water-binding properties of other products (Pietrasik and Jarmoluk, 2003).

According to some studies (Lanier et al., 2005), only 5 U (one unit of MTGase activity is defined as the formation of 1 μmol of hydroxamic acid/min at 37 ºC (Shimba et al., 2002)) per g of surimi protein is effective in increasing the gel strength during setting. Hence, for products containing 10-20 g of protein per 100 g of wet weight and enzyme products with an activity of 100 U/g, incorporation levels of the enzyme product from 0.5 to 1.0 % (w/w) may be advisable. A level of 0.5 % (w/w) MTGase added to giant squid (*Dosidicus gigas*) surimi showed to be enough for achieving a good textural quality, including an improved cohesiveness (Moreno et al., 2009). However, lower MTGase concentrations have been tested successfully. For example, it was possible to improve the properties (gel strength, hardness, cohesiveness and chewiness) of low salt restructured products from minced striped mullet with 0.3 % (w/w) MTGase (Ramírez et al., 2007b). Higher MTGase concentrations may bring about textural disadvantages. Some authors (Lanier et al., 2005) have claimed that excessive cross-linking can produce a much less deformable gel. This may be a similar effect to that of the natural cross-linking of collagen molecules that occurs in bones and skin as animals age. The accumulating cross-links make the collagen steadily less deformable and more brittle (Figure 1.30).

The extent of the MTGase action may also depend on the substrate, namely, on the number of reactive Gln residues on the myosin molecules. It has been reported (Maruyama et al., 1995) that the myosin heavy chain (MHC) of carp (*Cyprinus carpio*) possessed less reactive Gln residues than rainbow trout (*Oncorhynchus mykiss*) and atka mackerel (*Pleurogrammus azonus*). As a result, only protein dimmers were formed from carp MHC, while MHC of rainbow trout and mackerel were able to form large polymers and at a faster rate.

Table 1.15 – Applications of MTGase to restructured seafood products.

Raw material	Effects	Reference
Alaska pollock	-MTGase addition strengthened gels from high-quality surimi	Lanier et al., 2005
Filleting wastes of silver carp	-MTGase incorporation (0.3 %, w/w) made texturally feasible the preparation of low-salt (1.0 %, w/w) products	Téllez-Luis et al., 2002
Striped mullet	-MTGase addition (0.9 %, w/w) enabled to achieve a higher surimi quality (shear stress of 156 kPa)	Ramírez et al., 2000
Giant squid	-MTGase addition (0.5 %, w/w) significantly improved cohesiveness of a cephalopod surimi	Moreno et al., 2009
Striped mullet	-MTGase addition (0.3 %, w/w) to a minced fish product balanced the negative effects (gel strength, hardness, cohesiveness and chewiness) of salt reduction	Ramírez et al., 2007b
In general	-Incorporation levels of MTGase (100 U/g) in the range 0.5-1.0 %, w/w, may be advantageous	Lanier et al., 2005

1.7 - Alternative Processing Technologies

Besides additives incorporation, another main strategy for the improvement and transformation of functional properties is the application of alternative processing technologies. Among these technologies, alternative gelation-inducing processes are of great importance for the preparation of restructured fish products. The potential of some of these technologies with respect to heat-induced gelation is enhanced by the fact that they require reduced amounts of or no heating in order to produce gelation phenomena. The importance of this fact results of the deleterious effects of heating, the higher activity of proteolytic enzymes that attack myofibrillar proteins, especially when the temperature is between 50 and 70 ºC (Uresti et al., 2006). As a consequence, an improved textural quality may be expected, especially if working with poor quality raw materials, and the nutritional value of the products' ingredients (mainly thermolabile components of fish) may be best preserved. Two main alternative gelation technologies will be discussed in this work, both limiting heating and, in what concerns fish products, both are new and still largely unexplored research fields.

1.7.1 - High Hydrostatic Pressure

High hydrostatic pressure (HHP) is a promising new food processing technology, applied currently to microbial inactivation and food preservation (Cheftel, 1995; Yuste et al., 2001). Other potential applications for this technology have been found, such as oyster schucking (He et al., 2002) and the production of convenient products with high quality and a fresh appearance similar to that of minimally processed food (Rastogi et al., 2007) (Table 1.16).

Table 1.16 – Examples of industrial applications of HHP to food products.

Product	Application	Reference
Jams, jellies, and sauces	-Packaging and processing without application of heat, but ensuring microbial safety & nutritional value	Thakur and Nelson, 1998
Vegetables (purees, etc.)	-Inactivation of microorganisms and enzymes, thereby extending their shelf life	Rastogi et al., 2007
Fruit juice (specially apple juice)	-Production of safe juices with higher nutritional content (for instance, vitamins) & lower enzymatic degradation	Hugas et al., 2002
Guacamole	-Inactivation of microorganisms and enzymes, with quality benefits	Palou et al., 2000
Oysters	-Non-thermal treatment of the fresh product for reduction of bacterial load, without changes in appearance, flavour, texture, & nutritional qualities	Hugas et al., 2002
Ham	-Production of safe and high-quality product	Hugas et al., 2002

HHP causes gelation of protein as well as polyssacharides, which may be used for the modification of functional properties of foods (Rastogi et al., 2007). For instance, the HHP treatment of carp resulted in the gelling of fish paste, which is useful for product development. The application of this technology as a strategy to upgrade underutilized fish species and fish processing by-products was tested in this work.

1.7.1.1 - Technology Description

HHP is a simple concept (Park, 2005b): the food product is sealed inside a plastic bag, inserted into a chamber, and subjected to pressure up to 700 MPa or even more (Figure 1.36). For comparison, a typical autoclave cooker is at 0.25 MPa and the deepest parts of the ocean have approximately 120 MPa of pressure (Morrissey et al., 1994). An HHP unit applies extremely high pressures, causing physical and chemical changes. This pressure affects cell membranes, microorganisms, and enzymes (Farr, 1990), all of which are of extreme importance in food processing. As a thermodynamic parameter, pressure has far-reaching effects on the conformation of macromolecules and affects a number of chemical reactions (Cheftel, 1992; Tauscher, 1995). Phenomena that are accompanied by a decrease in volume are enhanced by pressure, and vice-versa (principle of Le Chatelier). Thus, under pressure, reaction equilibriums are shifted towards the most compact state.

The non-availability of suitable equipment encumbered early applications of high pressure. However, recent progress in equipment design has ensured worldwide recognition of the potential for such a technology in food processing (Rastogi et al., 2007).

The key advantages of this technology can be summarized in the following points:

- HHP enables food processing at room temperature or even lower temperatures;

- HHP ensures instant transmittance of pressure throughout the system, irrespective of size and geometry (the problems of spatial variations observed in treatments associated with heat, microwave or radiation penetration are avoided);

- HHP causes microbial death whilst virtually eliminating heat damage and the use of chemical preservatives/additives;

- HHP can be used to create products with new functional properties.

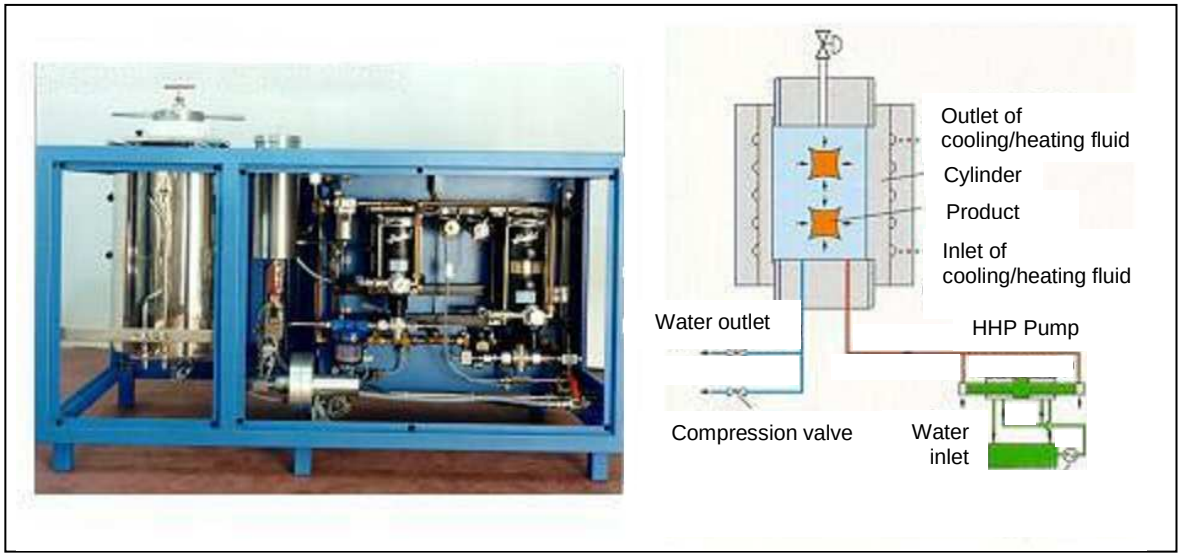

Figure 1.36 – Example of HHP equipment and diagram presenting the operational principle.

1.7.1.2 - Applications

Over the past two decades, this technology has attracted substantial research attention, mainly relating to three different areas of application. First, food quality preservation (Farkas and Hoover, 2001), particularly given its bactericidal effect. Second, alterations of the water phase transitions under extreme pressures, for instance, lowering of freezing point with increasing pressure (Knorr et al., 1998). Finally, changes of the physical and functional properties of food systems (Cheftel, 1992). Regarding this last area, a number of food materials, such as egg yolk, meat, and soy protein, have been shown to gel under HHP (Park, 2005b). Concerning fish products (Table 1.17), surimi readily gels at pressures as low as 200 MPa. For instance, gelation of pollock surimi by HHP was attributed to increased cross-linkage of the MHC (Shoji et al., 1990). Some authors (Chung et al., 1994) showed that gels from Pacific whiting and pollock surimi were texturally (significant increases in both strain and stress values) improved by HHP treatments except for those run at 50 ºC. It must be noticed that this temperature is near the optimum temperature (55 ºC) of some protease enzymes, for instance, in Pacific whiting (Morrissey et al., 1995). HHP may achieve some degree of gelation at low temperatures, even below 10 ºC, for instance, using blue whiting washed mince as raw

material (Fernández-Martín et al., 1998). Pressure-induced gels may present a textural quality similar or, even, regarding some properties, superior to heat-induced gels, as reported by various authors working with different seafood raw materials, such as giant squid surimi (Moreno et al., 2009), cod (*Gadus morhua*) or horse mackerel mince (Montero et al., 2005a). Pressure-induced fish gels have been characterized as softer, but more elastic than heat-induced gels. This positive effect has been likened to the favorable protein aggregation induced at low temperature by MTGase (Uresti et al., 2006). Information about the contribution of MTGase activity to pressure-induced gels is scarce. Nonetheless, some authors have claimed that the combination of MTGase and HHP (600 MPa, 5 min) has improved the texture of arrowtooth flounder gels (Uresti et al., 2006). But, these results were attained with cooked gels, since HHP treated samples were afterwards heated at 90 ºC for 15 min. A recent work (Moreno et al., 2009) has shown that MTGase presented a greater effect on low temperature (raw) pressure-induced (300 MPa, 30 min) gels than in cooked gels. It must be stressed that the effect of pressure is highly dependent on the HHP treatment conditions and the product matrix. The pressure level is of critical importance, with a greater pressure favouring a higher degree of restructuring, thus enabling the improvement of some texture properties, namely, hardness. But, it has been reported that pressures higher than 200-300 MPa could bring about a cooked appearance to squid gel products (Moreno et al., 2009), which can represent a disadvantage, if a fresh appearance is intended. Studies concerning the effects of HHP treatment in this lower pressure range would be useful.

Table 1.17 – Applications of HHP to restructured seafood products.

Raw material	Effects	Reference
Alaska pollock	-HHP treatment (<300 Mpa) produced surimi gels of acceptable quality	Shoji et al., 1990
Alaska pollock & Pacific whiting	-HHP treated surimi gels texturally (strain and stress values) improved (except when T = 50 ºC)	Chung et al., 1994
Blue whiting	-HHP treatment (200 MPa) ensured some degree of gelation of washed fish mince at T< 10 ºC	Fernández-Martín et al., 1998
Giant squid	-Pressure-induced gels (300 MPa) of surimi presented some textural advantages over heat-induced surimi gels	Moreno et al., 2009
Horse mackerel	-HHP treatment (300 MPa) of fish mince after a prior setting yielded gels with textural properties similar to those of heat-induced gels	Montero et al., 2005a
Arrowtooth flounder	-Combination of MTGase and HHP treatment (600 MPa) improved texture of minced fish gels	Uresti et al., 2006

1.7.2 - Ultraviolet Irradiation

Ultraviolet irradiation (UVI) is another alternative food processing technology. Regarding processed fish products, it has found application as a sterilization method applied to surimi with the purpose of reducing surface contamination and extending the products' shelf life (Su et al., 2005). Studies have shown that *Escherichia coli* contamination on fish product surfaces could be destroyed with a 5 s exposure to UVI, at an intensity of 40 mW/cm^2 (Hirose et al., 1982). As with HHP, another potential application for this technology may be the production of convenient products with a fresh appearance similar to that of minimally processed food, since heating is very limited. Alternatively, combination of UVI with a subsequent thermal treatment may achieve better results than those of heat-induced gels or similar results, but with less heating. According to some authors (Ishizaki et al., 1994; Taguchi et al., 1989), the gel strength of restructured fish products can be improved by UVI. Hence, the application of this technology as a strategy to upgrade underutilized fish species and fish processing by-products was also tested in this work.

1.7.2.1 - Technology Description

UVI is a simple and of straightforward application technology. The product required to be irradiated, must be even and thinly distributed on a surface and subjected to the photons irradiated by an UV lamp (Figure 1.37) (Jiang et al., 1998). The wavelength (254 and 365 nm are very common) and intensity of the UVI must be the adequate for the intended technological application. For instance, whereas sterilization may require 40 mW/cm^2 (Hirose et al., 1982), for the textural improvement of fish gelation lower values are proposed, such as 1.1 mW/cm^2 (Jiang et al., 1998). Another important issue is the thickness of the irradiated product, being advisable a depth of about 0.5 cm for the latter application. This is perfectly possible, when fish mince is to be the irradiated material. The whole set of sample and device must be put under refrigeration, preferably at a temperature not exceeding 10 ºC (Jiang et al., 1998).

Figure 1.37 – Example of UVI equipment (front view and opened chamber).

The possible action of UVI as a gelling promoting technology is related to the well known fact that UVI alters the structural conformation of proteins (Kato et al., 1992). It has been reported (Ishizaki et al., 1993; Taguchi et al., 1988) that UVI causes conformational changes in the myofibrillar proteins, thereby reinforcing subsequent thermal gelation.

1.7.2.2 - Applications

Besides sterilization (Su et al., 2005), UVI has been tested with respect to its gelling ability. It has been the object of some scattered works (Ishizaki et al., 1993; Ishizaki et al., 1994; Jiang et al., 1998; Taguchi et al., 1988) (Table 1.18).

Table 1.18 – Applications of UVI to restructured seafood products.

Raw material	Effects	Reference
Sardine	-UVI treatment (8.0 mW/cm^2 at 10 ºC) enhanced thermal gelation (elasticity & viscosity) of products prepared from washed fish mince	Ishizaki et al., 1993
Mackerel	-UVI treatment (1.1 mW/cm^2 at 10 ºC) increased the gel strength of gels produced from minced fish with added MTGase	Jiang et al., 1998

For instance, UVI was tested in washed fish mince from sardine (*Sardinops melanostictus*) (Ishizaki et al., 1993). Although the effect of UVI on protein structures is not fully understood, there is evidence that it can promote polymerization of proteins (Jiang et al., 1998) and, as a result, change protein functionality. It was demonstrated that UVI fragmented flying fish (*prognichthys agoo*) myosin and caused an increase in surface hydrophobicity (Ishizaki et al., 1994). Therefore, hydrophobic interactions (see 1.4.2) may also play an important role in the observed strengthening of surimi gels exposed to UVI. Other authors (Jiang et al., 1998), studying the interaction between UVI and MTGase, claimed that UVI (1.1 mW/cm^2 at 10 ºC) for 20 min of MTGase-supplemented minced mackerel increased gel strength by 25 %. Electrophoresis suggested that MTGase catalysis of the cross-linking (ε-amino-(γ-glutamyl) lysine bonds) of MHC was enhanced by UVI (Jiang et al., 1998). Hence, the application of UVI to various restructured fish products as well as the possible interaction between MTGase and UVI is a subject requiring further research.

2 - Objectives

In accordance with the Ph.D. programme and taking into account that many fish species and processing by-products remain underutilized —being additional research much needed for the development of marketable and high value added products from these raw materials—, four main objectives were defined for this study:

- development of new fish mince products through the incorporation of additives;
- use of new processing technologies as a way of improving the quality of fish products and/or of creating new processed products altogether;
- fish substitution in traditional meat products;
- storage stability of the products developed in the previous objectives.

Five fish species were selected: hake (*Merluccius capensis*), gilthead sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), chub (*Scomber japonicus*) and Atlantic mackerel (*Scomber scombrus*). They are underutilized fish species (particularly Atlantic mackerel) or largely wasted animal protein resource —for instance, the trimmings of frozen hake processing. Although chub mackerel is used as a raw material by the fish canning industry, leaner fish of this species are not used. A large portion of the captured mackerel is discarded by fishermen, since there is scarce demand for these fish in the market, making them low commercial value species (Cabral et al., 2003). It has been reported that this fishery targets bigger fish, thus, discarding marketable albeit smaller fish (ICES, 2006).

2.1 – New Fish Mince Products – Effect of Additives

This objective comprised the creation of innovative heat-induced gel products involving fish mince as raw material and using additives capable of improving textural quality and adding value to the products.

This objective encompassed various points:

- study of the effect of the incorporation of dietary fibre on the biochemical, nutritional, textural and sensory properties of the products;
- understanding the effect of microbial transglutaminase on the biochemical, nutritional, textural and sensory properties of the products and of the interaction between the enzyme and the dietary fibre;

- understanding of the effects of the additives at the molecular level, with special emphasis on protein bonding;
- evaluation of the advantages and drawbacks associated to different raw materials, namely, comparison between surimi and unwashed fish minces.

2.2 – New Processing Technologies

Under this theme, the effects of new processing technologies, such as HHP and UV, upon the upgrading of underutilized fish species and/or by-products of fish processing were investigated as well as the:

- the operational parameters associated to the application of these new processing technologies and their optimization;
- the effects of the processes at the molecular level, with special emphasis on protein bonding.

2.3 – Fish Substitution in Meat Products

The viability of substituting fish in traditional meat foods was studied. Taking sausages as a food model, a healthy low fat fish sausage containing dietary fibre was developed. The knowledge acquired with the results of the previous objectives was used for this purpose. The sensory properties of the fish sausages were evaluated and subjected to a process of optimization.

2.4 – Storage Stability Study

This objective encompassed a thorough study of the storage stability of minced fish and fish sausage products. This entailed the measurement over refrigerated storage time of various physical, chemical, sensory and microbiological parameters in order to know the variation of the products' quality and to establish the products' shelf life.

Moreover, two more points were addressed:

- assessment of the potential benefits and costs for the storage stability of the incorporation of $\omega 3$ fatty acids in the fish products;
- evaluation of the potential benefits and costs for the storage stability of the incorporation of dietary fibre in the fish products.

3 – Materials and Methods

3.1- Materials

3.1.1 - Utilized Fish Species

Five fish species were studied with the purpose of achieving a comprehensive understanding of the specific technical problems posed by each one. For hake (*Merluccius capensis*) and the farmed species, gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), the focus was on the utilization of by-products of the fish processing operations, namely, trimmings of the portioning and filleting operations (mainly muscle joined to the bones and skin). Concerning chub (*Scomber japonicus*) and Atlantic mackerel (*Scomber scombrus*), whole fish were used. These are two abundant fish species in European waters, being sometimes caught together (ICES, 2006) off the Iberian coast.

3.1.1.1 - Hake (*Merluccius capensis*)

Frozen South African hake (*Merluccius capensis*) were bought already headed and gutted from a local frozen fish processor. Each fish batch was kept frozen at –28 ºC and processed within three to four weeks after its arrival at the laboratory. For the production of sausages and other minced fish products, fillets were used, whereas for the HHP experiments trimmings of the cutting operations were utilized. The main reason for these choices was the commercial concept associated to the developed products (Frankfurt sausages or minced products with shellfish flavour), which presupposed the creation of value-added products, possibly targeting the more exquisite 'gourmet' market of consumers willing to trade higher prices for extra quality.

3.1.1.2 - Gilthead Sea Bream (*Sparus aurata*)

Farmed gilthead seabream (*Sparus aurata*) were bought in a local supermarket and kept frozen at –20 ºC until processing. Individual weight varied between 300 and 400 g. Fish were processed (headed, tailed, gutted and filleted) at low temperature (< 10 ºC) within one week after purchase. The trimmings (mainly muscle joined to the bones and skin) of the cutting operations were collected and used as raw material.

3.1.1.3 - Sea Bass (*Dicentrarchus labrax*)

Farmed fish, sea bass (*Dicentrarchus labrax*), were bought in a local supermarket and processed (headed, tailed, gutted and filleted) at low temperature (< 10 ºC). The trimmings of the cutting operations (mainly muscle joined to the bones and skin) were collected and used as raw material. Individual fish weight varied between 300 and 400 g.

3.1.1.4 - Chub (*Scomber japonicus*) & Atlantic Mackerel (*Scomber scombrus*)

Chub (*Scomber japonicus*) and Atlantic Mackerel (*Scomber scombrus*) were fished off the Portuguese coast, frozen on board and sent to L-IPIMAR. Fish were stored at –28 ºC and processed within two months after its arrival at the laboratory. Chub mackerel weight varied between 180 and 220 g, whereas Atlantic mackerel were smaller, weighing 70 to 200 g.

3.1.2 - Additives, Ingredients, and Chemical Reagents

Several additives, ingredients and chemical reagents were used. The distinction between these categories is given by their purposes, nature and function. Additives and ingredients are substances generally regarded as safe and, as such, usable in the food industry. Chemical reagents are substances used for the analyses of the food products and not in their preparation. Between additives and ingredients, the distinction is more blurred and lies mainly in their purpose. Sometimes, the term ingredient is applied in a broad sense and also encompasses the additives. Whereas ingredients are necessary and typical constituents of a food product (for instance, smoke aroma in Frankfurt sausages), additives are not. The latter are incorporated in food in order to enhance one or more characteristics of the product.

3.1.2.1 - Additives

In this study, seven dietary fibres (DF), two food grade oils —vegetable and fish—, and microbial transglutaminase (MTGase) were used:

- inner pea fibre - Swelite® (Swe);
- outer pea fibre - Exafine® 250;
- chicory root inulin - Fibruline® INSTANT (Fib);
- chicory root oligofructose - Fibrulose® 97;
- apple fibre - Vitacel® AF 401;
- carrageenan - CEAMGEL® 1830 (Carr);
- konjac flour - Nutricol® GP 312 (Kjc).

The first four DF were provided by Cosucra (Warcoing, Belgium), Vitacel® AF 401 was supplied by Rettenmaier (Holzmühle, Germany), CEAMGEL® 1830 was provided by Ceamsa (Porriño, Spain) and Nutricol® GP 312 by FMC Biopolymer (Philadelphia, USA).

The particular composition (dry matter) and other properties are presented in Table 3.1.

Table 3.1 – Properties of the dietary fibre products*.

PROPERTIES	Swelite®	Exafine® 250	Fibrulose® 97	Fibruline® INSTANT
Composition (D.M.)				
Total Carbohydrates (%)	93 ± 3	min. 85	min. 99.7	min. 99.7
Total DF (%)	48 ± 3	min. 85	97 ± 2	min. 90
Starch (%)	min. 36	max. 5	—	—
Protein (%)	max. 7	max. 6.5	—	—
Fat (%)	max. 0.5	max. 0.5	—	—
Ash (%)	max. 2	max. 3	max. 0.3	max. 0.3
Granulometry (µm)	< 400	85 %< 300	< 700	< 700
Colour	white	cream	white	white
Taste	neutral	slight vegetable	neutral to slightly sweet	neutral to slightly sweet

PROPERTIES	Vitacel® AF 401	CEAMGEL® 1830	Nutricol® GP 312
Composition (D.M.)			
Total Carbohydrates (%)	—†	—†	—†
Total DF (%)	55 ± 5	>90 %	>90 %
Starch (%)	—	—	—
Protein (%)	4.6	—†	—†
Fat (%)	2.5	—†	—†
Ash (%)	max. 3	—†	—†
Granulometry (µm)	90 %< 300	< 250	< 250
Colour	red brown	pale yellow	tan
Taste	fruity	neutral	neutral

* Values are manufacturer's claims (Anonymous, 2004a; Anonymous, 2004b; Anonymous, 2006; Nutricol® GP 312 Product Sheet, 2008).
† Not indicated by manufacturer.

These DF products also differ with respect to the type of fibre. The DF components of Swelite and Exafine were mixtures of insoluble cellulose and soluble pectic material, 2/3 and 1/3 for Swelite and for Exafine, respectively. The main DF of Fibruline and Fibrulose were inulins and oligofructoses, respectively. The DF component of Vitacel was mainly pectic material. The DF of the CEAMGEL product was a mixture of iota and kappa carrageenans (each approximately 50 %, w/w) from red seaweeds and, for Nutricol, glucomannan from the konjac plant was the main DF component.

Regarding the used oils, vegetable oil (VO), Fula® (Sovena, S.A., Oeiras, Portugal), was a mixture of soya, sunflower, peanut and corn oils, while fish oil (Winterisation, Fécamp, France) was a deodourized cod liver oil (CLO) without added antioxidant.

Microbial transglutaminase (MTGase) ACTIVA® (WM/GS) was supplied by Ajinomoto Japan, Inc. (Tokyo, Japan) as a mixture containing 99 % maltodextrine and 1 % transglutaminase. The transglutaminase activity was determined and expressed in units.g^{-1} (Folk, 1970).

3.1.2.2 - Ingredients

The main ingredients, besides fish, were different for each product. There were two main developed products: a Frankfurter sausage and a minced fish product containing DF.

For the various Frankfurt sausages, water, ice, pork meat and fat (bought at a local supermarket), potato starch from Emsland-Stärke GmbH (Emlichheim, Germany), TARIPROT® 1010, emulsifying soy protein from BK Giulini (Ladenburg, Germany), SOLCON/MAICON 70, soy protein concentrate powder from Solbar Hatzor Ltd. (Ashdod, Israel), Dextropam 100, dextrose from Copam, S.A. (Loures, Portugal), TARI® K7, Di-, Tri- and Polyphosphates from BK Giulini (Ladenburg, Germany), VATEL® Salt, common salt from VATEL (Alverca, Portugal), Palatinata Cure, curing salt from BK Giulini (Ladenburg, Germany), TARIMIX® Frankfurt, sausage seasoning from BK Giulini (Ladenburg, Germany), and TAROMA® Smoke, smoke aroma from BK Giulini (Ladenburg, Germany) were mixed.

For the minced fish products, water, food grade salt from VATEL (Alverca, Portugal), shellfish flavour from Givaudan Schweiz AG (Kemptthal, Switzerland), sodium polyphosphate from BK Giulini (Ladenburg, Germany), sodium caseinate EM6® from DMV International BV (Veghel, The Netherlands), and monosodium glutamate from Ajinomoto Japan, Inc. (Tokyo, Japan) were used.

As cryoprotectants, sorbitol, sucrose, and sodium polyphosphate from Merck KGaA (Darmstadt, Germany) were applied.

3.1.2.3 - Chemical Reagents

Chemicals for the analytical determinations (acetone, bromophenol blue, calcium hydroxide, chloroform, dissodium hydrogen phosphate, ethylenediamine tetraacetic acid, formaldehyde, glutaraldehyde, glycerol, methanol, peptone, phosphoric acid, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, sodium sulphate anhydrous, tetrassodium pyrophosphate, trichloroacetic acid, tris(hydroxymethyl)-aminomethane, urea) as well for the production of the so-called informational products (sodium dodecylsulphate, SDS, dithiothreitol, DTT, and N-ethylmaleimide, NEM) were of analytical grade and from Merck KGaA (Darmstadt, Germany).

3.1.2.4 – Microbiological Media

Whereas Plate Count Agar, Man Rogosa Sharp Agar, and Rose Bengal Chloramphenicol Agar were from Oxoid Ltd. (Hampshire, UK), Violet Red Bile Agar and Sulphadiazine Polymyxin Sulphite Agar were supplied by Merck KGaA (Darmstadt, Germany).

3.2 - Methods

The methods used in the experimental works comprise four main groups: preparation of the raw material, preparation and storage of the products, the analytical (physical, biochemical, microbiological and sensory) evaluation, and the statistical treatment of results.

3.2.1 - Preparation of the Raw Material

The raw material was prepared for the various experiments in one of two ways, either fish (fillets or trimmings) was only minced and, immediately, used in the trials or it underwent mincing, washing and other operations (the surimi preparation process), being frozen and frozen stored till the preparation of the products.

3.2.1.1 – Production of Fish Mince

Fish fillets or trimmings were mechanically processed in two different equipments, depending on the particular requirements of each study: a Baader 694 deboning machine (Baader, Lübeck, Germany) fitted with a drum with 3 mm diameter circular holes, medium pressure (Figure 1.6) or a model 84145 meat grinder (Hobart, Troy, OH, USA), equipped with 2 cm grind blades and a metallic screen with 6 mm diameter circular holes.

3.2.1.2 – Production of Surimi

Frozen fish were thawed overnight in a refrigerator. Then, fish were headed, gutted, washed and skin and bones were mechanically removed in a Baader 694 deboning machine (Baader, Lübeck, Germany) fitted with a drum with 3 mm diameter circular holes (temperature of the fish and of the resulting mince never exceeded 5 ºC). Afterwards, minced fish was washed with a solution (0-3 ºC) of sodium bicarbonate (5 g/l) and tetrassodium pyrophosphate (2.5 g/l) during 20 min, using the ratio 1 mince part:3 solution parts. Then, pH was adjusted to 6.5 with phosphoric acid. After, mince was dewatered in a New-Tech surimi pilot plant (Bibun, Fukuyama, Japan), a second washing with an identical ratio was done with a solution (0-3 ºC) of sodium chloride (3 g/l) during 10 min, ensuring a good dehydration of the mince. After dewatering the mince again, produced surimi was mixed with cryoprotectants —sorbitol (4.0 %, w/w), sucrose (4.0 %, w/w) and

sodium polyphosphate (0.5 %, w/w)— in a model UM12 refrigerated vacuum homogeniser (Stephan, Hameln, Germany). Mixing was performed for 1 min. at 2800 r.p.m. under vacuum and refrigeration (< 5 ºC). Then, surimi was weighed and vacuum-packed in plastic bags with a model A300/52 vacuum packager (Multivac, Wolfertschwenden, Germany). Finally, surimi was frozen down to -28 ºC in a model 2581 forced air freezer (Köttermann, Hänigsen, Germany) and stored at the same temperature for 1 week.

3.2.2 - Preparation and Storage of the Products

3.2.2.1 – Production of Heat-induced Gel Product

The appropriate quantities of mince/surimi, chilled water, salt (2.5 %, w/w) and other ingredients/additives, depending on each study for approximately 1.3-1.5 kg batches were weighed (Figure 3.1). The fish mince or surimi were mixed with the other ingredients/additives for 2 min at 1420 rpm and 2 (or 3) min at 2800 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Throughout all process, mixing was performed always under vacuum and temperature below 7 ºC. The batters attained were put inside a model EB-12 hydraulic filler (Mainca Equipamientos Carnicos, S.L., Granollers, Spain) and 'sausages' produced with cellulose casings. 'Sausages' were vacuum-packed in low-oxygen permeable barrier bags (O_2 transmission, <2.1 $cm^3/(m^2.day.bar)$ at 23 ºC, Colamin XX 100e, Obermühle Polymertechnik GmbH, Pössneck, Germany) with a model A300/52 vacuum packager (Multivac Sepp Haggenmüller GmbH, Wolfertschwenden, Germany). Then, these packages were immersed in water at 35 ºC for one hour (setting) and moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and steam cooked at 90 ºC for one hour (cooking). In order to guarantee these conditions even in the innermost part of the product, the oven's digital thermometer was placed in the centre of one 'sausage' through a hole in the bag. Finally, gel products were immediately cooled in iced water and stored overnight in a refrigerated room (at 5 ± 1 ºC with a relative humidity of 90-95 %) in the dark until further analysis.

3.2.2.2 – Production of Pressure-induced Gel Products

The pressure-induced gel products were prepared only from hake mince (Figure 3.2). For each experimental set, approximately 2.5 kg of mince from hake trimmings was used. The appropriate quantities of mince, salt (2.5 %, w/w), MTGase (0.0 or 0.5 %, w/w) and water (in order to guarantee final gel products with the same moisture level, 80 %) were weighed for approximately 1.5 kg batches.

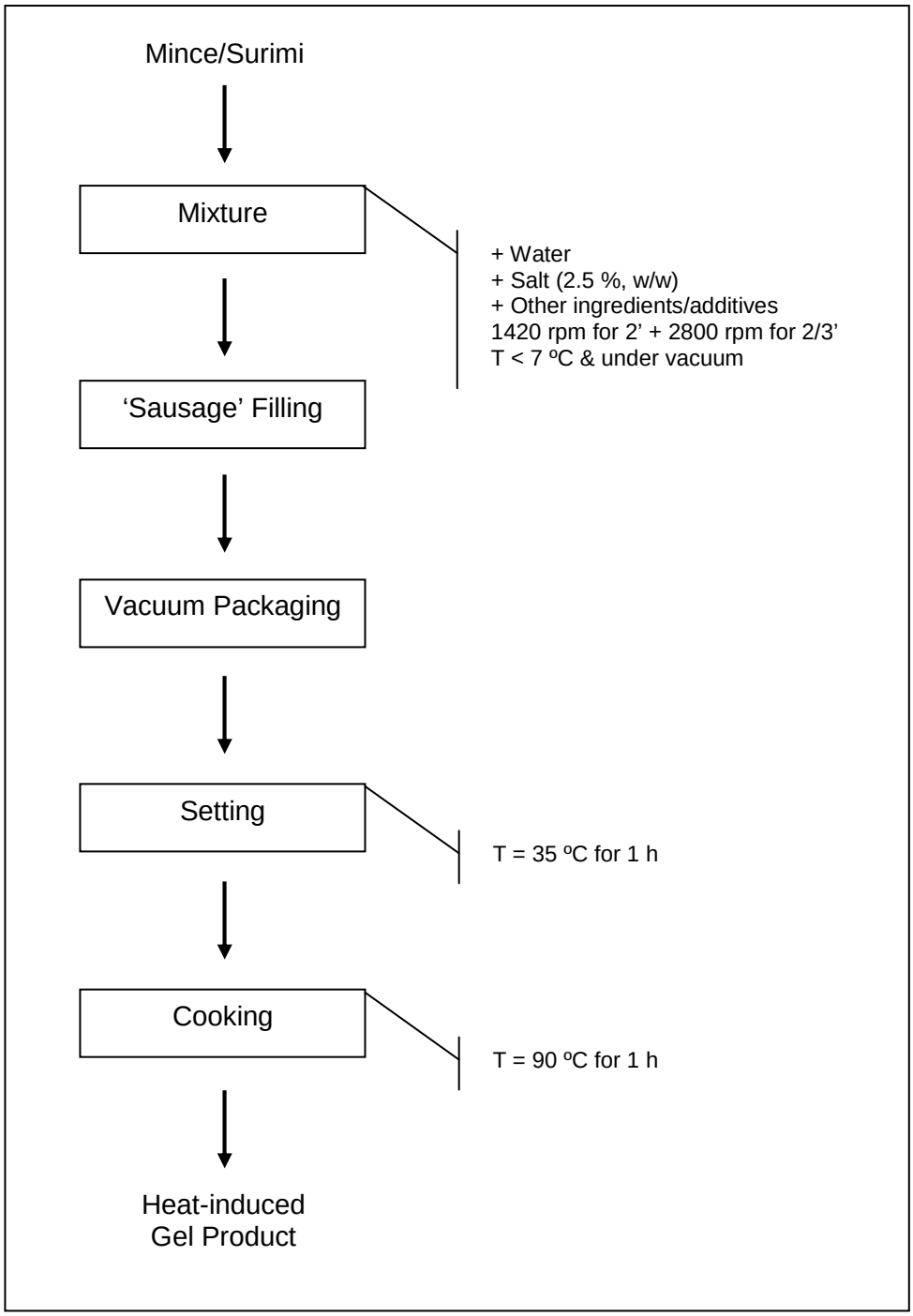

Figure 3.1 – Scheme of the production of heat-induced gel products.

Mince and ingredients were mixed for 2 min at 1420 rpm and 3 min at 2800 rpm in a refrigerated vacuum chopper (model UM12, Stephan & Söhne, Germany). During sample processing, mixing was performed always under vacuum at temperature below 7 ºC. The resulting pastes were stuffed into cellulose casings (25 mm diameter and about 85 mm length) using a hydraulic filler (model EB-12, Mainca, Spain). Each link was vacuum-packed in a low-oxygen permeable barrier bag (Colamin XX 100e, Germany) with a vacuum packager (model A300/52, Multivac, Germany). Then, these packages were left

at low temperature (5 ± 1 ºC) overnight. The next day, they were immersed in water at 35 ºC (temperature ensuring best possible gelation according to previous experiments, data not shown) for one half hour (setting) and subjected to high pressure.

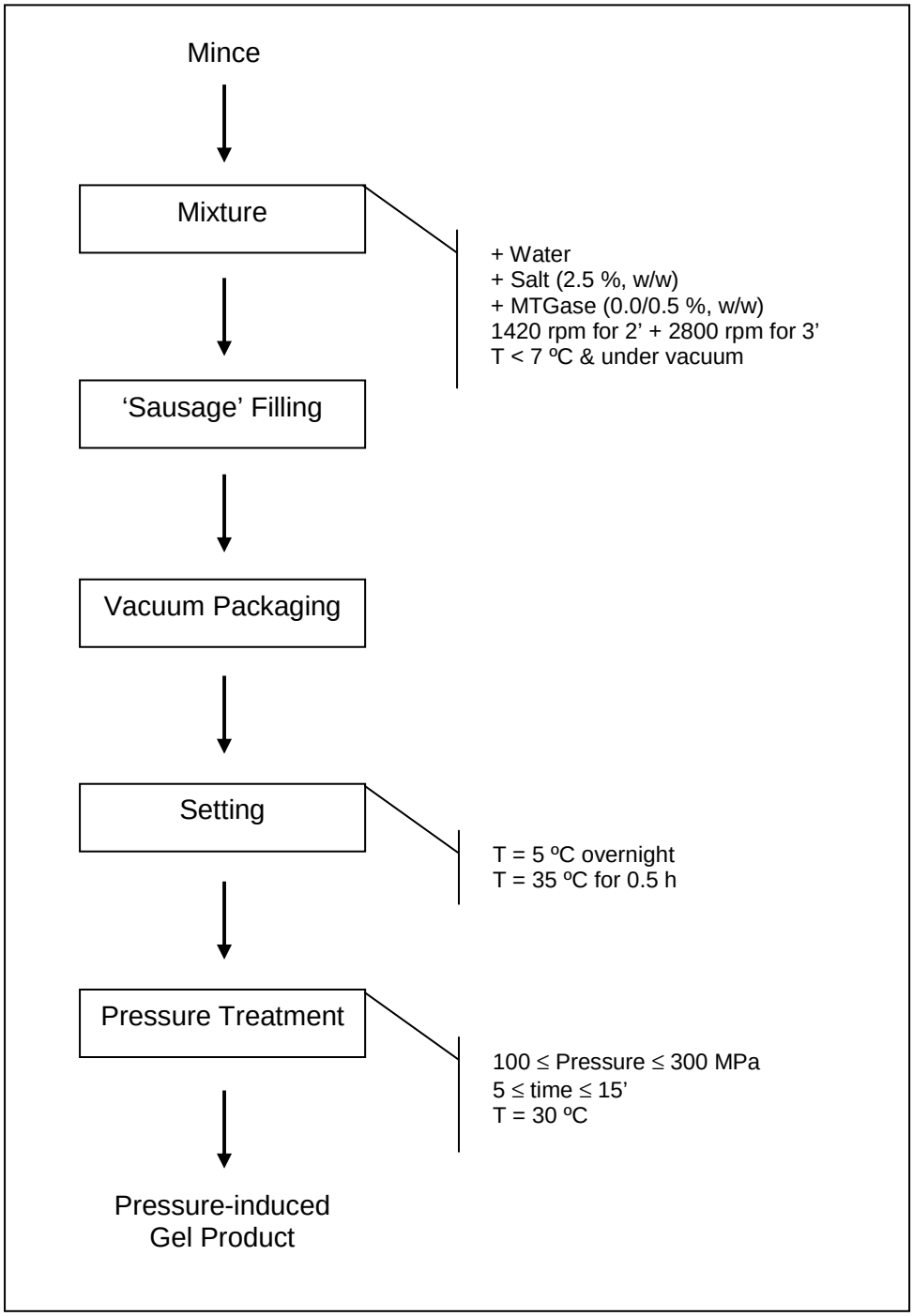

Figure 3.2 – Scheme of the production of pressure-induced gel products.

Pressure treatment was carried out using a hydrostatic press (model U33, Unipress Equipment, Poland). This equipment has a pressure vessel of 35 mm diameter and 100 mm height, surrounded by an external jacket, connected to a thermostatic bath to control the temperature (30 ºC), using a mixture of propylene glycol and water (1:1) as

pressurizing fluid. Pressure increase was between 240 (for 100 MPa) and 360 MPa/min (for 300 MPa). In average of all pressure treatments, temperature (equipment reading) increased 1-2 ºC with pressurization and decreased 1.5 ºC with depressurization, due to adiabatic heating/cooling. After treatment, samples were left at low temperature (5 ± 1 ºC) in the dark overnight until analysis.

3.2.2.3 – Production of UV Irradiation-induced Gel Products

The UV irradiation-induced gel products were prepared only from gilthead sea bream mince (Figure 3.3). Approximately 6 kg of gilthead sea bream mince from trimmings were used. The appropriate quantities of mince, Kjc (0.0 or 1.0 %, w/w), MTGase (0.0 or 0.5 %, w/w), salt (2.5 %, w/w), $Ca(OH)_2$ (0.15 %, w/w) and water (in order to guarantee final gel products with the same moisture level, 80 %) for approximately 1.4 kg batches were weighed. In order to hydrate Kjc before mixing, chilled water was added to Kjc in a ratio near 10:1 and mixed with Kjc for 1 min. Afterwards, the seabream mince were mixed with the other ingredients for 2 min at 1420 rpm and 2.5 min at 2800 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). In order to promote deacetylation of the konjac flour paste, a necessary step for gel formation to occur, $Ca(OH)_2$ solubilised in water was added to the batter and mixing proceeded for further 30 s at 2800 rpm. Throughout all process, mixing was performed always under vacuum and temperature below 7 ºC. The batters attained were submitted to one of two alternative treatments of the same duration (40 min) and at the same temperature (10 ºC): UV irradiation or storage in the dark. In both cases, the batter was spread to a depth of 0.5 cm on a plate. The difference was that, for each formulation, one of the spread batters was irradiated under a model 51438 UV lamp (Gelman Instruments Company, Ann Arbor, USA) at a wavelength of 254 nm and an intensity of 3300 $\mu W/cm^2$. After irradiation, the batters were put inside a model EB-12 hydraulic filler (Mainca Equipamientos Carnicos, S.L., Granollers, Spain) and 'sausages' with a diameter of 25 mm and a length of about 20 cm produced with cellulose casings. 'Sausages' were vacuum-packed in low-oxygen permeable barrier bags (O_2 transmission, <2.1 $cm^3/(m^2.day.bar)$ at 23 ºC, Colamin XX 100e, Obermühle Polymertechnik GmbH, Pössneck, Germany) with a model A300/52 vacuum packager (Multivac Sepp Haggenmüller GmbH & Co. KG, Wolfertschwenden, Germany). Then, these packages were immersed in water at 35 ºC for one hour (setting) and moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and steam cooked at 90 ºC for one hour (cooking). The oven's digital thermometer was placed in the centre of one 'sausage' through a hole in the bag. Finally, products were cooled and stored in a refrigerated room (at 5 ± 1 ºC) until analysis.

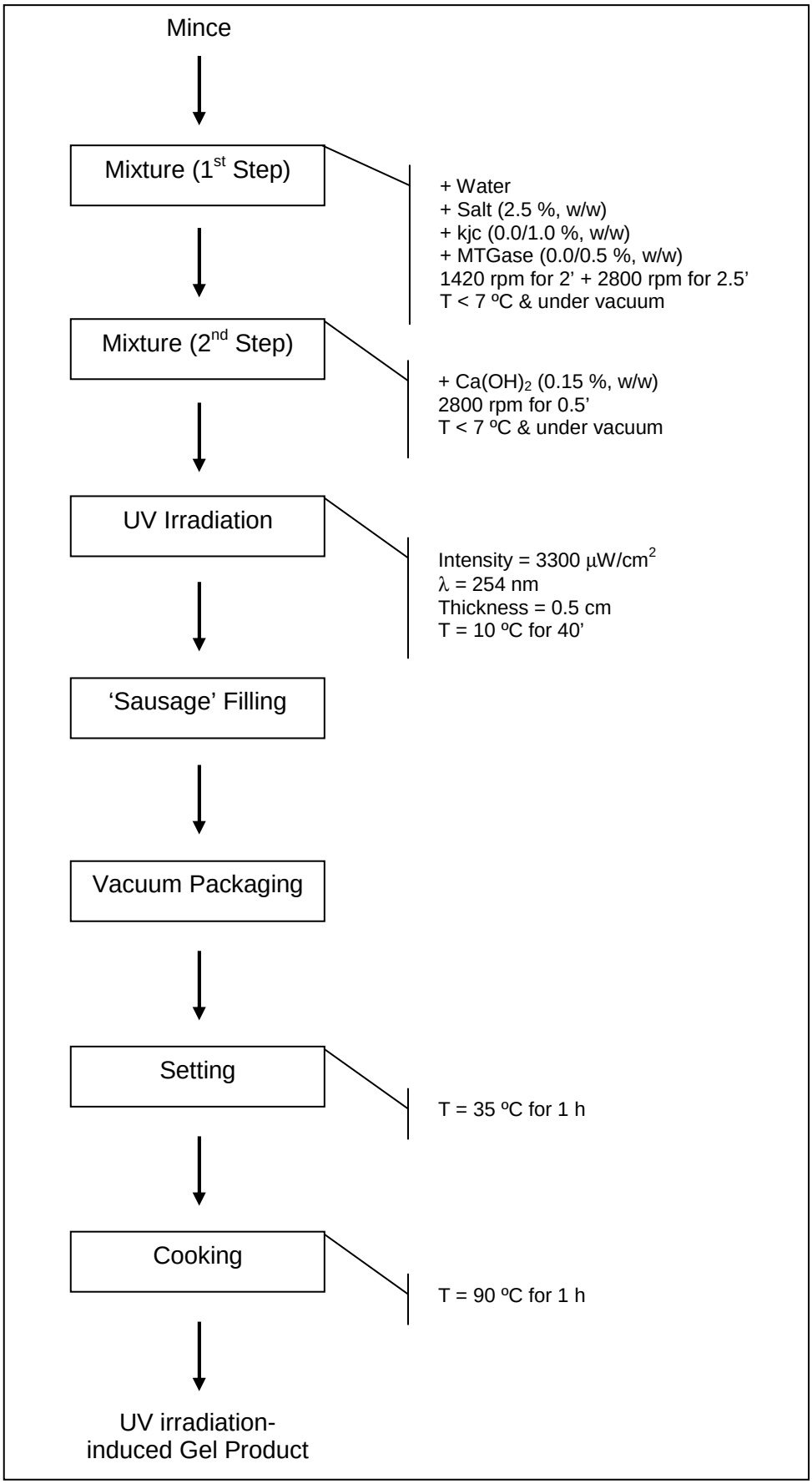

Figure 3.3 – Scheme of the production of UV irradiation-induced gel products.

3.2.2.4 – Production of Minced Fish Products with Shellfish Flavour

Five kg batches were prepared for each formulation and each storage temperature according to the procedure described in Figure 3.4.

Regarding the preparation of the minced fish batters, three sequential steps were always followed:

1) Previously, an emulsion was prepared with salt (0.2 %, w/w of final product), sodium caseinate, emulsifying protein (0.5 %, w/w), water (3.7 %, w/w) and oil (5.6 % VO, w/w, or 2.7 % VO + 2.9 % CLO, w/w) (1:2:19:28, w/w): sodium caseinate was mixed with hot water (approximately at 60 ºC) in a cup with a magnetic stirrer; oil was added and mixed with the other components for 15 minutes at 40 ºC and, then, salt was added and dissolved and mixing went on for 15 more minutes at 40 ºC. Afterwards, the produced emulsion was left overnight at 2 ± 1 ºC. Finally, it was minced one single time in the model 84145 meat grinder (Hobart, Troy, OH, USA).

2) Hake mince (80.0 %, w/w) was mixed with salt (1.3 %, w/w) and sodium polyphosphate (0.5 %, w/w) for 2 minutes at 1420 rpm and 1 minute at 2800 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Throughout all process, mixing was performed always under vacuum and temperature below 7 ºC.

3) The emulsion (10.0 %, w/w), inner pea fibre (2.0 %, w/w), carrageenan (1.0 %, w/w), monosodium glutamate (0.3 %, w/w), shellfish flavour (1.0 %, w/w), MTGase (0.1 %, w/w) and water (3.8 %, w/w) were added and mixed for 2 minute at 2800 rpm.

The batters attained were put inside a model EB-12 hydraulic filler (Mainca Equipamientos Carnicos, S.L., Granollers, Spain) and ‘sausages’ with a diameter of 25 mm and a length of about 20 cm produced with cellulose casings.

Minced fish products were vacuum-packed (two ‘sausages’ per package) in low-oxygen permeable barrier bags (O_2 transmission, <2.1 $cm^3/(m^2.day.bar)$ at 23 ºC, Colamin XX 100e, Obermühle Polymertechnik GmbH, Pössneck, Germany) with a model A300/52 vacuum packager (Multivac Sepp Haggenmüller GmbH & Co. KG, Wolfertschwenden, Germany). Then, these packages were immersed in water at 35 ºC for one hour (setting), moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and subjected to a steam cooking at 90 ºC for one hour (cooking). In order to guarantee these conditions even in the innermost part of the product, the oven’s digital thermometer was placed in the centre of a product through a hole in the bag (approximately 10 minutes until reaching 90 ºC). After, minced fish products were immediately cooled in iced water.

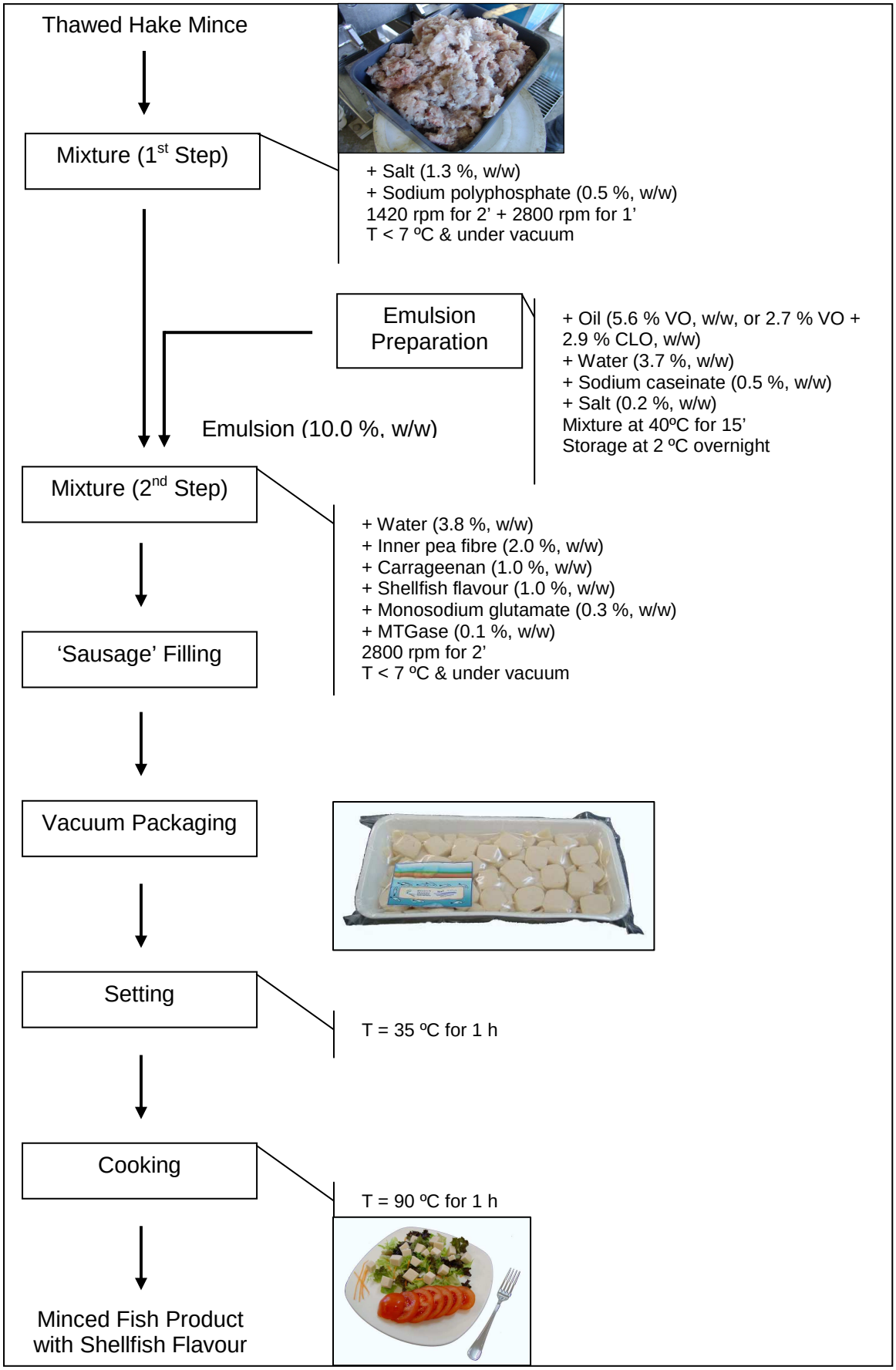

Figure 3.4 – Scheme of the production of minced fish products with shellfish flavour (all ingredients and additives quantities are related to the weight of the final product).

3.2.2.5 – Production of Sausages

First, the appropriate quantities of the various ingredients were weighed in order to produce 2 kg (Figure 3.5). Afterwards, for the preparation of the sausage batters, five sequential steps were always followed. Pork meat and/or hake mince was mixed with salt and phosphates for 1 minute at 1420 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Throughout all process, mixing was performed always under vacuum and refrigeration (temperature below 7 ºC). In a second step, ice (70 % of the total amount of ice and water), dextrose and curing salt were added and there was additional mixing for 1 minute at the same speed. Afterwards, the emulsifying protein and the soy protein concentrate were also added and further mixing at the same speed took place for 1 minute. Thereafter, pork fat or Fibruline was added and mixed for 1 minute at 2800 rpm. Meanwhile, Swelite was hydrated before mixing it with the other ingredients (chilled water was added to this dietary fibre in a ratio near 2:1). The fifth and last step involved the addition of the remaining ingredients, potato starch, the hydrated fibre (not added in the case of pork sausage without Swelite), sausage seasoning and smoke aroma and, moreover, mixing all for 2 minutes at 2800 rpm.

The batters attained were put inside a model EB-12 hydraulic filler (Mainca Equipamientos Carnicos, S.L., Granollers, Spain) and encased under pressure into cellulose sausage casings mounted over the end of the stuffing horn. Afterwards, these cellulose casings were twisted and tied, thereby, shaping sausages with a diameter of 25 mm and a length of about 20 cm. In the next step, sausages were moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and subjected to a steam cooking at 75 ºC for 15 minutes (cooking). Immediately after, sausages were taken from the oven and cooled with a mixture of water and ice (1:1, v/v). The cellulose casings were removed, sausages separated from one another and vacuum-packed in plastic bags with a model A300/52 vacuum packager (Multivac Sepp Haggenmüller GmbH & Co. KG, Wolfertschwenden, Germany). Following this operation, sausages were put in the same oven and subjected to a steam cooking at 85 ºC for 5 minutes (pasteurisation). Once again, they were immediately cooled in iced water. Afterwards, sausages were kept in a refrigerator overnight until further analysis.

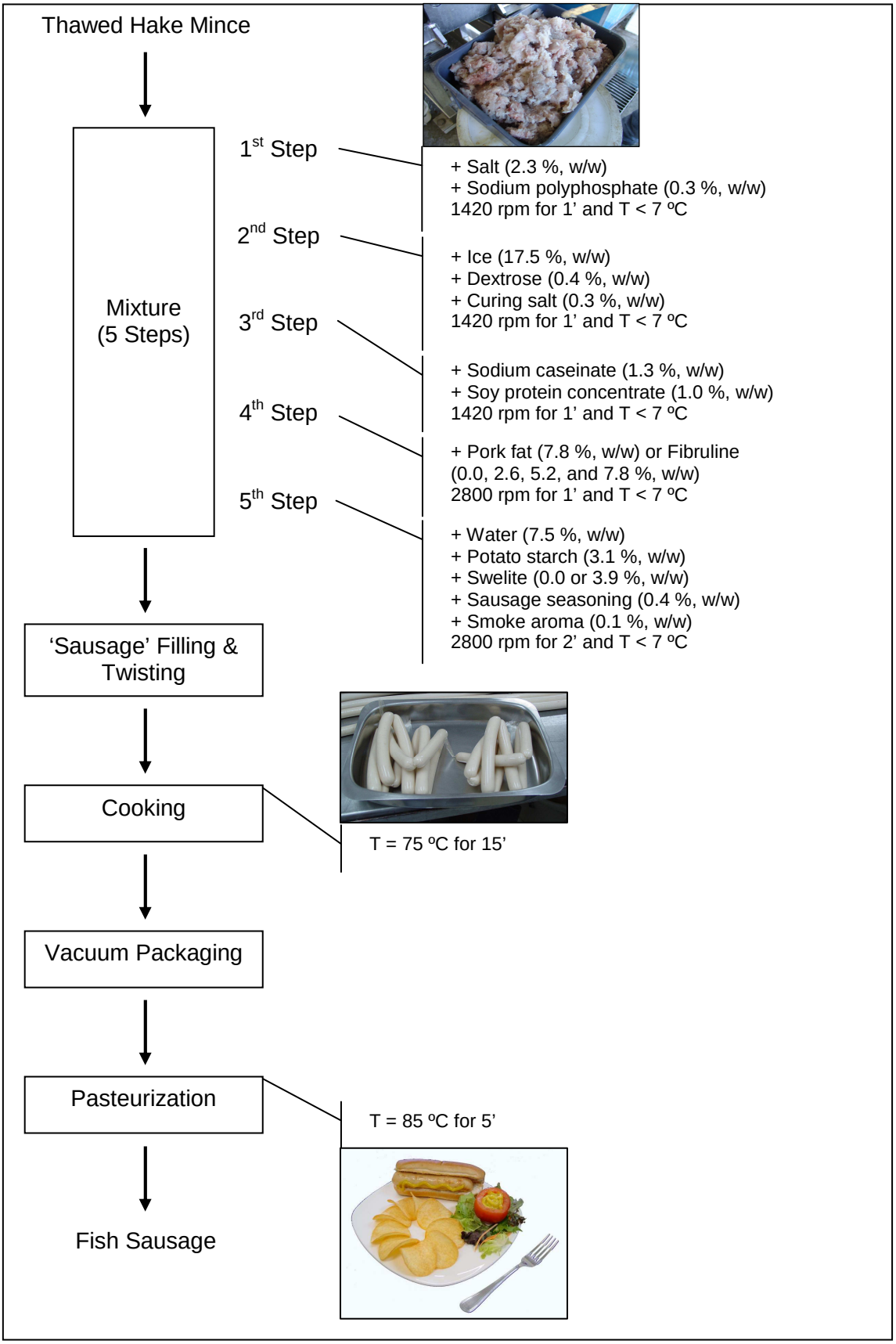

Figure 3.5 – Scheme of the production of fish sausages.

3.2.2.6 – Storage of Minced Fish Products (Shellfish Flavour) and Sausages

The minced fish products with a shellfish flavour were stored at two temperatures in refrigerated rooms (2 ± 1 ºC and 10 ± 1 ºC, relative humidity, 90-95 %) in the dark during approximately 105 days. The sausages were kept in a refrigerated room at 2 ± 1 ºC (relative humidity, 90-95 %) in the dark during approximately 80 days. For both studies, an electronic panel controlled the refrigerated rooms' temperature, turning on the refrigeration system whenever temperature rose above 2 or 10 ºC. Additionally, temperature was permanently monitored through a model MonoLog T temperature data logger (Digitron, Torquay, UK).

3.2.3 - Analytical Methods

3.2.3.1 - Proximate Composition

Moisture, protein, fat and ash were attained by the methods mentioned below. Regarding total carbohydrate content, it was determined by difference.

Moisture was determined gravimetrically by standard procedures (AOAC, 1984). Crude protein was analysed by standard procedures (AOAC, 1984) or, alternatively, in a model FP-528 LECO nitrogen analyser (LECO, St. Joseph, USA), calibrated with EDTA (Dumas method) (Saint-Denis and Goupy, 2004). Crude fat was determined by a rapid method of total lipid extraction (Bligh and Dyer, 1959). Ash was quantified gravimetrically by standard procedures (AOAC, 1984)

3.2.3.2 – Fatty Acid Profile

The fatty acids profile was determined by using an acid hydrolysis method according to Bandarra et al. (1999).

3.2.3.3 - Water Activity (a_w)

Water activity (a_W) was determined using a Hygropalm AW1 a_W meter (Rotronic, Bassersdorf, Switzerland). Samples were taken from the packages, the cellulose skin removed in the case of the sausage products, cut into small pieces, smashed inside a Rotronic a_W plate (1.5 cm depth and 5 cm diameter) and left for 1 hour closed inside the plate at room temperature. Then, the lid of the plate was taken off and the plate was immediately put inside the measuring compartment of the device. Measurements were only taken after 15 minutes stabilization.

3.2.3.4 - Water Holding Capacity (WHC)

WHC was measured by a modification of a published method (Sánchez-González et al., 2008). Coarsely chopped sample (≈2 g) (W_s), wrapped in two overlayed Whatman nº1 filter papers (also weighed, W_i), was placed in a centrifuge tube and submitted to 3,000×g for 10 min. at 20 ºC in a model 6800 centrifuge (Kubota corp., Tokyo, Japan). After centrifugation, sample was removed and filter papers were weighed again (W_f). Measurements were in triplicate. WHC is expressed as grams of water in sample after centrifugation per 100 g of water initially present in sample:

$$WHC = \frac{Ws \times (H/100) - (Wf - Wi)}{Ws \times (H/100)} \times 100$$

where,

H – sample moisture (%).

3.2.3.5 - pH

pH was measured using a Sen-Tix 21 surface pH electrode (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) on a model pH 539 pH meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Samples were taken from the packages, left for 1 hour at room temperature and, only then, the surface electrode was introduced in the products' interior and the measurements made. This determination was carried out in triplicate.

3.2.3.6 - Texture

The textural analysis comprised a wealth of different tests aiming at different physical aspects of texture. Whereas, folding test is a simple manual test used in the surimi industry, the other tests involve operation of a specific equipment, a texturometer.

For the folding test, the test piece was a 3 mm slice cut from the samples (with cylindrical or sausage shape) middle portion. The evaluation was performed in accordance with a 5-point grade system as follows. Grade 5, no crack when folded into quadrants; Grade 4, no cracks when folded in half; Grade 3, crack develops gradually when folded in half; Grade 2, crack develops immediately when folded in half; Grade 1, crumbles when pressed by finger.

Regarding the puncture test, samples were tempered to about 20 ℃ and cut into pieces of 25 mm diameter and 25 mm high. Each sample was penetrated to the breaking point with a metal probe equipped with a 5 mm diameter spherical head, using a model Instron 4301 texturometer (Instron Engineering Corp., Canton, MA, USA). The cross speed head was 10 mm/min and the load cell was 1000 N. Breaking force (N) and

breaking deformation (mm) were measured. Gel strength (N.mm) was determined by multiplying these two parameters.

Samples for texture profile analysis were tempered to about 20 ℃ and cut into pieces of 25 mm diameter and 25 mm high. For the texture profile analysis, samples (diameter, 25 mm and height, 25 mm) were compressed on the flat plate of the Instron texturometer with a cylindrical plunger (50 mm diameter) adapted to a 1000 N load cell at a deformation rate of 50 mm/min. On the basis of preliminary trials to establish a compression limit that would ensure no cracking and recoverability of most samples, it was decided to compress samples to 60 % of their height (40 % compression). In the test, each sample was compressed twice. The following parameters were determined: hardness (N), maximum height of first peak on first compression (in terms of eating quality, food's resistance at first bite); cohesiveness (A_2/A_1), ratio of second-compression to first-compression positive areas (maintenance of food resistance during chew down); gumminess (N), product of hardness and cohesiveness (strength required in the chew down process); springiness (L_2/L_1), ratio of the detected height of the product on the second compression to the original compression distance (ability of food to reacquire its initial shape and size after a first bite); chewiness (N), product of gumminess and springiness (albeit expressed in N, a measure of the energy spent in the chew down process).

Furthermore, according to above mentioned conditions, a more drastic compression (80 %) that causes gel rupture by compression of the whole sample was applied in order to measure force and distance at rupture. An 80 % compression was chosen because high compression levels imitate the effects of the mastication process upon food (Bourne, 1994).

For compression-relaxation test/elasticity, the samples were tempered, prior to analysis, to about 20 ℃ and cut into pieces of 25 mm diameter and 25 mm high. For the compression-relaxation test, the compression procedure was as for the texture profile analysis except that the sample was compressed only once for one minute and the force exerted on the sample was recorded. Relaxation (%) was calculated as $Y_T=100\times(F_0-F_1)/F_0$, where F_0 is the force registered at the onset of relaxation immediately after sample compression and F_1 is the force registered after one minute of relaxation. Thus, $(100-Y_T)$ is taken as an index of elasticity and is expressed as the percentage elasticity of the gel.

In what concerns the compression-relaxation test for the determination of viscoelastic parameters, another stress-relaxation test was conducted under conditions similar to those described above. However, in order to prevent even the weakest linkage rupture,

samples were compressed by only 10 % with a 50 mm diameter cylindrical probe and a load cell of 1 kN at a crosshead speed of 0.8 mm/s, the deformation being kept constant for 10 min. Initial stress (σ_0) was obtained, relaxation of stress was monitored as a function of time and the curves were fitted to a simplified Maxwell model, given by equation (Hamann and McDonald, 1992):

$$\sigma = \sigma_e + (\sigma_0 - \sigma_e) \times e^{\left(-\frac{t}{\tau_1}\right)}$$

where,

σ- decaying stress (kPa);

σ_e- stress at equilibrium (kPa);

t- time (s);

τ_1 - relaxation time (s).

For each fitting, starting σ_e and τ_1 values included into nonlinear regression equation were 1.1 kPa and 200 s, respectively. The viscous (η_1) and elastic (E_e and E_1) moduli were calculated: $\eta_1 = \tau_1 \times E_1$, $E_1 = (\sigma_0 - \sigma_e)$/deformation and $E_e = \sigma_e$/deformation.

3.2.3.7 - Dynamic Rheological Measurement (DRM)

Sets of batters of various fish species were prepared, using the same ingredients' and additives' proportions of the products. Afterwards, they were heated in a serrated plate geometry of a model RS 75 controlled-stress rheometer (Haake, Karlsruhe, Germany) and small amplitude oscillatory shear measurements were performed. The gap between the two plates was 1 mm. A layer of paraffin oil was placed around and over the batters to avoid evaporation during heating. The changes in the viscoelastic properties of the batters were monitored during heating using the rheometer in oscillatory mode at a frequency of 1 Hz and a shear stress of 100 Pa. The batters were heated from 20 to 85 ºC at a rate of 1 ºC.min^{-1}, held at 85 ºC for 30 min, and finally cooled from 85 to 20 ºC at 2 ºC.min^{-1}. The storage modulus, G', of the heated batters was measured every minute during the heating cycle. At the end of a 20 min holding at 20 ºC, a frequency sweep (from 0.001592 to 15.92 Hz) followed by a shear stress sweep (from 1 to 1000 Pa) was carried out on the batters. The frequency sweep and shear stress sweep data showed that the conditions of measurement were within the range of viscoelastic behaviour of these batters. Each batter was tested, at least, in duplicate.

3.2.3.8 - Colour

Samples were cut and put into Petri dishes, covering the entire bottom. A model MACBETH COLOUR-EYE® 3000 colourimeter (Macbeth, New Windsor, NY, USA) was used and, prior to measurements, standardized to a specific colour blank (CIELAB system: L*, 92.4; a*, -1.0; b*, 1.5). The attained values for L*, a* and b* (Figure 3.6) of the CIELAB system were always the means of ten measurements on each Petri dish. Furthermore, for a better assessment of colour, the three mentioned coordinates were combined in order to obtain the chroma and whiteness values:

$$Chroma = \sqrt{a^{*2} + b^{*2}}$$

$$Whiteness = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

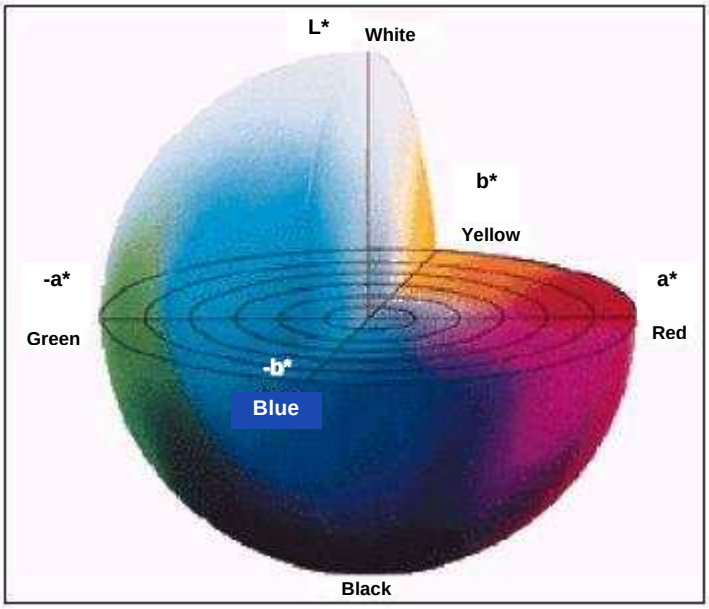

Figure 3.6 – Colours associated to the CIELAB system parameters.

3.2.3.9 - Sensory Evaluation

The minced fish product with shellfish flavour and the Frankfurt sausage, as products with a commercial potential, were sensory evaluated in accordance with ISO standards (ISO 6658, 2005). This assessment provided also insight into the different correlations between some sensory attributes and specific colour and textural properties measured instrumentally.

3.2.3.9.A - Minced fish products with shellfish flavour

Sensory evaluation was conducted by ten trained panellists, which participated in preliminary trials and in the experiments that led to the development of minced fish

products containing inner pea fibre and with shellfish flavour. Panellists were further prepared in more than five training sessions. Following evaluation of the products in individual booths with help of a preliminary sensory evaluation sheet, results were discussed by the panellists with a twofold purpose, to develop adequate sensory parameters for the sensory features of the product and to reach common concepts and standards of judgment for each parameter. In this way, panellists became completely familiar with the sensory scheme and capable of conducting a structured scaling of products (Nielsen et al., 2002).

Products were taken out from the package, their cellulose skin removed, tempered to about 20 ℃ and cut into 2 cm long slices. These slices were distributed in white plates and presented to the panellist in random order for evaluation. The assessment was performed in a room specifically conceived for sensory analysis and with adequate lighting (ISO 8589, 2007).

Panellists were asked to score several sensory parameters of the product, using a 0-5 scale: colour (0 – light to 5 – dark); fish aroma, shellfish aroma and unpleasant aroma (0 – absent to 5 - excessive); elasticity (0 – plastic to 5 – elastic), hardness (0 – soft to 5 – hard), cohesiveness (0 – scarcely cohesive to 5 – very cohesive), succulence (0 – dry to 5 - succulent), unpleasant texture (0 – absent to 5 - excessive); saltiness, bitterness, fish, shellfish and unpleasant flavour (0 – absent to 5 - excessive); and salty and astringent aftertaste (0 – absent to 5 - excessive).

3.2.3.9.B - Sausages

Sensory evaluation was conducted by five panellists from INRB, I.P./IPIMAR, extensively trained with the sensory scheme for Frankfurter sausage evaluation and capable of conducting a structured scaling of products (Nielsen et al., 2002). Panellists participated in preliminary trials and in the experiments that led to the development of Frankfurter fish sausages containing inner pea fibre.

Sausages were taken out from the package, tempered to about 20 ℃ and cut into 2 cm long slices. These slices were distributed in white plates and presented to the panellist in random order for evaluation. Mineral water was supplied to the panellists for mouth rinsing between samples. The evaluation was performed in a room specifically conceived for sensory analysis and with adequate lighting.

Panellists were asked to score several sensory parameters of the product, using a 0-5 scale: colour (0 – light to 5 – dark); smoke and unpleasant aroma (0 – absent to 5 - excessive); elasticity (0 – plastic to 5 – elastic), hardness (0 – soft to 5 – hard), cohesiveness (0 – scarcely cohesive to 5 – very cohesive), succulence (0 – dry to 5 - succulent) and fat mouth feel (0 – slightly oily to 5 – very oily); saltiness, smoke and

unpleasant flavour (0 – absent to 5 - excessive); and astringent aftertaste (0 – absent to 5 - excessive).

3.2.3.10 - Microbiological Analysis

Bacterial counts were determined by pour plate method over storage. The first dilution was prepared by aseptically homogenising 10 g of sausage with 90 ml of a peptone solution (1 g/l) for 1 min. in a Stomacher blender (Seward, London, UK).

Adequate serial dilutions were prepared and inoculations performed in duplicate. Plate Count Agar (PCA, Oxoid Ltd., Hampshire, UK) was used for total mesophilic aerobic count, incubated at 30 ºC for 3 days, and, for total psychrotrophic aerobic count, incubated at 6.5 ºC for 10 days. For *Enterobacteriaceae*, incubation was conducted at 37 ºC for 1 day in a Violet Red Bile Agar (Merck KGaA, Darmstadt, Germany), while, for lactic acid bacteria, incubation was done at 25 ºC for 3 days in a Man Rogosa Sharp Agar (MRS, Oxoid Ltd., Hampshire, UK). Regarding spores of sulfite-reducing clostridia, incubation was done at 37 ºC for 5 days in a Sulphadiazine Polymyxin Sulphite Agar (SPS, Merck KGaA, Darmstadt, Germany) under anaerobic conditions after treatment of serial decimal dilution volumes for 10 min. at 80 ºC. Albeit only black colonies were considered as positive result, plates were also examined with respect to presence of any other colonies. Finally, for mould and yeast, incubation was conducted at 25 ºC for 5 days in a Rose Bengal Chloramphenicol Agar (RB, Oxoid Ltd., Hampshire, UK). Results were expressed as log colony forming units (CFU)/g sample.

Whereas for the sausages all count determinations described above were performed, for the minced fish products with shellfish flavour only some of them were carried out, taking into account all previous experience acquired with the sausage product, which was likewise vacuum-packed and submitted to a thermal treatment.

3.2.3.11 - Thiobarbituric Acid Reactive Substances (TBARS)

Two different methods were used for the determination of TBARS during storage time: a trichloroacetic acid extraction followed by a spectrophotometric determination (Vyncke, 1970) for the sausage product and a HPLC separation on 7.5 % TCA extracts also followed by a spectrophotometric determination, as described by Seljeskog et al*.* (2006), for the minced products with shellfish flavour. This new method was chosen for its higher accuracy, in order to ensure a higher degree of reliability in the evolution of TBARS.

For each fish sausage or minced product type, two extracts were prepared, each one from a different package. Results were expressed as mg of malonaldehyde (MA) per 1000 g of product.

3.2.3.12 - Protein Solubility

Different extraction solutions were used (Table 3.2). The choice of the various possible combinations of extraction solutions was based on the purpose of each particular experimental work.

An amount of 600 mg of chopped sample, including raw material, the mackerel surimi, was homogenized in 8 ml of each extracting solution using a model Polytron® PT-MR 3000 homogenizer (Kinematica, Littau, Switzerland) equipped with a small rod for 60 s at low speed to avoid foaming. Then, samples were boiled in a water bath (100 ºC) for 2 min. and, afterwards, homogenized while hot for 30 s. Finally, samples were centrifuged (20,000×g at 20 ºC for 15 min.) in a model 6800 centrifuge (Kubota, Tokyo, Japan). Protein quantitation in the supernatants was performed through OD measurement at 280 nm (Piñeiro et al., 1999). Results are average of three determinations, calculated as percent protein solubilised with respect to total protein in the sample and divided by the protein solubility of a reference sample, thus being expressed as relative protein solubility.

Table 3.2– Composition of the protein extraction solutions and targeted chemical interactions.

CHARACTERISTICS	Extracting Solution					
	SDS+DTT+U	DTT+U	SDS+U	SDS+DTT	SDS	U
COMPONENTS						
Sodium dodecyl sulphate, SDS (%, w/v)	2	---	2	2	2	---
DL-Dithiothreitol, DTT (M)	0.1	0.1	---	0.1	---	---
Urea (M)	8	8	8	---	---	8
Tris(hidroxymethyl)-aminomethane, Tris(mM)	60	60	60	60	60	60
ACIDITY-ALCALINITY						
pH (adjusted with HCl)	7.5	7.5	7.5	7.5	7.5	7.5
TARGETED CHEMICAL INTERACTIONS (Liu and Hsieh, 2008)	Non-covalent bonds and disulphide bridges	Predominantly hydrogen bonds and disulphide bridges	Non-covalent bonds	Non-covalent bonds and disulphide bridges	Non-covalent bonds	Non-covalent bonds (more efficient in breaking hydrogen bonds)

3.2.3.13 - Electrophoresis

The supernatant (soluble protein) obtained was diluted to 0.3 mg/ml with a pH 6.8 Laemmli buffer (4.8 g/100 ml SDS + 1 mM EDTA + 0.1 mM DTT + 20 ml/100 ml glycerol + 125 mM Tris-HCl + 0.05 g/100 ml bromophenol blue), centrifuged (20,000g at 20 ºC for 10 min.) in a model Sigma 3K30 centrifuge (SIGMA, Osterode, Germany) and then heated for 2 min. in a boiling bath. Samples were analysed by SDS-PAGE in a model 2117 Multiphor II electrophoresis unit (LKB, Bromma, Sweden), using Excel Gel™ 15 % polyacrilamide gels and Excel Gel™ buffer strips (Amersham, Uppsala, Sweden). Electrophoresis conditions were 600 V, 30 mA and 30 W and temperature was kept at

15 ºC by a model 2219 Multitemp II thermostatic circulator (LKB, Bromma, Sweden). The protein bands were silver stained with a PlusOne™ kit (Amersham, Uppsala, Sweden). As reference for molecular weights, two standard (high and low molecular weight) electrophoresis calibration kits (Pharmacia Biotech, Uppsala, Sweden) were used. For optical density measurement, gels were analysed in a model GS-800 calibrated densitometer (Bio-Rad, Hercules, USA) with software Quantity One® (Bio-Rad, Hercules, USA).

3.2.3.14 - Scanning Electronic Microscopy (SEM)

Cubes of 2-3 mm were cut from inner part of the product samples for microscopic examination. Samples were fixed with a mixture (1:1, v/v) of 5 g/100 ml formaldehyde and 0.2 g/100 ml glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and dehydrated in increasing series of acetone (10-100 ml/100 ml). Afterwards, they were critical-point dried with CO_2 as transition fluid in a model Polaron 3000 critical-point dryer (Polaron, Hertfordshire, UK) and mounted on metallic holders, followed by gold sputter-coating in a model JFC-1100E ion sputtering device (JEOL, Tokyo, Japan). Samples were kept in a dryer until examination by a model JSM-5200 scanning microscope (JEOL, Tokyo, Japan) at 20 kV. Micrographs were taken at different magnifications.

3.2.4 - Statistical Analysis

Factorial analysis of variance (general linear model, factorial ANOVA, full factorial design) was carried out using the STATISTICA© software (StatSoft, Tulsa, USA), version 6.1, 2003. The difference of means between pairs was resolved by confidence intervals in a Tukey HSD test. Level of significance was set for $p<0.05$.

4. Experimental Design

The experimental design was elaborated in accordance with the four main objectives set by the Ph.D. programme. This led to the planning of various experimental trials, some focusing upon the molecular mechanisms underlying the food quality properties, others concerning these properties themselves and the effects of additives and different processing technologies on them. There were a total of ten experimental works, whose results were presented and discussed in ten papers, all published in indexed scientific journals with peer review. These papers were converted to chapters, forming the 5th part of this thesis, Results and Discussion (accordingly the chapters are numbered as 5.). All these works were interconnected and followed a logical order, as shown in Figure 4.1. In fact, a first group of works aimed to study the effects and action mechanisms of additives incorporated in heat-induced gel products (objective 1) and a second evaluated the effects and action mechanisms of alternative gelation technologies, thus studying pressure-induced and UV irradiation-induced gel products (objective 2). The results of these works were used in the production of innovative and marketable fish products (objectives 3 and 4). That is, the knowledge of the property changes imparted to fish products by different additives and gelation technologies helped to design products with specific desirable properties. Among these, with the purpose of assessing the viability of substituting fish in traditional meat foods (objective 3), a healthy low fat fish sausage containing dietary fibre was developed. Finally, quality changes during storage of those products with higher commercial potential were studied (objective 4). They were the fish sausage as well as the minced fish product with shellfish flavour.

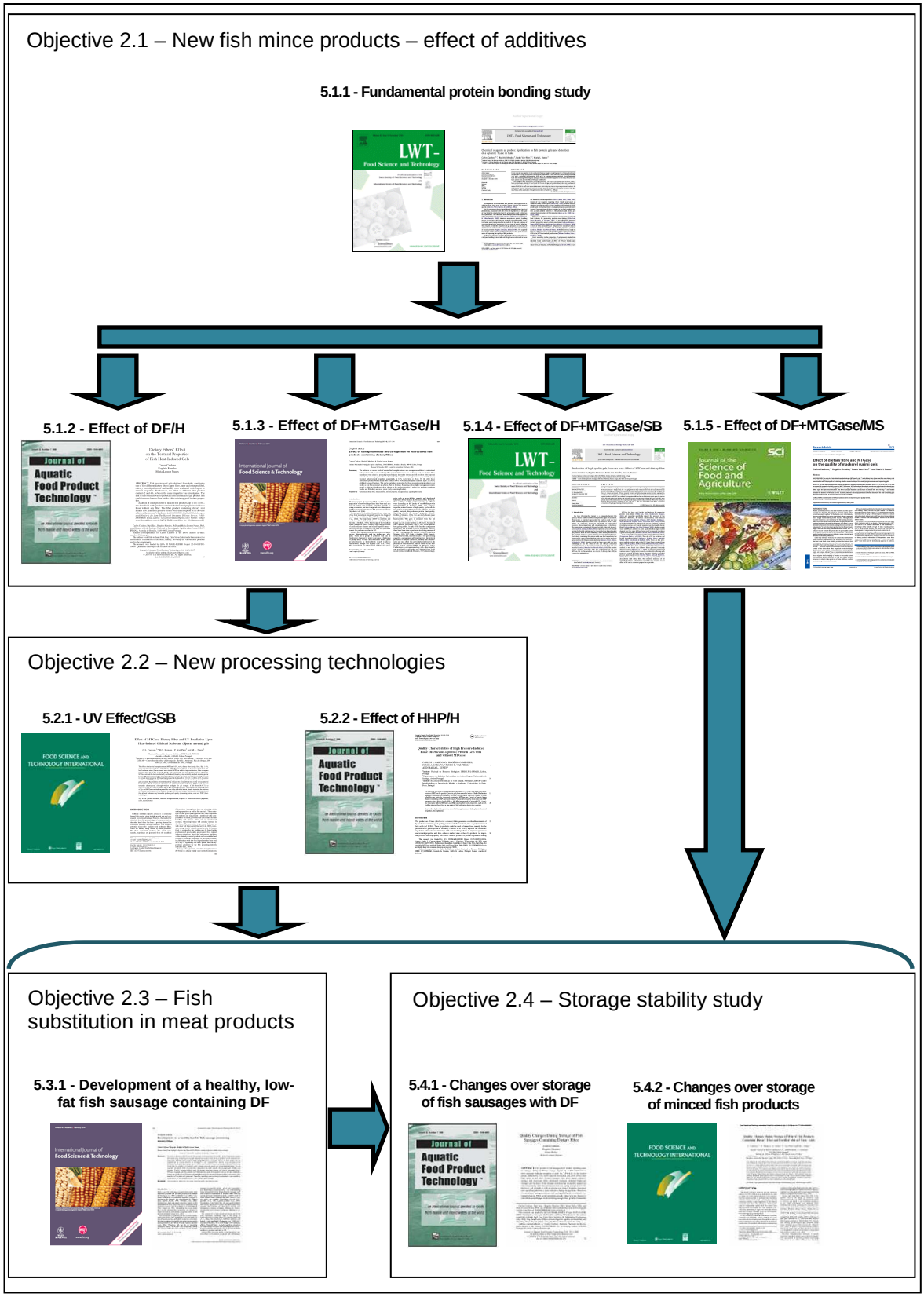

Figure 4.1 – Experimental design of the Ph.D. study.

5 – Results and Discussion

5.1 - New Fish Mince Products – Effect of Additives

5.1.1 – Chemical Reagents as Probes: Application to Fish Protein Gels and Detection of a Cysteine TGase in Hake

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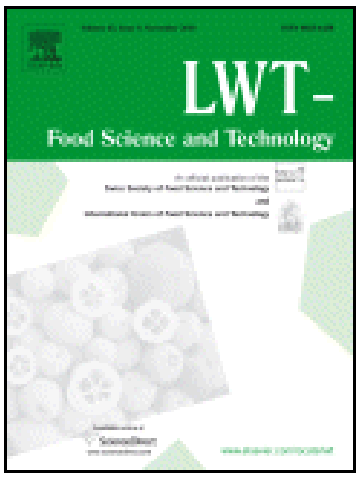

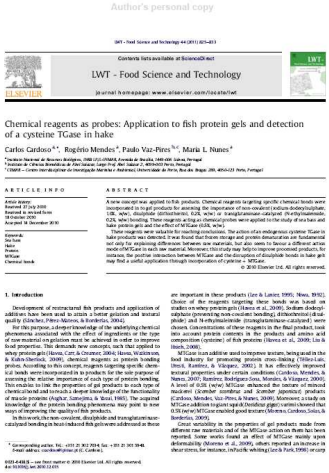

LWT - Food Science and Technology

Chemical reagents as probes: Application to fish protein gels and detection of a cysteine TGase in hake

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Abstract

A new concept was applied to fish products. Additives targeting specific chemical bonds were incorporated in to gel products for assessing the importance of non-covalent (sodium dodecylsulphate, 1.0 %, w/w), disulphide (dithiothreitol, 0.2 %, w/w) or transglutaminase-catalyzed (N-ethylmaleimide, 0.2 %, w/w) bonding. These additives acting as chemical probes were applied to the study of sea bass and hake protein gels and the effect of MTGase (0.5 %, w/w).

These additives were valuable for reaching conclusions. The action of an endogenous cysteine TGase in hake products was detected. It was found that frozen storage and protein denaturation are fundamental not only for explaining differences between raw materials, but also seem to favour a different action mode of MTGase in each raw material. Moreover, this study may help to improve processed products, for instance, the positive interaction between MTGase and the disruption of disulphide bonds in hake gels may find a useful application through incorporation of cysteine+MTGase.

Keywords: Sea bass, hake, protein, microbial transglutaminase, non-covalent bonds, disulphide bonds

Introduction

The development of restructured fish products and the application of additives have been used as a way to attain a better gelation and textural quality and to reach young and health-conscious consumers (Sánchez et al., 2004).

For this purpose, a deeper knowledge of the underlying chemical phenomena associated with the effect of the ingredients or the type of raw material on gelation must be achieved in order to improve food properties. This demands new concepts, such that applied to whey protein gels (Havea et al., 2004; Havea et al., 2009), additives as chemical probes. According to this concept, additives targeting specific chemical bonds were incorporated in to products for the sole purpose of assessing the relative importance of each type of bonding between proteins. This enables to link the properties of gel products (such as hardness) to each type of chemical bond. The acquired knowledge of the protein bonding phenomena may allow for the proposal of new ways of improving the quality of processed fish products.

In this work, the non-covalent, disulphide and transglutaminase-catalyzed bonding in heat-induced fish gels were addressed as these are expected to be important in these products (Niwa, 1992; Lee and Lanier, 1995). Choice of the additives targeting these bonds was based on previous works on whey protein gels (Havea et al., 2009). Therefore, sodium dodecylsulphate (preventing non-covalent bonding), dithiothreitol (disulphide) and

N-ethylmaleimide (transglutaminase-catalyzed) were chosen. The concentrations of these additives in the final product, took into account the protein contents in the products and the amino acid composition (cysteine) of fish proteins (Liu and Hsieh, 2008; Havea et al., 2009). The products with these additives enabled to study the importance of microbial transglutaminase (MTGase) and raw material.

MTGase is an additive used to improve texture, it has been used in the food industry for promoting protein cross-linking (Téllez-Luis et al., 2002). It has effectively improved textural properties under certain circumstances (Ramírez et al., 2000; chapter 5.1.3). A level of 0.5 % (w/w) MTGase enhanced the texture of minced mackerel (*Scomber scombrus* and *Scomber japonicus*) products prepared with dietary fibre (5.1.5). A study on MTGase addition to giant squid (*Dosidicus gigas*) surimi showed that the same MTGase level was enough to achieve good textural quality (Moreno et al., 2009).

Great variability in the properties of gel products made from different raw materials and of the MTGase action on them has been reported. Some works found an effect of MTGase mainly upon deformability (Moreno et al., 2009), others reported an increase in breaking force or shear stress, without affecting shear strain, for instance, in gels from Pacific whiting (Lee and Park, 1998) or carp (Tsukamasa et al., 2000). The choice of sea bass and hake in this work was prompted by the observed differences in the quality of gel products obtained from these species in previous studies (5.1.3; Cardoso et al., 2010b). With the same conditions, sea bass products had a higher textural quality. Therefore, a better understanding of the gelation process in these species is important.

The aim of this work was to apply additives as chemical probes in order to study the protein reactions that underlie the effects of MTGase addition and of the type of raw material on the properties of heat-induced gels.

Material and Methods

Materials

Fresh farmed sea bass (*Dicentrarchus labrax*) were bought in a supermarket and headed, tailed, gutted and filleted at < 10 ºC. Fish weight varied between 300 and 400 g.

Frozen South African hake (*Merluccius capensis*) was bought already headed and gutted from a local frozen fish processor after two months storage. Each fish batch was kept frozen at –28 ºC and processed within one week after its arrival at the laboratory.

Microbial transglutaminase TG-K (MTGase) ACTIVA® GS (Anonymous, 2009) was supplied by Ajinomoto (Tokyo, Japan) and presented an activity of about 100 $U.g^{-1}$.

Sodium dodecylsulphate (SDS), dithiothreitol (DTT), N-ethylmaleimide (NEM) and other chemicals were of analytical grade and from Merck (Darmstadt, Germany).

Proximate Composition

Moisture and ash were determined by standard AOAC procedures (AOAC, 1984), whereas crude protein was determined by the Dumas method in a model FP-528 LECO protein/nitrogen analyser (LECO Corp., St. Joseph, Mi, USA) and crude fat determined by Bligh and Dyer's rapid method of total lipid extraction and purification (Bligh and Dyer, 1959).

Mechanical Properties

Folding test, gel strength, elasticity and texture profile analysis (TPA), namely hardness, cohesiveness, gumminess, springiness, and chewiness, were done as in previous work (5.4.1). Gel strength, elasticity, and TPA were evaluated using an Instron model 4301 texturometer (Instron Corp., Canton, Ma, USA).

Force and distance at rupture were also done as in previous work (5.1.5).

Water Holding Capacity (WHC)

WHC was measured by a modification of a published method (Sánchez-González et al., 2008), exactly as described in a previous work (Cardoso et al., 2010b).

Protein Solubility

To follow protein-protein chemical interactions, protein solubility in four different extracting solutions was determined (Table 5.1.1.1). Soluble protein was separated as described in a previous work (Cardoso et al., 2010b). Protein quantitation in the supernatants was performed through absorbance measurement at 280 nm (Piñeiro et al., 1999). Results are the average of three determinations, calculated as percent protein solubilised with respect to total protein in the sample and divided by the protein solubility in SDS+DTT+U, to give a relative protein solubility. For each sample, comparison to the solubility in SDS+DTT+U (100 %) enabled assessment of the relative importance of the chemical interactions targeted by the missing component.

Electrophoresis

Changes in individual proteins were followed by SDS-PAGE in 15 % Excel-Gel™ (Amersham, Uppsala, Sweden) according to previous work (5.1.5). For optical density measurements, silver stained gels were analysed in a model GS-800 calibrated densitometer (Bio-Rad, Hercules, USA) with software Quantity One® (Bio-Rad).

Table 5.1.1.1 – Composition of the protein extracting solutions and targeted chemical interactions.

CHARACTERISTICS	Extracting Solution			
	SDS+DTT+U	SDS+DTT	SDS+U	DTT+U
COMPONENTS				
Sodium dodecyl sulphate, SDS (%, w/v)	2	2	2	---
DL-Dithiothreitol, DTT (M)	0.1	0.1	---	0.1
Urea (M)	8	---	8	8
Tris(hidroxymethyl)-aminomethane, Tris(mM)	60	60	60	60
ACIDITY-ALCALINITY				
pH (adjusted with HCl)	7.5	7.5	7.5	7.5
TARGETED CHEMICAL INTERACTIONS*:	Non-covalent bonds and disulphide bridges	Non-covalent bonds and disulphide bridges	Non-covalent bonds	Non-covalent bonds (more efficient in breaking hydrogen bonds) and disulphide bridges
Extracting solutions to be compared (shaded areas) in order to assess the importance of:				
Non-covalent (hydrogen) bonds†				
Disulphide bridges				
Non-covalent bonds				

* Based on Liu and Hsieh (2008).
† Urea breaks more efficiently hydrogen bonds (Liu and Hsieh, 2008).

Dynamic Rheological Measurements (DRM)

Two sets of batters, one of sea bass and the other of hake, were prepared, using the same ingredients' and additives' proportions as the gel products (Table 5.1.1.2). Afterwards, they were heated within a serrated plate geometry of a model RS 75 controlled-stress rheometer (Haake, Karlsruhe, Germany) and small amplitude oscillatory shear measurements were performed. The gap between the two plates was 1 mm. A layer of paraffin oil was placed around and over the batters to avoid evaporation during heating. The changes in the viscoelastic properties of the batters were monitored during heating using the rheometer in oscillatory mode at a frequency of 1 Hz and a shear stress of 100 Pa. The batters were heated from 20 to 85 ºC at a rate of 1 ºC.min^{-1}, held at 85 ºC for 30 min, and finally cooled from 85 to 20 ºC at 2 ºC.min^{-1}. The storage modulus, G', of the heated batters was measured every minute during the heating cycle. At the end of a 20 min holding at 20 ºC, a frequency sweep (from 0.001592 to 15.92 Hz) followed by a shear stress sweep (from 1 to 1000 Pa) was done on the cooked batters. The frequency and shear stress sweep data showed that measurement conditions were within the range of viscoelastic behaviour of the batters. Each batter was tested, at least, in duplicate.

Scanning Electron Microscopy (SEM)

SEM was performed as in previous work (5.1.5).

Statistical Analysis

Factorial analysis of variance (general linear model, three-dimensional ANOVA, full factorial design) was carried out using the STATISTICA© software (StatSoft, Inc., Tulsa, USA), version 6.1, 2003. This methodology enabled to analyse each of the studied effects

per se (raw material, MTGase, and each additive) as well the interaction between effects. The difference of means between pairs was resolved by using confidence intervals in a Tukey HSD test. Level of significance was set for $p<0.05$.

Experimental

Experimental Design

Sea bass and hake mince were used with other ingredients in the preparation of gels. The used sample coding and the ingredients' quantities are presented in Table 5.1.1.2. For each fish mince, the effects of the incorporation of 0.5 % (w/w) MTGase (a) were tested and compared with control products without MTGase (0). For both products with and without MTGase, the effects of the addition of 1.0 % (w/w) SDS, 0.2 % DTT or 0.2 % (w/w) NEM were assessed and compared to the products with no additives (CTL). SDS was incorporated as a chemical that ruptures all non-covalent bonds (Liu and Hsieh, 2008), thus putting in evidence the importance of the covalent bonds in the products. DTT ruptures a specific type of covalent bonds, disulphide bridges, therefore its addition puts in evidence the role of non-covalent bonds. Moreover, the combined information of SDS and DTT gels reveals the importance of the other covalent bonds, besides S-S bridges. Finally, addition of NEM, an inhibitor of cysteine TGases, enables to detect the presence of such enzymes in the fish species used as raw materials.

Table 5.1.1.2 – Sample coding and main ingredients (%, w/w) of the various minced fish products.

BATCH	Sea bass mince	Water	Salt	MTGase	SDS	DTT	NEM	Total
$SB0_{CTL}$	79.1	18.4	2.5	0.0	0.0	0.0	0.0	100.0
$SB0_{SDS}$	75.8	20.7	2.5	0.0	1.0	0.0	0.0	100.0
$SB0_{DTT}$	78.4	18.9	2.5	0.0	0.0	0.2	0.0	100.0
$SB0_{NEM}$	78.4	18.9	2.5	0.0	0.0	0.0	0.2	100.0
SBa_{CTL}	77.5	19.5	2.5	0.5	0.0	0.0	0.0	100.0
SBa_{SDS}	74.2	21.8	2.5	0.5	1.0	0.0	0.0	100.0
SBa_{DTT}	76.7	20.1	2.5	0.5	0.0	0.2	0.0	100.0
SBa_{NEM}	76.7	20.1	2.5	0.5	0.0	0.0	0.2	100.0
BATCH	Hake mince	Water	Salt	MTGase	SDS	DTT	NEM	Total
$H0_{CTL}$	96.8	0.7	2.5	0.0	0.0	0.0	0.0	100.0
$H0_{SDS}$	92.9	3.6	2.5	0.0	1.0	0.0	0.0	100.0
$H0_{DTT}$	96.1	1.2	2.5	0.0	0.0	0.2	0.0	100.0
$H0_{NEM}$	96.1	1.2	2.5	0.0	0.0	0.0	0.2	100.0
Ha_{CTL}	94.9	2.1	2.5	0.5	0.0	0.0	0.0	100.0
Ha_{SDS}	90.9	5.1	2.5	0.5	1.0	0.0	0.0	100.0
Ha_{DTT}	94.1	2.7	2.5	0.5	0.0	0.2	0.0	100.0
Ha_{NEM}	94.1	2.7	2.5	0.5	0.0	0.0	0.2	100.0

Gels Production

About 10 kg of sea bass mince were mechanically obtained in a Baader 694 deboning machine (Baader, Lübeck, Germany) fitted with a drum with 3 mm diameter circular holes.

Hake, about 10 kg of frozen fish, were thawed overnight in a refrigerator. Afterwards, skin and bones were manually removed. Resulting fish flesh was minced one single time in a model 84145 meat grinder (Hobart, Troy, OH, USA), equipped with 2 cm grind blades and a metallic screen with 6 mm diameter circular holes.

The appropriate quantities of mince, chilled water, salt (2.5 %, w/w), MTGase, SDS, DTT and NEM for approximately 1.3-1.4 kg batches were weighed (Table 5.1.1.2), to ensure a similar protein:water proportion. A portion of chilled water was added to SDS/DTT/NEM and mixed with the additive for 1 min. The sea bass or hake minces were mixed with the other ingredients/additives for 2 min at 1420 rpm and 2 min at 2800 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Throughout, mixing was always performed under vacuum and at a temperature below 7 ºC. Following procedure was carried out as described in a previous study (Cardoso et al., 2010b).

Results and Discussion

The use of chemical additives (SDS, DTT, and NEM) in the fish gel products aimed to attain more insight with respect to the underlying protein bonding phenomena in the gels, particularly regarding the effect of MTGase and raw material. Thus, the effect of each additive on sea bass and hake products (with or without MTGase) was not studied as a potential food treatment.

MTGase Effect

Folding test (FT), gel strength (GS), elasticity and TPA results are found in Tables 5.1.1.3 and 5.1.1.4, while force and distance at rupture are presented in Tables 5.1.1.5 and 5.1.1.6. SDS and DTT had no effect on the textural properties of the restructured products prepared from sea bass, whether with or without MTGase. However, for hake products, both additives changed the texture. For the products without MTGase, SDS significantly ($p<0.05$) reduced GS, elasticity, hardness, cohesiveness and the other properties related to hardness, such as force at rupture —obtained by 80 % compression. This deleterious effect upon the texture almost disappeared with MTGase addition, SDS induced only some softening of the products (from 28.1 ± 2.4 to 23.5 ± 1.3 N). For DTT and the samples without MTGase, a smaller negative effect on texture was measured. Particularly, GS and force at rupture were not different from those same properties of the control samples. For the products containing MTGase, DTT improved ($p<0.05$) texture with respect to the control, namely GS increased from 18.2 ± 3.1 to 28.9 ± 2.1 N.mm and force at rupture more than doubled from 32.7 ± 0.4 to 70.2 ± 1.2 N. NEM incorporation had no effect on sea bass gel products without MTGase. However, unlike SDS and DTT,

all products containing MTGase whether from sea bass or hake were negatively affected by NEM. Moreover, for hake, texture of NEM samples with and without MTGase was similar. NEM incorporation had a very negative effect upon the texture of hake products without MTGase, surpassing the effect of SDS. Hence, when samples with and without MTGase are taken together (averaged), NEM has a stronger impact on hake than on sea bass (sea bass samples' chewiness decreases from 12.7 ± 2.2 to 8.7 ± 1.8 N, for hake, chewiness is sharply reduced from 11.0 ± 1.3 to 1.5 ± 0.2 N).

Therefore, MTGase (0.5 %, w/w) had a positive effect on the textural quality of both sea bass and hake gel products. This enzyme increases cross-linking of myosin heavy chains (MHC) during setting, since it catalyzes covalent ε-amino-(γ-glutamyl)-lysine bonds (Ramírez-Suárez et al., 2001). However, the effect of MTGase differed between sea bass and hake. Whereas, in the former, GS (particularly, the breaking deformation component) and force at rupture were strongly improved, for hake, a hardening was observed (GS increased only its breaking force component). This suggests that MTGase promoted the deformability of sea bass gels and the hardness of hake gels. Similar differences in the effect of MTGase are reported in the literature (Tsukamasa et al., 2000; 5.1.5). Moreover, the negative effect on texture (in the hake products) of SDS and DTT was reversed by MTGase addition (DTT). The combination of DTT and MTGase was synergistic (for instance, chewiness decreased from 10.3 ± 0.1 to 6.7 ± 1.8 N with DTT and no MTGase, was basically left unchanged at 11.8 ± 1.5 N with MTGase, and increased to 17.6 ± 1.2 N with MTGase+DTT). There is a possible explanation for this. The covalent bonds catalyzed by MTGase lead to the exposure of more hydrophobic areas of myosin and other myofibrillar proteins, thus reinforcing the DTT action. Of course, the covalent bonds catalyzed by MTGase also had a role, but this does not explain the difference between Ha_{CTL} and Ha_{DTT}. However, there is another hypothesis: the toughening effect was not due to more hydrophobic (and other non-covalent) interactions, but to a different action of MTGase, as a function of the raw material and DTT presence (see below, raw material effect), which, in turn, was enhanced by DTT. The positive effect of MTGase was absent in the samples containing NEM. This phenomenon is due to the inhibition of MTGase by NEM (Kanaji et al., 1993). This is because the sole cysteine residue of MTGase, essential for enzymatic activity, is S-1,2-dicarboxyethylated by NEM. However, NEM also had a negative effect on the texture of hake gels without MTGase. Therefore, taking into account that other TGases (Kanaji et al., 1993), including fish TGases (Noguchi et al., 2001), also have a catalytic cysteine residue, it can be hypothesized that hake gels contained a hake endogenous TGase, which lost catalytic action as a result of NEM addition.

Table 5.1.1.3 – Folding test, gel strength, elasticity and TPA (texture profile analysis with 40 % compression) properties of the sea bass gels*.

Sample	Chemical probe	MTGase (%, w/w)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
$SB0_{CTL}$	---	0.0	3.0 ± 0.0^{b}	17.1 ± 1.4^{a}	55.0 ± 0.5^{a}	25.5 ± 1.0^{a}	0.50 ± 0.01^{b}	13.1 ± 0.5^{b}	0.83 ± 0.02^{ab}	10.9 ± 0.6^{c}
$SB0_{SDS}$	SDS	0.0	2.7 ± 0.6^{ab}	14.1 ± 2.9^{a}	55.1 ± 0.3^{a}	25.6 ± 2.5^{a}	0.50 ± 0.00^{b}	13.2 ± 0.9^{b}	0.81 ± 0.01^{b}	10.7 ± 0.8^{c}
$SB0_{DTT}$	DTT	0.0	2.7 ± 0.6^{ab}	14.0 ± 2.3^{a}	54.8 ± 0.6^{a}	25.1 ± 1.2^{a}	0.49 ± 0.01^{b}	12.8 ± 0.4^{b}	0.81 ± 0.01^{b}	10.3 ± 0.4^{c}
$SB0_{NEM}$	NEM	0.0	2.3 ± 0.6^{ab}	16.0 ± 0.6^{a}	53.4 ± 0.5^{a}	26.8 ± 1.4^{a}	0.46 ± 0.01^{a}	12.8 ± 0.1^{b}	0.81 ± 0.02^{b}	10.3 ± 0.2^{c}
SBa_{CTL}	---	0.5	5.0 ± 0.0^{c}	43.2 ± 8.4^{bc}	59.9 ± 0.5^{b}	28.4 ± 3.4^{a}	0.58 ± 0.01^{c}	17.2 ± 1.1^{c}	0.85 ± 0.00^{a}	14.6 ± 0.9^{b}
SBa_{SDS}	SDS	0.5	5.0 ± 0.0^{c}	41.5 ± 7.8^{b}	60.3 ± 0.5^{b}	29.0 ± 1.5^{a}	0.60 ± 0.01^{cd}	18.0 ± 0.5^{cd}	0.84 ± 0.01^{a}	15.2 ± 0.3^{ab}
SBa_{DTT}	DTT	0.5	5.0 ± 0.0^{c}	54.6 ± 6.6^{c}	61.5 ± 1.0^{b}	29.4 ± 3.9^{a}	0.61 ± 0.01^{d}	19.4 ± 0.5^{d}	0.86 ± 0.01^{a}	16.6 ± 0.5^{a}
SBa_{NEM}	NEM	0.5	2.0 ± 0.0^{a}	10.1 ± 2.3^{a}	52.8 ± 1.1^{a}	20.2 ± 1.0^{b}	0.47 ± 0.01^{a}	9.4 ± 0.6^{a}	0.76 ± 0.01^{c}	7.2 ± 0.5^{d}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Table 5.1.1.4 – Folding test, gel strength, elasticity and TPA (texture profile analysis with 40 % compression) properties of the hake gels*.

Sample	Chemical probe	MTGase (%, w/w)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
$H0_{CTL}$	---	0.0	2.7 ± 0.6^{a}	18.9 ± 6.5^{d}	54.1 ± 1.3^{a}	25.6 ± 2.8^{bc}	0.50 ± 0.01^{c}	13.0 ± 0.0^{c}	0.79 ± 0.01^{ab}	10.3 ± 0.1^{b}
$H0_{SDS}$	SDS	0.0	2.7 ± 0.6^{a}	7.5 ± 0.9^{a}	32.8 ± 3.0^{c}	14.3 ± 2.7^{e}	0.42 ± 0.02^{b}	6.8 ± 0.9^{b}	0.75 ± 0.00^{b}	5.1 ± 0.7^{c}
$H0_{DTT}$	DTT	0.0	2.7 ± 0.6^{a}	15.3 ± 3.2^{bcd}	44.3 ± 1.2^{b}	20.8 ± 2.2^{d}	0.41 ± 0.05^{b}	8.8 ± 2.1^{b}	0.75 ± 0.02^{b}	6.7 ± 1.8^{c}
$H0_{NEM}$	NEM	0.0	2.3 ± 0.6^{a}	10.6 ± 0.6^{ab}	29.6 ± 1.0^{c}	10.1 ± 1.2^{f}	0.26 ± 0.04^{a}	2.8 ± 0.2^{a}	0.58 ± 0.05^{c}	1.6 ± 0.2^{d}
Ha_{CTL}	---	0.5	3.0 ± 0.0^{a}	18.2 ± 3.1^{cd}	55.1 ± 0.9^{a}	28.1 ± 2.4^{b}	0.53 ± 0.01^{cd}	14.3 ± 1.6^{c}	0.82 ± 0.01^{a}	11.8 ± 1.5^{b}
Ha_{SDS}	SDS	0.5	2.7 ± 0.6^{a}	15.4 ± 2.3^{bcd}	58.5 ± 0.1^{a}	23.5 ± 1.3^{cd}	0.58 ± 0.00^{d}	13.4 ± 0.9^{c}	0.82 ± 0.00^{a}	11.0 ± 0.8^{b}
Ha_{DTT}	DTT	0.5	3.3 ± 0.6^{a}	28.9 ± 2.1^{e}	58.8 ± 0.7^{a}	35.8 ± 1.9^{a}	0.58 ± 0.01^{d}	21.0 ± 1.4^{d}	0.84 ± 0.01^{a}	17.6 ± 1.2^{a}
Ha_{NEM}	NEM	0.5	2.0 ± 0.0^{a}	11.1 ± 1.5^{abc}	31.5 ± 4.2^{c}	9.9 ± 0.9^{f}	0.24 ± 0.01^{a}	2.5 ± 0.2^{a}	0.55 ± 0.01^{c}	1.3 ± 0.1^{d}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Table 5.1.1.5 – Force and distance at rupture, WHC and relative protein solubility in different media of the sea bass gels*.

Sample	Chemical probe	MTGase (%, w/w)	Force at rupture (N)	Distance at rupture (mm)	WHC (%)	Protein solubility in SDS+DTT (%)	Protein solubility in SDS+U (%)	Protein solubility in DTT+U (%)
$SB0_{CTL}$	---	0.0	70.3 ± 14.1^{a}	16.9 ± 0.0^{bc}	69.9 ± 2.3^{a}	110.5 ± 6.2^{a}	103.8 ± 10.6^{a}	108.2 ± 0.8^{a}
$SB0_{SDS}$	SDS	0.0	53.2 ± 6.3^{a}	14.8 ± 0.6^{ab}	64.8 ± 2.9^{a}	86.1 ± 4.8^{c}	66.1 ± 8.9^{c}	83.4 ± 8.2^{c}
$SB0_{DTT}$	DTT	0.0	76.4 ± 9.0^{a}	17.4 ± 0.5^{bc}	65.9 ± 1.3^{a}	99.0 ± 0.7^{ab}	91.8 ± 2.6^{ab}	94.1 ± 2.4^{b}
$SB0_{NEM}$	NEM	0.0	40.7 ± 1.4^{a}	14.6 ± 1.5^{ab}	66.2 ± 1.0^{a}	107.7 ± 1.0^{a}	96.4 ± 3.5^{ab}	109.7 ± 1.0^{a}
SBa_{CTL}	---	0.5	160.0 ± 14.1^{ab}	19.0 ± 0.0^{c}	65.2 ± 3.3^{a}	82.4 ± 3.6^{c}	81.9 ± 1.1^{b}	54.7 ± 3.8^{d}
SBa_{SDS}	SDS	0.5	165.3 ± 65.8^{ab}	18.9 ± 1.6^{c}	65.7 ± 7.8^{a}	95.8 ± 7.2^{b}	67.7 ± 4.2^{c}	44.6 ± 0.4^{e}
SBa_{DTT}	DTT	0.5	255.4 ± 83.4^{b}	20.0 ± 0.0^{c}	70.5 ± 2.2^{a}	107.9 ± 6.9^{a}	97.2 ± 10.6^{ab}	54.8 ± 1.2^{d}
SBa_{NEM}	NEM	0.5	28.7 ± 2.3^{a}	13.4 ± 0.3^{a}	62.9 ± 5.1^{a}	93.1 ± 7.5^{b}	89.3 ± 5.0^{ab}	93.6 ± 1.6^{b}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Table 5.1.1.6 – Force and distance at rupture, WHC and relative protein solubility in different media of the hake gels*.

Sample	Chemical probe	MTGase (%, w/w)	Force at rupture (N)	Distance at rupture (mm)	WHC (%)	Protein solubility in SDS+DTT (%)	Protein solubility in SDS+U (%)	Protein solubility in DTT+U (%)
$H0_{CTL}$	---	0.0	28.7 ± 2.8^{bc}	12.3 ± 0.6^{bc}	57.1 ± 1.8^{a}	90.0 ± 3.3^{c}	75.4 ± 1.7^{a}	49.6 ± 9.0^{a}
$H0_{SDS}$	SDS	0.0	14.7 ± 4.7^{a}	10.4 ± 0.9^{b}	49.8 ± 2.3^{cde}	105.1 ± 3.8^{a}	89.1 ± 1.5^{b}	71.8 ± 8.6^{c}
$H0_{DTT}$	DTT	0.0	19.7 ± 2.3^{ab}	11.0 ± 1.2^{b}	46.3 ± 1.0^{e}	106.3 ± 2.5^{a}	96.6 ± 1.0^{c}	55.6 ± 4.9^{ab}
$H0_{NEM}$	NEM	0.0	11.0 ± 0.3^{a}	7.9 ± 0.3^{a}	48.4 ± 2.2^{de}	98.4 ± 2.9^{b}	90.5 ± 3.3^{b}	56.9 ± 1.5^{ab}
Ha_{CTL}	---	0.5	32.7 ± 0.4^{c}	12.5 ± 0.3^{bc}	51.7 ± 1.5^{bcd}	107.1 ± 2.2^{a}	81.2 ± 0.4^{ab}	45.9 ± 0.9^{a}
Ha_{SDS}	SDS	0.5	38.3 ± 4.7^{c}	13.5 ± 0.6^{cd}	54.5 ± 1.2^{ab}	106.4 ± 1.4^{a}	79.9 ± 1.5^{a}	62.2 ± 2.5^{b}
Ha_{DTT}	DTT	0.5	70.2 ± 1.2^{d}	15.6 ± 0.0^{d}	53.7 ± 1.4^{abc}	91.0 ± 4.5^{c}	107.9 ± 1.4^{d}	67.4 ± 2.9^{bc}
Ha_{NEM}	NEM	0.5	10.7 ± 0.4^{a}	7.9 ± 0.3^{a}	48.9 ± 0.9^{de}	110.2 ± 2.4^{a}	99.5 ± 3.9^{c}	66.2 ± 4.4^{bc}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

None of the additives had any effect upon WHC of sea bass products (Tables 5.1.1.5 and 5.1.1.6). However, all additives reduced the WHC of the hake gels without MTGase (DTT addition had the most negative effect). On the other hand, with hake products containing MTGase, no effect was detected. On the whole, MTGase incorporation had no clear effect. This differs from other studies, which have found a favourable action of MTGase on WHC/extracted water (Ramírez et al., 2007b; 5.1.5). However, in some products, no significant effect of MTGase on water-binding properties has been reported (Pietrasik and Jarmoluk, 2003). So, it seems that raw material characteristics (protein quality) may lead to different effects of the MTGase on WHC.

Protein solubility values in different extracting media are presented in Tables 5.1.1.5 and 5.1.1.6. All these percent values are compared to solubility in SDS+DTT+U (100 %). For sea bass products and in the absence of MTGase, the solubility of samples containing SDS had a substantial reduction when extracted with SDS+DTT (86.1 ± 4.8 %) and DTT+U (83.4 ± 8.2 %), but even more with SDS+U (66.1 ± 8.9 %). Hence, DTT addition to SDS+U largely increases solubility, showing the importance of the disulphides bridges (Table 5.1.1.1). For sea bass products containing MTGase, different effects were observed: the largest solubility reductions were attained with the removal of SDS for all samples. For hake, regardless of the incorporation of MTGase, the removal of SDS had the largest effect, followed by that of DTT and that of U. MTGase addition caused significant ($p<0.05$) solubility reductions only in sea bass products.

Electrophoresis profiles of the soluble proteins of all gel products in two extracting media, SDS+DTT and DTT+U, are shown in Figures 5.1.1.1 and 5.1.1.2. Differences were only detected for protein extracted with DTT+U. MTGase caused the disappearance of high molecular weight (HMW) protein bands, namely, bands assigned to MHC and M- and C-proteins. However, this effect of MTGase was not observed in the sample containing NEM, since its profile was similar to that of the samples without MTGase.

Whereas, no MTGase effect on protein solubility was found for hake gels, incorporation of MTGase in sea bass gels reduced protein solubility in all extracting media, but especially in DTT+U. This effect on solubility can be related to cross-linking of MHC during setting (Ashie and Lanier, 1999). However, this does not entirely explain the phenomenon, because all solubility values are relative to SDS+DTT+U and, accordingly, exclude the covalent bonds catalyzed by MTGase. A comparison of the sea bass electrophoretic profiles in SDS+DTT and DTT+U shows that while in SDS+DTT the HMW bands of the gels containing MTGase are largely preserved, in DTT+U these same bands disappear with MTGase addition. So, from these results it follows that MTGase action favoured hydrophobic and, to a lesser degree, other interactions (hydrogen bonds and disulphide bridges) in sea bass gels. In fact, if MTGase direct action (covalent bonds) were the sole

underlying phenomenon, then MTGase should have caused a similar disappearance of HMW bands in SDS+DTT. Taking into account that SDS targets non-covalent bonds, but U predominantly targets hydrogen bonds (Liu and Hsieh, 2008), the contrast between DTT+SDS and DTT+U must be ascribed predominantly to hydrophobic bonds. Therefore, this suggests that MTGase may favour the establishment of hydrophobic interactions in sea bass gels, thus, its direct catalytic action is compounded by indirect effects. Moreover, this idea is reinforced by the SBa_{NEM} product, whose solubility in SDS+U or DTT+U was almost identical to that in SDS+DTT+U and whose electrophoretic profile in DTT+U contained all HMW bands. This means that the inhibition of MTGase by NEM reduced the importance of non-covalent interactions and disulphide bridges.

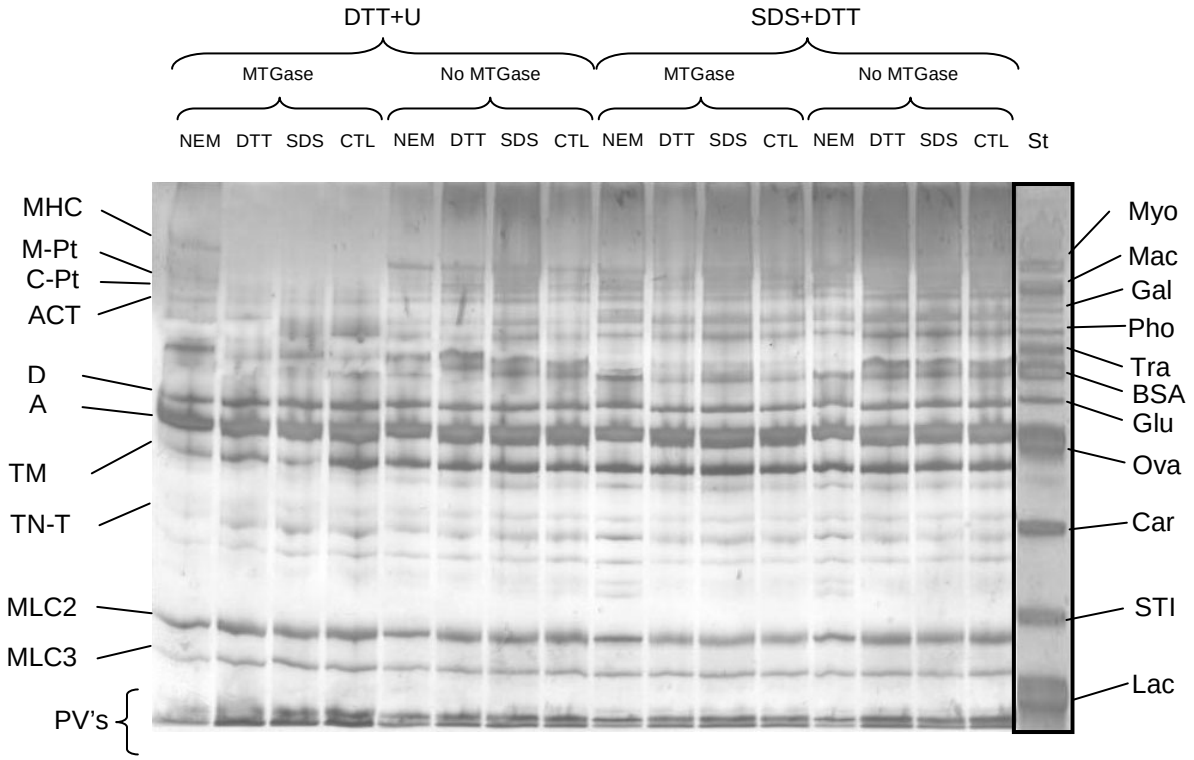

Figure 5.1.1.1 – 15 % Excel-Gel SDS-PAGE of soluble protein homogenates in DTT+U and SDS+DTT of the sea bass gels. MHC, myosin heavy chain; M-Pt, M-protein; C-Pt, C-protein; ACT, actinin; D, desmin; A, actin; TM, tropomyosin; TNT, troponin-T; MLC2, myosin light chain 2; MLC3, myosin light chain 3; Pv's, parvalbumins. Standard names and molecular weights: Myo, myosin (212.0 kDa); Mac, α2-macroglobulin (170.0 kDa); Gal, β-galactosidase (116.0 kDa); Pho, phosphorylase B (94.0 kDa); Tra, transferrin (76.0 kDa); BSA, bovine serum albumine (67.0 kDa); Glu, glutamic dehydrogenase (53.0 kDa); Ova, ovalbumin (43.0 kDa); Car, carbonic anhydrase (30.0 kDa); STI, soybean trypsin inhibitor (20.1 kDa); Lac, α-lactalbumin (14.4 kDa).

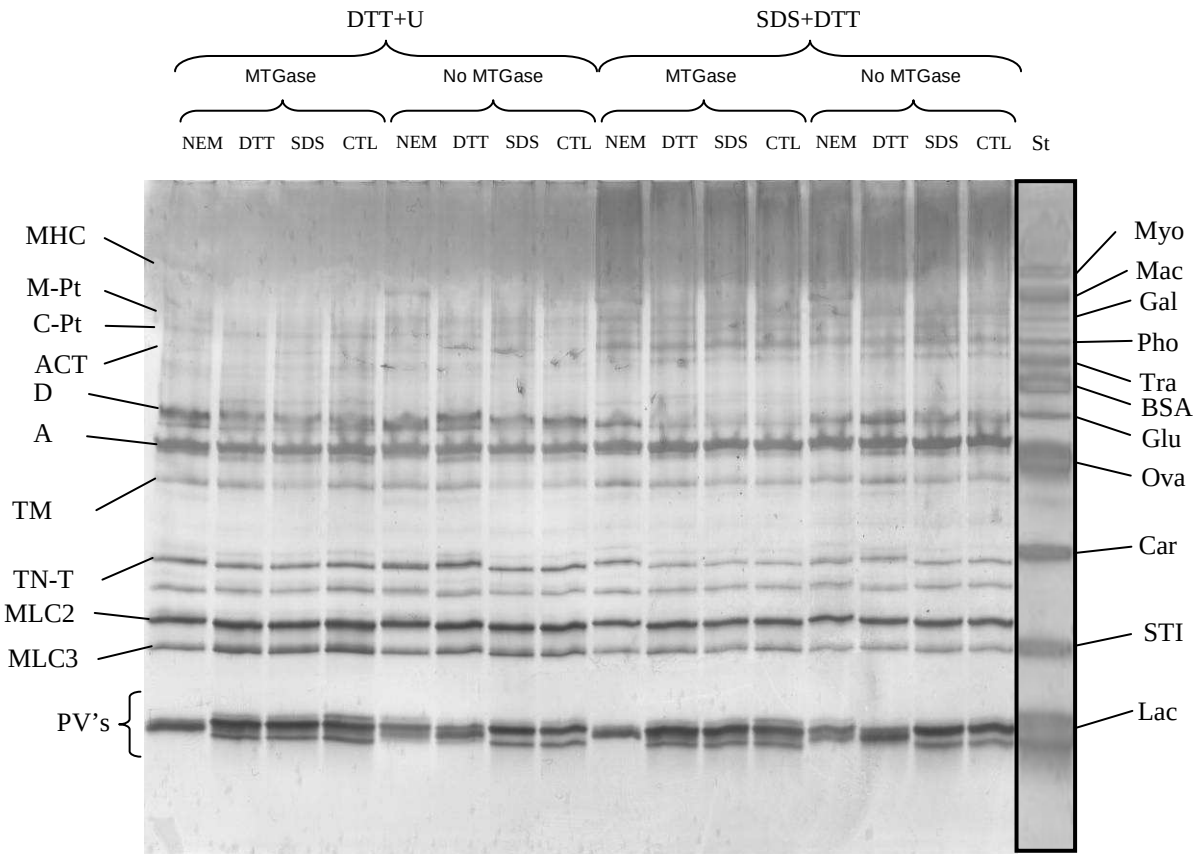

Figure 5.1.1.2 – 15 % Excel-Gel SDS-PAGE of soluble protein homogenates in DTT+U and SDS+DTT of the hake gels. Standard proteins as in Figure 5.1.1.1.

Gels microstructure was analyzed by SEM. MTGase incorporation in sea bass products had a more clear effect than any of the additives (Figure 5.1.1.3), since it favoured a more homogeneous and porous microstructure, with more evenly distributed pores ($SB0_{CTL}$ *vs* SBa_{CTL}). Other authors (Tammatinna et al., 2007) also reported a more ordered network structure with MTGase addition. Gels containing SDS and DTT had similar microstructures that did not differ much from the control, except for more uniformity with DTT incorporation (SBa_{CTL} *vs* SBa_{DTT}). Moreover, NEM incorporation caused some loss of uniformity and organization.

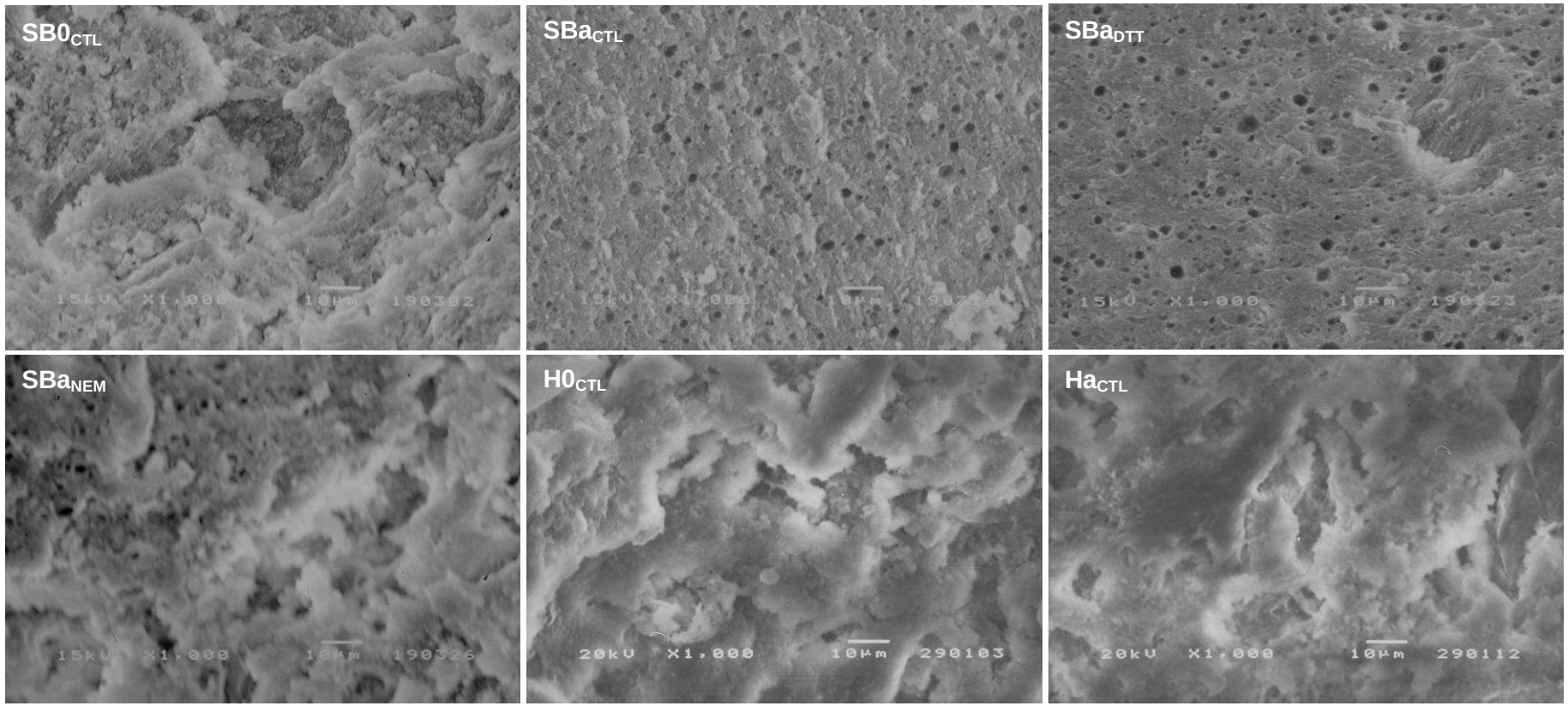

Figure 5.1.1.3 – Scanning electron microscopy (SEM) micrographs of some of the produced gels: $SB0_{CTL}$, SBa_{CTL}, SBa_{DTT}, SBa_{NEM}, $H0_{CTL}$ and Ha_{CTL}.

Raw Material Effect

Concerning proximate composition, whereas ash (3 %) and protein (16-18 %) contents of the gels were similar, the average moisture of the sea bass and hake gels differed, approximately 73 and 78 %, respectively. The higher moisture of the hake products was coupled with a low fat content of only 1 % and sea bass products had a fat level of 7 %.

The positive textural impact of MTGase was detected in both sea bass and hake products. However, the effect was different in each species (Tables 5.1.1.3-6). Namely, GS was increased much more in sea bass than in hake products. The MTGase effect of increasing the force at rupture was only significant ($p<0.05$) for sea bass samples. On the other hand, while the hardness of sea bass products was unaffected by MTGase, a toughening effect due to MTGase was found with hake samples. Finally, the overall comparison between fish species showed that the texture of sea bass gel products was much better than that of hake gel products. This difference was less important without MTGase incorporation (for instance, the folding test and GS of both products were similar), but was enhanced by MTGase. For WHC, raw material type also had influence, since the WHC of sea bass gels was quite higher ($p<0.05$) than that of hake gels.

Therefore, the quality of sea bass gels was better than that of hake gels, either without or with MTGase. Moreover, MTGase addition improved the texture of sea bass products much more, namely, their FT, GS and force at rupture. Only the hardness of hake gels was improved by MTGase. These two aspects, the better texture of sea bass gels and the differences concerning the action of MTGase are intertwined. In the literature, similar phenomena have been reported: some authors found an effect of MTGase, mainly upon deformability (Moreno et al., 2009), which has been related to a higher degree of compactness or density of the actomyosin network formed (Lee and Chung, 1989), others reported an increase in breaking force or shear stress, without affecting shear strain, for instance, in gels from Pacific whiting (Lee and Park, 1998), carp (Tsukamasa et al., 2000) or mackerel surimi (5.1.5). Precisely, one advantage of these additives (SDS, DTT or NEM) as chemical probes is to offer extra insight in to these matters. Each additive had a different effect on the products according to used raw material. Without MTGase, textural quality of hake gels was more sensitive to each additive. This indicates the important role of non-covalent interactions and S-S bridges in hake.

Moreover, the importance of hydrophobic bonds as assessed by the comparison between SDS+DTT+U and DTT+U was higher in hake than in sea bass products. This was due to a very marked solubility reduction in DTT+U for hake products without MTGase. For electrophoresis, solubility in SDS+DTT of hake samples led to slight changes as a result of additive or MTGase incorporation. But, for DTT+U, the addition of NEM (with or without MTGase) prevented the disappearance of the HMW bands. These

bands were much fainter in the profiles of the other samples, regardless of the incorporation of MTGase. In contrast, for sea bass samples, HMW bands were preserved in the absence of MTGase.

Effectively, the sensitivity to NEM combined with the electrophoretic profile of $H0_{NEM}$ (which in contrast to $H0_{CTL}$, $H0_{SDS}$ and $H0_{DTT}$, maintained its HMW bands) indicates an active endogenous TGase in hake. However, the texture of hake gels is worse than the texture of sea bass gels, in spite of the activity of this enzyme in hake. The electrophoretic profiles of hake gels without MTGase in DTT+U also contrast with the equivalent ones for sea bass gels, reinforcing the idea that hydrophobic interactions between proteins are more important in hake than in sea bass. As discussed above, whereas MTGase in hake products hardens the gels without altering protein solubility, it improves the deformability of sea bass gels and reduces protein solubility, thereby indicating a greater role of non-covalent (particularly, hydrophobic) and disulphide bonding. Effectively, some authors (Havea et al., 2004) have claimed that S-S bonding is related to higher deformability. However, other authors (Pérez-Mateos et al., 1997) have associated S-S bonding to higher hardness and lower elasticity. Nonetheless, the different effects of MTGase on each product as a function of raw material remain unexplained, since the indirect promotion of S-S bonding by MTGase solely in sea bass gels has not been elucidated. Moreover, according to some studies (Havea et al., 2009), the greater importance of the non-covalent (mainly hydrophobic) bonding should harden sea bass gels (with MTGase addition).

The formation of protein gels during heating was studied by recording the continuous changes in the viscoelastic properties of the sea bass and hake batters at small deformation (DRM), thus avoiding the problems with gel fracture during measurement. The storage modulus, G', a measure of gel rigidity, was followed during the heating cycle (Figure 5.1.1.4). Only the rheological curves of the products without MTGase are shown, since no important changes occurred with MTGase addition, with the exception of higher G' levels. The general behaviour of the curves was similar. There was an increase of G' during the initial heating phase, followed by a stagnation or even reduction of G' and a new and stronger upward trend, which was retained (with some loss of rate of increase in some cases) during the holding phase at 85 ºC. However, there were important differences regarding G' level and G' *vs* temperature. Whereas, for sea bass batters (Figure 5.1.1.4A), strong initial increases of G' from 20 to 30-35 ºC were followed by steep decreases until the 40-50 ºC range, for hake (Figure 5.1.1.4B), initial increases were slight and the downward slope was less intense. Afterwards, the G' of sea bass samples increased with a steeper slope than hake samples, but subsequently showed inflexion points at 70-78 ºC (lower slopes) and at the beginning of the holding phase (higher

slopes). Moreover, G' values were lower for sea bass samples. For hake samples, G' values were higher for the control and lower with DTT and NEM, with the SDS curve between the other curves. The control, SDS and DTT were characterized by a clear decreasing phase, with minima at 44.2, 40.9 and 42.8 ºC, respectively. As with sea bass batters, the DTT curve mimicked the control, in spite of varying within a lower range. The NEM curve was the most atypical, with no significant decreasing phase and a much lower slope in the following phase.

Hence, rheological curves also showed important differences between hake and sea bass. The initial increase of the G' up to 37 ºC, which is associated to the setting phenomenon (Gómez-Guillén et al., 1997b), was much steeper in the sea bass batters. This suggests a larger contribution of the cross-linking between proteins to the viscoelasticity of the products. For hake, this contribution seemed less important, in spite of the TGase activity, whose inhibition by NEM may explain the atypical curve of $H0_{NEM}$. Afterwards, the reduction of G', related to the denaturation of myosin molecules as α-helices in the tail portion unfold (Romero et al., 2009), was clearer in the sea bass samples. This indicates difficulty in the unfolding of hake proteins. The third phase, characterized by a continuous increase of G', is connected to protein aggregation (Romero et al., 2009). In the literature, this aggregation is considered to be dependent on the oxidation of sulfhydril groups (Acton and Dick, 1989). This phase is also relatively more pronounced for sea bass gels. However, the proposed explanation based on the oxidation of sulfhydril groups is not corroborated by the sea bass curves, since the slopes of $SB0_{DTT}$ and $SB0_{NEM}$ are almost identical to $SB0_{CTL}$ (DTT disrupts the S-S bonding and NEM inhibits the formation of new S-S bonds). For hake, $H0_{DTT}$ and $H0_{NEM}$ showed less steep slopes than the other two samples. So, it seems that, while S-S bonding augmented during heating of hake gels, in the sea bass gels neither S-S nor non-covalent bonding (similar slopes of $SB0_{CTL}$ and $SB0_{SDS}$) had an important role on protein aggregation.

Regarding SEM, sea bass gels were more porous and uniform than hake gels. This difference between raw materials was clearer with MTGase, since the microstructure of hake products was unaffected by the addition of the enzyme ($H0_{CTL}$ *vs* Ha_{CTL}).

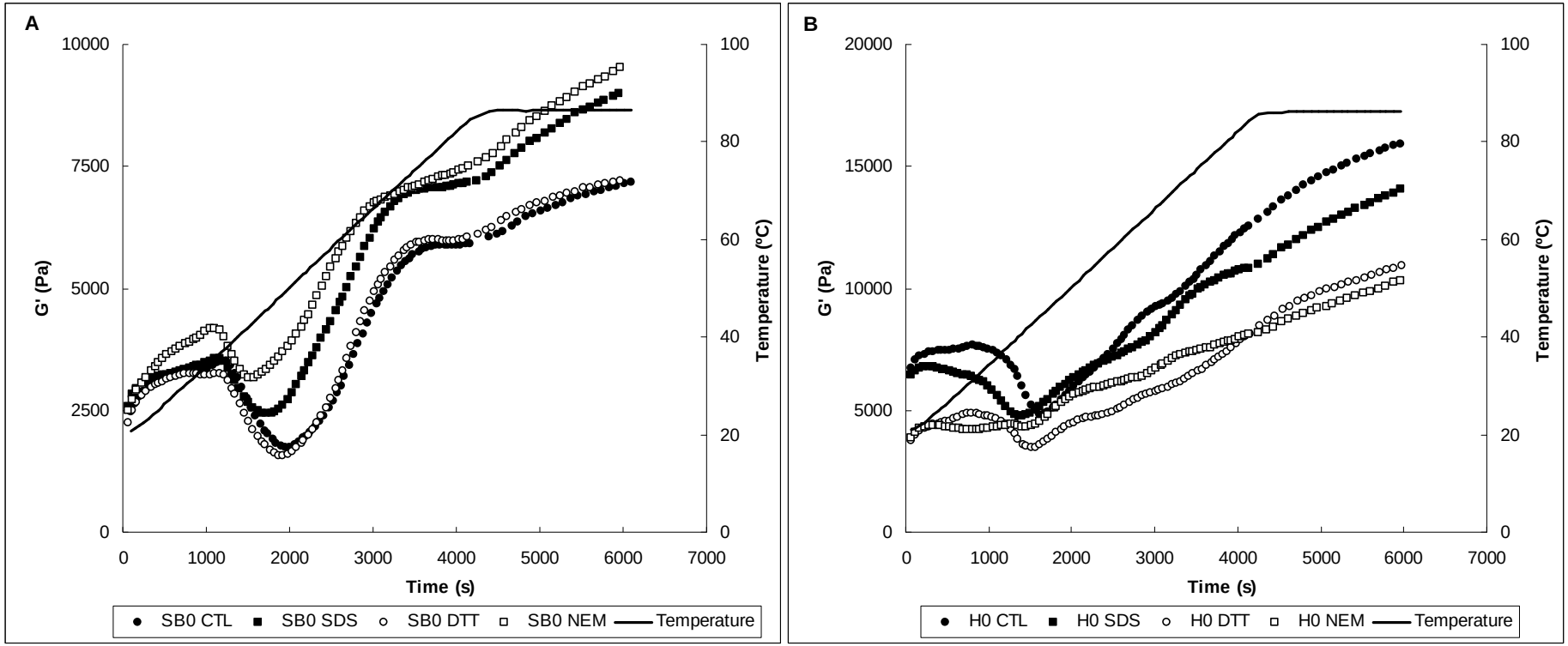

Figure 5.1.1.4 – Changes in the rheological properties of the sea bass (A) and hake (B) batters during heating. Batters with composition identical to the prepared batches were heated from 20 to 85 ºC at 1 ºC/min and held at 85 ºC for 30 min. G' values were measured every minute. Measuring conditions: oscillatory mode, frequency of 1 Hz and shear stress of 100 Pa.

All these results taken together show the inadequacy of a simple association between stiffness and non-covalent (hydrophobic) bonding or between deformability and S-S bonding (Havea et al., 2009) as explanation for the phenomena. Particularly, the different effect of MTGase on sea bass and hake is unexplained. It can be hypothesized that MTGase operates differently in each raw material. For hake, MTGase catalyzes the cross-linking between the small number of Gln and Lys residues exposed at the surface of proteins (DRM showed less unfolding of hake proteins) and, as such, the covalent ε-amino-(γ-glutamyl)-lysine bonds only link a small number of molecules and have a shorter distance effect. For sea bass, due to a greater unfolding of proteins, almost all Gln and Lys residues become exposed and the MTGase is able to link a large number of these residues, thereby uniting a large number of molecules and succeeding in the establishment of a vast network structure (as also shown by SEM). This difference in the action of MTGase is more important than any variation in hydrogen, hydrophobic or S-S bonding. This is shown by the absence of a correlation between hydrophobic bonding and hardness in sea bass and hake gels, which were hardened by MTGase, without any variation in the solubility in DTT+U. Finally, the reason for a lower degree of protein unfolding in hake as well as for a lower WHC (which also results from the insufficient unfolding and the consequent failure of network structure formation) is to be found in the denaturation of protein due to frozen storage. Frozen storage prior to processing is relevant, since hake was frozen and sea bass not. In fact, according to another study, breaking force and deformation of surimi gels produced from frozen fish decreased continuously with increasing time (mainly up to 10-12 weeks) of frozen storage (Benjakul et al., 2005). Moreover, the decline in gel-forming ability was associated with the decrease in Ca^{2+}-ATPase activity and with formaldehyde formation (from the decomposition of trimethylamine N-oxide). This may have contributed to the formation of intramolecular covalent bonds, which were left unscathed by the used chemicals. Future research should also use other additives for the targeting of these bonds.

Conclusions

The additives as chemical probes were a valuable tool for reaching conclusions regarding the underlying molecular phenomena in the studied protein gels. MTGase enhanced deformability of sea bass gels and hardened hake gels. Furthermore, the action of an endogenous TGase is presumed to be important in hake gels. This work strongly suggests that frozen storage and associated protein denaturation are fundamental for explaining observed differences between raw materials or the different effects of MTGase on each raw material. Finally, this study may find practical application, for instance, the positive interaction between MTGase and the disruption of disulphide bonds in hake gels

suggests a combination of cysteine and MTGase for the amelioration of poor quality hake products.

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5.1.2- Dietary Fibre's Effect on the Textural Properties of Fish Heat-Induced Gels

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Dietary Fibers' Effect
on the Textural Properties
of Fish Heat-Induced Gels

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ABSTRACT. Fish heat-induced gels obtained from hake, containing one of five different dietary fibers (apple fiber, inner and outer pea fiber, chicory root oligofructose and inulin), were evaluated with respect to textural properties. Furthermore, the effect of different fiber product content (2 and 4%, w/w) on the same properties was investigated. The aim of this research was to produce a fish heat-induced gel product that could increase human fiber intake while retaining good texture properties.

Addition of inner pea fiber to minced fish products--up to 4% (w/w)--was beneficial as the products retained their textural properties similar to those without any fiber. The fiber product containing chicory root inulins also generated positive results with the exception of its adverse effect on the product's hardness. doi:10.1300/J030v16n03_03 *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress.com> © 2007 by The Haworth Press, Inc. All rights reserved.]*

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Abstract

Fish heat-induced gels obtained from hake, containing one of five different dietary fibres (apple fibre, inner and outer pea fibre, chicory root oligofructose and inulin), were evaluated with respect to textural properties. Furthermore, the effect of different fibre product content (2 and 4%, w/w) on the same properties was investigated. The aim of this research was to produce a fish heat-induced gel product that could increase human fibre intake while retaining good texture properties.

Addition of inner pea fibre to minced fish products —up to 4% (w/w)— was beneficial as the products retained their textural properties similar to those without any fibre. The fibre product containing chicory root inulins also generated positive results with the exception of its adverse effect on the product's hardness.

Keywords: Minced fish, hake**,** pea fibre, chicory fibre

Introduction

The fish processing industry generates considerable amounts of waste, containing good quality proteins and other nutrients. These wastes originate mostly in fish filleting operations and offer a potential use as human food instead of being used in the production of fish meal for animal feed. In an effort to use such wastes, various methods of restructuring low-value cuts and trimmings involving recovery of meat as mince and new food ingredients have been developed to improve the appearance and textural properties.

Because of its considerable nutritional value, one of the food ingredients commonly used in the design of functional foods is dietary fibre, DF (Puupponen-Pimïa et al., 2002).

DF is defined as lignin plus the polysaccharide components of plants that are indigestible by enzymes in the human gastrointestinal tract (Bennink, 1994). The components of DF include cellulose, hemicellulose, pectins, hydrocolloids, lignin and oligosaccharides such as the inulins. With respect to water solubility these components are typically divided into two groups: soluble (pectic substances, inulins and hydrocolloids) and insoluble (cellulose, hemicellulose and lignin).

To assess the potential acceptability of new products by consumers, textural properties are of great importance. The texture of fish products varies as a function of species and processing methods. In the preparation of products based on the gelation of myofibrillar protein, additives are commonly used to modify texture properties. These additives produce structural changes in the protein matrix, depending on their composition, distribution, physical state, volume fraction and interaction with the continuous protein matrix (Okada, 1974; Filipi and Lee, 1998).

It was found that the addition of different DF to fish products generated texturally diverse products (Montero et al., 2000). It can be mentioned that addition of xanthan gum at 0.5% caused a considerable reduction in hardness compared to control (Montero et al., 2000). On the other hand, amidated low methoxyl (ALM) pectin was used in fish products with positive results on the mechanical properties, in particular, hardness increased by adding 10 g/kg ALM pectins (Ramírez et al., 2007a). Among the various possible fish species, hake has been among those tried, for instance, it was found that addition of wheat fibre to minced hake increased water holding capacity albeit reducing chewiness (Sánchez-Alonso et al., 2007a). Many other fibres have been added to fishery products: chitosan, gums, hydrocolloids (Borderías et al., 2005). Furthermore, there are other advantages associated with DF addition to fish products, for instance, prevention of lipid oxidation in minced fish through incorporation of grape antioxidant dietary fibre (Sánchez-Alonso et al., 2007b).

Therefore, to determine the effect of the addition of DF on minced hake products, their textural properties were determined.

Material and Methods

Raw materials and additives

To obtain a consistent raw material, frozen head and gutted South African hake (*Merluccius capensis*) —with approximately 30 days storage at -20ºC and weighing on average 1.5 kg each— was obtained from a local fish processor and stored at –28 ºC until processed within three to four weeks after its arrival at the laboratory. Five different hake batches were used in the experiments. These were received in July, September, October, November and December of 2004.

Five different dietary fibres were used. Four were supplied by Cosucra (Warcoing, Belgium): inner pea fibre-Swelite®; outer pea fibre-Exafine® 250; chicory root oligofructose-Fibrulose 97 and chicory root inulin-Fibruline INSTANT. The other fibre product, apple fibre-Vitacel AF 401, was supplied by Rettenmaier (Holzmühle, Germany). The composition (based on dry matter, D.M.) and other properties are shown in Table 5.1.2.1.

Microbial transglutaminase (MTGase) ACTIVA® was supplied by Ajinomoto Japan, Inc. (Tokyo, Japan) as a mixture containing 99 % maltodextrine and 1 % transglutaminase. The transglutaminase activity was approximately 67 ± 10 units.g^{-1} (Folk, 1970).

Table 5.1.2.1 – Properties of the five dietary fibre products*.

PROPERTIES	Swelite®	Exafine® 250	Fibrulose 97	Fibruline INSTANT	Vitacel AF 401
Composition (D.M.)					
Total Carbohydrates (%)	93 ± 3	min. 85	min. 99.7	min. 99.7	—†
Total DF (%)	48 ± 3	min. 85	97 ± 2	min. 90	55 ± 5
Starch (%)	min. 36	max. 5	—	—	—
Protein (%)	max. 7	max. 6.5	—	—	4.6
Fat (%)	max. 0.5	max. 0.5	—	—	2.5
Ash (%)	max. 2	max. 3	max. 0.3	max. 0.3	max. 3
Granulometry (µm)	< 400	85 %< 300	< 700	< 700	90 %< 300
Colour	white	cream	white	white	red brown
Taste	neutral	slight vegetable	neutral to slightly sweet	neutral to slightly sweet	fruity

* Values are manufacturer's claims (Anonymous, 2004a; Anonymous, 2004b).
† not analysed.

Reagents

All chemicals used were of analytical grade and were obtained from Merck KGaA (Darmstadt, Germany).

Experimental design

There were two sets of experiments: the first, encompassing five independent experiments, dealt with the effect of 2 and 4 % w/w of the five different DF products on the texture of fish mince. These experiments were done with four different hake batches: the first experiment, involving Swelite, used a batch received in July 2004; the Exafine experiment, another one received in September 2004; the third, with Fibruline, an October 2004 batch and, finally, the experiment conducted with Fibrulose and Vitacel used a batch received in November 2004. The second set, comprising one single experiment, involved a general textural comparison between hake products containing equal amounts (%, w/w) of the various dietary fibres. For this experiment a hake batch received in December 2004 was used. All experiments included a control (fish mince only) to minimize the possible variability arising from different fish batches.

Production of hake heat-induced gel products

Six to 12 kg of frozen hake were thawed overnight in a refrigerator and skin and bones were removed manually. The resulting fish flesh was minced once in a model 84145 meat grinder (Hobart, Troy, OH, USA) with a 6 mm plate. The appropriate quantities of fish mince (Table 5.1.2.2) were weighed to guarantee final hake heat-induced gel products with the same water content —equal to the previously determined hake moisture value (AOAC, 1984)— regardless of the quantity of added fibre (more fibre entailed a greater addition of water and, for a constant total weight of all ingredients, a smaller amount of mince). Fish mince was mixed with previously hydrated fibre (chilled water was added to the dry dietary fibre in a ratio 4:1), salt (2.5% of the final product weight) and MTGase

(0.1%) in a model UM12 refrigerated vacuum homogenizer (Stephan and Söhne, Hameln, Germany). The mixing was done for 5 min (2 min at 1420 r.p.m. followed by 3 min at 2800 r.p.m.) under vacuum and refrigeration (temperature below 7ºC). The resulting batters were packed into stainless steel tubes (diameter 30 mm, length 30 mm). The tubes were capped and immersed in a 35 ± 1 ºC thermo-regulated water bath for 1 hour for setting, then moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik, Landsberg am Lech, Germany) equipped with a digital thermometer and steam cooked at 90 ºC for 1 hr (cooking). The heat-induced gels were cooled immediately in ice water and kept in a refrigerator overnight until analysis.

Table 5.1.2.2 – Quantities of ingredients used in the manufacturing of the various hake heat-induced gel products.

INGREDIENTS	1st Set of experiments Dietary fibre level (%, w/w)			2nd Set of experiments Dietary fibre level (%, w/w)	
	0	2	4	0	4
Fish mince (g)	1500.0	1338.0	1176.0	1500.0	1176.0
Dry dietary fibre (g)	0.0	30.0	60.0	0.0	60.0
Water added (g)	0.0	132.0	264.0	0.0	264.0
Salt (g)	38.5	38.5	38.5	38.5	38.5
MTGase (g)	1.5	1.5	1.5	1.5	1.5
Total (g)	1540.0	1540.0	1540.0	1540.0	1540.0

Texture measurements

Folding Test. The test piece was a 3 mm thick slice cut from the gel cylinder (after removal from the steel tube). The evaluation was performed in accordance with the standard 5-point grade system as follows: Grade 5, no crack when folded into quadrants; Grade 4, no cracks when folded in half; Grade 3, crack develops gradually when folded in half; Grade 2, crack develops immediately when folded in half; Grade 1, crumbles when pressed with a finger.

Puncture test. Prior to analysis, samples were tempered to about 20 ℃, removed from the tube and cut into pieces 25 mm high. The gel was penetrated to the breaking-point with a metal probe equipped with a 5 mm diameter spherical head, using a model Instron 4301 texturometer (Instron Engineering, Canton, MA, USA). The crosshead speed was 10 mm/min and the load cell was 1 kN. Breaking force (N) and breaking deformation (mm) were measured. Gel strength (N.mm) was determined by multiplying these two parameters.

Compression tests. For texture profile analysis, samples (the same as for the puncture test) were compressed with a cylindrical plunger (50 mm diameter) adapted to a 1 kN load cell at a deformation rate of 50 mm/min. On the basis of preliminary trials to establish a compression limit that would ensure recoverability of samples without rupture, it was

decided to compress samples to 60 % of their height. In the test, each sample was compressed twice. The following parameters were determined: hardness (N), maximum height of first peak on first compression; cohesiveness (A_2/A_1), ratio of second-compression to first-compression positive areas; gumminess (N), product of hardness and cohesiveness; springiness (L_2/L_1), the ratio of the detected height of the product on the second compression to the original compression distance; chewiness (N), the product of gumminess and springiness.

For the compression-relaxation test, the compression procedure was the same as for the texture profile analysis except that the sample was compressed only once for one min and the force exerted on the sample recorded. Relaxation (%) was calculated as $Y_T=100\times(F_0-F_1)/F_0$, where F_0 is force registered at the onset of relaxation immediately after sample compression and F_1 is force registered after one min of relaxation. Thus, $(100-Y_T)$ is taken as an index of elasticity and is expressed as the percentage elasticity of the gel.

Statistical analysis

Folding test determinations were performed in duplicate, the gel strength results are the average of six measurements and all other determinations were done in triplicate. A general linear model (one-way ANOVA) was used to determine significant differences ($p<0.05$) among minced fish products with different added fibre and different levels of each fibre. Significant differences were established by the Tukey HSD test. All statistical treatment was done with the STATISTICA© software from StatSoft (Tulsa, OK, USA), version 6.1, 2003.

Results and Discussion

Given the great importance of the products' moisture for the textural properties, the comparison of fibre performance was done in formulations of equal moisture content (Pérez-Mateos and Montero, 2000).

Effect of fibre content

In the first trials the effects associated with the addition of different amounts of a specific fibre were analysed. All five fibre products were used at 2 and 4 %. The textural parameters (Table 5.1.2.3) showed some statistically significant trends.

Table 5.1.2.3 – Textural properties of fish heat-induced gel products containing different amounts of fibre*.

ADDED FIBRE	Fibre Level (%w/w)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
Swelite®	0	3.7 ± 0.6^{a}	45.9 ± 7.6^{a}	55.1 ± 0.4^{a}	45.2 ± 3.8^{a}	0.61 ± 0.01^{a}	28.3 ± 2.5^{a}	0.84 ± 0.01^{a}	23.8 ± 2.1^{a}
	2	4.3 ± 0.6^{a}	38.3 ± 4.8^{ab}	56.6 ± 0.7^{a}	42.0 ± 4.2^{a}	0.62 ± 0.06^{a}	25.3 ± 1.5^{a}	0.83 ± 0.04^{a}	21.0 ± 1.9^{a}
	4	3.3 ± 0.6^{a}	30.2 ± 3.6^{b}	54.8 ± 4.0^{a}	41.0 ± 5.6^{a}	0.60 ± 0.03^{a}	24.2 ± 3.4^{a}	0.81 ± 0.01^{a}	19.6 ± 2.9^{a}
Exafine® 250	0	3.0 ± 0.0^{a}	30.6 ± 5.4^{a}	44.7 ± 2.8^{ab}	48.3 ± 4.1^{a}	0.34 ± 0.02^{a}	16.3 ± 2.3^{a}	n.a.	n.a.
	2	3.0 ± 0.0^{a}	24.9 ± 4.0^{a}	48.4 ± 0.4^{a}	44.2 ± 2.0^{b}	0.31 ± 0.03^{a}	13.9 ± 1.4^{a}	n.a.	n.a.
	4	3.0± 0.0^{a}	18.2 ± 2.1^{b}	40.9 ± 2.4^{b}	32.3 ± 2.4^{c}	0.24 ± 0.05^{b}	7.5 ± 1.7^{b}	n.a.	n.a.
Fibrulose 97	0	3.0 ± 0.0^{a}	28.6 ± 2.2^{a}	46.4 ± 2.8^{a}	44.5 ± 2.3^{a}	0.35 ± 0.03^{a}	15.8 ± 0.4^{a}	n.a.	n.a.
	2	3.0 ± 0.0^{a}	23.1 ± 1.1^{b}	47.5 ± 3.4^{ab}	39.9 ± 3.2^{ab}	0.33 ± 0.02^{a}	13.3 ± 0.4^{b}	n.a.	n.a.
	4	3.0 ± 0.0^{a}	25.3 ± 5.2^{ab}	53.1 ± 0.5^{b}	36.2 ± 4.9^{b}	0.36 ± 0.05^{a}	13.3 ± 0.7^{b}	n.a.	n.a.
Fibruline INSTANT	0	4.0 ± 0.0^{a}	50.1 ± 5.4^{a}	56.7 ± 0.1^{a}	45.5 ± 2.4^{a}	0.63 ± 0.01^{a}	28.7 ± 1.5^{a}	0.91 ± 0.01^{a}	26.1 ± 1.3^{a}
	2	5.0± 0.0^{b}	44.6 ± 11.0^{a}	56.3 ± 0.8^{a}	38.3 ± 4.9^{b}	0.59 ± 0.01^{b}	23.5 ± 0.9^{b}	0.82 ± 0.00^{b}	19.2 ± 0.7^{b}
	4	5.0 ± 0.0^{b}	51.9 ± 13.5^{a}	58.0 ± 0.3^{b}	27.0 ± 2.2^{c}	0.65 ± 0.01^{a}	17.0 ± 0.7^{c}	0.83 ± 0.01^{b}	14.0 ± 0.6^{c}
Vitacel AF 401	0	3.0 ± 0.0^{a}	28.6 ± 2.2^{a}	46.4 ± 2.8^{a}	44.5 ± 2.3^{a}	0.35 ± 0.03^{a}	15.8 ± 0.4^{a}	n.a.	n.a.
	2	3.0 ± 0.0^{a}	31.3 ± 3.1^{a}	43.4 ± 5.5^{a}	40.4 ± 1.1^{b}	0.32 ± 0.05^{a}	13.1 ± 2.1^{ab}	n.a.	n.a.
	4	3.0 ± 0.0^{a}	28.9 ± 2.5^{a}	44.0 ± 3.6^{a}	38.1 ± 3.2^{b}	0.30 ± 0.02^{a}	11.0 ± 1.6^{b}	n.a.	n.a.

* Values correspond to mean ± standard deviation.
For each fibre, means within a column with different letters are significantly different ($p<0.05$).
n.a., not analysed.

Fibruline was the only fibre which had a considerable influence upon the products folding test, raising the scores for both levels from 4.0 to 5.0. Chicory root fibres (Fibruline and Fibrulose) reduced hardness while increasing the elasticity of the gels. However, the latter parameter only increased significantly ($p<0.05$) when 4 % (w/w) of Fibruline or Fibrulose was added. On the other hand, there was a significant decreasing trend for the hardness with increasing Fibruline content. It is also worth mentioning that Swelite was the only DF whose addition did not cause any significant change ($p\geq0.05$) in the hardness or other properties except for a decrease in gel strength.

In general, the addition of fibre gave gels with less favourable properties. However, this is hardly surprising since higher fibre content meant lower protein content in the final product, resulting in less firm gels (Park, 2000). Also, a progressive hardness reduction with increasing fibre concentration has been observed (Tudorica et al., 2002), which could be associated with the disruption of the protein matrix microstructure. Thus, Swelite products are unusual for retaining their hardness values. The positive results achieved with Swelite could be due to its starch content (min. 36 % of dry weight) which is known to promote a firmer and slightly more cohesive gel matrix (Lee and Kim, 1986; Lee et al., 1992). Therefore, Swelite was able to make up for the loss of protein content and its deleterious effect (Park, 2000). However, as mentioned above, gel strength decreased with the addition of more Swelite. An analysis of the effect of Swelite fibre content on breaking force and breaking deformation —the two factors whose product generated the

gel strength results— showed that Swelite containing gels were less deformable and, as a consequence, the force at the breaking point was necessarily lower (Figure 5.1.2.1).

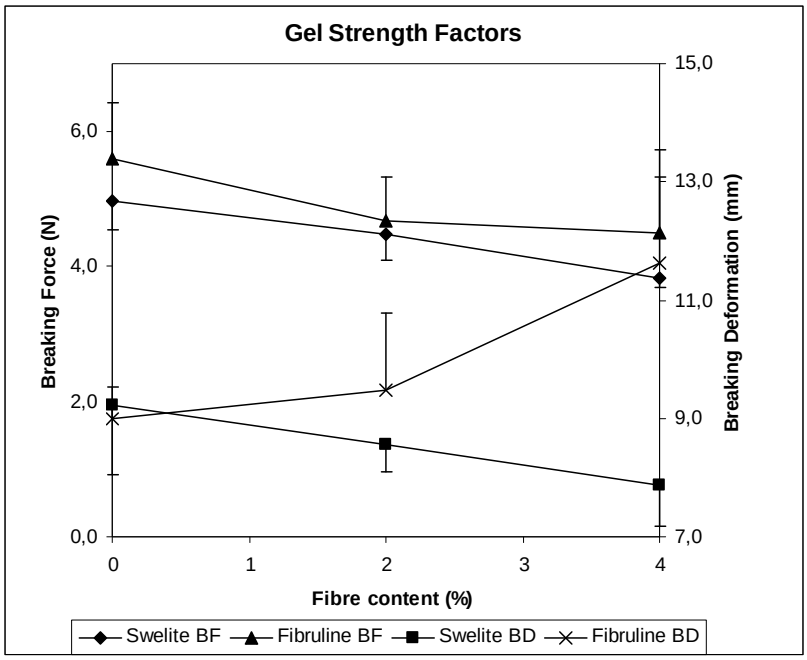

Figure 5.1.2.1 – Effect of Swelite and Fibruline fibre contents on the breaking force and breaking deformation.

It is interesting to note that Fibruline fibre —which was able to maintain the control product's gel strength in spite of producing very soft gels— had a large increase in the breaking deformation values. This trend compensated for the breaking force reduction and explains the gel strength results. Therefore, Fibruline seems to have the opposite effect of Swelite in that it generates soft and deformable gels.

Another important aspect of the study was the great textural variability in the control products (0% fibre (w/w)) obtained from the different hake batches (Table 5.1.2.3). Such variability is due to different storage conditions histories and to different biological conditions at the time of capture. This made it impossible to compare between the various types of fibre in this first set of experiments. Nevertheless, for each particular fibre, it was possible to compare different incorporation levels and, furthermore, for Fibruline and Swelite, the comparison showed clear trends.

Effect of fibre type

The simultaneous comparison between hake heat-induced gel products containing different dietary fibres present at the same level of 4 % (w/w) enabled an assessment of the advantages and drawbacks associated with each fibre. In this way the observed variability of the raw material due to frozen storage did not affect the comparison among different fibres, as it occurred in the fibres content study, where for each fibre there was a

different batch of hake. The various textural properties measured showed some interesting differences among products (Table 5.1.2.4).

Table 5.1.2.4 – Textural properties of fish heat-induced gel products containing 4 % (w/w) of different fibres*.

ADDED FIBRE	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiv.	Gumminess (N)	Springiness	Chewiness (N)
Control (no fibre)	4.7 ± 0.6^{a}	46.3 ± 12.0^{a}	55.2 ± 0.9^{a}	36.5 ± 5.2^{ab}	0.61 ± 0.05^{a}	23.9 ± 1.8^{a}	0.83 ± 0.04^{a}	19.9 ± 0.6^{a}
Swelite®	4.7 ± 0.6^{a}	34.3 ± 5.8^{ab}	54.4 ± 2.5^{a}	40.7 ± 5.7^{a}	0.59 ± 0.03^{a}	22.8 ± 1.5^{ab}	0.81 ± 0.01^{a}	18.4 ± 1.2^{ab}
Exafine® 250	3.3 ± 0.6^{bc}	23.2 ± 7.0^{b}	47.8 ± 0.7^{b}	40.0 ± 7.9^{a}	0.54 ± 0.08^{a}	18.6 ± 2.3^{bc}	0.79 ± 0.05^{a}	14.8 ± 1.0^{bc}
Fibrulose 97	4.3 ± 0.6^{ab}	30.4 ± 7.9^{b}	54.3 ± 0.4^{a}	24.6 ± 5.4^{c}	0.63 ± 0.02^{a}	13.7 ± 2.3^{c}	0.80 ± 0.02^{a}	11.0 ± 1.6^{c}
Fibruline INSTANT	5.0 ± 0.0^{a}	44.8 ± 9.5^{a}	55.8 ± 0.5^{a}	26.1 ± 4.4^{bc}	0.58 ± 0.03^{a}	15.7 ± 1.8^{c}	0.78 ± 0.03^{a}	12.2 ± 1.8^{c}
Vitacel AF 401	3.0 ± 0.0^{c}	46.4 ± 7.2^{a}	53.8 ± 0.0^{a}	41.5 ± 1.9^{a}	0.54 ± 0.07^{a}	21.6 ± 2.1^{ab}	0.81 ± 0.04^{a}	17.2 ± 2.6^{ab}

* Values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

The folding test showed that Swelite and Fibruline products had a score higher than 4.7 while Exafine and Vitacel products displayed significantly ($p<0.05$) lower values. With respect to gel strength, there were also two significantly distinct groups ($p<0.05$), a first one of strong gels comprising the control, Fibruline and Vitacel products and a second group of more fragile gels produced with Exafine and Fibrulose. These results are in accordance with those from the first group of trials (Table 5.1.2.3). The products containing pea or apple fibres were significantly ($p<0.05$) harder than those enriched with fibres obtained from chicory root. The control samples as well those enriched with Swelite and Vitacel had the highest gumminess and chewiness values and were the only ones not significantly different from the control.

Only the addition of Swelite led to hake gel products with textural properties similar to the control products. The low folding score of Vitacel excludes it from the previous group. On the other hand, Exafine products had the lowest values for the various textural properties, except for the hardness and, therefore, adding this fibre to minced fish products seems quite disadvantageous. The addition of chicory root fibres had a negative effect on the hardness parameter, however, softer products may be appropriate for pâtés and other similar applications. Furthermore, Fibruline might be better in some applications than Fibrulose since it had a higher gel strength.

Overall, Swelite, even at 4 %, was the best dietary fibre because its presence in minced fish gel products did not affect the overall texture evaluation compared to the control, probably due to its starch content. Starch acts as a simple filler in the myofibrillar protein gel, not directly interacting with the surimi protein matrix nor significantly affecting

its formation, as starch swelling occurs later in the cook cycle than protein gelation (Lee et al., 1992; Wu et al., 1985).

Conclusions

Results indicated that Swelite fibre addition to hake heat-induced gel products, up to 4 % (w/w), was beneficial in keeping the textural properties of the product without any fibre.

Furthermore, Fibruline addition was also favourable regarding some textural properties, namely, gel strength. The fibre's main drawback was its adverse effect on the hardness parameter. However, such a textural profile could be advantageous for some applications as, for instance, pâtés.

Moreover, the raw material's quality is a crucial aspect since the use of different hake batches gave rather different final products with respect to textural parameters.

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5.1.3- Effect of Transglutaminase and Carrageenan on Restructured Fish Products Containing Dietary Fibres

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Original article

Effect of transglutaminase and carrageenan on restructured fish products containing dietary fibres

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Summary The inclusion of various levels of a microbial transglutaminase or a carrageenan additive to restructured hake products with or without dietary fibre (obtained from inner pea or chicory root) was studied. These experiments were aimed at producing technologically innovative restructured fish products with improved textural and colour properties. It was found that the addition of transglutaminase, up to 0.5% (w/w), favoured better textural properties, particularly, at 0.1% (w/w) level or above. On the other hand, restructured product with a dietary fibre content of 4% (w/w) and carrageenan addition, up to 2% (w/w), exhibited increased hardness. This can be particularly interesting for those products containing chicory root inulins as this fibre presented an adverse effect on hardness. Regarding colour, higher carrageenan contents caused a slight but significant colour change in the product, making it redder (for products containing Swellite and Fibruline) and more yellow (only concerning Swellite).

Keywords Carrageenan, dietary fibre, restructured fish, textural properties, transglutaminase, upgrading fish wastes.

Introduction

The development of restructured fish products and the application of new food ingredients have been used as a way of creating new products, attractive, namely, to young consumers, but also to upgrade low-value species and the waste generated by the fish processing industry (Sánchez *et al.*, 2004).

On account of its considerable nutritional value, one of the food ingredients frequently used in the design of functional foods is dietary fibre, DF (Puupponen-Pimia *et al.*, 2002). The importance of DF in nutrition and health is well established (Anderson *et al.*, 1990; Kritchevsky & Bonfield, 1995). Knowledge of the beneficial effects of high DF diets – namely, regarding prevention of cardiovascular diseases and several types of cancer – has promoted the development of a large and profitable market for products enriched in DF.

Dietary fibre includes cellulose, hemicellulose, lignin, pectins, oligosaccharides such the inulins and, also, gums. These are a group of polymers that can be dissolved or dispersed in water to produce a thickening or gelling effect. As they are colloidal in nature, gums are also known as hydrocolloids (starch is also a hydrocolloid, though not a gum) (Lee *et al.*, 1992). They are selected for enhancement of functional properties such as water-holding capacity and rheological behaviour (Pérez-Mateos & Montero, 2000). However, these additives modify gel characteristics in different ways depending on the quality of myofibrillar protein (Gómez-Guillén & Montero, 1998). For instance, regarding minced muscle of high quality, hydrocolloids may reduce gel strength and hardness, whereas, for poor quality material, they enhance protein functionality, improving thereby the textural properties (Gómez-Guillén & Montero, 1998). For our study, besides inner pea and chicory root fibres, carrageenan was used as a hydrocolloid. This gum is extracted from the red seaweeds and available in three main types, namely, kappa (κ), iota (ι) and lambda (λ). However, because of their chemical differences, only ι- and κ-carrageenans have gelling ability. In spite of the fact that carrageenan addition to fish products is not a widespread practice, there have been some experiments involving presence of ι- and κ-carrageenan in seafood (Gómez-Guillén & Montero, 1996, 1997; Ortiz & Aguilera, 2004). Namely, it was observed that, in a fish muscle of low gel-forming capacity, ι-carrageenan addition improved the textural properties of samples (hardness, elasticity and cohesiveness) and such effect was particularly reinforced in the presence of other additives, such as starch or/and non-muscle protein (Gómez-Guillén & Montero, 1996). Furthermore, ι-carrageenan along with other ingredients was found to strengthen gels obtained from squid muscle (Gómez-Guillén & Montero, 1997). Interestingly,

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Abstract

The inclusion of various levels of a microbial transglutaminase or a carrageenan additive to restructured hake products with or without dietary fibre (obtained from inner pea or chicory root) was studied. These experiments were aimed at producing technologically innovative restructured fish products with improved textural and colour properties. It was found that the addition of transglutaminase, up to 0.5 % (w/w), favoured better textural properties, particularly, at 0.1 % (w/w) level or above. On the other hand, restructured product with a dietary fibre content of 4 % (w/w) and carrageenan addition, up to 2 % (w/w), exhibited increased hardness. This can be particularly interesting for those products containing chicory root inulins since this fibre presented an adverse effect on hardness. Regarding colour, higher carrageenan contents caused a slight but significant colour change in the product, making it redder (for products containing Swelite and Fibruline) and more yellow (only concerning Swelite).

Keywords: Restructured fish, tranglutaminase, carrageenan, dietary fibre, textural properties, upgrading fish wastes

Introduction

The development of restructured fish products and the application of new food ingredients have been used as a way of creating new products, attractive, namely, to young consumers, but also to upgrade low-value species and the waste generated by the fish processing industry (Sánchez et al., 2004).

On account of its considerable nutritional value, one of the food ingredients frequently used in the design of functional foods is dietary fibre, DF (Puupponen-Pimïa et al., 2002). The importance of DF in nutrition and health is well established (Anderson et al., 1990; Kritchevsky and Bonfield, 1995). Knowledge of the beneficial effects of high dietary fibre diets —namely, regarding prevention of cardiovascular diseases and several types of cancer— has promoted the development of a large and profitable market for products enriched in DF.

DF includes cellulose, hemicellulose, lignin, pectins, oligosaccharides such the inulins and, also, gums. These are a group of polymers that can be dissolved or dispersed in water to produce a thickening or gelling effect. Since they are colloidal in nature, gums are also known as hydrocolloids (starch is also a hydrocolloid, though not a gum) (Lee et al., 1992). They are selected for enhancement of functional properties such as water holding capacity and rheological behaviour (Pérez-Mateos and Montero, 2000). However, these additives modify gel characteristics in different ways depending on the quality of myofibrillar protein (Gómez-Guillén and Montero, 1996). For instance, regarding minced

muscle of high quality, hydrocolloids may reduce gel strength and hardness, whereas, for poor quality material, they enhance protein functionality, improving thereby the textural properties (Gómez-Guillén and Montero, 1996). For our study, besides inner pea and chicory root fibres, carrageenan was used as a hydrocolloid. This gum is extracted from the red seaweeds and available in three main types, namely, kappa (κ), iota (ι) and lambda (λ). However, due to their chemical differences, only iota and kappa carrageenans have gelling ability. In spite of the fact that carrageenan addition to fish products is not a widespread practice, there have been some experiments involving presence of iota and kappa carrageenan in seafood (Gómez-Guillén and Montero, 1996; Gómez-Guillén and Montero, 1997; Ortiz and Aguilera, 2004). Namely, it was observed that, in a fish muscle of low gel-forming capacity, ι-carrageenan addition improved the textural properties of samples (hardness, elasticity and cohesiveness) and such effect was particularly reinforced in the presence of other additives, such as starch or/and non-muscle protein (Gómez-Guillén and Montero, 1996). Furthermore, ι-carrageenan along with other ingredients was found to strengthen gels obtained from squid muscle (Gómez-Guillén and Montero, 1997). Interestingly, some authors have detected a positive interaction of carrageenan with starch, favouring stronger gels (Tye, 1988; Gómez-Guillén et al., 1992).

Microbial transglutaminase (MTGase) is widely used in the food industry with the purpose of promoting protein cross-linking (Téllez-Luís et al., 2002). For this reason MTGase has been studied as a means of improving textural characteristics and mechanical properties of fish products. Although transglutaminase (TGase) is known to be present in fish species (Tsukamasa and Shimizu, 1990, Ramírez et al., 2000), commercial production of TGase from fish is not as yet initiated and, therefore, it is only commercially available from microorganisms (Téllez-Luís et al., 2002). Despite this exogenous origin, MTGase has effectively improved, in various circumstances, the textural properties of restructured fish products (Téllez-Luís et al., 2002, Ramírez et al., 2000).

Firstly, this study aimed to assess the textural effect of MTGase addition to fish products with 4 % (w/w) of a dietary fibre (obtained from inner pea) or without any fibre. Secondly, to evaluate the effect of carrageenan on the textural and colour properties of restructured fish products containing 4 % (w/w) of a dietary fibre (obtained from inner pea or chicory root) and 0.1 % MTGase (w/w). Furthermore, concerning carrageenan and MTGase contents in the final product, tested ranges were chosen taking into account typical values mentioned in the literature (Gómez-Guillén and Montero 1996, Ramírez et al 2000).

Material and Methods

Raw materials and additives

Frozen South African hake (*Merluccius capensis*) was bought already headed and gutted from a local frozen fish processor. Each fish batch was kept frozen at –28 ºC and processed within three to four weeks after its arrival at the laboratory.

Regarding dietary fibre (DF) products, besides carrageenan (Ceamgel 1830) from Ceamsa (Porriño, Spain), two other fibre products were used. These were chosen for their favourable effect on the textural properties of the final products (data not published): inner pea fibre (Swelite®) and chicory root inulin (Fibruline INSTANT), both supplied by Cosucra, S.A. (Warcoing, Belgium).

CEAMGEL 1830 is a mixture of iota and kappa carrageenans extracted from red seaweeds of the class *Rhodophyceae*, containing additionally locust bean gum, potassium chloride and dextrose. This product presents itself as a pale yellow powder whose grains (more precisely, 98 % of them) are smaller than 250 μm. Concerning the other two fibre products, their particular composition (based on dry matter, D.M.) as well other properties are presented in Table 5.1.3.1.

Microbial transglutaminase (MTGase) ACTIVA® WM was supplied by Ajinomoto Japan, Inc. (Tokyo, Japan). Transglutaminase activity was approximately 67 ± 10 units.g^{-1}.

Table 5.1.3.1 – Relevant properties of the used dietary fibre products.

PROPERTIES	Swelite®	Fibruline INSTANT
Composition (D.M.)		
Total Carbohydrates (%)	93 ± 3	min. 99.7
Of which: *Total DF (%)*	48 ± 3	min. 90
Starch (%)	min. 36	—
Protein (%)	max. 7	—
Fat (%)	max. 0.5	—
Ash (%)	max. 2	max. 0.3
Granulometry (μm)	< 400	< 700
Colour	white	white
Taste	neutral	neutral to slightly sweet

Reagents

The salt was of analytical grade and was obtained from Merck KGaA (Darmstadt, Germany).

Experimental design

In a first set of experiments, the mean textural properties of restructured hake products —with 4 % (w/w) Swelite or without this fibre— containing various levels of MTGase — ranging from 0.0 to 0.5 % (w/w)— were determined. Swelite was chosen since previous trials with this dietary fibre had produced very positive results. Afterwards, in a second set of experiments, the mean textural properties and colour parameters of restructured hake

products containining 4 % (w/w) fibre —Swelite or Fibruline—, 0.1 % (w/w) MTGase and different levels of carrageenan —varying from 0 to 2 % (w/w)— were measured. Error assessment was derived from replication of the various analyses performed.

Production of restructured hake products

About 10 kg of frozen hake were thawed overnight in a refrigerator. Afterwards, skin and bones were manually removed. The resulting fish flesh was minced one single time in a model 84145 meat grinder (Hobart, Troy, OH, USA), equipped with 2 cm grind blades and a metallic screen with 6 mm diameter circular holes. The appropriate quantities of fish mince (Table 5.1.3.2) were weighed in order to guarantee final restructured hake products with the same water content regardless of the quantity of added fibre (more fibre entailed a greater addition of water and, for a constant total weight of all ingredients, a smaller amount of mince). Concerning MTGase, fish mince was mixed with previously hydrated fibre (chilled water was added to the dietary fibre in a ratio 4:1), salt (2.5 % of the final product weight) and MTGase (at a variable level) in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Whereas, regarding carrageenan addition, the procedure was identical except for the addition of carrageenan to the other dietary fibre prior to hydration and the inclusion of MTGase at a constant level, 0.1 %. The mixing was performed for 5 minutes (2 minutes at 1420 r.p.m. followed by 3 minutes at 2800 r.p.m.) under vacuum and refrigeration (temperature below 7 ºC). The batters attained were packed into stainless steel tubes (diameter 30 mm, length 30 mm). The tubes were capped and immersed in water at 35 ºC for 1 hour (setting), then moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and subjected to a steam cooking at 90 ºC for 1 hour (cooking). The heat-induced gels were cooled immediately in iced water and kept in a refrigerator overnight until analysis.

Table 5.1.3.2 – Main ingredient quantities used in the manufacturing of the various restructured hake products.

INGREDIENTS	1st Set of experiments		2nd Set of experiments Carrageenan level in the products (%, w/w)				
	No Fibre	Fibre	0	0.4	0.8	1.2	2
Fish mince (g)	1500	1176	1089	1063	1037	1011	959
Swelite/Fibruline (g)	0	60	54	54	54	54	54
Carrageenan (g)	0	0	0.0	5.4	10.8	16.2	27.0
Water (g)	0	264	207	228	248	269	310

Texture measurements

Folding test. The test piece was a 3 mm slice cut from the stainless steel tubes. The evaluation was performed in accordance with a 5-point grade system as follows. Grade 5, no crack when folded into quadrants; Grade 4, no cracks when folded in half; Grade 3,

crack develops gradually when folded in half; Grade 2, crack develops immediately when folded in half; Grade 1, crumbles when pressed by finger.

Puncture test. Prior to analysis, samples were tempered to about 20 ℃, removed from the tubes and cut into pieces of 30 mm diameter and 25 mm high. The gel was penetrated to the breaking point with a metal probe equipped with a 5 mm diameter spherical head, using a model Instron 4301 texturometer (Instron Engineering Corp., Canton, MA, USA). The cross speed head was 10 mm/min and the load cell was 1000 N. Breaking force (N) and breaking deformation (mm) were measured. Gel strength (N.mm) was determined by multiplying these two parameters.

Compression tests. For the texture profile analysis, samples (diameter, 30 mm and height, 25 mm) were compressed on the flat plate of the Instron texturometer with a cylindrical plunger (50 mm diameter) adapted to a 1000N load cell at a deformation rate of 50 mm/min. On the basis of preliminary trials to establish a compression limit that would ensure no cracking and recoverability of most samples, it was decided to compress samples to 60 % of their height. In the test, each sample was compressed twice. The following parameters were determined: hardness (N), maximum height of first peak on first compression (in terms of eating quality, food's resistance at first bite); cohesiveness (A_2/A_1), ratio of second-compression to first-compression positive areas (maintenance of food resistance during chew down); gumminess (N), product of hardness and cohesiveness (strength required in the chew down process); springiness (L_2/L_1), ratio of the detected height of the product on the second compression to the original compression distance (ability of food to reacquire its initial shape and size after a first bite); chewiness (N), product of gumminess and springiness (albeit expressed in N, a measure of the energy spent in the chew down process).

For the compression-relaxation test, the compression procedure was as for the texture profile analysis except that the sample was compressed only once for one minute and the force exerted on the sample was recorded. Relaxation (%) was calculated as $Y_T=100\times(F_0-F_1)/F_0$, where F_0 is the force registered at the onset of relaxation immediately after sample compression and F_1 is the force registered after one minute of relaxation. Thus, $(100-Y_T)$ is taken as an index of elasticity and is expressed as the percentage elasticity of the gel.

Colour measurements

The colour measurements were performed on the product prior to setting and cooking. After two days in chilled storage, the product was put into Petri dishes, covering the entire bottom. A model MACBETH COLOUR-EYE® 3000 colourimeter (Macbeth, New Windsor, NY, USA) was used and, prior to measurements, standardized to a specific colour blank (CIELAB system: L*, 92.4; a*, -1.0; b*, 1.5). The attained values for L*, a* and b* of the CIELAB system were always the means of ten measurements on each Petri dish. Furthermore, for a better assessment of colour, the three mentioned coordinates were combined in order to obtain the chroma and whiteness values:

$$Chroma = \sqrt{a^{*2} + b^{*2}}$$

$$Whiteness = 100 - \sqrt{(100 - L^{*})^{2} + a^{*2} + b^{*2}}$$

Statistical analysis

Colour and folding test determinations were performed in duplicate, the gel strength results are the average of six measurements and all other determinations were done in triplicate. A general linear model —one-way ANOVA— was used to determine significant differences ($p<0.05$) among restructured fish products with different levels of MTGase and carrageenan. Multiple comparisons were done by the Tukey HSD test. All statistical treatment was done with the software STATISTICA© from StatSoft, Inc. (Tulsa, OK, USA), version 6.1, 2003.

Results and Discussion

Effect of MTGase content

Texture evaluation. In a first group of trials, the consequences of distinct MTGase levels in the restructured hake products were analysed. The effect of six different enzyme concentrations (0.0, 0.02, 0.05, 0.1, 0.2 and 0.5 % w/w) on some relevant textural parameters (folding test, gel strength, elasticity and hardness) of products, with 4 % (w/w) Swelite or without any fibre, was assessed (Table 5.1.3.3).

Table 5.1.3.3 – Mean textural properties of restructured hake products (with and without fibre) and containing various levels of MTGase*.

MTGase LEVEL (% w/w)	NO FIBRE ADDED Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	SWELITE 4 % ADDED Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)
0.00	3.0 ± 0.0^{a}	15.8 ± 2.9^{ab}	38.0 ± 1.5^{a}	18.9 ± 0.4^{a}	3.0 ± 0.0^{ab}	17.9 ± 4.2^{a}	51.7 ± 1.4^{ab}	17.6 ± 1.1^{a}
0.02	2.5 ± 0.7^{a}	14.9 ± 2.8^{a}	52.4 ± 0.4^{b}	18.9 ± 0.4^{a}	2.5 ± 0.7^{a}	13.5 ± 1.0^{a}	53.7 ± 0.6^{bc}	17.6 ± 1.1^{a}
0.05	3.0 ± 0.0^{a}	16.8 ± 5.3^{ab}	51.4 ± 3.6^{b}	20.6 ± 2.4^{ab}	3.0 ± 0.0^{ab}	14.5 ± 1.2^{a}	47.2 ± 1.8^{a}	19.7 ± 0.1^{a}
0.10	2.5 ± 0.7^{a}	17.0 ± 4.0^{ab}	53.1 ± 1.3^{b}	19.7 ± 2.3^{a}	4.0 ± 0.0^{b}	25.4 ± 10.4^{a}	56.8 ± 1.1^{bc}	20.2 ± 2.3^{a}
0.20	3.0 ± 0.0^{a}	21.9 ± 4.9^{ab}	53.6 ± 0.8^{b}	20.5 ± 1.6^{ab}	4.0 ± 0.0^{b}	26.7 ± 7.2^{a}	57.6 ± 1.6^{c}	20.3 ± 0.9^{a}
0.50	3.0 ± 0.0^{a}	24.6 ± 4.6^{b}	52.1 ± 0.5^{b}	25.9 ± 0.3^{b}	4.0 ± 0.0^{b}	22.9 ± 7.0^{a}	58.5 ± 0.8^{c}	19.5 ± 1.0^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

The presence of MTGase, whatever its level, did not alter the folding test (FT) of the products without fibre, however, for those products containing Swelite, the enzyme showed a clear influence upon the FT. Indeed, MTGase levels equal to or higher than 0.1 % increased the FT score from 3.0 to 4.0. Regarding gel strength, on the contrary, there was no statistically significant effect ($p \geq 0.05$) on fibre-containing products, whereas 0.5 % MTGase, the highest content tested in the experiment, significantly strengthened ($p<0.05$) the gels of the products without fibre, increasing the aforementioned textural property from 14.9 ± 2.8 N.mm, at 0.02 % level, to 24.6 ± 4.6 N.mm. For both types of products, with and without Swelite, addition of MTGase increased significantly ($p<0.05$) elasticity with respect to controls (0.0 % level), however, the critical concentration was different in each case: for the latter products, 0.02 % MTGase was enough to significantly augment ($p<0.05$) elasticity from 38.0 ± 1.5 % to 52.4 ± 0.4 %, whereas the former products required a higher concentration, 0.2 % MTGase. Finally, it must be noticed that TGase, at a 0.5 % level, made products without Swelite significantly harder ($p<0.05$) with respect to the control, 25.9 ± 0.3 N *vs* 18.9 ± 0.4 N, while no hardness variation was detected in the products with Swelite.

On the whole, the results expressed above show that there is advantage on using MTGase in the restructured fish products, regardless the presence of fibre. Moreover, for products with Swelite, the enzyme improved textural parameters with the exception of gel strength and hardness. It is worth mentioning that Swelite contains a considerable starch percentage (Table 5.1.3.1), which improves gel-forming capacity by acting as a simple filler of the myofibrillar protein gel (Lee et al., 1992) and, on the other hand, MTGase favours the cross-linking of proteins through the formation of covalent bonds between protein molecules (Park, 2000). Therefore, the two additives can be seen as complementary. Therefore, in future experiments involving restructured fish products containing fibre, it seems advisable to include MTGase. Regarding the enzyme

concentration, it must be stressed that one has to take into account the enzyme activity. Thus, for transglutaminases with activities similar to the one known for our enzyme, 67 ± 10 units.g^{-1}, a level of 0.1 % (w/w) is quite advantageous since it ensured a better folding test and an elasticity not significantly different ($p \geq 0.05$) from that obtained with the highest MTGase levels.

Effect of carrageenan content in combination with Swelite

Texture evaluation. This group of trials was conducted with the purpose of studying the influence of five carrageenan levels (0, 0.4, 0.8, 1.2 and 2 %, w/w) in combination with 4 % (w/w) Swelite on the textural properties of restructured hake products. The results attained from the determination of these properties are shown in Table 5.1.3.4.

Table 5.1.3.4 – Mean textural properties of restructured hake products containing 4 % (w/w) Swelite, 0.1 % (w/w) MTGase and various levels of carrageenan*.

CARRAGEENAN LEVEL (%w/w)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
0.0	2.8 ± 0.5^{a}	26.4 ± 4.5^{a}	55.4 ± 0.3^{a}	44.6 ± 6.0^{a}	0.53 ± 0.02^{a}	25.9 ± 1.7^{a}	0.79 ± 0.02^{a}	20.5 ± 1.9^{a}
0.4	3.0 ± 0.0^{a}	23.2 ± 3.4^{ab}	54.5 ± 0.7^{a}	46.4 ± 2.3^{ab}	0.54 ± 0.01^{a}	24.5 ± 1.0^{a}	0.83 ± 0.02^{a}	20.2 ± 1.3^{a}
0.8	3.0 ± 0.0^{a}	20.7 ± 3.1^{ab}	50.1 ± 1.7^{ab}	52.2 ± 3.9^{bc}	0.50 ± 0.03^{a}	27.8 ± 1.4^{ab}	0.79 ± 0.01^{a}	22.0 ± 1.0^{ab}
1.2	2.8 ± 0.5^{a}	17.5 ± 3.1^{b}	52.1 ± 2.4^{ab}	52.9 ± 2.5^{c}	0.50 ± 0.05^{a}	26.9 ± 1.9^{ab}	0.80 ± 0.01^{a}	21.4 ± 1.8^{a}
2.0	3.0 ± 0.0^{a}	17.7 ± 1.7^{b}	46.5 ± 3.7^{b}	62.7 ± 3.6^{d}	0.48 ± 0.03^{a}	31.2 ± 3.0^{b}	0.83 ± 0.02^{a}	25.9 ± 2.9^{b}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p < 0.05$).

FT, cohesiveness and springiness results did not present any significant difference ($p \geq 0.05$). But, on the contrary, gel strength values showed a clear decreasing trend with increasing carrageenan concentration. Gels of products with 1.2 or 2 % carrageenan, were weaker ($p \leq 0.05$) than the control gels. A similar significant ($p < 0.05$) downward trend was observed in the elasticity parameter (from 55.4 ± 0.3 to 46.5 ± 3.7 %) with increasing carrageenan content. However, hardness, gumminess and chewiness contrasted strongly with the two previous textural properties in that higher levels of the gum brought about significantly ($p < 0.05$) harder and, as a consequence of the other two parameters dependence on the hardness measurement, gummier and chewier products. Indeed, regarding hardness, this trend was particularly notorious since there was a relentless hardening with growing carrageenan concentration, from 44.6 ± 6.0 to 62.7 ± 3.6 N.

The reported textural results highlighted two main effects of increasing carrageenan levels: loss of elasticity and hardening of the restructured fish products containing Swelite. Analysing the components of gel strength (see Material and Methods, puncture test), the gel strength reduction was a consequence of the loss of deformability since the breaking deformation component was the only one to decrease, from 7.5 mm (control) to 5.1 mm

(2 % carrageenan), while breaking force remained constant at 3.5 N. The hardness increase agrees with other authors' findings, namely, those working on sardine mince, which reported a considerable rigidity increase due to carrageenan addition (Gómez-Guillén et al., 1997b) and, specifically, a hardening effect of the gels prepared from low-quality mince (Gómez-Guillén and Montero, 1996). This effect is quite remarkable because, for equal moisture and Swelite levels, a greater carrageenan content means a substantially lower protein content in the final product and, as stated (Park, 2000), such a reduction entails less firm gels. Clearly, the so-called 'diluting' effect seems to be offset by the action of the added hydrocolloid. However, concerning the loss of elasticity, there is disagreement with the results presented by the same authors (Gómez-Guillén and Montero, 1996), this could be due to the used fish species, sardine, a small pelagic fish, which is rather different from hake, namely, on its fat and dark muscle content.

The carrageenan action could be similar to that of starch —one of the main components of Swelite and as well a hydrocolloid—, i.e., acting as a simple filler of the myofibrillar protein gel. Nonetheless, it is interesting to note that while increasing carrageenan, in the presence of Swelite, hardened considerably the products (Table 5.1.3.4), previous results not published showed that augmenting Swelite (in products without carrageenan) did not present any hardening effect, meaning that the hardening power of carrageenan combined with Swelite seems to be superior to the one exhibited by Swelite per se. This raises the possibility that the carrageenan product —which is a mixture of iota and kappa carrageenans— has good synergy with starch as happens with the iota type (Tye, 1988; Tye, 1991; Gómez-Guillén et al., 1992). Furthermore, it has been mentioned that the formation of two interpenetrating gel networks (from fish protein and carbohydrate) is a distinct possibility for gums having gelling ability (Lee et al., 1992). In this model, the network formed by the gum does not interact directly with the continuous matrix, instead it cooperates structurally, due to the entwining of the two gel networks. Such a model was observed to occur with ι-carrageenan and fish protein, in that ι-carrageenan formed an independent network that supported the main structured formed by the fish protein, while starch was incorporated into the network in granular form (Montero et al., 2000; Gómez-Guillén et al., 1996).

Colour evaluation. Besides textural parameters, the effect of carrageenan content on the colour of the restructured fish products containing Swelite was also analysed (Table 5.1.3.5).

Concerning a* and b* values, there was a consistent increasing trend with growing carrageenan levels. In spite of the slight differences between control and 1.2 and 2 % carrageenan products, they were less green ($p<0.05$) for both levels when compared to

the control. b* also increased significantly ($p<0.05$) with growing carrageenan levels. Thus, higher carrageenan contents made the fish products less green or more red and more yellow. On the contrary, Chroma and Whiteness values did not show any significant difference ($p\geq0.05$) regarding the control sample. However, it is important to remember that for Chroma only the absolute values matter, and, as such, taking a* negative values, the absolute values associated to this parameter decreased and offset the increasingly positive values of b*.

Table 5.1.3.5 – Mean colour parameters of restructured hake products containing 4 % (w/w) Swelite, 0.1 % (w/w) MTGase and various levels of carrageenan*.

CARRAGEENAN LEVEL (% w/w)	a*	b*	Chroma	Whiteness
0.0	-2.47 ± 0.01^{a}	1.21 ± 0.07^{a}	2.75 ± 0.02^{a}	66.8 ± 0.3^{a}
0.4	-2.46 ± 0.02^{a}	1.24 ± 0.17^{a}	2.76 ± 0.06^{a}	67.2 ± 0.1^{a}
0.8	-2.38 ± 0.02^{ab}	1.41 ± 0.06^{ab}	2.77 ± 0.01^{a}	67.1 ± 0.2^{a}
1.2	-2.28 ± 0.03^{b}	1.60 ± 0.11^{ab}	2.79 ± 0.03^{a}	65.9 ± 0.6^{a}
2.0	-2.26 ± 0.06^{b}	1.77 ± 0.06^{b}	2.87 ± 0.06^{a}	66.4 ± 0.1^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Effect of carrageenan content in combination with Fibruline

Texture evaluation. The influence of increasing carrageenan contents (0, 0.4, 0.8, 1.2 and 2 %, w/w) upon the textural properties of products already containing 4 % (w/w) of Fibruline was also assessed (Table 5.1.3.6).

Table 5.1.3.6 – Mean textural properties of restructured hake products containing 4 % (w/w) Fibruline, 0.1 % (w/w) MTGase and various levels of carrageenan*.

CARRAGEENAN LEVEL (%w/w)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
0.0	3.3 ± 0.5^{a}	32.7 ± 5.3^{a}	53.0 ± 2.7^{a}	33.5 ± 5.0^{a}	0.61 ± 0.02^{a}	19.4 ± 2.1^{a}	0.82 ± 0.02^{a}	15.8 ± 2.0^{a}
0.4	3.3 ± 0.5^{a}	25.2 ± 1.5^{a}	54.8 ± 1.4^{ab}	33.6 ± 2.9^{a}	0.59 ± 0.01^{a}	19.3 ± 0.9^{a}	0.81 ± 0.01^{a}	15.6± 0.7^{a}
0.8	4.3 ± 0.5^{ab}	23.9 ± 7.7^{a}	56.5 ± 0.5^{ab}	36.3 ± 3.1^{ab}	0.58 ± 0.03^{a}	21.0 ± 1.1^{ab}	0.79 ± 0.02^{a}	16.6 ± 0.7^{ab}
1.2	4.0 ± 0.0^{ab}	26.2 ± 5.4^{a}	56.2 ± 0.3^{ab}	40.3 ± 3.6^{ab}	0.57 ± 0.02^{a}	22.1 ± 1.6^{ab}	0.79 ± 0.01^{a}	17.5 ± 1.1^{ab}
2.0	4.5 ± 0.6^{b}	27.1 ± 4.3^{a}	57.5 ± 1.2^{b}	42.0 ± 6.6^{b}	0.59 ± 0.05^{a}	24.4 ± 2.9^{b}	0.81 ± 0.02^{a}	19.9 ± 1.9^{b}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Gel strength, cohesiveness and springiness results did not reveal any significant difference ($p\geq0.05$). On the other hand, FT increased significantly ($p<0.05$) with growing carrageenan concentration, from 3.3 ± 0.5, at 0 %, to 4.5 ± 0.6, at 2 %. A similar upward trend was registered in the elasticity values, which increased significantly ($p<0.05$) from the control to the 2 % carrageenan product, 53.0 ± 2.7 and 57.5 ± 1.2 %, respectively. Moreover, the restructured product richest in carrageenan (2 %) was significantly ($p<0.05$) harder than the control (42.0 ± 6.6 *vs* 33.5 ± 5.0 N). Given the cohesiveness and

springiness invariability, the trends observed for the gumminess and chewiness properties were practically identical to the hardness rising trend.

The effects of carrageenan on restructured hake products containing Fibruline were somewhat different from those found in the products including Swelite. Namely, FT and elasticity both increased with higher carrageenan incorporation, while those products with Swelite presented an elasticity reduction and no variation in the FT. Moreover, hardness and associated parameters increased in response to carrageenan concentration (similarly to the Swelite trials). Concerning Fibruline, this is rather important since it was found that Fibruline alone softened the products (data not published). Thus, adding carrageenan made up for that deleterious effect and, also, for the protein level reduction, enabling, at a 2 % level, to achieve a hardness result similar to the one exhibited by products with 4 % Swelite and no carrageenan (Table 5.1.3.4). Therefore, regarding the tested textural properties, carrageenan addition to products containing Fibruline presented only beneficial effects. The main drawback associated to Fibruline —its softening influence— was overcome and some of this fibre interesting effects, for instance, increasing elasticity (data not published), were further enhanced. Such a positive interaction of carrageenan with Fibruline should deserve future studies.

Colour evaluation. With the purpose of analysing the influence of carrageenan incorporation upon colour, a*, b*, Chroma and Whiteness of products with 4 % Fibruline were determined (Table 5.1.3.7).

Table 5.1.3.7 – Mean colour parameters of restructured hake products containing 4 % (w/w) Fibruline, 0.1 % (w/w) MTGase and various levels of carrageenan[*].

CARRAGEENAN LEVEL (% w/w)	a*	b*	Chroma	Whiteness
0.0	-2.89 ± 0.00^{a}	-0.50 ± 0.10^{a}	2.93 ± 0.02^{a}	64.0 ± 0.1^{a}
0.4	-2.79 ± 0.01^{b}	-0.44 ± 0.19^{a}	2.83 ± 0.04^{ab}	63.9 ± 0.8^{a}
0.8	-2.83 ± 0.04^{ab}	-0.40 ± 0.04^{a}	2.86 ± 0.03^{ab}	64.6 ± 0.1^{a}
1.2	-2.77 ± 0.04^{b}	-0.45 ± 0.06^{a}	2.81 ± 0.04^{b}	64.9 ± 0.1^{a}
2.0	-2.67 ± 0.01^{c}	-0.13 ± 0.04^{a}	2.67 ± 0.01^{c}	64.9 ± 0.0^{a}

[*] Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Albeit a small b* increase for 2 % carrageenan, b* and, also, Whiteness did not show any significant trend ($p\geq0.05$). However, the parameter a* increased significantly ($p<0.05$) in response to higher carrageenan contents, varying slightly from –2.89 ± 0.0 (0 %) to -2.67 ± 0.01 (2 %). The significant reduction ($p<0.05$) observed in Chroma was only a consequence of the parameter a* variation.

Conclusions

It was observed that the addition of transglutaminase, up to 0.5 % (w/w), favoured better textural properties, particularly, at 0.1 % (w/w) level or above. On the other hand, concerning restructured products with a significant dietary fibre content (4 % w/w fibre), carrageenan addition, up to 2 % (w/w), increased products hardness. This can be particularly interesting for those products containing chicory root inulins like Fibruline since this fibre presented an adverse effect on hardness.

Regarding colour, higher carrageenan contents caused a slight but significant colour change in the product, making it redder (for products containing Swelite and Fibruline) and more yellow (only concerning Swelite).

A comparison between Swelite and Fibruline shows interesting differences, mainly, at textural level. Specifically, Fibruline favoured higher FT score, superior gel strength and cohesiveness, whereas Swelite, even without carrageenan addition, ensured harder products and, as a consequence, higher values for the gumminess and chewiness parameters.

On the whole, these findings underline the practical advantages of combining carrageenan with other dietary fibres in order to create new tailor-made products with specific textural properties.

These innovative restructured fish products containing fibre can also be very useful in the upgrading of fish waste —from fish portioning and filleting— provided that its quality is ensured through proper collecting and preservation, in accordance with good manufacture practices and hygiene.

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5.1.4 – Production of High Quality Gels from Sea Bass: Effect of MTGase and Dietary Fibre

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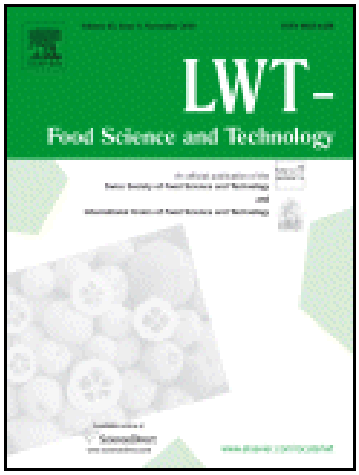

LWT - Food Science and Technology

Production of high quality gels from sea bass: Effect of MTGase and dietary fibre

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Abstract

The effects of MTGase (5 g/kg, w/w) and dietary fibre (inner pea fibre, 40 g/kg, w/w, carrageenan, 10 g/kg, w/w, carrageenan + konjac flour, 10 + 10 g/kg, w/w) on heat-induced gels from sea bass (*Dicentrarchus labrax*) trimmings were studied. MTGase incorporation had a positive effect on texture: gel strength and force at rupture increased. MTGase reduced protein solubility, meaning greater protein aggregation, according to electropherograms. Pea fibre had no positive effects: texture was quite similar to that of the control products and WHC was reduced. Carrageenan addition had no deleterious effect upon the texture of the gels and made them harder and springier. Force at rupture (even without MTGase) and WHC were greatly improved. Combination of carrageenan and konjac further enhanced these effects. For those gels without MTGase, protein solubility in urea and urea + DTT was reduced by both fibres, suggesting a reduction of hydrogen bonding between proteins.

Keywords: Sea bass, texture, transglutaminase, dietary fibre, functional properties

Introduction

Sea bass (*Dicentrarchus labrax*) is a commonly farmed fish species, whose market for whole fish presents signs of saturation. This creates a potential opportunity for ready-to-cook sea bass fillets. But these products entail costs: by-products, such as trimmings, are generated. These are potentially an undervalued resource, whose importance will certainly increase in the future. The conversion to powdered fish meal as with other fish by-products (Guerard et al., 2002), represents a loss of valuable protein for food. A solution may be the production of good quality gel products from sea bass trimmings, since sensory attributes of the attained products can be directed to consumer preferences. Particularly, combining fish minces with new food ingredients has been used as a way of upgrading low-value species and by-products generated by the fish processing industry (Sánchez et al., 2004). In fact, the authors have already done previous work with sea bass trimmings, testing the effects of salt and MTGase on heat-induced gels prepared from this raw material (Cardoso et al., 2010b). The positive results attained, especially with the combination of salt and MTGase, led to this work on the effects of dietary fibre (DF) in connection with MTGase.

MTGase has been used in the food industry for promoting protein cross-linking (Téllez-Luis et al., 2002) and regarding specifically minced fish products, it has effectively improved, in various circumstances, their textural properties (Téllez-Luis et al., 2002; Ramírez et al., 2000; 5.1.3). A level of 5 g/kg (w/w) MTGase enhanced the texture of minced mackerel (*Scomber scombrus* and *Scomber japonicus*) products with dietary fibre

(5.1.5). Another recent study on MTGase addition to giant squid (*Dosidicus gigas*) surimi showed that the same MTGase level could be enough to achieve a good textural quality (Moreno et al., 2009).

DF is a commonly used additive in the design of functional foods (Puupponen-Pimïa et al., 2002). The role of DF in nutrition and health is well established (Anderson et al., 1990; Kritchevsky and Bonfield, 1995). There are few references on fish products with added DF. Some fibres obtained from algae (Ortiz and Aguilera, 2004) or seeds (Montero et al., 2000) have been used for technological purposes in seafood. It was found that different fibres generated texturally diverse products (Montero et al., 2000), for instance, presence of xanthan gum at 5 g/kg (w/w) caused a considerable softening respect to control. Recently, wheat fibre was added to a seafood product, giant squid surimi (Sánchez-Alonso et al., 2007). Texture, colour and water binding capacity (WBC) were also altered with wheat fibre. The observed reduction of gel strength, hardness, cohesiveness and WBC was assigned to the effect of DF and to a smaller proportion of protein.

Among DFs, konjac flour, the generic name of the milled tuber from *Amorphophallus konjac* (Park, 1996), has been used in the development of low fat products (Lin and Huang, 2008) and its application to fish is receiving progressively more attention (Park, 1996; Xiong et al., 2009). It contains a high molecular weight glucomannan, whose D-glucose and D-mannose units (molar ratio ~3:2) are bonded by β-1,4-linkages (Tye, 1991). Acetyl groups are randomly scattered along the essentially linear polymer with an occurrence of ~1 per 19 monomers (glucose or mannose). In the presence of alkali konjac glucomannan (KGM) will deacetylate and form a thermo-irreversible and highly heat-stable gel, the basis of many traditional oriental foods (Nishinari et al., 1992). It has been shown that konjac (at least 10 g/kg, w/w) can improve texture of fish products, namely reinforcing shear stress of gels in both whiting and pollock surimi (Park, 1996). Other authors have proposed that the optimum addition level of konjac glucomannan to surimi gels from grass carp (*Ctenopharyngodon idella*) would be 10 g/kg, w/w (Xiong et al., 2009). This level ensured good mechanical and water-holding properties. KGM can also generate a gel by synergistic interaction with other hydrocolloids (carrageenan, starch and gellan gum) (Fernández-Martín et al., 2009).

Carrageenan is another DF potentially beneficial for fish products. It is extracted from the red seaweeds and of the three main types, namely, kappa (κ), iota (ι) and lambda (λ), only ι- and κ- carrageenans have gelling ability (5.1.3). There have been some experiments involving presence of ι- and κ- carrageenan in seafood (Ortiz and Aguilera, 2004; Gómez-Guillén and Montero 1996; Gómez-Guillén and Montero 1997). Particularly, it was found that incorporation of, at least, 8-12 g/kg (w/w) of carrageenan into minced hake (*Merluccius capensis*) products hardened the heat-induced gels and had a

deleterious effect on the elasticity and gel strength, since gels deformability was reduced (5.1.3). Carrageenan also affected colour, making the products redder. The combined incorporation of carrageenan and KGM into fish products was not experimented until now, thus any possible synergistic effects of the two fibres remain untested.

Inner pea fibre (Swelite®) has also been identified as a promising ingredient (5.3.1). Its DF composition is approximately 2/3 cellulose and 1/3 pectic material (Anderson and Berry, 2001). Swelite® also contains a significant amount of starch. Studies have shown that incorporation of low levels (≤ 40 g/kg, w/w) of this DF may alter texture of minced hake (*Merluccius capensis*) products, namely, this DF favoured greater gel strength and hardness (5.3.1). However, effects can differ between fish species, for instance, this DF did not improve the gel strength of mackerel (*Scomber scombrus* & *Scomber japonicus*) surimi gels (5.1.5).

This work was carried out with two purposes: production of high quality gel products from minced sea bass trimmings and change of their functional properties through incorporation of additives.

Materials and Methods

Materials

Farmed fish, sea bass (*Dicentrarchus labrax*), were bought in a local supermarket and processed (headed, tailed, gutted and filleted) at low temperature (< 10 ºC). The trimmings (muscle joined to the bones and skin) of the cutting operations were collected and used as raw material. Individual fish weight varied between 300 and 400 g.

A new package of microbial transglutaminase TG-K (MTGase) ACTIVA® GS was supplied by Ajinomoto (Tokyo, Japan), presenting an activity of about 100 $U.g^{-1}$.

Three DF were used in this study: inner pea fibre/Swelite® (Swe) supplied by Cosucra, S.A. (Warcoing, Belgium), carrageenan/CEAMGEL 1830 (Carr) by Ceamsa (Porriño, Spain) and konjac flour/Nutricol® GP 312 (Kjc) provided by FMC Biopolymer (Philadelphia, USA). Swe contains 480 g/kg (w/w) of DF (composed of 2/3 insoluble cellulose and 1/3 soluble pectic material), 360 g/kg (w/w) of starch, 90 g/kg (w/w) of other carbohydrates and a maximum of 70 g/kg (w/w) of protein. It is a white powder whose grains are smaller than 400 μm (Anderson and Berry, 2001; *Swelite® Product Sheet*, 2005). Carr is a mixture of iota and kappa carrageenans (each approximately 500 g/kg, w/w) from red seaweeds, containing additionally potassium chloride and dextrose. Its pale yellow grains are smaller than 250 μm (*CEAMGEL1830® Product Sheet*, 2006). Kjc contains KGM extracted from the konjac plant and its tan grains are also smaller than

250 μm (*Nutricol® GP 312 Product Sheet*, 2008). Chemicals were of analytical grade and from Merck (Darmstadt, Germany).

Proximate Composition

Moisture and ash were determined by standard AOAC procedures (AOAC, 1984), whereas crude protein was determined by Dumas method in a model FP-528 LECO protein/nitrogen analyser (LECO Corp., St. Joseph, USA) and crude fat determined by Bligh and Dyer's rapid method of total lipid extraction and purification (Bligh and Dyer, 1959).

Texture

Folding test was done according to previous work (5.4.1). Gel strength, elasticity and texture profile analysis (TPA), namely hardness, cohesiveness, gumminess, springiness and chewiness were evaluated using a model Instron 4301 texturometer (Instron Engineering Corp., Canton, USA) and a 40 % compression (for elasticity and TPA parameters), according to previous work (5.4.1). Gel strength was calculated as the product of breaking force and breaking deformation.

Furthermore, a more drastic compression (80 %) was also applied in order to measure force and distance at rupture (5.1.5).

pH

pH was measured using a Sen-Tix 21 surface pH electrode (WTW, Weilheim, Germany) on a model pH 539 pH meter (WTW, Weilheim, Germany). This determination was carried out in triplicate.

Water Holding Capacity (WHC)

WHC was measured by a modification of a published method (Sánchez-González et al., 2008), exactly as described in a previous work (Cardoso et al., 2010b).

Colour

L*, a* and b*, chroma and whiteness were determined on a model MACBETH COLOUR-EYE® 3000 colourimeter (Macbeth, New Windsor, USA) as described by a previous work (5.4.1). This determination was carried out in triplicate.

Protein Solubility

In order to follow protein-protein chemical interactions, protein solubility in four different extracting solutions was determined (Table 5.1.4.1). Soluble protein was separated as described in a previous work (Cardoso et al., 2010b). Protein quantitation in the supernatants was performed through absorbance measurement at 280 nm (Piñeiro et al.,

1999). Results are average of three determinations, calculated as percent protein solubilised with respect to total protein in the sample and divided by FCKa protein solubility in SDS+DTT, expressed as relative protein solubility.

Table 5.1.4.1 – Composition of the protein extracting solutions and targeted chemical interactions.

CHARACTERISTICS	Extracting Solution			
	SDS	SDS+DTT	Urea	Urea+DTT
COMPONENTS				
Sodium dodecyl sulphate, SDS (g/l, w/v)	20	20	---	---
DL-Dithiothreitol, DTT (mol/l)	---	0.1	---	0.1
Urea (mol/l)	---	---	8	8
Tris(hidroxymethyl)-aminomethane, Tris(mmol/l)	60	60	60	60
ACIDITY-ALCALINITY				
pH (adjusted with HCl)	7.5	7.5	7.5	7.5
TARGETED CHEMICAL INTERACTIONS*:	Non-covalent bonds	Non-covalent bonds and dissulphide bridges	Non-covalent bonds (more efficient in breaking hydrogen bonds)	Non-covalent bonds (more efficient in breaking hydrogen bonds) and disulphide bridges

* Based on Liu and Hsieh (2008).

Electrophoresis

Changes in individual proteins were followed by SDS-PAGE in 15 % Excel-Gel™ (Amersham, Uppsala, Sweden) according to previous work (5.1.5). For optical density measurement, silver stained gels were analysed in a model GS-800 calibrated densitometer (Bio-Rad, Hercules, USA) with software Quantity One® (Bio-Rad, Hercules, USA).

Scanning Electron Microscopy (SEM)

Cubes of 2-3 mm were cut from inner part of the gels for microscopic examination. Samples were fixed with a mixture (1:1, v/v) of 50 $g.l^{-1}$ formaldehyde and 2 $g.l^{-1}$ glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and dehydrated in increasing series of acetone (100-1000 $ml.l^{-1}$). Afterwards, they were critical-point dried with CO_2 as transition fluid in a model Polaron 3000 critical-point dryer (Polaron, Hertfordshire, UK) and mounted on metallic holders, followed by gold sputter-coating in a model JFC-1100E ion sputtering device (JEOL, Tokyo, Japan). Samples were kept in a dryer until examination by a model JSM-5200 scanning microscope (JEOL, Tokyo, Japan) at 20 kV. Micrographs were taken at different magnifications.

Statistical Analysis

Analysis of variance (general linear model, two-way ANOVA) was carried out using the STATISTICA© software (StatSoft, Inc., Tulsa, USA), version 6.1, 2003. This statistical methodology enabled to analyse each one of the studied effects *per se* (MTGase and

each DF addition) as well as the interaction between effects. The difference of means between pairs was resolved by using confidence intervals in a Tukey HSD test. Level of significance was set for $p<0.05$.

Experimental

Experimental Design

Sea bass mince was used with other ingredients in the preparation of gels (Table 5.1.4.2). Eight batches were prepared: four without MTGase (identified by 0) and four with 5 g/kg (w/w) MTGase (a). These four pairs of batches comprised a no DF batch (C), a 40 g/kg (w/w) Swe batch (FS), a 10 g/kg (w/w) Carr batch (FC), and a batch containing 10 g/kg (w/w) Carr + 10 g/kg (w/w) Kjc (FCK, the combination of Carr and Kjc is required for their synergistic interaction). Error assessment was derived from replication of the various analyses performed.

Table 5.1.4.2 – Main ingredients (g/kg, w/w), nomenclature and protein content (g/kg, w/w) for the various minced sea bass products.

Batch	Sea bass mince	Water	Salt	NaOH sol.	MTGase	Swe	Carr	Kjc	Total	Protein (%, w/w)*
C0	628	327	25	20	0	0	0	0	1000	107 ± 5^{ab}
Ca	611	339	25	20	5	0	0	0	1000	119 ± 5^{a}
FS0	484	431	25	20	0	40	0	0	1000	89 ± 7^{cd}
FSa	466	444	25	20	5	40	0	0	1000	80 ± 2^{d}
FC0	592	353	25	20	0	0	10	0	1000	98 ± 2^{bc}
FCa	574	366	25	20	5	0	10	0	1000	97 ± 5^{bc}
FCK0	557	378	25	20	0	0	10	10	1000	93 ± 2^{cd}
FCKa	539	391	25	20	5	0	10	10	1000	89 ± 3^{cd}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Gels Production

Approximately 6 kg of sea bass mince from trimmings were mechanically obtained in a Baader 694 deboning machine (Baader, Lübeck, Germany) fitted with a drum with 3 mm diameter circular holes. The appropriate quantities of mince, water (in order to guarantee final gel products with the same moisture level, about 800 g/kg, w/w), salt (25 g/kg, w/w), 20 g/kg (w/v) NaOH aqueous solution (required for Kjc deacetylation —in order to form a thermoirreversible and highly heat-stable gel—, but also added to the other batches with the purpose of avoiding pH differences among the batches), MTGase, Swe, Carr and Kjc for approximately 1.3-1.4 kg batches were weighed (Table 5.1.4.2). In order to hydrate DF (Swe and Carr) before mixing, chilled water was added to DF in a ratio near 10:1 and mixed with DF for 1 min. For the FCK batches, a different procedure was used in order to generate a gel by synergistic interaction of Kjc and Carr. Instead of adding the hydrated

DF to the other ingredients, a previously prepared mixed Kjc-Carr gel was added. To make the gel, konjac flour was homogenized in water in a ratio 1:13 and mixed with carrageenan powder previously dispersed in water (ratio 1:5), then, a volume of 20 g/l (w/v) NaOH aqueous solution was added with gentle stirring at room temperature in order to ensure 20 g of this solution in each kg of final product. The admixture was cast and allowed to stand in appropriate containers at 2 ºC (overnight) to gel, yielding a hard and solid gum (Osburn and Keeton, 1994). Only afterwards, the sea bass minces were mixed with the other ingredients for 2 min at 1420 rpm and 2 min at 2800 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Throughout all process, mixing was performed always under vacuum and temperature below 7 ºC. Following procedure was carried out as described in a previous study (Cardoso et al., 2010b).

Results

Proximate Composition

Proximate composition of gels was quite similar and, for moisture, fat and ash, mean values of 770, 50 and 30 g/kg (w/w), respectively, were determined. Differences ($p<0.05$) were detected in protein content (Table 5.1.4.2), which ranged from 110-120 g/kg (w/w) in C0 and Ca to near 80 g/kg (w/w) in FSa. This reduction was due to the choice for an adjustment to an identical moisture level in all products, thus leading to higher water addition in the samples containing higher amounts of DF, such as the FS batches. Hence, there is a protein dilution effect, which shows correlation with the amount of added fibre.

Texture

Folding test (FT), gel strength (GS), elasticity and TPA results are found in Table 5.1.4.3, while force and distance at rupture are presented in Table 5.1.4.4. FT scores, generally used for a quick evaluation of gel quality, were all very good, only two (C0 and FS0) were below the maximum score, but not significantly ($p\geq0.05$). On the other hand, GS showed significant differences between products: MTGase augmented GS in samples with DF and in those without DF, due to MTGase effect upon the breaking force component. Gels without MTGase containing Swe (FS0) showed a lower GS than FCK0 gels (11.3 ± 1.7 *vs* 22.2 ± 3.5 N.mm). These variations were mainly due to the breaking force component of the GS, which, for instance, between FS0 and FCK0, augmented from 1.4 ± 0.1 to 2.5 ± 0.2 N. This compares with a slight increase of the breaking deformation component from 7.9 ± 0.1 to 8.8 ± 0.8 mm. Elasticity was enhanced by MTGase and the combined addition of Carr and Kjc, regardless of MTGase addition. All TPA parameters showed significant ($p<0.05$) textural advantages with MTGase incorporation: gels became

harder with exception of FC0 and FCa samples and cohesiveness improved from 0.51-0.56 to 0.59-0.61. Swe had no effect on TPA properties with exception of higher springiness and chewiness in samples without MTGase.

Contrastingly, Carr and Carr+Kjc had a greater effect: hardness and related properties were increased even in those harder gels containing MTGase (from 17.2 ± 1.7 N in Ca to 23.0 ± 2.1 N in FCa and 30.2 ± 1.8 N in FCKa). Moreover, for products without MTGase, springiness also benefited from addition of these fibres. Chewiness was highest for FCKa, thus, showing that a combination of Carr, Kjc and MTGase produced better quality gels. Finally, with respect to force and distance at rupture —obtained by 80 % compression (imitating mastication in human mouth (Bourne, 1994))—, there was an impressive 1.5 to 3.6 times increase of the force and a higher distance (with exception of the pair FCK0 and FCKa) as a result of MTGase incorporation. Strengthening of the gels without MTGase through Carr and Carr+Kjc addition was also observed (force at rupture more than doubled from 39.7 ± 3.4 N in C0 to 92.8 ± 20.7 N in FC0 and 96.2 ± 8.5 N in FCK0) (Table 5.1.4.4).

pH

Regarding pH values (Table 5.1.4.4), there were only two major effects: a pH reduction through Carr+Kjc incorporation in products without MTGase and a higher pH with MTGase addition to the FCK products. pH changes must be seen as indirect effects of DF and MTGase, probably, resulting from exposure of more or less acidic groups. A pH increase with MTGase addition was reported in previous works (5.1.5). These variations were all inside a narrow range (7.10-7.38), meaning that inclusion of an equal amount of NaOH solution in all formulations yielded products with a similar slightly alkaline pH and that any differences in properties can not be assigned to pH.

WHC

MTGase had no effect upon WHC (Table 5.1.4.4), regardless of the DF added. On the other hand, products containing Swe held less water than those containing Carr or the combination of Carr and Kjc (55.5-56.5 % *vs* 69.0-70.7 %).

Colour

Only some colour values (Table 5.1.4.5) were slightly changed by MTGase or DF addition. While, b* and whiteness remained unaltered, a* was reduced in samples FSa, FCa and FCK0 and chroma only showed a higher colour intensity of FCa samples with respect to Ca ones. These variations show that MTGase changed colour towards the green end of the scale, especially when combined with DF.

Table 5.1.4.3 – Folding test, gel strength, elasticity and TPA (texture profile analysis with 40 % compression) properties of the gels*.

Sample	Dietary fibre (type and g/kg, w/w)	MTGase (g/kg,w/w)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Springiness	Chewiness (N)
C0	0	0	4.7 ± 0.6^{a}	13.3 ± 1.2^{ab}	54.7 ± 0.1^{a}	10.2 ± 0.9^{a}	0.52 ± 0.01^{a}	0.72 ± 0.00^{a}	4.0 ± 0.3^{a}
Ca	0	5	5.0 ± 0.0^{a}	38.3 ± 6.4^{e}	61.2 ± 0.6^{bc}	17.2 ± 1.7^{bc}	0.61 ± 0.01^{d}	0.83 ± 0.01^{cd}	8.8 ± 0.6^{c}
FS0	Swe, 40	0	4.7 ± 0.6^{a}	11.3 ± 1.7^{a}	56.1 ± 0.7^{a}	13.8 ± 0.8^{ab}	0.52 ± 0.00^{ab}	0.80 ± 0.01^{b}	6.0 ± 0.2^{b}
FSa	Swe, 40	5	5.0 ± 0.0^{a}	28.2 ± 3.7^{cd}	62.5 ± 1.8^{bc}	18.2 ± 1.6^{c}	0.59 ± 0.00^{cd}	0.84 ± 0.01^{cd}	9.6 ± 0.3^{cd}
FC0	Carr, 10	0	5.0 ± 0.0^{a}	20.0 ± 2.1^{abc}	56.7 ± 0.5^{a}	24.2 ± 2.1^{d}	0.51 ± 0.00^{a}	0.83 ± 0.00^{cd}	10.9 ± 0.3^{de}
FCa	Carr, 10	5	5.0 ± 0.0^{a}	31.9 ± 7.2^{de}	63.0 ± 0.9^{bc}	23.0 ± 2.1^{d}	0.60 ± 0.01^{d}	0.85 ± 0.01^{cd}	12.3 ± 0.3^{e}
FCK0	Carr+Kjc, 10+10	0	5.0 ± 0.0^{a}	22.2 ± 3.5^{bc}	60.6 ± 0.3^{b}	23.8 ± 2.6^{d}	0.56 ± 0.03^{bc}	0.82 ± 0.01^{bc}	10.3 ± 1.0^{d}
FCKa	Carr+Kjc, 10+10	5	5.0 ± 0.0^{a}	33.6 ± 2.0^{de}	64.0 ± 0.9^{c}	30.2 ± 1.8^{e}	0.59 ± 0.01^{d}	0.85 ± 0.01^{d}	15.3 ± 0.7^{f}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Table 5.1.4.4 – Rupture (at 80 % compression) properties, pH and WHC of the gels*.

Sample	Dietary fibre (type and g/kg, w/w)	MTGase (g/kg,w/w)	Force at rupture (N)	Distance at rupture (mm)	pH	WHC (%)
C0	0	0	39.7 ± 3.4^{a}	18.0 ± 1.0^{ab}	7.26 ± 0.05^{ab}	62.9 ± 7.2^{ab}
Ca	0	5	141.0 ± 9.3^{c}	20.0 ± 0.0^{d}	7.24 ± 0.04^{b}	62.8 ± 1.9^{ab}
FS0	Swe, 40	0	35.4 ± 1.1^{a}	17.1 ± 0.3^{a}	7.22 ± 0.06^{bc}	55.5 ± 3.1^{b}
FSa	Swe, 40	5	122.3 ± 1.6^{bc}	19.7 ± 0.4^{d}	7.28 ± 0.01^{ab}	56.5 ± 4.8^{b}
FC0	Carr, 10	0	92.8 ± 20.7^{b}	18.5 ± 0.5^{bc}	7.27 ± 0.01^{ab}	69.7 ± 1.7^{a}
FCa	Carr, 10	5	139.9 ± 29.3^{c}	19.8 ± 0.3^{d}	7.27 ± 0.01^{ab}	69.0 ± 3.0^{a}
FCK0	Carr+Kjc, 10+10	0	96.2 ± 8.5^{b}	20.0 ± 0.0^{d}	7.10 ± 0.09^{c}	70.1 ± 2.3^{a}
FCKa	Carr+Kjc, 10+10	5	126.1 ± 1.2^{bc}	19.3 ± 1.0^{cd}	7.38 ± 0.05^{a}	70.7 ± 2.1^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Table 5.1.4.5 – Colour parameters of the gels*.

Sample	Dietary fibre (type and g/kg, w/w)	MTGase (g/kg,w/w)	a*	b*	Whiteness	Chroma
C0	0	0	-1.36 ± 0.09^{a}	4.18 ± 0.48^{a}	72.9 ± 0.9^{a}	4.40 ± 0.48^{ab}
Ca	0	5	-1.41 ± 0.05^{ab}	4.02 ± 0.11^{a}	72.9 ± 0.3^{a}	4.26 ± 0.12^{a}
FS0	Swe, 40	0	-1.31 ± 0.19^{a}	4.27 ± 0.28^{a}	72.9 ± 1.0^{a}	4.46 ± 0.32^{ab}
FSa	Swe, 40	5	-1.81 ± 0.21^{bcd}	4.65 ± 0.47^{a}	72.8 ± 0.8^{a}	4.99 ± 0.51^{ab}
FC0	Carr, 10	0	-1.25 ± 0.05^{a}	4.22 ± 0.04^{a}	72.7 ± 0.5^{a}	4.41 ± 0.05^{ab}
FCa	Carr, 10	5	-2.03 ± 0.00^{d}	4.98 ± 0.02^{a}	74.6 ± 0.7^{a}	5.38 ± 0.02^{b}
FCK0	Carr+Kjc, 10+10	0	-1.84 ± 0.01^{cd}	4.77 ± 0.11^{a}	74.6 ± 1.0^{a}	5.11 ± 0.11^{ab}
FCKa	Carr+Kjc, 10+10	5	-1.54 ± 0.03^{abc}	4.97 ± 0.09^{a}	73.2 ± 0.4^{a}	5.21 ± 0.10^{ab}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Protein Solubility

The proteins of sea bass products had higher solubility in SDS+DTT and lower solubility in urea (Table 5.1.4.6). MTGase addition reduced solubility in SDS for gels containing Carr and in all other media for control gels (without DF). However, a clearer evaluation of the effect of MTGase may be achieved with a statistical comparison of the averages of all the products with and without MTGase, regardless of the DF added. This analysis revealed that MTGase reduced ($p<0.05$) protein solubility in SDS, from 71.1 ± 12.2 to 51.1 ± 7.3 %. The same kind of analysis did not show any similar effects concerning the remaining extracting solutions. Swe increased protein solubility of the products containing MTGase in SDS+DTT and urea, but reduced solubility in urea of the products without enzyme. These opposite variations may lack significance, since a statistical comparison of averages (as done above) showed that P samples were not different from control samples. Carr and Carr+Kjc had a similar effect: solubility of products without MTGase was reduced in urea and urea+DTT. The reduction of solubility in urea+DTT largely reflected the variation observed in urea. Furthermore, for samples containing MTGase, addition of Carr or Carr+Kjc caused no solubility loss.

Table 5.1.4.6 – Relative protein solubility of the gels in different extracting solutions*.

Sample	Dietary fibre (type and g/kg, w/w)	MTGase (g/kg,w/w)	Protein solubility in SDS (%)	Protein solubility in SDS+DTT (%)	Protein solubility in urea (%)	Protein solubility in urea+DTT (%)
C0	0	0	64.7 ± 13.2^{ab}	86.3 ± 8.9^{acd}	43.5 ± 2.2^{a}	66.7 ± 2.8^{a}
Ca	0	5	47.7 ± 3.6^{b}	62.7 ± 4.8^{b}	27.1 ± 4.2^{b}	37.9 ± 4.2^{b}
FS0	Swe, 40	0	77.6 ± 12.1^{a}	90.7 ± 2.9^{cd}	29.6 ± 3.6^{b}	52.0 ± 10.3^{ab}
FSa	Swe, 40	5	58.7 ± 10.5^{ab}	85.4 ± 5.6^{acd}	48.1 ± 7.0^{a}	52.1 ± 6.8^{ab}
FC0	Carr, 10	0	76.7 ± 13.0^{a}	81.1 ± 2.8^{ac}	28.7 ± 2.2^{b}	45.0 ± 3.4^{b}
FCa	Carr, 10	5	45.6 ± 3.4^{b}	74.0 ± 2.1^{ab}	24.9 ± 2.7^{b}	46.5 ± 7.6^{b}
FCK0	Carr+Kjc, 10+10	0	65.5 ± 10.4^{ab}	80.4 ± 4.7^{ac}	26.0 ± 0.7^{b}	48.2 ± 1.0^{b}
FCKa	Carr+Kjc, 10+10	5	52.3 ± 3.2^{ab}	100.0 ± 7.7^{d}	27.3 ± 2.0^{b}	43.3 ± 5.9^{b}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Electrophoresis

Only the electrophoresis profiles of the proteins extracted with U+DTT are shown, since differences between samples were more evident in this extracting medium (Figure 5.1.4.1). MTGase caused the disappearance of high molecular weight (HMW) protein bands, namely, bands assigned to the myosin heavy chain (MHC) and M- and C-proteins. For the gels without MTGase, addition of DF also weakened the intensity of the MHC band. However, whereas Swe had a very slight effect, Carr and Carr+Kjc made the MHC band much fainter than in the C0 lane. Particularly, the combination of Carr and Kjc caused the almost complete disappearance of the MHC band.

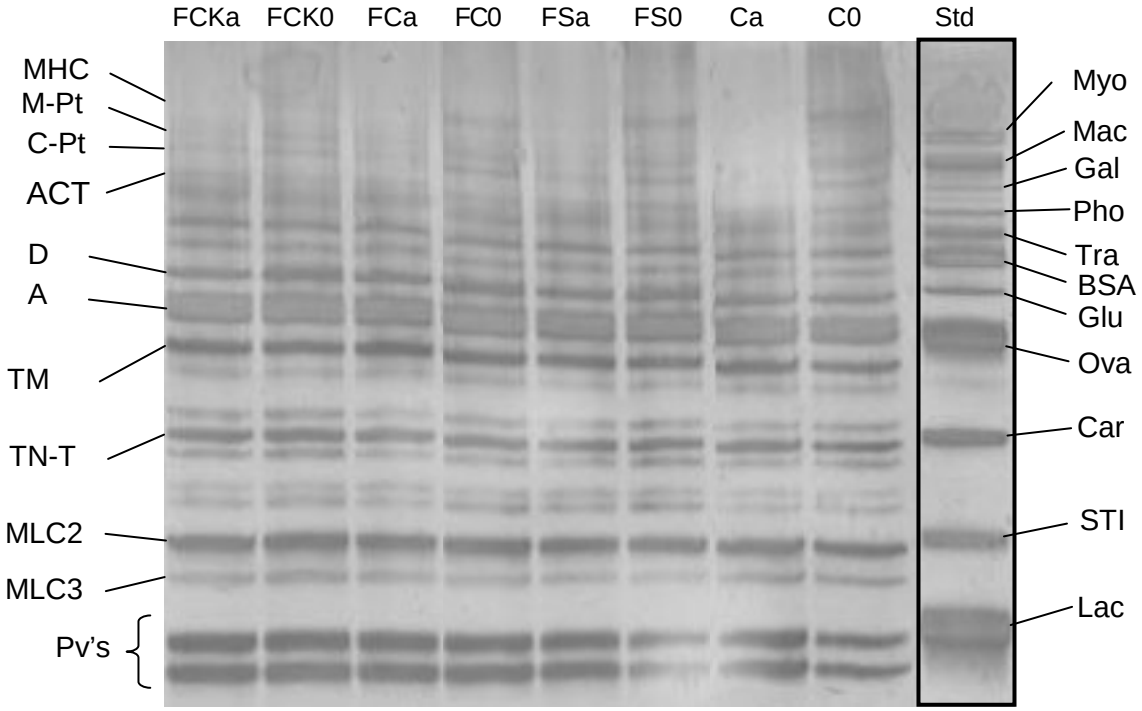

Figure 5.1.4.1 – 15 % Excel-Gel SDS-PAGE of soluble protein homogenates in Urea+DTT of gels. Sample references as in Table 1. MHC, myosin heavy chain; M-Pt, M-protein; C-Pt, C-protein; ACT, actinin; D, desmin; A, actin; TM, tropomyosin; TNT, troponin-T; MLC2, myosin light chain 2; MLC3, myosin light chain 3; Pv's, parvalbumins. Standard names and molecular weights: Myo, myosin (212.0 kDa); Mac, α2-macroglobulin (170.0 kDa); Gal, β-galactosidase (116.0 kDa); Pho, phosphorylase B (94.0 kDa); Tra, transferrin (76.0 kDa); BSA, bovine serum albumine (67.0 kDa); Glu, glutamic dehydrogenase (53.0 kDa); Ova, ovalbumin (43.0 kDa); Car, carbonic anhydrase (30.0 kDa); STI, soybean trypsin inhibitor (20.1 kDa); Lac, α-lactalbumin (14.4 kDa)

SEM

Gels microstructure was analyzed by SEM. MTGase incorporation had a more clear effect upon those gels without DF, since it favoured a more homogeneous microstructure with more evenly distributed pores (Figure 5.1.4.2).

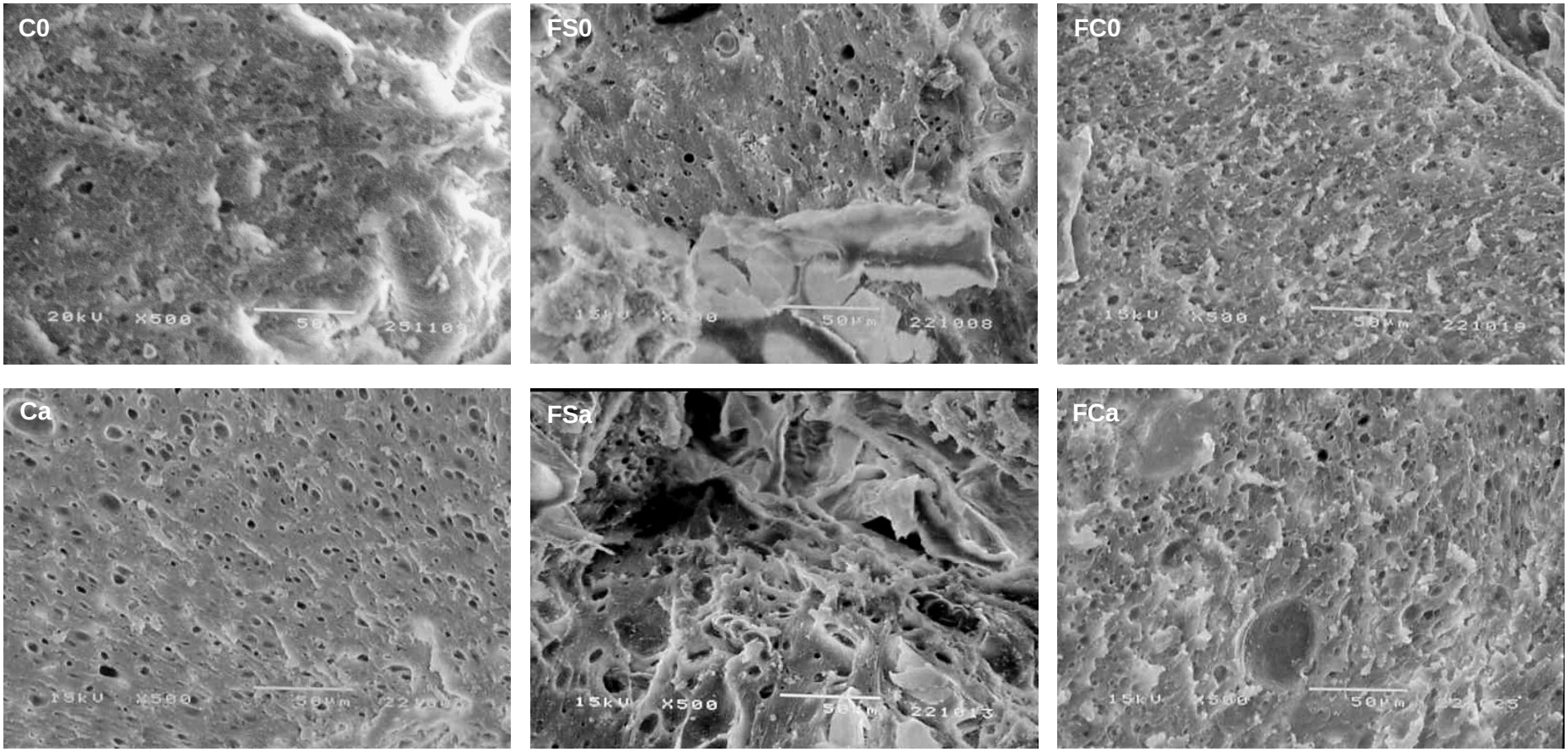

Figure 5.1.4.2 – Scanning electron microscopy (SEM) micrographs of some of the produced gels: C0, FS0, FC0, Ca, FSa and FCa.

Swe incorporation caused a microstructural rearrangement, originating large pores, undulating surfaces and a less compact morphology. Finally, gels containing Carr and Carr+Kjc had similar microstructures and did not differ much from control. However, they presented a denser network of small pores (compare C0 and FC0) and some superficial whitish globular structures, which may be carrageenan.

Discussion

According to the knowledge of the authors, the production of heat-induced gels from sea bass (*Dicentrarchus labrax*) trimmings or from any other sea bass portions (namely, fillets) was only tested in one previous work (Cardoso et al., 2010b). This may be explained by the high commercial value of the whole fresh fish. However, the market for the whole fish presents signs of saturation and there is a potential market for ready-to-cook sea bass fillets, which, in turn, may generate by-products. Gel fish products could offer an outlet for these by-products.

The quality of the heat-induced sea bass gels was very good either with respect to texture or to WHC, even without the addition of MTGase or DF. This finding becomes more remarkable when properties of these unwashed gels are compared with those of other gels produced from surimi of fat fish species. For instance, gels from mackerel (*Scomber scombrus* and *Scomber japonicus*) surimi without improving additives had a GS of 8.1 ± 0.8 N.mm (13.3 ± 1.2 N.mm for C0 in present work) or a hardness of 6.7 ± 0.4 N (10.2 ± 0.9 N) (5.1.5). However, the comparison with gels from another farmed fish species, gilthead seabream (*Sparus aurata*), prepared under identical conditions, reveals a quality similar to that found in sea bass gels (5.2.1). This suggests that farmed fish species present good quality protein and may yield good quality gels. A possible explanation for this phenomenon may lie in a lower level of protein denaturation of marketed farmed fish with respect to wild fish. The harvest techniques and the time elapsed between capture and marketing may be influential. Nonetheless, other factors may be involved. Other authors working with filleting wastes of a typically farmed fish species, silver carp (*Hypophthalmichthys molitrix*), reported low cohesiveness values, 0.21 ± 0.01 (Téllez-Luis et al., 2002), which compares to 0.52 ± 0.01 in this study. Frozen storage prior to processing may also be important, since in this work fish were not frozen stored during several weeks, as frequently happens with fish used in surimi production. In fact, according to another study, breaking force and deformation of surimi gels produced from frozen fish decreased continuously with increasing time of frozen storage (Benjakul et al., 2005). Moreover, the decline in gel-forming ability was associated with the decrease in Ca^{2+}-ATPase activity and mainly with formaldehyde formation.

Concerning MTGase, textural benefits have been found in many other fish products (Téllez-Luis et al., 2002; Ramírez et al., 2000; 5.1.3). Likewise, 50 g/kg (w/w) MTGase addition improved all textural properties of heat-induced sea bass gels with exception of FT (which was already near the upper limit of the scale in C0). The action of this enzyme is well known and was already discussed in a previous study (Cardoso et al., 2010b). It increases cross-linking of myosin heavy chains (MHC) during setting, since it catalyzes covalent ε-amino-(γ-glutamyl)-lysine bonds (Kumazawa et al., 1993). The absence of effect of MTGase on WHC was already observed in heat-induced gels from sea bass trimmings by the authors (Cardoso et al., 2010b).

The decrease of soluble protein with MTGase addition is due to cross-linking of MHC during setting, since it catalyzes covalent bonds, which lead to the formation of insoluble polymeric aggregates (Ashie and Lanier, 1999). Hence, those chemicals that break non-covalent bonds or disulphide bridges extract much less protein. In fact, a reduction of the percentage of extracted protein, as a result of MTGase addition, was also reported by other authors (Gómez-Guillén et al., 2005). Based on the statistical comparison of the averages of all the products with and without MTGase, results show that only those products without added DF presented such loss of solubility for all extracting media. For products containing DF, such phenomenon only occurred in SDS, which contrasted with the absence of it in SDS+DTT. This contrast enables to hypothesize that MTGase may promote the establishment of disulphide bonds (Table 5.1.4.1) in sea bass gels, thus, being its direct catalytic action compounded by indirect effects. Nevertheless, these are disulphide bonds between proteins with extensive exposure of their hydrophobic areas — with urea+DTT *vs* urea no similar phenomenon was detected— and only occurred in the presence of DF. A similar hypothesis was admitted for mackerel surimi (5.1.5). However, the absence of any significant effect of the MTGase upon protein solubility in SDS+DTT for gels containing DF —already observed with the addition of 4 % (w/w) pea fibre to mackerel surimi— suggests that DF may hinder MTGase action, leading to less ε-amino-(γ-glutamyl) lysine bonds. Furthermore, the electrophoretic study revealed that samples containing DF presented a smaller reduction of the intensity of the MHC band as a result of MTGase incorporation. Although, there is weakening of the MHC band with DF addition, it does not exclude a disturbance of the MTGase action on MHC. Nonetheless, MTGase effect on solubility results are consistent with texture parameters, since they show that GS or TPA properties increases are correlated with important chemical alterations in the gels, which favoured a denser covalent cross-linking (with admixture of disulphide bonds in the presence of DF) between myofibrillar proteins. Moreover, texture improvement by MTGase was less notorious in samples containing DF, which reinforces the hypothesis of a thwarting effect of DF upon MTGase activity. The correlation between

textural effects and MTGase found in this study was strong for breaking force, force at rupture and hardness and less so for elasticity or cohesiveness. This can be due to the acceptable cohesiveness and elasticity values of the control, which left less space for improvement, but also may indicate an overwhelming hardening effect of MTGase. On this subject, there are different views, while some authors have related S-S and other covalent bonding with higher hardness and lower elasticity (Pérez-Mateos et al., 1997), others sustain the opposite (Havea et al., 2004).

Used DF (Swe, Carr and Carr+Kjc) had different effects upon sea bass gels, enabling to consider two groups, a first comprising Swe and a second the other two fibres.

Swe had a negative effect: texture was quite similar to that of the C products, but WHC was reduced. Among all added fibres, it had the smallest impact on the electrophoresis profile. This was quite different from the referenced Swe effect on other fish gel products (5.3.1; 5.1.5). Specifically, it was shown that incorporation of similar levels (40 g/kg, w/w) of inner pea fibre significantly improved texture (gel strength and hardness) of minced hake (*Merluccius capensis*) products (5.3.1). Of course, other studies showed that advantages of Swe were more limited, for instance, gel strength of mackerel (*Scomber scombrus* and *Scomber japonicus*) surimi gels was unaffected by Swe (5.1.5). Although variability among species may have a role, present results and those from the literature suggest also another phenomenon. As seen in Table 5.1.4.2, protein content of the sea bass products fell with Swe incorporation from 107 ± 5 to 89 ± 7 g/kg (w/w). This is a substantial variation (1/6 of the C value) and is due to the objective of producing gels with the same moisture level —a protein dilution effect. Hardness reduction with increasing fibre concentration has also been observed and associated to dilution and disruption of the protein matrix (Tudorica et al., 2002). However, in other studies (5.3.1; 5.1.5), the same dilution effect was present and results were different. This apparent contradiction led to the following hypothesis: if fish protein is of poor quality, replacing protein by Swe has a beneficial effect, but, if protein presents adequate properties, Swe substitution has a negative net effect. That is, the advantages accrued by Swe addition are smaller than the costs associated to a lower concentration of protein in the product. This seems to be the case in the present study, since C products with no additive had high textural quality. Loss of rheological quality when the minced muscle is of high quality has been reported (Pérez-Mateos and Montero, 2000).

A positive net effect can only be achieved if substituted fibre imparts more textural (and WHC) quality to the product than protein. Carr or Carr+Kjc seem to belong to this category. Texture was generally improved, particularly, hardness and chewiness and, for products without MTGase, springiness, force and distance at rupture were enhanced. WHC also increased. Only cohesiveness (with the exception of Carr+Kjc in gels without

MTGase) and breaking deformation (a component of gel strength) remained unaltered. Therefore, the dilution effect (more important for Carr+Kjc, FCK0 had a protein content of 93 ± 2 g/kg, w/w) was more than countervailed by these two fibres. Thus, higher incorporation levels of these fibres may improve even more the texture. It has been reported that while compression test measures overall binding property of the gel material, penetration test (GS) evaluates degree of compactness or density of actomyosin (Lee and Chung, 1989). According to this interpretation, Carr, alone or combined with Kjc, increased strongly overall binding, but, unlike MTGase, failed to produce a higher degree of compactness in the gel network. However, this does not explain the contrast to Swe, which contains starch and, as such, may improve gel-forming capacity by acting as a filler of the myofibrillar protein gel (Lee et al., 1992). Observed differences between Swe and Carr (or Carr+Kjc) may lie in the nature of the DF. Whereas, Swe has cellulose (2/3) and pectic material (1/3) as main DF components (Anderson & Berry, 2001), Carr is a mixture of iota and kappa carrageenans and Kjc contains glucomannan (*CEAMGEL1830*® *Product Sheet*, 2006; *Nutricol*® *GP 312 Product Sheet*, 2008). The latter fibres may form *per se* gels and, as such, act as gelling agents in food systems (Fernández-Martín et al., 2009; Candogan and Kolsarici, 2003), while cellulose not (Borderías et al., 2005). Therefore, Carr and Kjc have an effect that goes beyond the sheer filling action and this may explain their favourable effect on the texture of sea bass gels. Different hypothesis regarding this have been put forth. It may be that, besides the protein gel network, there is a second (polyssacharide) network enhancing hardness and related parameters. Other authors found that ι-carrageenan formed an independent network, which established connections between adjacent structures supporting the main structure formed by the fish protein (Gómez-Guillén et al., 1996). However, this hypothesis does not exclude the existence of important polyssacharide-protein interactions, evidenced namely in the improvement of the gel-forming capacity of Alaska Pollock surimi by the addition of ι- and κ-carrageenans as a result of the interaction of carrageenan sulphate groups and fish proteins (Llanto et al., 1990). Furthermore, other authors' findings also support the view that such fibres may act upon the protein network through their water holding capacity, which reduces the water content of the mesh and increases the density of the surrounding protein matrix (Niwa et al., 1988; Niwa et al., 1989). In fact, regarding WHC, a strong water absorptivity of carrageenan and KGM agrees perfectly with results, which are similar to others reported in the literature (Xiong et al., 2009; Gómez-Guillén and Montero, 1996). The reduction of protein solubility in urea (and urea+DTT) of Carr or Carr+Kjc samples extracts suggests that hydrogen bonds between proteins were less important for protein aggregation in the presence of DF. A possible cause could be the involvement of

some protonated amino acids in electrostatic interactions with the sulphate groups of carrageenan. However, a similar loss of total protein solubility was observed for Swe. On the other hand, electrophoresis showed that solubility of the MHC decreased when Swe was replaced by Carr and, even more, for products containing Carr+Kjc (total protein solubility results were not affected, probably, as a result of two opposite effects: aggregation of MHC and lesser aggregation of other proteins), thus indicating that these fibres acted upon the main network protein. However, the nature of the action is not clear, since it may be direct or indirect. Though this effect on MHC was observed for all extracting media —which targeted various chemical interactions—, a strong electrostatic interaction can not be overruled (Montero et al., 2000). An indirect action would be an enhancement of the density of the protein matrix by Carr or Carr+Kjc (Niwa et al., 1988; 1989), this would mean a higher degree of covalent bonding between MHC molecules and, accordingly, a lower solubility. The microstructural images do not enable to decide which hypothesis is more suitable. They only suggest that Carr or Carr+Kjc favour a more orderly protein matrix. However, their effect was much less intense than Swe. A strong microstructural effect of this DF was already observed in previous work (5.1.5).

Conclusions

MTGase incorporation had a positive effect on texture (gel strength and force at rupture were greatly improved) but not on WHC. Regarding the interaction of MTGase and DF, protein solubility results suggested that DF may hinder MTGase action, leading to less ε-amino-(γ-glutamyl) lysine bonds. Pea fibre had no positive effects: the texture was quite similar to that of the control products (no DF) and the WHC was reduced. On the other hand, carrageenan and KGM addition markedly improved quality of the gels. The different outcomes may be due to different interactions between DF and protein.

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5.1.5- Effect of Dietary Fibre and MTGase on the Quality of Mackerel Surimi Gels

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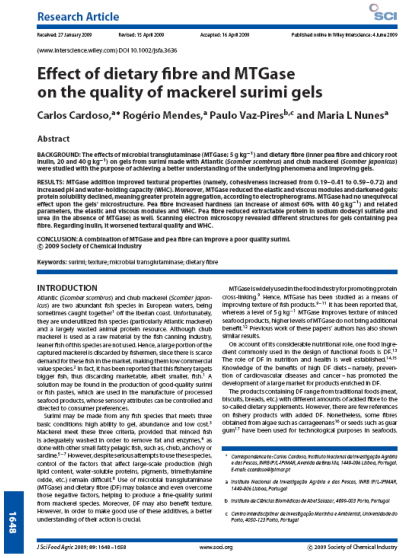

Research Article

Effect of dietary fibre and MTGase on the quality of mackerel surimi gels

Carlos Cardoso,* Rogério Mendes, Paulo Vaz-Pires and Maria L Nunes

Abstract

BACKGROUND: The effects of microbial transglutaminase (MTGase; 5 g kg^{-1}) and dietary fibre (inner pea fibre and chicory root inulin, 20 and 40 g kg^{-1}) on gels from surimi made with Atlantic (*Scomber scombrus*) and chub mackerel (*Scomber japonicus*) were studied with the purpose of achieving a better understanding of the underlying phenomena and improving gels.

RESULTS: MTGase addition improved textural properties (namely, cohesiveness increased from 0.19–0.41 to 0.59–0.72) and increased pH and water-holding capacity (WHC). Moreover, MTGase reduced the elastic and viscous modulus and darkened gels; protein solubility declined, meaning greater protein aggregation, according to electropherograms. MTGase had no unequivocal effect upon the gels' microstructure. Pea fibre increased hardness (an increase of almost 60% with 40 g kg^{-1}) and related parameters, the elastic and viscous modules and WHC. Pea fibre reduced extractable protein in sodium dodecyl sulfate and urea (in the absence of MTGase) as well. Scanning electron microscopy revealed different structures for gels containing pea fibre. Regarding inulin, it worsened textural quality and WHC.

CONCLUSION: A combination of MTGase and pea fibre can improve a poor quality surimi.
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Keywords: surimi; texture; microbial transglutaminase; dietary fibre

INTRODUCTION

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Abstract

Background

The effects of MTGase (0.5 %, w/w) and dietary fibre (inner pea fibre and chicory root inulin, 2.0 and 4.0 %, w/w) on gels from surimi made with Atlantic (*Scomber scombrus*) and chub mackerel (*Scomber japonicus*) were studied with the purpose of achieving a better understanding of the underlying phenomena and improving gels.

Results

MTGase addition improved textural properties (namely, cohesiveness increased from 0.19-0.41 to 0.59-0.72) and increased pH and water holding capacity (WHC). Moreover, MTGase reduced the elastic and viscous modules and darkened gels; protein solubility declined, meaning greater protein aggregation, according to electropherograms. MTGase had no unequivocal effect upon the gels' microstructure.

Pea fibre increased hardness (an increase of almost 60 % with 4.0 %, w/w) and related parameters, the elastic and viscous modules and WHC. Pea fibre reduced extractable protein in SDS and urea (in the absence of MTGase) as well. Scanning electron microscopy revealed different structures for gels containing pea fibre. Regarding inulin, it worsened textural quality and WHC.

Conclusion

Therefore, a combination of MTGase and pea fibre can improve a poor quality surimi.

Keywords: Surimi, texture, microbial transglutaminase, dietary fibre

Introduction

Atlantic (*Scomber scombrus*) and chub mackerel (*Scomber japonicus*) are two abundant fish species in European waters, being sometimes caught together (ICES, 2006) off the Iberian coast. Unfortunately, they are underutilized fish species (particularly Atlantic mackerel) and a largely wasted animal protein resource. Although chub mackerel is used as a raw material by the fish canning industry, leaner fish of this species are not used. Hence, a large portion of the captured mackerel is discarded by fishermen, since there is scarce demand for these fish in the market, making them low commercial value species (Cabral et al., 2003). In fact, it has been reported that this fishery targets bigger fish, thus, discarding marketable albeit smaller fish (ICES, 2006). A solution may be found in the production of good quality surimi or fish pastes, which are used in the manufacture of processed seafood products, whose sensory attributes can be controlled and directed to consumer preferences.

Surimi may be made from any fish species that meets three basic conditions: high ability to gel, abundance and low cost (Aguad et al., 1989). Mackerel meet these three

criteria, provided that minced fish is adequately washed in order to remove fat and enzymes (Shimizu et al., 1992), as done with other small fatty pelagic fish, such as, chub, anchovy, or sardine (Bentis et al., 2005; Gómez-Guillén et al., 1997b; Kaba, 2006). However, despite serious attempts to use these species, control of the factors that affect large-scale production (high lipid content, water soluble proteins, pigments, trimethylamine oxide, etc.) remain difficult (Chen et al., 1997). Use of microbial transglutaminase (MTGase) and dietary fibre (DF) may balance and even overcome those negative factors, helping to produce a fine quality surimi from mackerel species. Moreover, DF may also benefit texture. However, in order to make good use of these additives, a better understanding of their action is crucial.

MTGase is widely used in the food industry for promoting protein cross-linking (Téllez-Luis et al., 2002). Hence, MTGase has been studied as a means of improving texture of fish products (Téllez-Luis et al., 2002; Ramírez et al., 2000). It has been reported that, whereas a level of 0.5 % (w/w) MTGase improves texture of minced seafood products, higher levels of MTGase do not bring additional benefit (Moreno et al., 2009). Previous work of these paper's authors has also shown similar results.

On account of its considerable nutritional role, one food ingredient commonly used in the design of functional foods is DF (Puupponen-Pimïa et al., 2002). The role of DF in nutrition and health is well established (Anderson et al., 1990; Kritchevsky and Bonfield, 1995). Knowledge of the benefits of high DF diets —namely, prevention of cardiovascular diseases and cancer— has promoted the development of a large market for products enriched in DF.

The products containing DF range from traditional foods (meat, biscuits, breads, etc.) with different amounts of added fibre to the so-called dietary supplements. However, there are few references on fishery products with added DF. Nonetheless, some fibres obtained from algae such as carrageenans (Ortiz and Aguilera, 2004) or seeds such as guar gum (Montero et al., 2000) have been used for technological purposes in seafoods. It was found that different fibres generated texturally diverse products (Montero et al., 2000), for instance, presence of xanthan gum at 0.5 % (w/w) caused a considerable hardness reduction with respect to control. Besides texture, colour was altered, turning more yellow with addition of some fibres. Furthermore, other studies have shown that incorporation of low levels (≤ 4.0 %, w/w) of inner pea fibre and chicory root inulin may alter texture of restructured fish products, namely, inner pea fibre favoured greater gel strength and hardness (chapter 5.3.1), whereas, the latter fibre, albeit reducing hardness, increased folding test in hake heat-induced gels (5.1.2).

This work aimed to improve the gel quality of mackerel surimi through incorporation of MTGase and DF and to understand better the effects of these additives and the possible underlying phenomena associated to them with an ample set of techniques.

Materials and Methods

Materials

Chub (*Scomber japonicus*) and Atlantic Mackerel (*Scomber scombrus*) were fished off the Portuguese coast, frozen on board and sent to L-IPIMAR. Fish were stored at –28 ºC and processed within two months after its arrival at the laboratory. Chub mackerel weight varied between 180 and 220 g, whereas Atlantic mackerel were smaller, weighing 70 to 200 g.

There were two DF used in this study: inner pea fibre-Swelite® (Swe) and chicory root inulin-Fibruline INSTANT (Fib), supplied by Cosucra, S.A. (Warcoing, Belgium). The particular composition (dry matter) and other properties are presented in Table 5.1.5.1.

Microbial transglutaminase (MTGase) ACTIVA® GS was supplied by Ajinomoto (Tokyo, Japan).

Chemicals were of analytical grade and from Merck (Darmstadt, Germany).

Table 5.1.5.1 – Relevant properties of the used dietary fibre products.

PROPERTIES	Swelite®*	Fibruline INSTANT†
Composition (D.M.)		
Total Carbohydrates (%)	93 ± 3	min. 99.7
Of which: *Total DF (%)*	48 ± 3	min. 90
Starch (%)	min. 36	—
Protein (%)	max. 7	—
Fat (%)	max. 0.5	—
Ash (%)	max. 2	max. 0.3
Granulometry (µm)	< 400	< 700
Colour	white	white
Taste	neutral	neutral to slightly sweet

* (Anonymous, 2005a).
† (Anonymous, 2005b).

Experimental design

Mackerel surimi was produced and used with other ingredients in the preparation of gels. Inner pea fibre, Swe, and chicory root inulin, Fib, were tested at two levels, 20 (2S and 2F, respectively) and 40 $g.kg^{-1}$ (4S and 4F), and compared with a control without added DF (C). The effect of MTGase was assessed for each of these formulations, producing ten groups of gel products: five without MTGase (C0, 2S0, 4S0, 2F0 and 4F0) and five with 5 $g.kg^{-1}$, MTGase (Ca, 2Sa, 4Sa, 2Fa and 4Fa). Error assessment was derived from replication of the analyses performed.

Surimi production

28 kg of frozen mackerel (approximately, 23 kg of Atlantic and 5 kg of chub mackerel, a ratio 4.5:1) were thawed overnight in a refrigerator. Then, mackerel were headed, gutted, washed and skin and bones were mechanically removed in a Baader 694 deboning machine (Baader, Lübeck, Germany) fitted with a drum with 3 mm diameter circular holes (temperature of the fish and of the resulting mince never exceeded 5 ºC). Afterwards, minced fish was washed with a solution (0-3 ºC) of sodium bicarbonate (5 g/l) and tetrassodium pyrophosphate (2.5 g/l) during 20 min., using the ratio 1 mince part:3 solution parts. Then, pH was adjusted to 6.5 with phosphoric acid. After, mince was dewatered in a New-Tech surimi pilot plant (Bibun, Fukuyama, Japan), a second washing with an identical ratio was done with a solution (0-3 ºC) of sodium chloride (3 g/l) during 10 min., ensuring a good dehydration of the mince. After dewatering the mince again, produced surimi was mixed with cryoprotectants —sorbitol (4.0 %, w/w), sucrose (4.0 %, w/w) and sodium polyphosphate (0.5 %, w/w)— in a model UM12 refrigerated vacuum homogeniser (Stephan, Hameln, Germany). Mixing was performed for 1 min. at 2800 r.p.m. under vacuum and refrigeration (< 5 ºC). Then, surimi was weighed (10.3 kg) and vacuum-packed in plastic bags (≈2 kg) with a model A300/52 vacuum packager (Multivac, Wolfertschwenden, Germany). Finally, surimi was frozen down to -28 ºC in a model 2581 forced air freezer (Köttermann, Hänigsen, Germany) and stored at the same temperature for 1 week.

Gels production

10 kg of frozen mackerel surimi were thawed over 12 hours in a refrigerator (2 ± 1 ºC). Surimi, DF, MTGase, salt (2.5 %, w/w) and water (in order to guarantee final products with the same moisture, 80.0 %, w/w) were weighed (1.7 kg batches). Then, in order to hydrate DF before mixing, chilled water was added to DF in a ratio 2:1 (w/w) and mixed with DF for 5 min. A suspension of MTGase and water (ratio 1:5, w/w) was also prepared. Afterwards, surimi was mixed with the other ingredients for 2 min. at 1420 rpm and 3 min. at 2800 rpm in the refrigerated vacuum homogeniser. Throughout all process, mixing was performed always under vacuum and temperature below 7 ºC. The batters attained were put inside a model EB-12 hydraulic filler (Mainca, Granollers, Spain) and ‘sausages’ with a diameter of 25 mm and a length of about 20 cm produced with cellulose casings. ‘Sausages’ were vacuum-packed in low-oxygen permeable barrier bags (O_2 transmission, <2.1 $cm^3/(m^2.day.bar)$ at 23 ºC, Colamin XX 100e, Obermühle, Pössneck, Germany) with a model A300/52 vacuum packager (Multivac, Wolfertschwenden, Germany). Then, these packages were immersed in water at 35 ºC for one hour (setting). For each gel product, a ‘sausage’ was taken apart and frozen at -80 ºC for determination of protein solubility and

electrophoresis. All other packages were moved to a model Combi-Master CM6 oven (Rational, Landsberg, Germany) equipped with a digital thermometer and subjected to steam cooking at 90 ºC for one hour. In order to guarantee these conditions in the innermost part of the product, the oven's digital thermometer was placed in the centre of one 'sausage' through a hole in the bag. Finally, gel products were immediately cooled in iced water and stored in a refrigerated room (5 ± 1 ºC – humidity, 90-95 %) in the dark until analysis.

Proximate composition

Moisture and ash were determined by standard procedures (AOAC, 1984), whereas crude protein was determined in a model FP-528 LECO nitrogen analyser (LECO, St. Joseph, USA), calibrated with EDTA (Dumas method) and crude fat was determined by a rapid method of total lipid extraction (Bligh and Dyer, 1959). Total carbohydrate was determined by difference.

Texture

Folding test (scoring scale from 1 to 5) and gel strength (puncture test) were done according to previous work (Cardoso et al., 2008a), namely, a metal probe equipped with a 5 mm diameter spherical head was used. Texture profile analysis (TPA), namely hardness, cohesiveness, gumminess, springiness and chewiness were also evaluated according to previous work (5.3.1), meaning a 40 % compression. A 50 mm diameter cylindrical probe was used for this purpose.

Furthermore, according to previously tested conditions (5.3.1), a more drastic compression (80 %) that causes gel rupture by compression of all sample was applied in order to measure force and distance at rupture. An 80 % compression was chosen because high compression levels imitate the effects of the mastication process upon food (Bourne, 1994).

A stress-relaxation test was also conducted. To prevent even the weakest linkage rupture, samples were compressed by 10 % with a 50 mm diameter cylindrical probe and a load cell of 1 kN at a crosshead speed of 0.8 mm/s, the deformation being kept constant for 10 min. Initial stress (σ_0) was obtained, relaxation of stress was monitored as a function of time and the curves were fitted to a simplified Maxwell model, given by equation (Hamann and McDonald, 1992):

$$\sigma = \sigma_e + (\sigma_0 - \sigma_e) \times e^{\left(-\frac{t}{\tau_1}\right)}$$

where,

σ - decaying stress (kPa);

σ_e - stress at equilibrium (kPa);

t- time (s);

τ_i- relaxation time (s).

For each fitting, starting σ_e and τ_1 values included into nonlinear regression equation were 1.1 kPa and 200 s, respectively. The viscous (η_1) and elastic (E_e and E_1) moduli were calculated: $\eta_1 = \tau_1 \times E_1$, $E_1 = (\sigma_0 - \sigma_e)$/deformation and $E_e = \sigma_e$/deformation.

pH

pH was measured using a Sen-Tix 21 surface pH electrode (WTW, Weilheim, Germany) on a model pH 539 pH meter (WTW, Weilheim, Germany). This determination was carried out in triplicate.

Water holding capacity (WHC)

WHC was measured by a modification of a published method (Sánchez-González et al., 2008). Coarsely chopped sample (≈2 g) (W_s), wrapped in two overlayed Whatman nº1 filter papers (also weighed, W_i), was placed in a centrifuge tube and submitted to 3,000×g for 10 min. at 20 ºC in a model 6800 centrifuge (Kubota corp., Tokyo, Japan). After centrifugation, sample was removed and filter papers were weighed again (W_f). Measurements were in triplicate. WHC is expressed as grams of water in sample after centrifugation per 100 g of water initially present in sample:

$$WHC = \frac{Ws \times (H/100) - (Wf - Wi)}{Ws \times (H/100)} \times 100$$

where,

H – moisture (%).

Colour

L*, a* and b*, chroma and whiteness were determined on a model MACBETH COLOUR-EYE® 3000 colourimeter (Macbeth, New Windsor, USA) as described by a previous work (5.3.1). This determination was carried out in triplicate.

Protein solubility

Four different extracting solutions were used (Table 5.1.5.2). An amount of 600 mg of chopped sample, including raw material, the mackerel surimi, was homogenized in 8 ml of each extracting solution using a model Polytron® PT-MR 3000 homogenizer (Kinematica, Littau, Switzerland) equipped with a small rod for 60 s at low speed to avoid foaming. Then, samples were boiled in a water bath (100 ºC) for 2 min. and, afterwards, homogenized while hot for 30 s. Finally, samples were centrifuged (20,000g at 20 ºC for

15 min.) in a model 6800 centrifuge (Kubota, Tokyo, Japan). Protein quantitation in the supernatants was performed through OD measurement at 280 nm (Piñeiro et al., 1999). Results are average of three determinations, calculated as percent protein solubilised with respect to total protein in the sample and divided by C0's protein solubility in urea+DTT, so expressed as relative protein solubility.

Table 5.1.5.2 – Composition of the protein extracting solutions and targeted chemical interactions.

CHARACTERISTICS	Extracting Solution			
	SDS	SDS+DTT	Urea	Urea+DTT
COMPONENTS				
Sodium dodecyl sulphate, SDS (%, w/v)	2	2	---	---
DL-Dithiothreitol, DTT (M)	---	0.1	---	0.1
Urea (M)	---	---	8	8
Tris(hidroxymethyl)-aminomethane, Tris(mM)	60	60	60	60
ACIDITY-ALCALINITY				
pH (adjusted with HCl)	7.5	7.5	7.5	7.5
TARGETED CHEMICAL INTERACTIONS	Non-covalent bonds	Non-covalent bonds and disssulphide bridges	Predominantly hydrogen bonds	Predominantly hydrogen bonds and disssulphide bridges

Electrophoresis

The supernatant (soluble protein) attained was diluted to 0.3 mg/ml with a pH 6.8 Laemmli buffer (4.8 g/100 ml SDS + 1 mM EDTA + 0.1 mM DTT + 20 ml/100 ml glycerol + 125 mM Tris-HCl + 0.05 g/100 ml bromophenol blue), centrifuged (20,000g at 20 ºC for 10 min.) in a model Sigma 3K30 centrifuge (SIGMA, Osterode, Germany) and then heated for 2 min. in a boiling bath. Samples were analysed by SDS-PAGE in a model 2117 Multiphor II electrophoresis unit (LKB, Bromma, Sweden), using Excel Gel™ 15 % polyacrilamide gels and Excel Gel™ buffer strips (Amersham, Uppsala, Sweden). Electrophoresis conditions were 600 V, 30 mA and 30 W and temperature was kept at 15 ºC by a model 2219 Multitemp II thermostatic circulator (LKB, Bromma, Sweden). The protein bands were silver stained with a PlusOne™ kit (Amersham, Uppsala, Sweden). As reference for molecular weights, two standard (high and low molecular weight) electrophoresis calibration kits (Pharmacia Biotech, Uppsala, Sweden) were used. For optical density measurement, gels were analysed in a model GS-800 calibrated densitometer (Bio-Rad, Hercules, USA) with software Quantity One® (Bio-Rad, Hercules, USA).

Scanning electron microscopy (SEM)

Cubes of 2-3 mm were cut from inner part of the gels for microscopic examination. Samples were fixed with a mixture (1:1, v/v) of 5 g/100 ml formaldehyde and 0.2 g/100 ml glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and dehydrated in increasing series of acetone (10-100 ml/100 ml). Afterwards, they were critical-point dried with CO_2 as

transition fluid in a model Polaron 3000 critical-point dryer (Polaron, Hertfordshire, UK) and mounted on metallic holders, followed by gold sputter-coating in a model JFC-1100E ion sputtering device (JEOL, Tokyo, Japan). Samples were kept in a dryer until examination by a model JSM-5200 scanning microscope (JEOL, Tokyo, Japan) at 20 kV. Micrographs were taken at different magnifications.

Statistical analysis

Factorial analysis of variance (general linear model, factorial ANOVA, full factorial design) was carried out using the STATISTICA© software (StatSoft, Tulsa, USA), version 6.1, 2003. The difference of means between pairs was resolved by confidence intervals in a Tukey HSD test. Level of significance was set for $p<0.05$.

Results

Proximate composition

Proximate composition of gels was quite similar, since moisture, protein, fat and ash values were near 80.0, 11.0, 1.0, and 2.5 % (w/w), respectively (Table 5.1.5.3).

Table 5.1.5.3 – Proximate composition of the raw material (Atlantic and chub mackerel), mackerel surimi and gels*.

Sample	MTGase (% w/w)	FIBRE LEVEL (type, % w/w)	Moisture (% w/w)	Protein (% w/w)	Fat (% w/w)	Ash (% w/w)
Atlantic mackerel	---	---	67.12 ± 0.44	19.01 ± 0.29	11.75 ± 0.37	1.37 ± 0.05
Chub mackerel	---	---	64.30 ± 0.36	20.33 ± 0.09	13.48 ± 0.19	1.42 ± 0.04
Surimi	---	---	73.95 ± 0.61	15.92 ± 0.38	1.54 ± 0.13	1.18 ± 0.01
C0	0.0	0.0	79.19 ± 0.35	12.06 ± 0.15	1.10 ± 0.04	2.25 ± 0.02
2S0	0.0	Swe, 2.0	79.44 ± 0.06	10.43 ± 0.48	1.02 ± 0.06	2.31 ± 0.01
4S0	0.0	Swe, 4.0	77.41 ± 0.57	10.99 ± 0.43	0.89 ± 0.01	2.71 ± 0.03
2F0	0.0	Fib, 2.0	79.42 ± 0.69	10.25 ± 0.12	1.24 ± 0.27	2.41 ± 0.02
4F0	0.0	Fib, 4.0	79.29 ± 0.15	11.94 ± 0.25	1.00 ± 0.02	2.50 ± 0.09
Ca	0.5	0.0	79.58 ± 0.30	12.54 ± 0.23	0.99 ± 0.03	2.38 ± 0.03
2Sa	0.5	Swe, 2.0	76.74 ± 0.79	11.41 ± 0.34	0.96 ± 0.00	3.07 ± 0.03
4Sa	0.5	Swe, 4.0	77.59 ± 0.04	10.48 ± 0.32	0.89 ± 0.00	2.61 ± 0.01
2Fa	0.5	Fib, 2.0	78.44 ± 0.33	10.69 ± 0.51	1.10 ± 0.09	2.64 ± 0.06
4Fa	0.5	Fib, 4.0	77.99 ± 0.95	10.48 ± 0.12	0.99 ± 0.05	2.72 ± 0.05

* Presented values correspond to mean ± standard deviation.

The low fat content reflected the composition of mackerel surimi, about 1.5 % (w/w) fat. This low fat level resulted mainly from the removal of skin and washing operations, since fish presented between 11.8 and 13.5 % (w/w) fat. So, even taking into account the dilution effect of additives, surimi preparation entailed the removal of almost 90 % of initial fat. Protein values suggested some dilution effect with the addition of DF, given their low protein content (Table 5.1.5.1), however only MTGase-containing gels showed a declining trend with higher DF incorporation. Regarding the estimated total carbohydrate content, differences were detected, since it ranged from around 5.0 % (w/w) in C0 and Ca (albeit

fibre was not added to these samples, mackerel surimi had a great amount of sorbitol/sucrose) to near 8.0 % (w/w) in 4S0, 4Sa, 4F0 and 4Fa. Hence, there was some correlation between this parameter and the amount of added fibre. Most important, these data reveal that only DF incorporation (and not MTGase addition) can be deemed as a possible formulation factor affecting analytical determinations.

Texture

Folding test, gel strength and TPA results are found in Table 5.1.5.4, while force and distance at rupture and stress-relaxation parameters are presented in Table 5.1.5.5.

Table 5.1.5.4 – Folding test, gel strength and TPA (texture profile analysis with 40 % compression) properties of the gels*.

Sample	Folding test	Gel strength (N.mm)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
C0	1.7 ± 0.6^{a}	8.1 ± 0.8^{ab}	6.7 ± 0.4^{ab}	0.27 ± 0.03^{ab}	1.8 ± 0.3^{ab}	0.52 ± 0.05^{ac}	0.9 ± 0.2^{ab}
2S0	2.0 ± 0.0^{ab}	5.9 ± 1.4^{ab}	7.6 ± 0.5^{ac}	0.41 ± 0.06^{a}	3.1 ± 0.6^{bc}	0.65 ± 0.02^{ab}	2.0 ± 0.4^{abc}
4S0	2.0 ± 0.0^{ab}	9.0 ± 2.0^{a}	10.6 ± 1.2^{cd}	0.38 ± 0.07^{a}	4.0 ± 0.8^{bc}	0.68 ± 0.06^{bd}	2.7 ± 0.6^{bc}
2F0	1.7 ± 0.6^{a}	5.3 ± 0.7^{ab}	5.7 ± 0.8^{ab}	0.29 ± 0.10^{ab}	1.7 ± 0.8^{ab}	0.51 ± 0.12^{c}	0.9 ± 0.6^{ab}
4F0	2.0 ± 0.0^{ab}	4.3 ± 0.9^{b}	3.7 ± 0.1^{b}	0.19 ± 0.05^{b}	0.7 ± 0.2^{a}	0.26 ± 0.05^{e}	0.2 ± 0.1^{a}
Ca	3.3 ± 0.6^{c}	14.8 ± 3.3^{c}	11.5 ± 0.9^{d}	0.72 ± 0.01^{c}	8.3 ± 0.5^{d}	0.85 ± 0.01^{f}	7.0 ± 0.4^{d}
2Sa	2.7 ± 0.6^{abc}	16.6 ± 3.3^{c}	13.4 ± 2.4^{d}	0.67 ± 0.02^{c}	9.0 ± 1.3^{de}	0.84 ± 0.01^{f}	7.5 ± 1.1^{de}
4Sa	3.0 ± 0.0^{bc}	14.1 ± 2.8^{c}	18.2 ± 2.5^{e}	0.61 ± 0.00^{c}	11.1 ± 1.5^{e}	0.84 ± 0.01^{f}	9.4 ± 1.4^{e}
2Fa	3.3 ± 0.6^{c}	7.7 ± 0.9^{ab}	13.4 ± 1.2^{d}	0.65 ± 0.02^{c}	8.7 ± 0.6^{d}	0.81 ± 0.01^{df}	7.0 ± 0.4^{d}
4Fa	2.3 ± 0.6^{abc}	6.0 ± 0.7^{ab}	7.4 ± 1.1^{abc}	0.59 ± 0.08^{c}	4.3 ± 0.7^{c}	0.72 ± 0.04^{bdf}	3.1 ± 0.6^{c}

* Presented values correspond to mean ± standard deviation.
For each fibre, means within a column with different letters are significantly different ($p<0.05$).

Table 5.1.5.5 – Rupture (at 80 % compression) and stress-relaxation properties of the gels*.

Sample	MTGase (% w/w)	FIBRE (type, % w/w)	Force at rupture (N)	Distance at rupture (mm)	Relaxation time (s)	Elastic module, E_1 (kPa)	Elastic module, E_e (kPa)	Viscous module, η_1 (kPa.s)
C0	0.0	0.0	6.6 ± 2.1^{ab}	9.6 ± 1.5^{a}	51.1 ± 3.0^{a}	20.3 ± 0.5^{a}	10.7 ± 2.4^{a}	1041 ± 85^{a}
2S0	0.0	Swe, 2.0	7.8 ± 0.6^{abc}	9.7 ± 0.7^{ab}	47.2 ± 1.3^{a}	19.1 ± 0.4^{a}	12.8 ± 0.2^{ab}	902 ± 7^{ac}
4S0	0.0	Swe, 4.0	12.3 ± 1.3^{c}	9.6 ± 0.3^{a}	49.1 ± 6.4^{a}	29.9 ± 2.8^{b}	20.5 ± 4.8^{b}	1460 ± 55^{b}
2F0	0.0	Fib, 2.0	5.3 ± 0.1^{ab}	8.1 ± 0.0^{a}	50.1 ± 0.5^{a}	19.6 ± 0.8^{a}	11.0 ± 0.8^{a}	981 ± 52^{a}
4F0	0.0	Fib, 4.0	3.8 ± 0.4^{a}	8.1 ± 0.0^{a}	47.7 ± 6.7^{a}	16.6 ± 0.8^{a}	8.6 ± 0.5^{a}	795 ± 147^{acd}
Ca	0.5	0.0	30.0 ± 0.2^{d}	14.3 ± 0.1^{cd}	62.6 ± 5.7^{a}	8.4 ± 0.6^{cd}	9.6 ± 1.9^{a}	525 ± 10^{de}
2Sa	0.5	Swe, 2.0	27.9 ± 1.7^{d}	15.0 ± 0.9^{d}	55.1 ± 5.2^{a}	11.8 ± 0.9^{c}	13.0 ± 0.2^{ab}	649 ± 108^{cd}
4Sa	0.5	Swe, 4.0	30.1 ± 0.7^{d}	14.4 ± 0.0^{cd}	54.1 ± 1.6^{a}	11.8 ± 0.3^{c}	10.7 ± 3.5^{a}	637 ± 1^{cd}
2Fa	0.5	Fib, 2.0	19.6 ± 2.8^{e}	12.7 ± 0.6^{cd}	55.2 ± 2.4^{a}	11.2 ± 0.4^{c}	12.2 ± 0.4^{ab}	617 ± 51^{d}
4Fa	0.5	Fib, 4.0	9.9 ± 0.3^{bc}	12.3 ± 0.6^{bc}	47.2 ± 0.0^{a}	4.9 ± 1.0^{d}	5.9 ± 1.5^{a}	322 ± 79^{e}

* Presented values correspond to mean ± standard deviation.
For each fibre, means within a column with different letters are significantly different ($p<0.05$).

Gels scored between 1.7 ± 0.6 (control) and 3.3 ± 0.6 (Ca and 2Fa) in the folding test. MTGase had a significantly ($p<0.05$) favourable effect upon the gels, meaning that this

enzyme is adequate for inducing gelation of the mackerel proteins. There was no sign of a negative interaction between DF and MTGase. Likewise, puncture test results revealed that strength of the gels containing MTGase was significantly ($p<0.05$) greater than that of the other samples. This was due to breaking force differences, since breaking deformation values were similar (data not shown). Thus, MTGase seems to have mainly a toughening effect. Gels containing Fib were weaker and breaking force was the cause. All TPA parameters showed significant ($p<0.05$) textural advantages with MTGase incorporation. Cohesiveness improvement, a fundamental indicator of gel quality, was impressive, from 0.19-0.41 to 0.59-0.72. Fib showed a negative effect, making gels softer, when incorporated at 4.0 % (w/w). But even in this case, MTGase largely compensated, producing cohesiveness or springiness values above other gels without MTGase. Swe increased ($p<0.05$) hardness, even in those harder gels containing MTGase (an increase of almost 60 % with 4.0 %, w/w, Swe). This DF also improved springiness in those gels without MTGase. Contrastingly, albeit not significant, Swe seemed to make gels with MTGase less cohesive. Nevertheless, chewiness (a parameter giving a global assessment of textural quality) was highest for 4Sa, thus, showing that this DF produces better quality gels. Furthermore, force and distance at rupture —obtained by 80 % compression (imitating mastication in human mouth (Bourne, 1994))— were significantly ($p<0.05$) enhanced by MTGase and reduced by Fib. Regarding stress-relaxation properties, used for evaluation of the viscoelastic behaviour of gels, relaxation time was higher in the MTGase containing products, taken as a whole (54.8 *vs* 49.1 s). However, elastic modules E_1 were halved or even more reduced by MTGase addition and this effect was also reflected by viscous module η_1. Fib also reduced elastic and viscous modules, whereas, Swe, at 4.0 %, w/w, (in the absence of MTGase) increased these modules.

pH, WHC and Colour

pH values (Table 5.1.5.6) were, generally, significantly ($p<0.05$) higher in those products containing MTGase. On the other hand, Swe lowered pH. Fib seemed to increase alkalinity, but only in the presence of MTGase and at a 4.0 %, w/w, incorporation. pH changes must be seen as indirect effects of DF and MTGase, probably, resulting from altered protein conformations, that is, exposure of more or less acidic groups. However, the released ammonia as a result of the reaction catalysed by MTGase may also have a role.

Table 5.1.5.6 – pH and WHC of the gels*.

Sample	MTGase (% w/w)	FIBRE (type, % w/w)	pH	WHC (%)
C0	0.0	0.0	6.40 ± 0.02^{a}	36.4 ± 4.6^{a}
2S0	0.0	Swe, 2.0	6.29 ± 0.04^{bc}	36.3 ± 3.6^{a}
4S0	0.0	Swe, 4.0	6.24 ± 0.03^{c}	50.0 ± 2.1^{b}
2F0	0.0	Fib, 2.0	6.25 ± 0.01^{c}	33.6 ± 1.7^{ac}
4F0	0.0	Fib, 4.0	6.37 ± 0.04^{a}	26.5 ± 4.3^{c}
Ca	0.5	0.0	6.40 ± 0.03^{a}	45.5 ± 0.0^{b}
2Sa	0.5	Swe, 2.0	6.38 ± 0.02^{a}	51.8 ± 0.3^{b}
4Sa	0.5	Swe, 4.0	6.35 ± 0.04^{ab}	49.1 ± 0.6^{b}
2Fa	0.5	Fib, 2.0	6.41 ± 0.01^{a}	38.1 ± 0.9^{a}
4Fa	0.5	Fib, 4.0	6.51 ± 0.02^{d}	36.5 ± 1.6^{a}

* Presented values correspond to mean ± standard deviation.
For each fibre, means within a column with different letters are significantly different ($p<0.05$).

MTGase also increased WHC (Table 5.1.5.6): the lower increase was observed in 2F samples —from 33.6 ± 1.7 (2F0) to 38.1 ± 0.9 % (2Fa)— and the highest in 2S samples— from 36.3 ± 3.6 (2S0) to 51.8 ± 0.3 % (2Sa). However, for 4S, no improvement was detected. This can be due to the large water holding effect of 4.0 %, w/w, Swe in those gels without MTGase, augmenting WHC from 36.4 ± 4.6 (C0) to 50.0 ± 2.1 % (4S0). On the contrary, Fib reduced ($p<0.05$) WHC.

Regarding colour (Table 5.1.5.7), whereas parameters a*, b* and chroma were unaltered, whiteness was significantly ($p<0.05$), albeit slightly, reduced by MTGase addition.

Table 5.1.5.7 – Colour parameters of the gels*.

Sample	MTGase (% w/w)	FIBRE (type, % w/w)	a*	b*	Whiteness	Chroma
C0	0.0	0.0	-1.77 ± 0.19^{a}	4.81 ± 0.08^{a}	69.0 ± 0.6^{abc}	5.13 ± 0.09^{a}
2S0	0.0	Swe, 2.0	-1.80 ± 0.16^{a}	5.12 ± 0.06^{a}	69.3 ± 0.3^{ab}	5.43 ± 0.01^{a}
4S0	0.0	Swe, 4.0	-1.89 ± 0.02^{a}	5.09 ± 0.08^{a}	69.8 ± 0.3^{a}	5.43 ± 0.08^{a}
2F0	0.0	Fib, 2.0	-1.99 ± 0.03^{a}	5.18 ± 0.06^{a}	69.2 ± 0.4^{ab}	5.55 ± 0.07^{a}
4F0	0.0	Fib, 4.0	-1.94 ± 0.18^{a}	4.90 ± 0.05^{a}	68.8 ± 0.2^{bcd}	5.28 ± 0.11^{a}
Ca	0.5	0.0	-1.89 ± 0.03^{a}	4.89 ± 0.11^{a}	68.6 ± 0.1^{bcd}	5.24 ± 0.09^{a}
2Sa	0.5	Swe, 2.0	-1.92 ± 0.03^{a}	5.00 ± 0.19^{a}	68.2 ± 0.1^{cd}	5.35 ± 0.18^{a}
4Sa	0.5	Swe, 4.0	-1.88 ± 0.01^{a}	4.86 ± 0.02^{a}	68.3 ± 0.2^{cd}	5.21 ± 0.02^{a}
2Fa	0.5	Fib, 2.0	-1.96 ± 0.06^{a}	5.01 ± 0.08^{a}	68.0 ± 0.5^{d}	5.38 ± 0.10^{a}
4Fa	0.5	Fib, 4.0	-2.00 ± 0.03^{a}	5.07 ± 0.04^{a}	67.9 ± 0.1^{d}	5.45 ± 0.04^{a}

* Presented values correspond to mean ± standard deviation.
For each fibre, means within a column with different letters are significantly different ($p<0.05$).

Protein solubility

Protein solubility in different extracting media is presented in Table 5.1.5.8. For almost all samples, protein solubility in the different extracting media followed the order: urea<SDS<urea+DTT<SDS+DTT. The two exceptions to this general rule were not of particular significance: 4Fa (SDS~urea+DTT) and C0 (urea+DTT~SDS+DTT). Both MTGase and DF reduced ($p<0.05$) solubility. MTGase had a weaker effect when protein was solubilized in SDS+DTT, particularly, in those gels containing DF, MTGase practically did not affect solubility. Without enzyme, Swe and Fib had a similar effect: only with urea or urea+DTT a steeper decline with Swe was observed. With MTGase, DF did not have a strong effect upon solubility, however with SDS+DTT, there were solubility increases with higher levels of DF incorporation. Moreover, whereas protein solubility of Sa and Fa samples was identical in urea, with addition of DTT to urea, solubility of Sa samples increased more than that of Fa samples.

Table 5.1.5.8 – Relative protein solubility of the gels in different extracting solutions*.

Sample	MTGase (% w/w)	FIBRE (type, % w/w)	Protein Solub. in SDS (%)	Protein Solub. in SDS+DTT (%)	Protein Solub. in Urea (%)	Protein Solub. in Urea+DTT (%)
C0	0.0	0.0	68.0 ± 3.1^{a}	96.2 ± 5.7^{a}	47.4 ± 2.1^{a}	100.0 ± 1.3^{a}
2S0	0.0	Swe, 2.0	69.9 ± 2.6^{a}	86.2 ± 5.5^{ab}	29.3 ± 0.3^{b}	80.6 ± 1.0^{b}
4S0	0.0	Swe, 4.0	55.3 ± 0.4^{b}	82.3 ± 2.6^{ab}	27.0 ± 1.5^{bc}	61.1 ± 6.3^{c}
2F0	0.0	Fib, 2.0	65.1 ± 1.3^{a}	90.5 ± 2.1^{ab}	41.8 ± 0.9^{d}	76.0 ± 1.9^{b}
4F0	0.0	Fib, 4.0	56.6 ± 0.6^{b}	78.7 ± 2.0^{b}	36.6 ± 1.4^{e}	73.4 ± 2.6^{b}
Ca	0.5	0.0	39.2 ± 1.1^{c}	78.3 ± 3.3^{bc}	21.4 ± 0.5^{f}	55.7 ± 1.3^{cd}
2Sa	0.5	Swe, 2.0	37.9 ± 0.8^{c}	70.3 ± 0.2^{c}	21.2 ± 0.5^{f}	56.2 ± 2.9^{c}
4Sa	0.5	Swe, 4.0	32.9 ± 0.4^{d}	87.5 ± 5.0^{ab}	25.5 ± 1.0^{c}	61.3 ± 0.8^{c}
2Fa	0.5	Fib, 2.0	39.3 ± 0.7^{c}	74.9 ± 1.3^{bc}	25.7 ± 0.7^{c}	44.6 ± 0.3^{e}
4Fa	0.5	Fib, 4.0	48.0 ± 0.8^{e}	86.3 ± 1.8^{ab}	24.8 ± 1.2^{c}	48.4 ± 0.5^{de}

* Presented values correspond to mean ± standard deviation.
For each fibre, means within a column with different letters are significantly different ($p<0.05$).

Electrophoresis

Electrophoresis only revealed differences due to MTGase. DF addition did not present effects. Therefore, only electrophoretic profiles for C0, Ca and raw material (mackerel surimi) are presented (Figure 5.1.5.1). MTGase caused the disappearance of high molecular weight (HMW) protein bands, namely, bands assigned to MHC and M- and C-proteins. All processing, in particular, heating over one hour caused loss of intensity in the bands of C0 and Ca with respect to raw material. In SDS extracted homogenates, MHC seemed to disappear after processing.

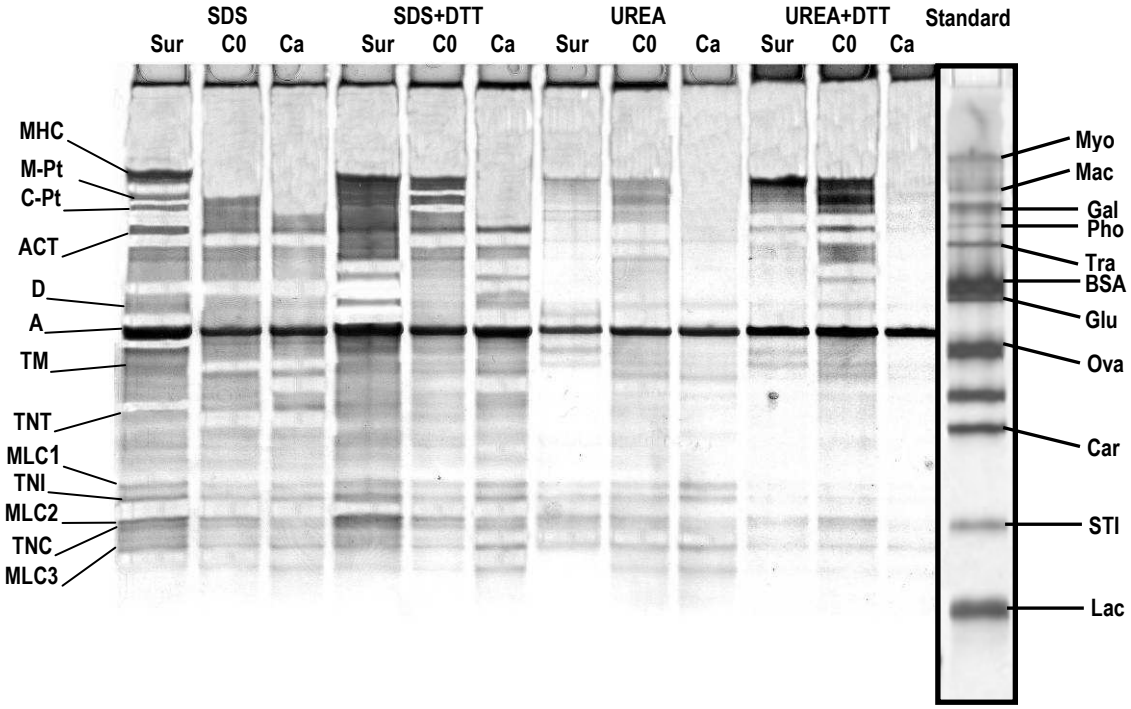

Figure 5.1.5.1 – SDS-PAGE of soluble protein homogenates in the four extracting media from raw material (sur) and produced gels without (C0) and with MTGase (Ca). MHC: myosin heavy chain; M-Pt: M-protein; C-Pt: C-protein; ACT: actinin; D: desmin; A: actin; TM: tropomyosin; TNT: troponin-T; MLC1: myosin light chain 1; TNI: troponin-I; MLC2: myosin light chain 2; TNC: troponin-C; MLC3: myosin light chain 3. Standards name and molecular weights: Myo: myosin (212.0 kDa); Mac: α_2-macroglobulin (170.0 kDa); Gal: β-galactosidase (116.0 kDa); Pho: phosphorylase B (94.0 kDa); Tra: transferring (76.0 kDa); BSA: bovine serum albumin (67.0 kDa); Glu: glutamic dehydrogenase (53.0 kDa); Ova: ovalbumin (43.0 kDa); Car: carbonic anhydrase (30.0 kDa); STI: soybean trypsin inhibitor (20.1 kDa); Lac: α-lactalbumin (14.4 kDa).

SEM

Microstructure was analyzed by SEM. Only those gels containing Swe exhibited clear differences (Figure 5.1.5.2). In other gels, areas presenting an aggregated morphology were overwhelmingly predominant. Differently from other scientific papers (Montero et al., 2005), no areas of a predominantly reticular structure were observed. In contrast to Fib, Swe incorporation caused a microstructural rearrangement, originating large pores, undulating surfaces and a less compact morphology. This was most obvious in the gels containing MTGase. Moreover, these samples seemed to present some starch granules within protein matrix (see right side of 4Sa image).

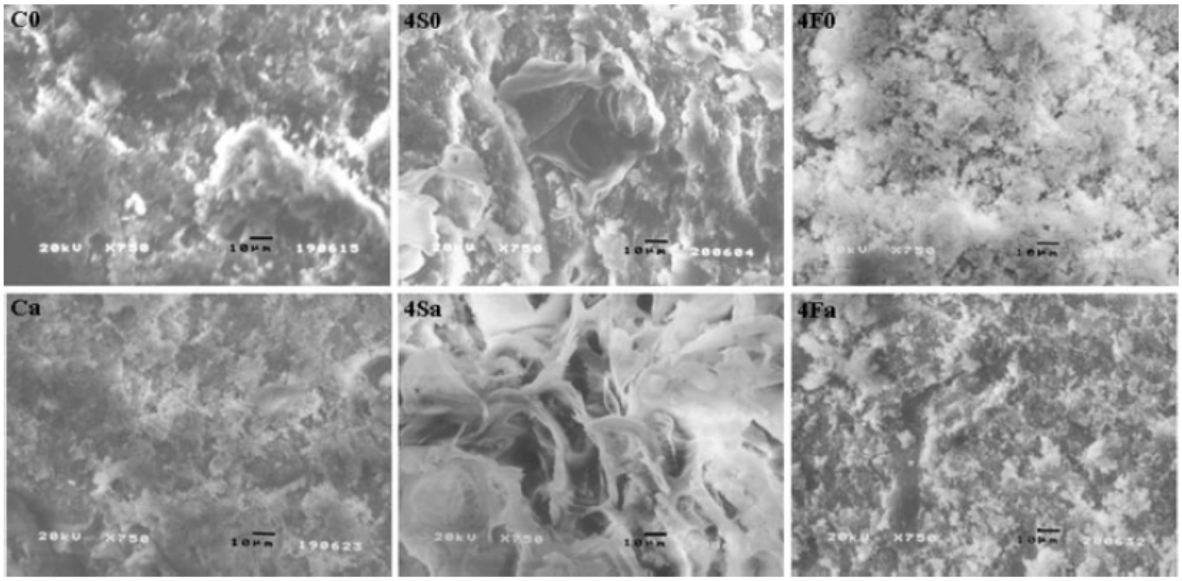

Figure 5.1.5.2 – Scanning electron micrographs of produced gels: C0, 4S0, 4F0, Ca, 4Sa and 4Fa.

Discussion

Textural benefits of MTGase have been found in many other fish products (Téllez-Luis et al., 2002; Ramírez et al., 2000; 5.1.3). Nonetheless, it deserves to be remarked that 0.5 % (w/w), MTGase addition to mackerel surimi improved almost all textural properties. MTGase increases cross-linking of myosin heavy chains (MHC) during setting, since it catalyzes covalent ε-amino-(γ-glutamyl)-lysine bonds (Kumazawa at al., 1993; Ramírez-Suárez et al., 2001). Therefore, it creates a denser bond network between proteins in gels. A relationship between MHC polymerization and an increase in breaking force or shear stress, without affecting shear strain, has also been found in gels from various fish species, such as Pacific whiting (Lee and Park, 1998) or carp (Tsukamasa et al., 2000). Furthermore, MTGase enabled to attain an acceptable product with a raw material which had been subjected to more than two months of frozen storage. Since freezing and frozen storage cause and/or not avoid important structural and physicochemical changes (Herrera et al., 2000), the results show that MTGase can be used as a tool to compensate protein alterations with negative textural consequences. Concerning stress-relaxation data, the decline of elastic modules E_1 with MTGase was apparently in contradiction to other measured properties. But, these results were systematically found in all gels containing MTGase and σ values were lower for these gels, meaning a softening effect. Stress-relaxation test involved a 10 % compression, so it seems that MTGase action reduces those chemical interactions that respond intensely at a slight compression but are progressively destroyed with larger compressions (40 % in TPA; 80 % in rupture test) and, on the other hand, increases stronger bonds, which appear to generate large forces only

with higher compression levels. So, there is a hardness rank inversion between gels with and without MTGase and as a function of compression. Similar absence of correlation between small and large deformation tests has been reported for other gels (Mitchell, 1980). It has been shown that consumer assessment of the gels hardness correlates better with the rupture force rather than the elastic modulus (Wood, 1979). The elastic modules E_e, which can be related to residual solidity of the system derived from permanent cross-links (Kim et al., 2005a), do not show a clear effect of MTGase. This enzyme also produced a reduction of viscous modules η_1, but less accentuated than with elastic modules E_1, as reflected by higher relaxation times. This means a relatively more important viscous element and that energy dissipation is slower, which may be due to the better alignment of protein chains and to the dissociation and re-association of non-covalent bonds as protein chains pass each other (Hamann and McDonald, 1992). Other authors reported, under similar conditions (10 % compression), higher values for surimi gels as a result of previous setting (E_1, 22.3 *vs* 19.5 kPa; E_e, 32.3 *vs* 19.1 kPa; η_1, 4453 *vs* 2731 kPa.s and τ_1, 200 *vs* 140 s) (Hamann and McDonald, 1992). Since is frequently assumed a predominant role of TGase in setting of surimi, these data seem to be in contradiction with this work's findings. However, setting effects may be found in highly washed surimi, revealing the importance of other factors than TGase (Lanier at al., 2005). Alternatively, contradiction may be explained by a somewhat different action of MTGase with respect to endogenous TGase. Hence, this subject needs further investigation.

Swe had also positive effects, however, unlike MTGase, it improved only some properties, while leaving others basically unchanged. It hardened the gels and turned those without MTGase springier, but did not increase gel strength. The apparent lack of correlation between the two types of rheological measurement (gel strength and hardness) has been reviewed (Lee and Chung, 1989). According to these authors, while compression test measures overall binding property of the gel material, penetration test evaluates degree of compactness or density of actomyosin. Following this interpretation, Swe increased strongly overall binding, but, unlike MTGase, failed to produce a higher degree of compactness in the gel network. It is worth mentioning that Swe contains starch (Table 5.1.5.1), which improves gel-forming capacity by acting as a filler of the myofibrillar protein gel (Lee et al., 1992). However, inner pea fibre (mostly insoluble) must have a great role, since it represents ~50.0 % (w/w) of Swe and textural improvements are found in other properties besides hardness.

Fib or inulin (soluble fibre) had an opposite effect, softening gel products and worsening other parameters. This DF neither improves degree of compactness nor overall binding capacity. This may be explained by its short chains, which are unable to make

connections over long distances and to create an effective network. Furthermore, there was no indirect evidence of any Fib interaction with myofibrillar proteins. Therefore, this DF only functioned as a diluting agent of the myofibrillar protein, specially, because, in order to ensure equal moisture for all batches, a greater quantity of water was added. Hardness reduction with increasing fibre concentration has been observed (Tudorica et al., 2002) and associated to disruption of the protein matrix.

Regarding WHC, different studies have also reported similar effects of MTGase upon WHC or upon extracted water (Ramírez et al., 2007). WHC values were correlated with gel strength (R^2=0.74) (Tables 5.1.5.4 and 5.1.5.6) or breaking force (R^2=0.71) as found by other authors (Chaijan et al., 2004). However, for Swe (in those gels with no MTGase), no good correlation was found between gel strength and WHC (R^2=0.53), while between force at rupture (Table 5.1.5.5) and WHC a better correlation was observed (R^2=0.96). The reason lies in Swe action, this DF not only hardens the gels (see Texture measurements, above), but also has, *per se*, a high WHC as a result of its binding action. The hardening effect may be due to the filling effect of starch (Lee et al., 1992), which is much more obvious in the compression test (force at rupture) than in the puncture test (gel strength). For WHC increase, a possible explanation may be found in the protein-polyssacharide matrix that is formed in a gel product containing starch, which may entrap water (Pérez-Mateos and Montero, 2000). However, Swe only produced WHC increases, when incorporated at 4.0 % (w/w), contrasting with other DF (alginate or xanthan), which increased WHC in 40 % when added at 0.5 %, w/w (Pérez-Mateos and Montero, 2000).

Increased whiteness can be related to degree of protein degradation, for instance, with cooking (Hwang et al., 2007). Therefore, the slight darkening effect of MTGase can indicate lower denaturation of those gels containing MTGase. The cross-links catalyzed by MTGase during setting may avoid degradation during cooking. Moreover, improved texture, namely, gel strength and cohesiveness, seem to reinforce this idea also for mackerel surimi gels.

Solubility results show that non-covalent interactions other than hydrogen bonds, such as hydrophobic interactions, are important between mackerel proteins, given the greater solubility values with SDS (with respect to urea) and with SDS+DTT (with respect to urea+DTT, Table 5.1.5.2). Regarding solubility reduction with MTGase, it can be related to cross-linking of MHC during setting, since it catalyzes covalent bonds (Kumazawa et al., 1993; Ramírez-Suárez et al., 2001), which cause formation of insoluble polymeric aggregates (Ashie and Lanier, 1999). As a result, those chemicals that rupture non-covalent bonds (hydrophobic interactions or hydrogen bridges) solubilize much less protein. A pronounced decrease of the percentage of soluble protein, as a result of MTGase addition, was also reported by other authors (Gómez-Guillén et al., 2005). But, in

SDS+DTT, this effect was weaker. This fact, when brought together with the rupture action of this extracting solution upon non-covalent and disulphide bonds, enables to hypothesize that MTGase may promote the establishment of disulphide bonds in mackerel surimi, thus, being its direct catalytic action compounded by indirect effects. However, these are disulphide bonds between proteins with extensive exposure of their hydrophobic areas, since with urea+DTT no similar phenomenon was detected. This fact suggests a greater exposure of some hydrophobic areas, which come in contact through cross-linking catalyzed by MTGase. This induced vicinity may also favour the establishment of disulphide bonds between cysteine residues of different proteins. For SDS+DTT extraction and gels containing DF, especially if at highest level, no differences were found as a consequence of MTGase. This suggests that Swe and Fib in larger amounts may affect MTGase action upon protein configuration, leading to less ε-amino-(γ-glutamyl) lysine bonds. However, electrophoretic study revealed a similar effect of MTGase on gels with and without DF. Solubility results show that DF *per se*, in the absence of MTGase, promoted protein aggregation and that hydrogen and disulphide bonds between proteins were reduced. The effect upon hydrogen bonds was stronger with Swe, suggesting a greater exposure of hydrophobic areas of proteins. It is possible that this DF (and the starch associated to it) competed with proteins for water, leaving less water available for protein hydration, thus generating a less hydrophilic environment. Other authors (Cai et al., 2008) have also mentioned a similar effect with addition of other polyssacharides. However, Swe also reduced (in a lesser degree) the share of proteins associated by other non-covalent bonds, which forces to admit the role of other chemical interactions. In fact, addition of polysaccharides to fish gels has led to contradictory results (Gómez-Guillén et al., 2005; Benjakul et al., 2001). Nevertheless, this can be explained by different gelling procedures, for instance, decreased protein solubility has been reported in suwari and kamaboko gels containing chitosan (Benjakul et al., 2001),whereas addition of this polysaccharide to pressure-induced gels did not change solubility (Gómez-Guillén et al., 2005). For MTGase, solubility results are consistent with texture parameters, since they show that hardness, gel strength or cohesiveness increases are correlated with important chemical alterations in the gels, favouring a denser cross-linking (whether more or less dependent upon a particular bond type) between myofibrillar proteins. Concerning DF, interpretation is not so clear, both Swe and Fib caused a lowering of solubility, but their effects on texture were different: Swe hardened and Fib softened gels. So, Fib caused protein aggregation, but in a way that must have reduced the degree of compactness of actomyosin. In this context, solubility results of the F samples may offer an explanation, since they point to a loss of importance of disulphide bonding (particularly when DTT is added to urea), a type of bonding associated by several authors to improved gel quality

(Havea et al., 2004). So, besides affecting MTGase action upon protein configuration, Fib may have had a chemical effect that led to less S-S bonds and, consequently, reduced protein cross-linking. Swe may have had a similar albeit smaller effect, but its components, namely, starch (Table 5.1.5.1), probably offset any negative effect, since starch gels due to hydrogen bonds formed during cooling of surimi foods (Lanier et al., 2005), thus contributing for greater hardness.

Electrophoresis phenomena may be explained by exclusion of heavy proteins from soluble protein fraction as result of aggregation: MTGase promotes the formation of intra- and intermolecular cross-linked products, converting MHC and other HMW proteins to insoluble polymers, which were too large to enter in the stacking gel. Actin was remarkably resistant to cross-linking, probably as a result of low exposure of the susceptible amino acid residues (Gln, Lys) in its globular conformation (Ramírez-Suárez et al., 2005). Regarding DF, Swe- or Fib-containing samples were identical to control, hence, solubility decreases possibly affected all proteins regardless of their molecular weight (for all samples, soluble protein was adjusted to 0.3 mg/ml, making uniform changes in profiles invisible). Regarding the effect of MTGase upon those gels containing DF, taking into account that HMW proteins disappeared in 4Sa and 4Fa lanes, it may seem contradictory to protein solubility results (discussed above) that no sign of any disturbance on protein cross-linking by MTGase was observed. In fact, these samples related to 4S0 and 4F0 in the same way as Ca to C0, so the absence of protein solubility variation can be interpreted as resulting of two opposite effects: aggregation of HMW proteins by MTGase and lesser aggregation of other proteins (compensating the reduction of solubility of HMW proteins that would be expected in all media). In this hypothesis, MTGase led proteins to interact in a way that hindered any aggregative effects of DF. Further research on this subject is necessary.

4S gels' microstructures are explained by DF composition: whereas Swe contains insoluble polysaccharides, Fib contains soluble oligosaccharides. Possibly, Swe's polysaccharides, namely its insoluble DF, may have formed a backbone structure, along which proteins established their interactions. These were probably not only among proteins, but also between proteins, starch and DF, thus reinforcing texturally the gels (Tables 5.1.5.4 and 5.1.5.5). However, these interactions were more important if combined with MTGase action. MTGase increased protein cross-linking, thus forcing a greater protein rearrangement. Moreover, some starch granules were found in those gels containing Swe and MTGase. A comparison with another work, which used higher starch incorporation (5.0 %, w/w, vs approximately 1.6 %, w/w, in this work) shows a considerable resemblance between these granules and non-gelatinized starch granules (Couso et al., 1998). According to these authors, whereas gelatinized granules are

amorphous, non-gelatinized granules have a globular shape. The latter are formed in heat-induced gels previously subjected to setting, just as the gels produced in present work. The setting ensures a previous development of protein cross-linking, which traps water and limits its availability for hydration and gelatinization of the starch. Something similar may have occurred in present experiments, particularly, in the 4Sa gels, since MTGase as a protein cross-linking enhancer may indirectly limit starch gelatinization even more.

Conclusion

MTGase addition improved textural properties and increased pH and WHC. On the other hand, MTGase reduced the elastic modules and the viscous module and, also, darkened slightly the gels; furthermore, protein solubility fell, meaning greater protein aggregation, particularly, according to electropherograms, for MHC. MTGase had no unequivocal effect upon gels' microstructure.

Swe increased hardness and related parameters, but, also, WHC, the elastic and viscous modules. Swe reduced extractable protein in SDS and urea (in the absence of MTGase). SEM revealed different structures for gels containing Swe. Fib effects were different, it worsened texture and WHC.

Therefore, it was shown that a combination of MTGase and Swe can improve quite significantly a poor quality surimi. Future work shall study possible products to be obtained from this improved surimi, namely, concerning organoleptic features and consumer satisfaction as well as frozen storage stability.

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5.2.- New Processing Technologies

5.2.1 – Effect of MTGase, Dietary Fibre and UV Irradiation upon Heat-Induced Gilthead Seabream (*Sparus aurata*) Gels

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Effect of MTGase, Dietary Fiber and UV Irradiation Upon Heat-Induced Gilthead Seabream (*Sparus aurata*) gels

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The effects of microbial transglutaminase (MTGase, 0.5%, w/w), dietary fiber (konjac flour, Kjc, 1.0%, w/w) and ultraviolet irradiation, UV (254 nm, 3300 μW/cm² and 60 min), on heat-induced gels from gilthead seabream mince (*Sparus aurata*) were studied. MTGase addition improved texture, force at rupture increased from 44.3 ± 18.1 to 131.9 ± 56.7 N, and increased pH and water-holding capacity. Moreover, MTGase reduced the elastic modulus E_i and darkened the gels; protein solubility declined, meaning greater protein aggregation, according to electrophoregrams. Evidence was found that disulfide bonding has a role in textural improvement by MTGase. Kjc increased the hardness from 15.1 ± 5.1 to 20.6 ± 4.7 N, the elastic modulus and WHC. Kjc itself and not its effect upon proteins may explain the hardening effect. However, gels containing Kjc were less deformable and Kjc reduced the extractable protein in the various selective bond media. UV did not present advantages as a gelation-promoting technology. MTGase and Kjc were texturally advantageous, although without synergies for gel strength: it fell from 56.9 ± 7.1 to 24.6 ± 5.9 N·mm as a result of adding Kjc to gels containing MTGase. Nevertheless, the hardening effect of Kjc and MTGase combined surpassed the sum of the individual effects, thereby indicating the existence of a mutual reinforcement of the hardness through MTGase and konjac. Additionally, this study showed that gilthead seabream may be used to produce good quality (concerning texture, color and WHC) heat-induced gels.

Key Words: gilthead seabream, microbial transglutaminase, konjac, UV irradiation, textural properties, color, electrophoresis

INTRODUCTION

Gilthead seabream (*Sparus aurata*) is a commonly farmed fish species, given its high growth rate and consequent economic advantage. However, the market for the whole fresh fish presents signs of saturation and, on the other hand there has been a growing demand for convenient products among consumers. This creates a potential market for ready-to-cook seabream fillets, which are already being offered by some producers. But these convenient products also entail costs, namely, byproducts are generated from the unutilized fish portions. Among these, there are trimmings of the cutting operations as well as fins and tails. These materials contain good-quality protein and other important fish nutrients and may present a nutritional value comparable to the fillets, provided that care is taken in their handling and storage. These are an undervalued resource, whose importance will certainly increase in the future. The conversion to powdered fish meal as with other fish byproducts (Guerard et al., 2002), represents a huge loss of valuable animal protein for human food. A solution for this problem may be found in the production of good-quality gel products from minced seabream trimmings, since taste and sensory attributes of the attained processed products can be controlled and directed to consumer preferences. In particular, combining fish minces with new food ingredients has been used as a way of upgrading low-value species and fish byproducts generated by the fish processing industry (Sánchez et al., 2004).

Among such ingredients, microbial transglutaminase (MTGase) is already widely used in the food industry

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Abstract

The effects of MTGase (0.5 %, w/w), dietary fibre (konjac flour, Kjc, 1.0 %, w/w) and ultraviolet irradiation, UV (254 nm, 3300 μW/cm^2, 40 min), on heat-induced gels from gilthead seabream mince (*Sparus aurata*) were studied. MTGase addition improved texture, force at rupture increased from 44.3 ± 18.1 to 131.9 ± 56.7 N, and increased pH and water holding capacity (WHC). Moreover, MTGase reduced the elastic modules E_1 and darkened gels; protein solubility declined, meaning greater protein aggregation, according to electropherograms. Evidence was found that disulphide bonding has a role in textural improvement by MTGase. Kjc increased hardness from 15.1 ± 3.1 to 20.6 ± 4.7 N, the elastic modules and WHC. Kjc itself and not its effect upon proteins may explain the hardening effect. However, gels containing Kjc were less deformable and Kjc reduced extractable protein in the various selective bond media. UV did not present advantages as a gelation-promoting technology. MTGase and Kjc were texturally advantageous, although without synergies for gel strength: it fell from 56.9 ± 7.1 to 24.6 ± 5.9 N.mm as a result of adding Kjc to gels containing MTGase. Nevertheless, the hardening effect of Kjc and MTGase combined surpassed the sum of the individual effects, thereby indicating the existence of a mutual reinforcement of the hardness through MTGase and konjac. Additionally, this work showed that gilthead seabream may be used to produce good quality (concerning texture, colour and WHC) heat-induced gels.

Keywords: Gilthead seabream, microbial transglutaminase, konjac, UV irradiation, textural properties, colour, electrophoresis

Introduction

Gilthead seabream (*Sparus aurata*) is a commonly farmed fish species, given its high growth rate and consequent economic advantage. However, the market for the whole fresh fish presents signs of saturation and, on the other hand there has been a growing demand for convenience products among consumers. This creates a potential market for ready-to-cook seabream fillets, which are already being offered by some producers. But these convenience products also entails costs, namely, by-products are generated from the unutilized fish portions. Among these, there are trimmings of the cutting operations as well as fins and tails. These materials contain good quality protein and other important fish nutrients and may present a nutritional value comparable to the fillets, provided that care is taken in their handling and storage. These are an undervalued resource, whose importance will certainly increase in the future. The conversion to powdered fish meal as with other fish by-products (Guerard et al., 2002), represents a huge loss of valuable animal protein for human food. A solution for this problem may be found in the production

of good quality gel products from minced seabream trimmings, since taste and sensory attributes of the attained processed products can be controlled and directed to consumer preferences. In particular, combining fish minces with new food ingredients has been used as a way of upgrading low-value species and fish by-products generated by the fish processing industry (Sánchez et al., 2004).

Among such ingredients, microbial transglutaminase (MTGase) is already widely used in the food industry with the purpose of promoting protein cross-linking (Téllez-Luís et al., 2002). MTGase has effectively improved, in various circumstances, the textural properties of minced fish products (Ramírez et al., 2000; Téllez-Luís et al., 2002; 5.1.2). A level of 0.5 % (w/w) MTGase enhanced the texture of minced mackerel (*Scomber scombrus and Scomber japonicus*) products with dietary fibre (5.1.5). Moreover, another recent study on MTGase addition to giant squid (*Dosidicus gigas*) surimi showed that 0.5 % (w/w) MTGase could be enough to achieve a good textural quality (Moreno et al., 2009).

Another potentially beneficial ingredient for texture is konjac flour, the generic name of the milled tuber from *Amorphophallus konjac* (Park, 1996). It contains a dietary fibre, high molecular weight glucomannan, whose D-glucose and D-mannose units (molar ratio ~3:2) are bonded by β-1,4-linkages (Tye, 1991). Acetyl groups are randomly scattered along the essentially linear polymer with an occurrence of ~1 per 19 monomers (glucose or mannose). The acetyl groups impart water solubility in an otherwise amylose-like molecule. This ingredient is generally recognized as safe, GRAS (Osburn and Keeton, 1994) and preliminary clinical evidence suggests that konjac may be beneficial against obesity (Keithley and Swanson, 2005). Low fat meat products containing konjac have been developed (Lin and Huang, 2008). Furthermore, it has been shown that konjac (at least 1.0 %, w/w) can improve texture of fish products, namely reinforcing shear stress of gels in both whiting and pollock surimi (Park, 1996). Furthermore, Xiong et al. (2009) have proposed that the optimum addition level of konjac glucomannan to surimi gels from grass carp (*Ctenopharyngodon idella*) would be 1 % (w/w). This level ensured a good cryoprotective effect, comparable to a conventional cryoprotectant and, also, good mechanical and water-holding properties. In fact, previous works have shown the importance of dietary fibre for the achievement of specific textural properties in minced fish products, specifically it was reported that inner pea fibre favoured greater gel strength and hardness (5.3.1), whereas, chicory's inulin, albeit reducing hardness, increased folding test in hake heat-induced gels (5.1.2). So, in the future, konjac may become a fundamental ingredient of processed fish products, namely, of fish minces and surimi, given the benefits to health, the cryoprotective action (very important for products which may be subjected to long frozen storage periods) and the improvement of texture it brings about.

Besides gel promoting ingredients, functional properties of fish minces can also be improved by new processing technologies and, among these, ultraviolet (UV) irradiation has been the object of some scattered works (Ishizaki et al., 1994; Jiang et al., 1998). Although the effect of UV irradiation on protein structures is not well understood, there is evidence that it can promote polymerization of proteins (Ishizaki et al., 1994) and, thereby, change protein functionality. In fact, it was demonstrated that UV irradiation fragmented flying fish myosin, caused an increase in surface hydrophobicity and the polymerization of myosin heavy chains (Ishizaki et al., 1994). Moreover, other authors claimed that UV irradiation for 20 min of MTGase-supplemented minced mackerel increased gel strength by 25 % (Jiang et al., 1998). Electrophoresis suggested that MTGase catalysis of the cross-linking (ε-amino-(γ-glutamyl) lysine bonds) of myosin heavy chains (MHC) was enhanced by UV irradiation (Jiang et al., 1998). The possible interaction between MTGase and UV irradiation is a subject requiring further research.

Therefore, in this work, MTGase and UV irradiation were brought together in order to examine the existence of any synergy among these two factors. The konjac, as an ingredient with great untapped potential for fish products and which may interact positively with MTGase and/or UV irradiation, was also chosen as an experimental factor. These three factors were studied with the twofold objective: improving the quality of gel products of minced gilthead seabream through incorporation of MTGase and/or konjac and UV irradiation and gaining a deeper knowledge of the underlying molecular phenomena.

Material and Methods

Raw Materials, Ingredients, and Reagents

Farmed gilthead seabream (*Sparus aurata*) were bought in a local supermarket and kept frozen at –20 ºC until processing. Individual weight varied between 300 and 400 g. Fish were processed (headed, tailed, gutted and filleted) at low temperature (< 10 ºC) within one week after purchase. The trimmings of the cutting operations were collected and used as raw material.

Konjac flour, Nutricol® GP 312 was provided by FMC Biopolymer (Philadelphia, USA) and microbial transglutaminase TG-K (MTGase) ACTIVA® GS was supplied by Ajinomoto Japan, Inc. (Tokyo, Japan), presenting an activity of about 100 $U.g^{-1}$.

All chemicals used were of analytical grade and were obtained from Merck KGaA (Darmstadt, Germany).

Experimental Design

Gilthead seabream mince was used with other ingredients in the preparation of gels. The effect of 1.0 % (w/w) konjac flour, Kjc, (samples K) and 0.5 % (w/w) MTGase addition (samples a) was tested and compared with products without Kjc (C) or without MTGase (0). Finally, for each of the four formulations (C0, Ca, K0 and Ka), one of two identical portions was submitted to UV irradiation for 40 min at 10 ºC, while the other was left in refrigeration (40 min at 10 ºC). Error assessment was derived from replication of the various analyses performed.

Production of Gels

Approximately 6 kg of gilthead seabream mince from trimmings were used. The appropriate quantities of mince, Kjc (0.0 or 1.0 %, w/w), MTGase (0.0 or 0.5 %, w/w), salt (2.5 %, w/w), $Ca(OH)_2$ (0.15 %, w/w) and water (in order to guarantee final gel products with the same moisture level, 80 %) for approximately 1.4 kg batches were weighed. In order to hydrate Kjc before mixing, chilled water was added to Kjc in a ratio near 10:1 and mixed with Kjc for 1 min. Afterwards, the seabream mince were mixed with the other ingredients for 2 min at 1420 rpm and 2.5 min at 2800 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). In order to promote deacetylation of the konjac flour paste, a necessary step for gel formation to occur, $Ca(OH)_2$ solubilised in water was added to the batter and mixing proceeded for further 30 s at 2800 rpm. Throughout all process, mixing was performed always under vacuum and temperature below 7 ºC. The batters attained were submitted to one of two alternative treatments of the same duration (40 min) and at the same temperature (10 ºC): UV irradiation or storage in the dark. In both cases, the batter was spread to a depth of 0.5 cm on a plate. The difference was that, for each formulation, one of the spread batters was irradiated under a model 51438 UV lamp (Gelman Instruments Company, Ann Arbor, USA) at a wavelength of 254 nm and an intensity of 3300 $\mu W/cm^2$. After irradiation, the batters were put inside a model EB-12 hydraulic filler (Mainca Equipamientos Carnicos, S.L., Granollers, Spain) and 'sausages' with a diameter of 25 mm and a length of about 20 cm produced with cellulose casings. 'Sausages' were vacuum-packed in low-oxygen permeable barrier bags (O_2 transmission, <2.1 $cm^3/(m^2.day.bar)$ at 23 ºC, Colamin XX 100e, Obermühle Polymertechnik GmbH, Pössneck, Germany) with a model A300/52 vacuum packager (Multivac Sepp Haggenmüller GmbH & Co. KG, Wolfertschwenden, Germany). Then, these packages were immersed in water at 35 ºC for one hour (setting) and moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and steam cooked at 90 ºC for one hour (cooking). In order to guarantee these conditions even in the innermost

part of the product, the oven's digital thermometer was placed in the centre of one 'sausage' through a hole in the bag. Finally, gel products were immediately cooled in iced water and stored in a refrigerated room (at 5 ± 1 ºC with a relative humidity of 90-95 %) in the dark until further analysis.

Proximate Composition

Moisture and ash were determined by standard procedures (AOAC, 1984), whereas crude protein was determined in a model FP-528 LECO protein/nitrogen analyser (LECO Corp., St. Joseph, USA), calibrated with EDTA (Dumas method) and crude fat was determined by a rapid method of total lipid extraction and purification (Bligh and Dyer, 1959).

Texture Measurements

Folding test and texture profile analysis (TPA), namely hardness, cohesiveness, gumminess, springiness and chewiness were evaluated according to description in chapter 5.4.1, using a 40 % compression level.

Furthermore, a more drastic compression (80 %) was applied in order to measure force and distance at rupture, keeping all other parameters unchanged as in chapter 5.4.1.

A stress-relaxation test was also conducted. Samples were compressed by 10 % with a 50 mm diameter cylindrical probe and a load cell of 1 kN at a crosshead speed of 0.8 mm/s, the deformation being kept constant for 10 min. Initial stress (σ_0) was obtained, relaxation of stress was monitored as a function of time and the curves were fitted to a simplified Maxwell model, given by the equation (Hamann and MacDonald, 1992):

$$\sigma = \sigma e + (\sigma 0 - \sigma e) \times e^{\left(-\frac{t}{\tau 1}\right)}$$

where,

σ - decaying stress (kPa);

σ_e- stress at equilibrium (kPa), when t= ∞;

t- time (s);

τ_i- relaxation time (s).

For each fitting, starting σ_e and τ_1 values included into nonlinear regression equation were 1.1 kPa and 200 s, respectively. The viscous (η_1) and elastic (E_e and E_1) moduli were calculated taking into account that $\eta_1 = \tau_1 \times E_1$, $E_1 = (\sigma_0 - \sigma_e)$/deformation and $E_e = \sigma_e$/deformation.

Gel strength was attained through a puncture test. Prior to analysis, 'sausages' were cut into pieces of 25 mm diameter and 25 mm high. Each sample was penetrated to the

breaking point with a metal probe equipped with a 5 mm diameter spherical head, using a model Instron 4301 texturometer (Instron Engineering Corp., Canton, MA, USA). The cross speed head was 10 mm/min and the load cell was 1000 N. Breaking force (N) and breaking deformation (mm) were measured. Gel strength (N.mm) was determined by multiplying these two parameters.

pH

pH was measured using a Sen-Tix 21 surface pH electrode and a model pH 539 pH meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). This determination was carried out in triplicate.

Water Holding Capacity (WHC)

WHC was measured in the gels (after heating) by a modification of Sánchez-González et al. (2008) method. Coarsely chopped sample (≈2 g) (W_s), wrapped in two overlayed Whatman nº1 filter papers (also weighed, W_i), was placed in a centrifuge tube and submitted to 3,000 × *g* for 10 min. at 20 ºC in a model 6800 centrifuge (Kubota Corp., Tokyo, Japan). After centrifugation, sample was removed and filter papers were weighed again (W_f). Measurements were made in triplicate. WHC is expressed as grams of water retained in the sample after centrifugation per 100 g of water initially present in the sample:

$$WHC = \frac{Ws \times (H/100) - (Wf - Wi)}{Ws \times (H/100)} \times 100$$

where,

H – moisture (%).

Colour

L*, a* and b*, chroma and whiteness were determined in the gels (after heating) on a model MACBETH COLOUR-EYE® 3000 colourimeter (Macbeth, New Windsor, NY, USA) as described in 5.4.1. This determination was carried out in triplicate.

Protein Solubility

In order to evaluate the importance of different chemical interactions between gilthead seabream proteins, four different extracting solutions targeting specific bonds were used (Table 5.2.1.1). Protein solubility was determined spectrophotometrically at 280 nm in accordance with description in chapter 5.1.5.

Table 5.2.1.1 – Composition of the protein extracting solutions and targeted chemical interactions.

CHARACTERISTICS	Extracting Solution			
	SDS	SDS+DTT	Urea	Urea+DTT
COMPONENTS				
Sodium dodecyl sulphate, SDS (%, w/v)	2	2	---	---
DL-Dithiothreitol, DTT (M)	---	0.1	---	0.1
Urea (M)	---	---	8	8
Tris(hidroxymethyl)-aminomethane, Tris(mM)	60	60	60	60
ACIDITY-ALCALINITY				
pH (adjusted with HCl)	7.5	7.5	7.5	7.5
TARGETED CHEMICAL INTERACTIONS (Liu and Hsieh, 2008)	Non-covalent (predominantly hydrophobic)	Non-covalent and disulphide bridges	Predominantly hydrogen bonds	Predominantly hydrogen bonds and disulphide bridges

Electrophoresis

SDS-PAGE electropherograms of the extracted proteins were attained as described in chapter 5.1.5. The protein bands were silver stained with a PlusOne™ silver staining kit (Amersham Biosciences AB, Uppsala, Sweden). As reference molecular weights, two standard (high and low molecular weight) electrophoresis calibration kits (Pharmacia Biotech, Uppsala, Sweden) were used. Gels' optical density was analysed in a model GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, USA) with the software Quantity One® (Bio-Rad Laboratories, Hercules, USA), version 4.5.2.

Statistical Analysis

Factorial analysis of variance (general linear model, factorial ANOVA, full factorial design) was carried out using the STATISTICA© software (StatSoft, Inc., Tulsa, USA), version 6.1, 2003. This statistical methodology enabled to analyse each one of the studied effects *per se* (Kjc, MTGase and UV irradiation) as well the interaction between effects. The difference of means between pairs was resolved by using confidence intervals in a Tukey HSD test. Level of significance was set for $p<0.05$.

Results and Discussion

Proximate Composition

Proximate composition of gels was quite similar, since moisture, protein, fat and ash were near 80 %, 12 %, 5 % and 2 %, respectively. Detected differences were not significant. Therefore, although incorporated Kjc amount was substantial (1.0 %, w/w), no significant dilution effect was observed in protein content. This means that any property variations between samples, namely texture modification, have to be assigned to other factors than protein quantity.

Texture Measurements

Folding test, gel strength and TPA results are found in Table 5.2.1.2, while force and distance at rupture and stress-relaxation parameters are presented in Table 5.2.1.3. In the folding test (FT), most gels scored the maximum, 5.0 ± 0.0, with exception of C0 UV, 3.0 ± 0.0, and C0 and K0 UV, 3.3 ± 0.6. MTGase had a significantly favourable effect upon the gels, meaning that this enzyme is adequate for inducing gelation of the gilthead seabream proteins. Likewise, Kjc presented a positive effect upon the FT. Puncture test results also showed that strength of the gels containing MTGase was significantly greater than that of the other samples. This was due to breaking deformation differences, being breaking force increases consequence of larger deformations (data not shown). Thus, MTGase seems to strongly enhance deformability and to moderately harden gilthead seabream gels. Those gels containing Kjc were weaker, mainly as a result of lower breaking deformation. However, this effect was clear only for formulations containing MTGase. Hence, MTGase and Kjc presented no synergies for gel strength, since it fell from 56.9 ± 7.1 (Ca/Ca UV) to 24.6 ± 5.9 N.mm (Ka/Ka UV) as a result of adding Kjc to those gels containing MTGase. UV irradiation produced no alteration. All TPA parameters showed significant textural advantages with MTGase incorporation (including springiness, if MTGase containing samples are taken together and compared with the other). Namely, cohesiveness, a fundamental indicator of gel quality, increased from values in the range 0.49-0.54 to 0.59-0.71. On the other hand, Kjc showed different effects, it decreased cohesiveness —once again, this negative variation occurred in those samples with MTGase— and hardened gels (global averages, 15.1 ± 3.1 *vs* 20.6 ± 4.7 N) —only clearly detected in non-irradiated samples. The combination of MTGase and Kjc yielded a greater hardening effect than each one of these ingredients *per se*, namely, hardness increased, in the absence of UV treatment, from 12.3 ± 1.0 (C0) to 26.4 ± 1.8 N (Ka), thus MTGase and Kjc together added 14.1 N, more than the sum of the increases observed with incorporation of MTGase (2.4 N) and Kjc (6.1 N). A similar synergistic effect was also detected with UV treatment. Gumminess and chewiness variations largely reflected these hardness variations. UV irradiation only affected cohesiveness, which declined from 0.60 ± 0.07 to 0.57 ± 0.07 (global averages) with UV treatment. Furthermore, force and distance at rupture —obtained by a 80 % compression, which imitates the effects of the mastication process upon food (Bourne, 1994)— were significantly augmented by MTGase (force at rupture, average of gels C, K, C UV and K UV, augmented from 44.3 ± 18.1 to 131.9 ± 56.7 N) and reduced by Kjc and UV, revealing a response to the studied factors very similar to cohesiveness. Regarding stress-relaxation properties, generally used for evaluation of the viscoelastic behaviour of gels, statistical analysis revealed that relaxation time was higher in the MTGase containing products, taken as a whole (67.1 *vs*

55.0 s). However, elastic modules E_1 were reduced by MTGase addition. Kjc exhibited an opposite effect upon E_1 and relaxation time and increased elastic modules E_e and viscous modules η_1. For UV irradiated samples, whereas relaxation time was unaffected, E_1 and η_1 were increased —especially in the absence of Kjc— and E_e decreased.

The textural benefits of MTGase have been reported for many other fish products (Ramírez et al. 2000; Téllez-Luís et al. 2002; 5.1.3). Nonetheless, it is remarkable that addition of 0.5 % (w/w) MTGase to gilthead seabream minces had a so positive effect on almost all textural properties. MTGase increases cross-linking of myosin heavy chains (MHC) during setting, since it catalyzes covalent ε-amino-(γ-glutamyl) lysine bonds (Kumazawa et al., 1993; Ramírez-Suárez et al., 2001). Therefore, it creates a denser bond network between proteins in the gels. Moreover, a relationship between MHC polymerization and an increase in breaking force or shear stress has also been found in gels from various fish species, such as Pacific whiting (Lee and Park, 1998) or carp (Tsukamasa et al., 2000). Concerning stress-relaxation data, the decline of elastic modules E_1 with MTGase was apparently in contradiction to all other measured properties. However, these results were systematically found in all gels containing MTGase and σ values were lower for these gels, meaning a softening effect. Similar variation was also observed with mackerel surimi gels (5.1.5). Taking into account that stress-relaxation test involved a 10 % compression, it seems that MTGase action reduces those chemical interactions that respond intensely at a slight compression but are progressively destroyed with larger compressions (40 % as in TPA or 80 % as in rupture test) and, on the other hand, increases stronger bonds, which appear to generate large forces only with higher compression levels. So, there is a hardness rank inversion between gels with and without MTGase as a consequence of different degrees of compression. Similar absence of correlation between small and large deformation tests has already been reported for other gels (Mitchell, 1980). Furthermore, it has been shown that the consumer assessment of the hardness of the gels correlates better with the force at rupture rather than the elastic modulus (Wood, 1979). The elastic modules E_e, which can be related to residual solidity of the system derived from permanent cross-links (Kim et al., 2005a), do not present a clear effect of MTGase. This enzyme produced no reduction of the viscous module η_1, since elastic module E_1 variation was compensated by a higher relaxation time. This means a relatively more important viscous element and that energy dissipation is slower, which may be due to the better alignment of protein chains and to the dissociation and re-association of non-covalent bonds as protein chains pass each other (Hamann et al., 1992).

Table 5.2.1.2 – Folding test and TPA (texture profile analysis with 40 % compression) properties of gels*.

Sample	UV treatment	Kjc (% w/w)	MTGase (% w/w)	Folding test	Gel strength (N.mm)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
C0	No	0.0	0.0	3.3 ± 0.6^{a}	13.6 ± 2.2^{ab}	12.3 ± 1.0^{a}	0.54 ± 0.01^{b}	6.6 ± 0.5^{a}	0.74 ± 0.01^{a}	4.9 ± 0.4^{a}
Ca	No	0.0	0.5	5.0 ± 0.0^{b}	50.6 ± 2.0^{e}	14.7 ± 3.1^{ab}	0.71 ± 0.01^{e}	10.4 ± 2.0^{c}	0.86 ± 0.00^{a}	8.9 ± 1.8^{cd}
K0	No	1.0	0.0	5.0 ± 0.0^{b}	16.2 ± 3.2^{bc}	18.4 ± 1.4^{bc}	0.54 ± 0.02^{b}	10.0 ± 0.7^{bc}	0.78 ± 0.01^{a}	7.8 ± 0.6^{bc}
Ka	No	1.0	0.5	5.0 ± 0.0^{b}	28.5 ± 4.0^{d}	26.4 ± 1.8^{d}	0.60 ± 0.01^{c}	15.8 ± 0.9^{e}	0.83 ± 0.01^{a}	13.2 ± 1.0^{e}
C0 UV	Yes	0.0	0.0	3.0 ± 0.0^{a}	11.1 ± 0.8^{ab}	14.3 ± 1.6^{ab}	0.52 ± 0.01^{ab}	7.3 ± 0.7^{ab}	0.82 ± 0.13^{a}	6.0 ± 1.4^{ab}
Ca UV	Yes	0.0	0.5	5.0 ± 0.0^{b}	63.2 ± 4.7^{f}	19.0 ± 1.6^{bc}	0.66 ± 0.01^{d}	12.6 ± 0.9^{cd}	0.84 ± 0.00^{a}	10.6 ± 0.7^{de}
K0 UV	Yes	1.0	0.0	3.3 ± 0.6^{a}	8.9 ± 1.3^{a}	15.0 ± 2.1^{ab}	0.49 ± 0.02^{a}	7.3 ± 0.8^{ab}	0.73 ± 0.01^{a}	5.3 ± 0.6^{ab}
Ka UV	Yes	1.0	0.5	5.0 ± 0.0^{b}	18.4 ± 2.9^{c}	22.7 ± 0.9^{cd}	0.59 ± 0.01^{c}	13.5 ± 0.4^{de}	0.81 ± 0.01^{a}	10.9 ± 0.2^{de}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different.

Table 5.2.1.3 – Rupture (at 80 % compression) and stress-relaxation properties of gels*.

Sample	UV treatment	Kjc (% w/w)	MTGase (% w/w)	Force at rupture (N)	Distance at rupture (mm)	Relaxation time (s)	Elastic module, E_1 (kPa)	Elastic module, E_e (kPa)	Viscous module, η_1 (kPa.s)
C0	No	0.0	0.0	47.4 ± 7.3^{a}	15.7 ± 0.5^{bc}	49.9 ± 3.5^{a}	18.3 ± 1.6^{a}	13.7 ± 2.2^{a}	914 ± 142^{a}
Ca	No	0.0	0.5	185.9 ± 8.3^{c}	19.9 ± 0.3^{f}	77.7 ± 0.0^{b}	12.7 ± 0.0^{a}	13.4 ± 0.0^{a}	983 ± 0^{ab}
K0	No	1.0	0.0	70.7 ± 1.8^{ab}	16.9 ± 0.5^{cd}	52.0 ± 4.8^{ab}	36.2 ± 1.0^{b}	24.5 ± 0.4^{d}	1887 ± 228^{b}
Ka	No	1.0	0.5	107.4 ± 23.2^{b}	17.8 ± 1.1^{de}	57.4 ± 3.1^{ab}	31.2 ± 0.8^{b}	22.8 ± 0.5^{cd}	1795 ± 141^{ab}
C0 UV	Yes	0.0	0.0	32.7 ± 0.6^{a}	14.4 ± 0.4^{ab}	62.5 ± 2.9^{ab}	30.7 ± 0.5^{b}	15.3 ± 0.5^{ab}	1917 ± 119^{b}
Ca UV	Yes	0.0	0.5	170.6 ± 48.8^{c}	19.2 ± 0.7^{ef}	75.0 ± 9.1^{ab}	17.2 ± 4.2^{a}	13.9 ± 0.2^{ab}	1308 ± 474^{ab}
K0 UV	Yes	1.0	0.0	26.3 ± 1.6^{a}	12.7 ± 1.1^{a}	55.7 ± 14.9^{ab}	33.5 ± 4.3^{b}	15.6 ± 2.7^{ab}	1832 ± 258^{ab}
Ka UV	Yes	1.0	0.5	63.6 ± 12.3^{ab}	15.9 ± 0.6^{bcd}	58.4 ± 4.3^{ab}	29.2 ± 2.3^{b}	19.0 ± 0.9^{bc}	1710 ± 259^{ab}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different.

Other authors reported, under similar conditions (10 % compression), higher values for surimi gels as a result of previous setting (E_1, 22.3 *vs* 19.5 kPa; E_e, 32.3 *vs* 19.1 kPa; η_1, 4453 *vs* 2731 kPa.s and τ_1, 200 *vs* 140 s) (Hamann et al., 1992). Since it is frequently assumed a predominant role of TGase in setting of surimi, these data, particularly regarding E_1 seem to be opposite to this work's findings. However, setting effects may be found in highly washed surimi (with a low TGase content), revealing the importance of other factors than TGase (Lanier et al., 2005). Alternatively, the contradiction may be explained by a somewhat different action of MTGase (decreased E_1) with respect to endogenous TGase (increased E_1). Hence, this subject needs further investigation.

Kjc had also positive effects, however, unlike MTGase, it improved only those properties linked to hardening of the gels and it had a negative effect upon those parameters concerning deformability/elasticity, namely the gel strength. The apparent lack of correlation between the two types of rheological measurement (gel strength and hardness) has been extensively reviewed by Lee and Chung (1989). According to these authors, while the compression test measures the overall binding property of the gel material, the penetration test evaluates the degree of compactness or density of actomyosin. Following this interpretation, Kjc increased strongly the overall binding, but, unlike MTGase, failed to promote a higher degree of compactness in the gel network. Moreover, it seemed to partially disrupt MTGase action. These effects are similar to the ones caused by inner pea fibre incorporation to hake mince (5.1.2) or mackerel surimi (5.1.5) gels. The observed synergistic effect between Kjc and MTGase upon hardness of the gels is quite noteworthy and may be used in future works as a way to improve very soft gels.

Among tested factors, UV presented the least effects upon texture and these effects, whenever significant, were generally deleterious for gel quality. Furthermore, as with Kjc, elastic modules E_1 were higher with UV treatment, but combination of Kjc and UV did not yield further gains of E_1. This means that some chemical interactions were indeed promoted by UV irradiation, enabling some toughening of samples at 10 % compression, but not those beneficial for gel quality. These findings oppose to other authors' results which point to an enhancement of gel strength with exposition to UV light (of a comparable source and intensity to the ones used in this work's experiments) of MTGase-supplemented fish mince (Jiang et al., 1998). One possible explanation may be given with the differences between myofibrillar proteins, namely myosin, of different species (Kawabata et al., 2000). Moreover, it can also be hypothesized that used intensity, 3300 $\mu W/cm^2$, may have been too low, especially if compared with a recent work on fish gelatins that reported a significant improvement of gel strength with higher intensity UV

irradiation (Bhat and Karim, 2009). In fact, it has been claimed that, depending on protein nature (different in gelatins and minces) and irradiation dosage, the net result of irradiating protein in the solid state could be cross-linking (aggregate formation) or molecular degradation (Urbain, 1977).

pH, WHC and Colour

pH values (Table 5.2.1.4) were, in general, significantly higher in those products containing MTGase. On the other hand, UV irradiation lowered pH.

These variations must be seen as indirect effects of MTGase and UV, probably resulting from altered protein conformations, that is, exposure of more or less acidic groups.

MTGase also increased WHC (Table 5.2.1.4), from 57.2 ± 10.7 to 62.7 ± 8.3 % (average values of C, K, C UV and K UV samples). However, the effect was stronger in the absence of Kjc, 47.5 ± 3.1 to 55.3 ± 3.2 % (averages of C and C UV). This can be due to the large water holding effect of Kjc, from 51.4 ± 5.1 to 68.6 ± 3.6 % (averages of C0, Ca, C0 UV and Ca UV) (a 33.5 % increase), which did not leave much room for progression concerning MTGase effect. UV had no effect.

Different studies have also reported similar effects of MTGase upon WHC or upon a related parameter, extracted water (Ramírez et al., 2007; 5.1.5). Kjc effect may be explained by the protein-polyssacharide matrix that is formed in a gel product and that entraps water (Pérez-Mateos and Montero, 2000). This was achieved with a low level of Kjc, 1.0 %, w/w, thus showing an effect upon WHC much similar to other dietary fibres, such as alginate or xanthan gum, which increased WHC in 40 % when added at a comparable low level, 0.5 % (Pérez-Mateos and Montero, 2000).

With respect to colour (Table 5.2.1.4) whiteness was always significantly, albeit slightly, reduced by MTGase addition. Effect of this enzyme upon other colour parameters was generally non-significant. Kjc had a significant effect upon all parameters, increasing a*, b* and chroma (essentially as a consequence of b* variation) and reducing whiteness. UV had no relevant effect.

The slight darkening effect of MTGase can be read as an indication of a higher cross-linking degree, catalyzed by MTGase. This more orderly net structure may have reduced opaqueness, leading to higher light absorption and lower whiteness (Shie and Park, 1999). Concerning Kjc, the tan colour of this ingredient must have imparted the detected colour changes to the gels, increasing b* and chroma.

Table 5.2.1.4 – pH, WHC and colour parameters of gels*.

Sample	UV treatment	Kjc (% w/w)	MTGase (% w/w)	pH	WHC (%)	a*	b*	Whiteness	Chroma
C0	No	0.0	0.0	7.40 ± 0.04^{cd}	47.8 ± 4.2^{a}	-1.86 ± 0.12^{bcd}	5.41 ± 0.27^{b}	77.6 ± 0.4^{c}	5.73 ± 0.22^{b}
Ca	No	0.0	0.5	7.48 ± 0.05^{d}	53.1 ± 2.3^{ab}	-2.09 ± 0.05^{a}	4.56 ± 0.12^{a}	75.7 ± 0.4^{b}	5.01 ± 0.10^{a}
K0	No	1.0	0.0	7.34 ± 0.03^{bc}	68.8 ± 2.6^{d}	-1.73 ± 0.04^{cd}	6.45 ± 0.09^{c}	76.9 ± 0.2^{c}	6.68 ± 0.08^{c}
Ka	No	1.0	0.5	7.38 ± 0.01^{c}	69.6 ± 2.5^{d}	-1.85 ± 0.07^{bcd}	6.05 ± 0.18^{c}	75.2 ± 0.3^{b}	6.33 ± 0.15^{c}
C0 UV	Yes	0.0	0.0	7.26 ± 0.01^{ab}	47.2 ± 2.4^{a}	-1.96 ± 0.03^{ab}	5.02 ± 0.11^{ab}	77.3 ± 0.2^{c}	5.38 ± 0.10^{ab}
Ca UV	Yes	0.0	0.5	7.28 ± 0.01^{ab}	57.5 ± 2.6^{bc}	-1.93 ± 0.07^{abc}	5.30 ± 0.16^{b}	75.4 ± 0.3^{b}	5.64 ± 0.13^{b}
K0 UV	Yes	1.0	0.0	7.23 ± 0.05^{a}	65.2 ± 3.9^{cd}	-1.71 ± 0.04^{d}	6.03 ± 0.13^{c}	75.5 ± 0.4^{b}	6.27 ± 0.11^{c}
Ka UV	Yes	1.0	0.5	7.32 ± 0.01^{bc}	70.6 ± 4.1^{d}	-1.68 ± 0.10^{d}	6.19 ± 0.28^{c}	74.2 ± 0.3^{a}	6.41 ± 0.25^{c}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different.

Table 5.2.1.5 – Effects of UV treatment, Kjc (1.0 %, w/w) and MTGase (0.5 %, w/w) addition on the relative protein solubility of the gels in different extracting solutions*.

Effect	Samples	Protein Solub. in SDS (%)	Protein Solub. in SDS+DTT (%)	Protein Solub. in Urea (%)	Protein Solub. in Urea+DTT (%)
UV treatment	No	76.2 ± 8.0^{a}	105.5 ± 8.4^{a}	40.7 ± 13.0^{a}	59.3 ± 12.5^{a}
	Yes	67.7 ± 14.2^{b}	100.1 ± 12.2^{a}	37.4 ± 3.7^{b}	57.3 ± 17.0^{a}
Kjc addition	No	78.1 ± 9.3^{a}	109.1 ± 6.6^{a}	44.5 ± 9.8^{a}	65.3 ± 15.7^{a}
	Yes	65.8 ± 11.6^{b}	96.5 ± 10.2^{b}	33.6 ± 5.4^{b}	51.4 ± 9.7^{b}
MTGase addition	No	76.9 ± 9.2^{a}	107.2 ± 8.0^{a}	43.3 ± 10.3^{a}	58.1 ± 13.0^{a}
	Yes	67.0 ± 12.9^{b}	98.4 ± 11.3^{b}	34.9 ± 6.6^{b}	58.6 ± 16.7^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different.

Protein Solubility and Electrophoresis

Protein solubility values in four different extracting media targeting specific bonds are presented in Table 5.2.1.5. Samples were grouped in order to highlight the effects of the studied variables (MTGase, Kjc and UV). Whereas, Kjc reduced protein solubility in all media, MTGase lowered extractability in all media apart from urea+DTT. UV had significant reducing effects on the solubility in SDS and urea.

Solubility reduction with MTGase can be related to cross-linking of myosin heavy chains (MHC) during setting, since it catalyzes covalent ε-amino-(γ-glutamyl) lysine bonds (Kumazawa et al., 1993, Ramírez-Suárez et al., 2001) which, in turn, cause the formation of insoluble polymeric aggregates (Ashie and Lanier, 1999). A pronounced decrease of the percentage of soluble protein, as a result of MTGase addition, was also reported with gels obtained from pelagic fish (Gómez-Guillén et al., 2005). However, in urea+DTT, no similar effect was found. This suggests that MTGase may promote the establishment of disulphide bonds, broken by DTT (Table 5.2.1.1), in gilthead seabream minces, thus, being its direct catalytic action compounded by indirect effects. Similar effect of promotion of disulphide bonds by MTGase was found in mackerel surimi (5.1.5). However, these disulphide bonds are predominantly associated to hydrogen bonding, i.e. hydrophilic protein regions, since a similar contrast was not found between solubility in SDS and in SDS+DTT. Taking into account that an increased degree of disulphide linkages between proteins is associated to rubberiness/deformability of heat-induced gels (Havea et al., 2004), these molecular phenomena may explain gel strength as well as rupture force and deformation increases, alongside with the covalent bonds catalysed by MTGase.

Moreover, solubility results show that Kjc promoted protein aggregation. This may be due to glucomannan, however, addition of polysaccharides to fish gels has led to contradictory results (Benjakul et al., 2001; Gómez-Guillén et al., 2005). Nevertheless, this can be explained by different gelling procedures, for instance, decreased protein solubility has been reported in suwari and heat-induced gels containing chitosan (Benjakul et al., 2001), whereas addition of this polysaccharide to pressure-induced gels did not change solubility values (Gómez-Guillén et al., 2005). Whereas, for MTGase, solubility results are consistent with the textural effects, concerning Kjc interpretation is not straightforward, thus requiring further research. Moreover, Kjc hardened gels (Table 5.2.1.2), in spite of the lower degree of non-covalent bonding (indirectly measured by protein solubility in SDS and urea). This seems to be in contradiction to other authors (Havea et al., 2004), who claim that non-covalent bonding is responsible for the hardness of gels. A possible solution for this contradiction may be the hardening effect of Kjc itself, which may have more than compensated the softening due to less non-covalent bonding between proteins. This hardening effect was found by other authors working with different matrices, such as

surimi gels from grass carp (Xiong et al., 2009). UV irradiated samples presented less non-covalent bonding, but this did not produce any loss of hardness. Besides, UV exhibited also an increase of E_1 modules. A possible explanation for these facts may be in the variation of the relative importance of each kind of non-covalent interaction. On the other hand, the lower cohesiveness and deformability of the UV treated gels can not be traced back to a reduction of disulphide bonds, leaving as conclusion that molecular degradation of an enzymatic nature, given the presence of sarcoplasmic proteins in the seabream minces, was stimulated by UV irradiation and surpassed the effect of any additionally induced cross-linking (Urbain, 1977).

Electrophoresis only revealed differences due to MTGase. Kjc addition and UV irradiation did not present any effect. Therefore, only electrophoretic profiles for C0, Ca and raw material (gilthead seabream mince) in the four extracting solutions are presented (Figure 5.2.1.1). Electrophoresis profiles of proteins extracted in urea and urea+DTT were all less intense in the zone of high molecular weight (HMW) protein bands. Processing, in particular heating over one hour caused loss of intensity in the protein bands of C0 and Ca with respect to raw material. MTGase caused the disappearance of HMW protein bands (assigned to MHC and M- and C-proteins) in the SDS and SDS+DTT extracted proteins. For urea and urea+DTT, electrophoresis profiles of C0 and Ca were similar.

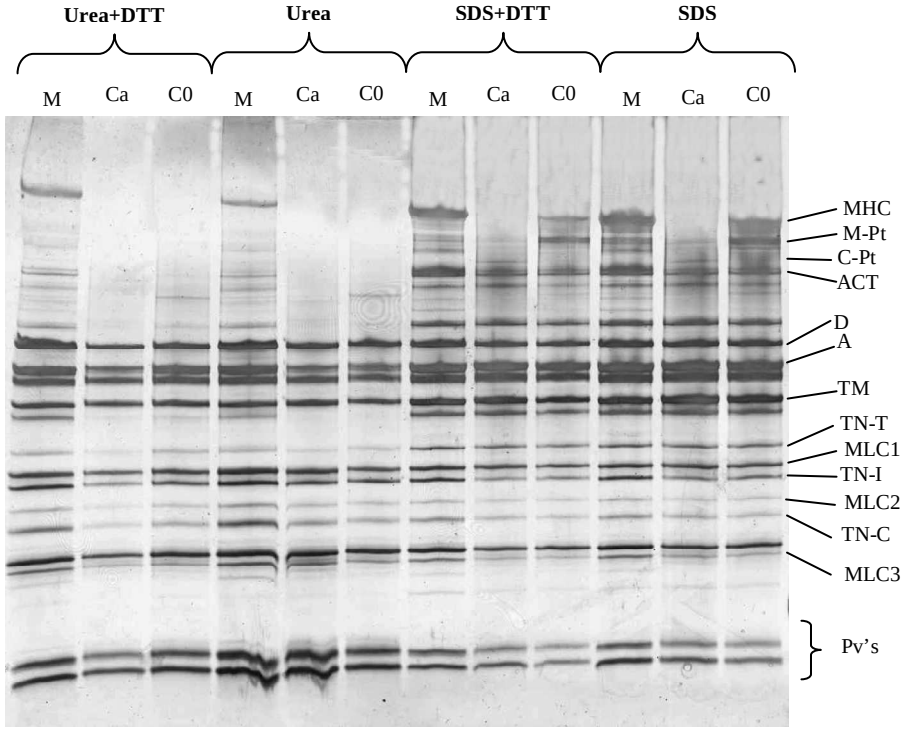

Figure 5.2.1.1 – SDS-PAGE of soluble protein homogenates in the four extracting media from raw material (M) and produced gels without (C0) and with MTGase (Ca). MHC: myosin heavy chain; M-Pt: M-protein; C-Pt: C-protein; ACT: actinin; D: desmin; A: actin; TM: tropomyosin; TNT: troponin-T; MLC1: myosin light chain 1; TNI: troponin-I; MLC2: myosin light chain 2; TNC: troponin-C; MLC3: myosin light chain 3; Pv's: parvalbumins.

Therefore, the electropherograms revealed that HMW proteins were largely aggregated by hydrophobic interactions targeted by SDS and also that even the raw material presented already a considerable level of aggregated HMW proteins. This level became even higher with mince processing, since almost no heavy proteins were extracted from the processed products in the absence of SDS. However, this aggregation was mostly of a hydrophobic non-covalent nature, since raw material and C0 had almost identical profiles when extracted with SDS.

The other observed phenomena may also be explained by exclusion of heavy proteins from the soluble protein fraction as a result of aggregation: MTGase promotes the formation of intra- and intermolecular cross-linked products, thus converting MHC and other HMW proteins to insoluble aggregates or polymers. This aggregation is of a covalent nature other than disulphide bonding, confirming the importance of the ε-amino-(γ-glutamyl) lysine bonds. On the other hand, actin was remarkably resistant to cross-linking, probably as a result of low exposure of the susceptible amino acid residues (Gln, Lys) in its globular conformation (Ramírez-Suárez et al., 2005).

Concerning Kjc-containing and UV irradiated samples, no differences were found with respect to controls (C0 or Ca). Hence, solubility decreases (see above) possibly affected all proteins, regardless of their molecular weight (for all samples, soluble protein was adjusted to 0.3 mg/ml, making any uniform changes along the profiles invisible).

Conclusions

MTGase addition improved texture and water holding capacity (WHC). Moreover, it reduced the elastic modules E_1 and darkened gels; protein solubility declined, meaning greater protein aggregation, according to electropherograms. Evidence was found that, besides ε-amino-(γ-glutamyl) lysine bonds, also disulphide bonding has a role in textural improvement by MTGase. Kjc incorporation yielded harder gels and increased the elastic modules and WHC. Kjc itself and not its effect upon proteins may explain the hardening effect. UV had no positive effect on textural quality and WHC and reduced protein solubility in SDS and urea. Hence, UV does not present significant advantages as a gelation-promoting technology, at least, for this species or at applied irradiation intensity. The interaction between these three factors was not of a synergistic nature, with the only major exception of the great hardness increase with the combination of MTGase and Kjc. Moreover, this work showed for the first time that mince from gilthead seabream (a commonly farmed species) trimmings may be used to produce good quality heat-induced gels.

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5.2.2 – Quality Characteristics of High Pressure-Induced Hake (*Merluccius capensis*) Protein Gels with and without MTGase

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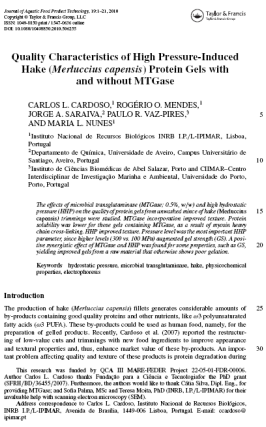

Quality Characteristics of High Pressure-Induced Hake (*Merluccius capensis*) Protein Gels with and without MTGase

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*The effects of microbial transglutaminase (MTGase; 0.5%, w/w) and high hydrostatic pressure (HHP) on the quality of protein gels from unwashed mince of hake (*Merluccius capensis*) trimmings were studied. MTGase incorporation improved texture. Protein solubility was lower for those gels containing MTGase, as a result of protein heavy chain cross-linking. HHP improved texture. Pressure level was the most important HHP parameter, since higher levels (300 vs. 100 MPa) augmented gel strength (GS). A positive synergistic effect of MTGase and HHP was found for some properties, such as GS, yielding improved gels from a raw material that otherwise shows poor gelation.*

Keywords hydrostatic pressure, microbial transglutaminase, hake, physicochemical properties, electrophoresis

Introduction

The production of hake (*Merluccius capensis*) fillets generates considerable amounts of by-products containing good quality proteins and other nutrients, like ω3 polyunsaturated fatty acids (ω3 PUFA). These by-products could be used as human food, namely, for the preparation of gelled products. Recently, Cardoso et al. (2007) reported the restructuring of low-value cuts and trimmings with new food ingredients to improve appearance and textural properties and, thus, enhance market value of these by-products. An important problem affecting quality and texture of these products is protein degradation during

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Abstract

The effects of microbial transglutaminase (MTGase) (0.5 %, w/w) and high hydrostatic pressure (HHP) on quality of protein gels from unwashed mince of hake (*Merluccius capensis*) trimmings were studied. MTGase incorporation improved texture. Protein solubility was lower for those gels containing MTGase, as a result of myosin heavy chain cross-linking. HHP improved texture. Pressure level was the most important HHP parameter, since higher levels (300 *vs* 100 MPa) augmented gel strength (GS). A positive synergistic effect of MTGase and HHP was found for some properties, such as GS, yielding improved gels from a raw material that otherwise shows poor gelation.

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Introduction

Hake (*Merluccius capensis*) fillets production generates considerable amounts of by-products, containing good quality proteins and other nutrients, like ω3 polyunsaturated fatty acids (ω3 PUFA). These by-products could be used as human food, namely, for the preparation of gelled products. In chapter 5.1.3, it was reported the restructuring of low-value cuts and trimmings with new food ingredients to improve appearance and textural properties and, thus, enhance market value of these by-products. An important problem affecting quality and texture of these products is protein degradation during the heating step used to promote gelation. Particularly, myosin, the myofibrillar protein responsible for functional and mechanical properties, is subjected to proteolytic degradation leading to the loss of textural quality (Ramírez et al., 2002). Heat-induced gelation at temperatures between 50 and 70 ºC results in maximum myofibrillar protein degradation in various fish species, namely, arrowtooth flounder (*Atheresthes stomias*) (Uresti et al., 2006). Furthermore, the long duration (0.5 to 1 h) of high temperature (85-95 ºC) gelation processes enhances the risks of lipid oxidation of the ω3 PUFA present in fish. Therefore, alternative gelation techniques to the usual thermal process (Gómez-Estaca et al., 2007) as well as the combination of ingredients and new techniques (Uresti et al., 2006) are of current interest.

Among such ingredients, MTGase has been widely used in the food industry with the purpose of promoting protein cross-linking. Although fish mince contains an endogenous TGase, largely responsible for the setting phenomenon which occurs typically at temperatures between 25 and 40 ºC, the addition of MTGase has effectively improved, in various cases, the textural properties of minced fish products, either washed, such as surimi (Nakahara et al., 1999), or not (5.1.3). For example, MTGase at 0.5 % (w/w)

improved the texture of mackerel surimi gel with dietary fibre (5.1.5). MTGase can also work at low temperatures (from 2-5 ºC), yielding thermostable gel from the giant squid surimi (Moreno et al., 2009) and hake (*Merluccius merluccius*) (Moreno et al., 2010b) with adequate properties.

Regarding alternative gelation techniques, high hydrostatic pressure (HHP) has been applied to some fish products (Rastogi et al., 2007) and can be used to produce restructured gelled fish products (Jiménez-Colmenero, 2002). Moreover, HHP may achieve some degree of gelation at low temperatures, even below 10 ºC (Uresti et al., 2003). A different mechanism was proposed for HHP-induced gelation from the heat-induced one (Uresti et al., 2006). It seems that HHP application to fish mince at low temperature promotes a kind of protein aggregation characterized by side-to-side interactions of proteins with reduced denaturation instead of large conformational changes as with thermal gelation. Such gelation process involved in pressure-induced gel may explain the formation of different mechanical properties as reported by various investigators. Besides, these gels may present a textural quality similar or, even, regarding some properties, superior to heat-induced gels, as reported by various authors working with different seafood raw materials, such as giant squid (*Dosidicus gigas*) surimi (Moreno et al., 2009), cod (*Gadus morhua*) or horse mackerel (*Trachurus trachurus*) mince (Montero et al., 2005a). Moreover, pressure-induced fish gels have been characterized as softer, but more elastic than heat-induced gels. This positive effect has been likened to the favorable protein aggregation induced at low temperature by MTGase (Uresti et al., 2006). Information about the contribution of MTGase activity to pressure-induced gels is scarce. Nonetheless, some authors have claimed that the combination of MTGase and HHP (600 MPa, 5 min) has improved the texture of arrowtooth flounder gels (Uresti et al., 2006). However, these results were attained with cooked gels, since HHP-treated samples were afterwards heated at 90 ºC for 15 min. Other authors working on the gel forming ability of horse mackerel (*Trachurus* spp.) muscle under high pressure found that MTGase led to an increase in hardness, but to a decrease in elasticity (Gómez-Guillén et al., 2005). Moreover, it was found that pressurization with previous setting induced more deformable and stronger gels as compared to high pressure without setting, or with a setting step following pressurization (Gómez-Guillén et al., 2005; Pérez-Mateos et al., 2002). The loss of MTGase activity with pressure treatment may be not too high, since it was reported a 40 % loss of the initial activity after 1 h at 600 MPa/37 ºC (Lee and Park, 2002). On the other hand, a recent work (Moreno et al., 2009) has shown that MTGase presented a greater effect on low temperature pressure-induced (300 MPa, 30 min) gels than in cooked gels. It must be stressed that the effect of pressure is highly dependent on the HHP treatment conditions and the product matrix. Particularly, the

pressure level is of critical importance, with a greater pressure favoring a higher degree of restructuring, thus enabling the improvement of some texture properties, namely, hardness. However, it has been reported that pressures higher than 200-300 MPa could bring about a cooked appearance to squid gel products (Moreno et al., 2009), which can represent a disadvantage, depending on the kind of product intended to be attained (for instance, sashimi/sushi like products or new products mimicking fresh or cold smoked fillets). Furthermore, higher pressure presents other disadvantages: it requires costlier equipments and higher operating costs, making HHP technology transfer to the industry more difficult from an economical point of view. Therefore, application of lower pressure ranges deserves more attention, that is, besides some studies showing an undiminished effect of TGase by lower pressure ranges (100-300 MPa) (Gilleland et al., 1997; Ashie and Lanier, 1999), further studies concerning the effects of HHP treatment in this range would be useful. With respect to other HHP parameters as well as to potential synergies between MTGase and HHP, more studies are needed in order to achieve a better understanding of the underlying molecular phenomena. Particularly, through the study of the influence of the number of compression cycles HHP effects can be better understood, since this can highlight which is the most important phase of the HHP treatment: whether the compression phase (increasing pressure) or the maintenance of a high pressure for some time period.

Therefore, the present work aimed to study the effect of HHP treatment as well as the effects of HHP parameters (pressure level, compression time and number of compression cycles) on the protein characteristics and physicochemical quality of a gel product (produced solely from hake, salt and water) with and without MTGase.

Material and Methods

Raw Materials, additives and Reagents

Trimmings (muscle joined to the bones and skin) of frozen South African hake (*Merluccius capensis*) were obtained from a local fish processor, kept frozen at –20 ºC until processed within one to two weeks. MTGase (ACTIVA® GS, supplied by Ajinomoto, Tokyo, Japan) with an activity of about 100 $U.g^{-1}$. All chemicals used were of analytical grade and were obtained from Merck KGaA (Germany).

Experimental Design

Approximately 5 kg of hake mince from trimmings were mechanically obtained in a deboning machine (model Baader 694, Baader, Lübeck, Germany) fitted with a drum with 3 mm diameter circular holes. Hake mince was mixed with salt and water in preparation of gels (samples with subscript 0) and 0.5 % (w/w) MTGase was added to half of the

resulting mince (samples with subscript a). A setting at 35 ºC after overnight storage was carried out in order to ensure a complete effect of the MTGase, since the activity of this enzyme in an unwashed mince attained from hake trimmings is lower than with surimi.

Two sets of experiments were carried out where the first set was to evaluate the effect of compression time (5 *vs* 15 min) and the second set to assess the effect of replacing a single cycle by multi-cycles of compression (1 cycle of 15 min *vs* 3 cycles of 5 min *vs* 5 cycles of 3 min). In both sets, the effect of pressure level (1st set: 100, 200 and 300 MPa; 2nd set: 100 and 300 MPa) was also studied. These pressure-induced gels (samples P) were compared with two controls, one without (S) and the other with the traditional thermal treatment (T). Table 5.2.2.1 shows the applied treatments and the samples codes used.

Production of Gels

For each experimental set, approximately 2.5 kg of mince from hake trimmings was used. The appropriate quantities of mince, salt (2.5 %, w/w), MTGase (0.0 or 0.5 %, w/w) and water (in order to guarantee final gel products with the same moisture level, 80 %) were weighed for approximately 1.5 kg batches. Mince and ingredients were mixed for 2 min at 1420 rpm and 3 min at 2800 rpm in a refrigerated vacuum chopper (model UM12, Stephan & Söhne, Germany). During sample processing, mixing was performed always under vacuum at temperature below 7 ºC. The resulting pastes were stuffed into cellulose casings (25 mm diameter and about 85 mm length) using a hydraulic filler (model EB-12, Mainca, Spain). Each link was vacuum-packed in a low-oxygen permeable barrier bag (Colamin XX 100e, Germany) with a vacuum packager (model A300/52, Multivac, Germany). Then, these packages were left at low temperature (5 ± 1 ºC) overnight. The next day, they were immersed in water at 35 ºC —temperature ensuring best possible gelation according to previous experiments (Mendes et al., 1997)— for 30 min (setting) and subjected to one of three different treatments (left in cold storage with no further treatment; thermal treated during 1 h at 90 ºC or subjected to high pressure). Pressure treatment was carried out using a hydrostatic press (model U33, Unipress Equipment, Poland). This equipment has a pressure vessel of 35 mm diameter and 100 mm height, surrounded by an external jacket, connected to a thermostatic bath to control the temperature (30 ºC), using a mixture of propylene glycol and water (1:1) as pressurizing fluid. Pressure increase was between 240 (for 100 MPa) and 360 MPa/min (for 300 MPa). In average of all pressure treatments, temperature (equipment reading) increased 1-2 ºC with pressurization and decreased 1.5 ºC with depressurization, due to adiabatic heating/cooling. After treatment, samples were left at low temperature (5 ± 1 ºC) in the dark overnight until analysis.

Table 5.2.2.1 – Various treatments for both sets of experiments[*†].

SAMPLE CODES		MTGase	Treatments 1st Set	SAMPLE CODES		MTGase	Treatments 2nd Set
S	S_0	No	Setting (35 ºC/30')	S	S_0	No	Setting (35 ºC/30')
	S_a	Yes	Setting (35 ºC/30')		S_a	Yes	Setting (35 ºC/30')
T	T_0	No	Setting (35 ºC/30') + Heating (90 ºC/1 h)	T	T_0	No	Setting (35 ºC/30') + Heating (90 ºC/1 h)
	T_a	Yes	Setting (35 ºC/30') + Heating (90 ºC/1 h)		T_a	Yes	Setting (35 ºC/30') + Heating (90 ºC/1 h)
P100-5	P100-5_0	No	Setting (35 ºC/30') + HHP (100 MPa/5'/1 cycle)	P100-1C	P100-$1C_0$	No	Setting (35 ºC/30') + HHP (100 MPa/15'/1 cycle)
	P100-5_a	Yes	Setting (35 ºC/30') + HHP (100 MPa/5'/1 cycle)		P100-$1C_a$	Yes	Setting (35 ºC/30') + HHP (100 MPa/15'/1 cycle)
P100-15	P100-15_0	No	Setting (35 ºC/30') + HHP (100 MPa/15'/1 cycle)	P100-3C	P100-$3C_0$	No	Setting (35 ºC/30') + HHP (100 MPa/15'/3 cycles)
	P100-15_a	Yes	Setting (35 ºC/30') + HHP (100 MPa/15'/1 cycle)		P100-$3C_a$	Yes	Setting (35 ºC/30') + HHP (100 MPa/15'/3 cycles)
P200-5	P200-5_0	No	Setting (35 ºC/30') + HHP (200 MPa/5'/1 cycle)	P100-5C	P100-$5C_0$	No	Setting (35 ºC/30') + HHP (100 MPa/15'/5 cycles)
	P200-5_a	Yes	Setting (35 ºC/30') + HHP (200 MPa/5'/1 cycle)		P100-$5C_a$	Yes	Setting (35 ºC/30') + HHP (100 MPa/15'/5 cycles)
P200-15	P200-15_0	No	Setting (35 ºC/30') + HHP (200 MPa/15'/1 cycle)	P300-1C	P300-$1C_0$	No	Setting (35 ºC/30') + HHP (300 MPa/15'/1 cycle)
	P200-15_a	Yes	Setting (35 ºC/30') + HHP (200 MPa/15'/1 cycle)		P300-$1C_a$	Yes	Setting (35 ºC/30') + HHP (300 MPa/15'/1 cycle)
P300-5	P300-5_0	No	Setting (35 ºC/30') + HHP (300 MPa/5'/1 cycle)	P300-3C	P300-$3C_0$	No	Setting (35 ºC/30') + HHP (300 MPa/15'/3 cycles)
	P300-5_a	Yes	Setting (35 ºC/30') + HHP (300 MPa/5'/1 cycle)		P300-$3C_a$	Yes	Setting (35 ºC/30') + HHP (300 MPa/15'/3 cycles)
P300-15	P300-15_0	No	Setting (35 ºC/30') + HHP (300 MPa/15'/1 cycle)	P300-5C	P300-$5C_0$	No	Setting (35 ºC/30') + HHP (300 MPa/15'/5 cycles)
	P300-15_a	Yes	Setting (35 ºC/30') + HHP (300 MPa/15'/1 cycle)		P300-$5C_a$	Yes	Setting (35 ºC/30') + HHP (300 MPa/15'/5 cycles)

[*] 1st set addressed the effect of compression time (5 *vs* 15 min) and the 2nd set the effect of replacing a single cycle by multi-cycles of compression (1 cycle of 15 min *vs* 3 cycles of 5 min *vs* 5 cycles of 3 min).
[†] The subscripts 0 and a refer, respectively, to without and with MTGase.

Proximate Composition

Moisture and ash were determined by standard AOAC procedures (AOAC, 1984), whereas crude protein was determined using LECO protein/nitrogen analyser (model FP-528, LECO Corp., St. Joseph, USA) and crude fat determined by Bligh and Dyer's rapid method of total lipid extraction and purification (Bligh and Dyer, 1959).

Texture Measurements

Sausages were cut with a sharp knife in order to produce cylindrical gel samples (25 mm diameter and 25 mm height). Gel samples were left 1 h at room temperature before texture measurements.

Folding test (scoring scale from 1 to 5), gel strength (puncture test using a metal probe with a 5-mm diameter spherical head) and texture profile analysis (TPA using a 50 mm diameter cylindrical probe and a 40 % compression level) were carried out following the methods described in chapter 5.4.1 and TPA was performed using a Instron texturometer (model 4301, USA).

Water Holding Capacity (WHC)

WHC was measured following the method of Sánchez-González et al. (2008) with some modification. One portion of coarsely chopped sample (≈2 g) (W_S), wrapped in two overlayed Whatman No. 1 filter paper (also weighed, W_i), was placed in a 50 ml centrifuge tube and centrifuged at 3,000×g for 10 min at 20 ºC in a Kubota centrifuge (model 6800, Japan). After centrifugation, sample was removed and filter papers were weighed again (W_f). Measurements were in triplicate. WHC is expressed as grams of water in sample after centrifugation per 100 g of water initially present in sample:

$$WHC = \frac{Ws \times (H/100) - (Wf - Wi)}{Ws \times (H/100)} \times 100$$

where,

H: moisture (%).

Colour

L*, a* and b*, chroma and whiteness were determined on a MACBETH colourimeter (model COLOUR-EYE® 3000, USA) as described in chapter 5.4.1.

Protein Solubility

In order to evaluate the extent of protein interactions, protein solubility in four different extracting solutions (SDS, SDS+DTT, Urea and Urea+DTT) was determined (Table 5.2.2.2). About 100 g of sample was chopped and a 600 mg portion was homogenized in 8 mL of each extraction solution for 60 s at a low speed to avoid foaming. Polytron

homogenizer (model PT-MR 3000, Switzerland) equipped with a small rod was used. Then, samples were boiled in a water bath (100 ºC) for 2 min and homogenized, while hot, for 30 s. Finally, samples were centrifuged at 20,000g at 20 ºC for 15 min in a Kubota centrifuge (model 6800, Japan). Protein in the supernatant was measured at 280 nm in triplicate through absorbance (Piñeiro *et al.*, 1999). Protein solubility was expressed as % protein solubilized with respect to total protein in the sample and divided by the protein solubility of S samples (cold set samples with and without MTGase without heating or HHP) for each extraction solution, expressed as relative protein solubility.

Table 5.2.2.2 – Composition of various protein extraction solutions and targeted chemical interactions.

CHARACTERISTICS	Extracting Solution			
	SDS	SDS+DTT	Urea	Urea+DTT
COMPONENTS				
Sodium dodecyl sulphate, SDS (%, w/v)	2	2	---	---
DL-Dithiothreitol, DTT (M)	---	0.1	---	0.1
Urea (M)	---	---	8	8
Tris(hidroxymethyl)-aminomethane, Tris(mM)	60	60	60	60
ACIDITY-ALCALINITY				
pH (adjusted with HCl)	7.5	7.5	7.5	7.5
TARGETED CHEMICAL INTERACTIONS	Non-covalent *	Non-covalent and disulphide bridges	Non-covalent (more efficient in breaking hydrogen bonds)	Predominantly hydrogen bonds and disulphide bridges

* The contrast between SDS and Urea enables to assess importance of the hydrophobic bonds.

Electrophoresis

SDS-PAGE (15% Excel Gel™) electropherograms of the extracted proteins were attained as described in chapter 5.1.5. The protein bands were silver stained with a silver staining kit (PlusOne™, Sweden). As reference molecular weights, two standards (high and low molecular weight) electrophoresis calibration kits (Pharmacia, Sweden) were used. The protein bands were quantified using a densitometer (model GS-800, USA) and appropriate software (Quantity One®, Bio-Rad).

Scanning Electron Microscopy (SEM)

SEM was performed as previously described (5.1.5). Cubes of 2-3 mm were cut from the inner part of the gels for microscopic examination. Samples were fixed with a mixture (1:1, v/v) of 50 $g.l^{-1}$ formaldehyde and 2 $g.l^{-1}$ glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and dehydrated in increasing series of acetone (100-1000 $ml.l^{-1}$). Afterwards, they were critical-point dried with CO_2 as transition fluid in a Polaron critical-point dryer (model 3000, UK) and mounted on metallic holders, followed by gold sputter-coating in a ion sputtering device (model JFC-1100E, Japan). Samples were kept in a dryer until

examination by a scanning microscope (model JSM-5200, Japan) at 20 kV. Micrographs were taken at different magnifications.

Statistical Analysis

Factorial analysis of variance (general linear model, factorial ANOVA, full factorial design) was carried out using a statistical analysis software (STATISTICA©, StatSoft, USA), version 6.1, 2003. This statistical methodology enabled us to analyse effects of MTGase, pressure level, compression time, multi-cycles, and their interactions. The significance of the difference between means was determined using a Tukey HSD test at $p<0.05$.

Results and Discussion

Effect of MTGase

Proximate composition of gels was quite similar, since moisture, protein, fat and ash were near 79 %, 17 %, 1 % and 3 %, respectively. Differences between the proximate compositions of the samples were not significant. Therefore, although MTGase incorporation (0.5 %, w/w) entailed a non-negligible water addition (2.0 %, w/w) in order to maintain a moisture level of 80 %, no significant dilution of the protein content was observed.

Folding test, gel strength, TPA and WHC results are found in Tables 5.2.2.3 and 5.2.2.4. It should be noted that the effect of MTGase can be seen in the rows No and Yes. For a clear evidence of the effect of MTGase, the results presented in these rows are the means of all samples (regardless of the pressure/temperature treatment) without and with, respectively, added MTGase. For each studied effect (MTGase or treatment), means within a column with different letters are significantly different ($p<0.05$).

In both sets of experiments, MTGase had no significant ($p\geq0.05$) effect on the folding test (FT) of the gels. However, MTGase increased significantly ($p<0.05$) gel strength, particularly the breaking force. Moreover, all TPA parameters showed significant ($p<0.05$) textural improvements with MTGase incorporation. Cohesiveness, an important indicator of gel quality, increased from values in the range 0.67-0.68 to 0.79. Concerning WHC, a significant ($p<0.05$) reduction was observed in the 1st set of experiments, but not in the 2nd set. The positive effects of MTGase on the texture of many other fish products, such as hake mince, have been reported (5.1.3). Nonetheless, it is remarkable that addition of 0.5 % (w/w) MTGase to hake minces had a so positive effect on almost all textural properties.

Table 5.2.2.3 – Effect of MTGase, pressure level and compression time (1st set of experiments) on folding test, gel strength, TPA parameters and WHC of the gels*.

MTGase†	Folding test	Gel strength (N.mm)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)	WHC (%)
No	4.5 ± 0.9^{a}	19.3 ± 8.8^{a}	9.7 ± 3.6^{a}	0.68 ± 0.09^{a}	6.4 ± 1.7^{a}	0.80 ± 0.05^{a}	5.2 ± 1.5^{a}	63.2 ± 8.4^{a}
Yes	4.3 ± 0.7^{a}	24.8 ± 7.9^{b}	17.8 ± 5.3^{b}	0.79 ± 0.09^{b}	13.9 ± 3.5^{b}	0.89 ± 0.05^{b}	12.5 ± 3.4^{b}	52.7 ± 5.5^{b}
Treatment								
S	4.8 ± 0.5^{a}	9.9 ± 3.4^{a}	6.8 ± 1.9^{a}	0.65 ± 0.06^{b}	4.5 ± 1.6^{a}	0.73 ± 0.05^{a}	3.4 ± 1.4^{a}	66.2 ± 6.5^{c}
T	2.8 ± 0.5^{b}	30.1 ± 7.5^{e}	22.9 ± 6.2^{c}	0.54 ± 0.06^{a}	12.5 ± 4.6^{b}	0.81 ± 0.04^{b}	10.3 ± 4.2^{b}	45.9 ± 1.3^{a}
P100-5	4.5 ± 0.6^{a}	17.8 ± 7.0^{bc}	13.5 ± 5.5^{b}	0.79 ± 0.08^{cd}	10.9 ± 5.3^{b}	0.87 ± 0.06^{c}	9.8 ± 5.3^{b}	58.8 ± 5.5^{bc}
P100-15	4.5 ± 0.6^{a}	13.7 ± 2.7^{ab}	12.9 ± 5.1^{b}	0.77 ± 0.06^{cd}	10.2 ± 4.7^{b}	0.86 ± 0.06^{c}	8.9 ± 4.6^{b}	58.3 ± 7.6^{bc}
P200-5	5.0 ± 0.0^{a}	24.8 ± 3.3^{de}	13.5 ± 6.0^{b}	0.78 ± 0.06^{cd}	10.7 ± 5.4^{b}	0.86 ± 0.05^{c}	9.5 ± 5.2^{b}	60.9 ± 13.1^{bc}
P200-15	4.5 ± 0.6^{a}	30.8 ± 4.4^{e}	13.6 ± 6.5^{b}	0.81 ± 0.07^{d}	11.3 ± 6.1^{b}	0.89 ± 0.06^{c}	10.3 ± 6.0^{b}	61.5 ± 10.0^{bc}
P300-5	4.8 ± 0.5^{a}	22.4 ± 4.4^{cd}	13.6 ± 4.5^{b}	0.75 ± 0.06^{c}	10.4 ± 4.3^{b}	0.86 ± 0.05^{c}	9.1 ± 4.2^{b}	55.0 ± 3.7^{ab}
P300-15	4.5 ± 0.6^{a}	27.2 ± 6.9^{de}	13.4 ± 3.1^{b}	0.78 ± 0.07^{cd}	10.6 ± 3.3^{b}	0.88 ± 0.05^{c}	9.4 ± 3.4^{b}	56.6 ± 7.4^{bc}

* Presented values correspond to mean ± standard deviation.
† In the rows No and Yes the means of all samples (regardless of the pressure/temperature treatment) without and with added MTGase, respectively, are presented. For each studied effect (MTGase or treatment), means within a column with different letters are significantly different ($p<0.05$).

Table 5.2.2.4 – Effect of MTGase, pressure level and multi-cycles (2nd set of experiments) on folding test, gel strength, TPA parameters and WHC of the gels*.

MTGase†	Folding test	Gel strength (N.mm)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)	WHC (%)
No	4.6 ± 0.9^{a}	11.6 ± 3.8^{a}	6.7 ± 2.8^{a}	0.67 ± 0.06^{a}	4.4 ± 1.4^{a}	0.78 ± 0.03^{a}	3.5 ± 1.0^{a}	64.5 ± 7.6^{a}
Yes	4.8 ± 0.4^{a}	33.9 ± 8.9^{b}	15.7 ± 3.9^{b}	0.79 ± 0.07^{b}	12.3 ± 2.8^{b}	0.88 ± 0.03^{b}	10.9 ± 2.7^{b}	66.5 ± 3.9^{a}
Treatment								
S	4.8 ± 0.5^{a}	14.5 ± 7.0^{a}	9.0 ± 5.7^{ab}	0.75 ± 0.05^{b}	6.9 ± 4.7^{ab}	0.82 ± 0.06^{b}	5.9 ± 4.2^{ab}	67.6 ± 5.1^{b}
T	3.3 ± 1.0^{b}	28.0 ± 15.5^{d}	16.1 ± 4.7^{c}	0.56 ± 0.06^{a}	9.2 ± 3.2^{ab}	0.78 ± 0.03^{a}	7.2 ± 2.8^{ab}	55.8 ± 5.9^{a}
P100-1C	4.8 ± 0.5^{a}	16.5 ± 9.3^{ab}	9.7 ± 4.6^{ab}	0.75 ± 0.08^{b}	7.5 ± 4.2^{ab}	0.82 ± 0.07^{bc}	6.4 ± 4.0^{ab}	69.5 ± 4.1^{b}
P100-3C	5.0 ± 0.0^{a}	20.6 ± 12.5^{bc}	9.1 ± 6.7^{ab}	0.76 ± 0.09^{b}	7.3 ± 5.8^{ab}	0.82 ± 0.09^{bcd}	6.6 ± 5.0^{ab}	66.8 ± 4.4^{b}
P100-5C	5.0 ± 0.0^{a}	18.7 ± 10.1^{ab}	7.7 ± 2.8^{a}	0.74 ± 0.08^{b}	5.8 ± 2.7^{a}	0.81 ± 0.07^{b}	4.9 ± 2.6^{a}	68.8± 4.8^{b}
P300-1C	5.0 ± 0.0^{a}	29.2 ± 15.0^{d}	12.8 ± 6.6^{abc}	0.76 ± 0.09^{b}	10.2 ± 6.1^{b}	0.86 ± 0.07^{e}	9.0 ± 5.9^{b}	64.6 ± 4.5^{ab}
P300-3C	5.0 ± 0.0^{a}	29.0 ± 13.7^{d}	13.4 ± 7.0^{bc}	0.75 ± 0.05^{b}	10.3 ± 6.0^{b}	0.85 ± 0.04^{cde}	9.0 ± 5.5^{b}	65.7 ± 7.2^{ab}
P300-5C	4.8 ± 0.5^{a}	25.2 ± 15.3^{cd}	11.8 ± 5.9^{abc}	0.77 ± 0.07^{b}	9.4 ± 5.4^{ab}	0.85 ± 0.06^{de}	8.3 ± 5.2^{b}	65.3 ± 3.3^{ab}

* Presented values correspond to mean ± standard deviation.
† In the rows No and Yes the means of all samples (regardless of the pressure/temperature treatment) without and with added MTGase, respectively, are presented. For each studied effect (MTGase or treatment), means within a column with different letters are significantly different ($p<0.05$).

MTGase promotes the cross-linking of myosin heavy chains (MHC) during setting since it catalyzes covalent ε-amino-(γ-glutamyl) lysine bonds (Ramírez-Suárez et al., 2001). Therefore, it creates a denser bond network between proteins in the gels. Moreover, a relationship between MHC polymerization and an increase in breaking force or shear stress has also been found in gels from various fish species, such as carp or Pacific whiting (Lee and Park, 1998). Different studies have reported a positive effect of MTGase on WHC or expressible moisture (Ramírez et al., 2007b; 5.1.5), thus displaying an effect

opposite to that observed in the 1st set of experiments. The attained results also show that WHC and breaking force/gel strength do not correlate well, not only for HHP-treated samples but also for S and T samples, another finding opposite to other empirical observations (Rawdkuen et al., 2009). However, in some products no significant effect of MTGase on water-binding properties was observed (Pietrasik and Jarmoluk, 2003). So, it seems that raw material variability may lead to different effects of the MTGase. While in the 1st set, WHC reduction by MTGase was accompanied by a lower gel strength, in the 2nd set the absence of a negative effect of MTGase on WHC resulted in a higher gel strength. So, though there is no clear correlation between WHC and gel strength, a raw material of higher quality with a lesser degree of protein denaturation (2nd set) seems to form more easily a gel network, thus presenting a more clear improvement with MTGase addition.

Concerning colour (Tables 5.2.2.5 and 5.2.2.6), for both sets of experiments, a*, b*, chroma and whiteness significantly increased with MTGase addition. Regarding whiteness, all gels with exception of T samples exhibited a slight increase. Generally, whiteness increases are associated to a higher denaturation of proteins, as it happens with cooking, or to a less orderly net structure (Hwang et al., 2007). Therefore, MTGase activity, given its positive effects on texture, promoting an orderly net micro-structure, should reduce whiteness. However, the opposite was observed, contradicting previous findings of these (5.1.5) and other authors (Ramírez et al., 2007b). Nevertheless, similar observations were made by authors working with gels prepared from white shrimp meat: L* increased with higher amounts (> 0.2 U/g of sample) of MTGase (Tammatinna et al., 2007).

It is also possible that the incorporation of 0.5 % (w/w) (0.5 U/g sample) of MTGase caused the formation of a very finely porous micro-structure (see below), whose smaller pores increased opaqueness, leading to smaller light absorption and, hence, to higher whiteness.

MTGase lowered ($p<0.05$) protein solubility (Tables 5.2.2.5 and 5.2.2.6) in all extractants. Reduced extractability with MTGase can be related to cross-linking of myosin heavy chains (MHC) during setting (Ramírez-Suárez et al., 2001), which generate insoluble polymeric aggregates (Ashie and Lanier, 1999). A large reduction of soluble proteins by MTGase was also observed with other gels prepared from fish (Gómez-Guillén et al., 2005).

Table 5.2.2.5 – Effect of MTGase, pressure level and compression time (1st set of experiments) on colour and relative protein solubility of the gels in the extractants*.

MTGase†	a*	b*	Whiteness	Chroma	Protein Solub. in SDS (%)	Protein Solub. in SDS+DTT (%)	Protein Solub. in Urea (%)	Protein Solub. in Urea+DTT (%)
No	-2.60 ± 0.20^{a}	2.04 ± 1.07^{a}	70.8 ± 2.5^{a}	3.40 ± 0.70^{a}	104.7 ± 10.3^{a}	113.0 ± 6.0^{a}	101.0 ± 12.7^{a}	113.9 ± 10.5^{a}
Yes	-2.38 ± 0.20^{b}	2.50 ± 0.80^{b}	71.7 ± 2.1^{b}	3.51 ± 0.56^{b}	78.7 ± 13.8^{b}	80.0 ± 15.9^{b}	74.3 ± 5.9^{b}	90.2 ± 3.8^{b}
Treatment								
S	-2.54 ± 0.16^{a}	1.23 ± 0.19^{a}	69.0 ± 0.9^{a}	2.83 ± 0.08^{a}	100.0 ± 19.1^{a}	100.0 ± 22.8^{a}	100.0 ± 18.0^{a}	100.0 ± 6.8^{a}
T	-2.15 ± 0.10^{b}	4.44 ± 0.31^{e}	75.7 ± 0.7^{e}	4.94 ± 0.26^{d}	104.2 ± 7.3^{a}	106.5 ± 2.9^{b}	79.4 ± 10.6^{b}	99.2 ± 11.7^{a}
P100-5	-2.55 ± 0.12^{a}	1.87 ± 0.34^{bc}	69.8 ± 1.6^{ab}	3.17 ± 0.15^{b}	83.4 ± 14.5^{b}	90.1 ± 27.9^{c}	90.2 ± 20.0^{c}	108.6 ± 22.8^{b}
P100-15	-2.60 ± 0.14^{a}	2.03 ± 0.36^{bc}	70.6 ± 1.0^{bc}	3.31 ± 0.20^{bc}	---	---	–	–
P200-5	-2.66 ± 0.16^{a}	1.75 ± 0.42^{b}	70.6 ± 0.7^{bc}	3.21 ± 0.12^{b}	---	---	–	–
P200-15	-2.57 ± 0.08^{a}	2.11 ± 0.46^{bcd}	71.2 ± 1.6^{c}	3.35 ± 0.25^{bc}	---	---	–	–
P300-5	-2.32 ± 0.34^{b}	2.23 ± 0.43^{cd}	69.9 ± 1.7^{abc}	3.25 ± 0.18^{b}	---	---	–	–
P300-15	-2.52 ± 0.11^{a}	2.50 ± 0.44^{d}	73.2 ± 1.0^{d}	3.56 ± 0.27^{c}	79.1 ± 16.8^{b}	89.6 ± 19.8^{c}	81.2 ± 11.3^{b}	100.4 ± 13.1^{a}

* Presented values correspond to mean ± standard deviation.
† In the rows No and Yes the means of all samples (regardless of the pressure/temperature treatment) without and with added MTGase, respectively, are presented. For each studied effect (MTGase or treatment), means within a column with different letters are significantly different ($p<0.05$).

Table 5.2.2.6 - Effect of MTGase, pressure level and multi-cycles (2nd set of experiments) on colour and relative protein solubility of the gels in the extractants*.

MTGase†	a*	b*	Whiteness	Chroma	Protein Solub. in SDS (%)	Protein Solub. in SDS+DTT (%)	Protein Solub. in Urea (%)	Protein Solub. in Urea+DTT (%)
No	-2.55 ± 0.19^{a}	1.64 ± 0.94^{a}	70.9 ± 2.3^{a}	3.13 ± 0.57^{a}	123.7 ± 9.2^{a}	108.2 ± 6.9^{a}	103.5 ± 20.8^{a}	98.6 ± 14.8^{a}
Yes	-2.49 ± 0.15^{b}	2.55 ± 0.68^{b}	71.9 ± 1.7^{b}	3.60 ± 0.45^{b}	85.8 ± 7.9^{b}	103.0 ± 7.1^{b}	74.7 ± 5.3^{b}	81.7 ± 17.4^{b}
Treatment								
S	-2.48 ± 0.09^{b}	1.57 ± 0.39^{a}	69.6 ± 1.5^{a}	2.95 ± 0.28^{a}	100.0 ± 24.1^{a}	100.0 ± 6.2^{a}	100.0 ± 24.2^{a}	100.0 ± 10.5^{a}
T	-2.15 ± 0.10^{a}	4.06 ± 0.08^{d}	74.5 ± 1.4^{e}	4.60 ± 0.10^{e}	105.5 ± 10.1^{a}	113.4 ± 7.5^{b}	70.2 ± 2.2^{b}	83.8 ± 6.5^{b}
P100-1C	-2.65 ± 0.11^{c}	1.68 ± 0.65^{ab}	70.1 ± 0.6^{ab}	3.18 ± 0.27^{bc}	---	---	–	–
P100-3C	-2.56 ± 0.05^{bc}	1.66 ± 0.50^{ab}	69.5 ± 0.8^{a}	3.07 ± 0.24^{ab}	---	---	–	–
P100-5C	-2.55 ± 0.11^{bc}	1.70 ± 0.47^{ab}	71.1 ± 1.0^{bc}	3.09 ± 0.25^{ab}	---	---	–	–
P300-1C	-2.64 ± 0.10^{bc}	2.03 ± 0.63^{c}	73.5 ± 0.5^{de}	3.36 ± 0.32^{cd}	---	---	–	–
P300-3C	-2.58 ± 0.11^{bc}	2.12 ± 0.69^{c}	72.3 ± 0.9^{cd}	3.38 ± 0.39^{d}	---	---	–	–
P300-5C	-2.57 ± 0.09^{bc}	1.94 ± 0.76^{bc}	70.8 ± 1.9^{b}	3.28 ± 0.38^{cd}	---	---	–	–

* Presented values correspond to mean ± standard deviation.
† In the rows No and Yes the means of all samples (regardless of the pressure/temperature treatment) without and with added MTGase, respectively, are presented. For each studied effect (MTGase or treatment), means within a column with different letters are significantly different ($p<0.05$).

Electrophoresis results showed similar patterns for the four extractants and for both experimental sets, so only the electropherogram of soluble protein in SDS+DTT (1st set) is presented (Figure 5.2.2.1).

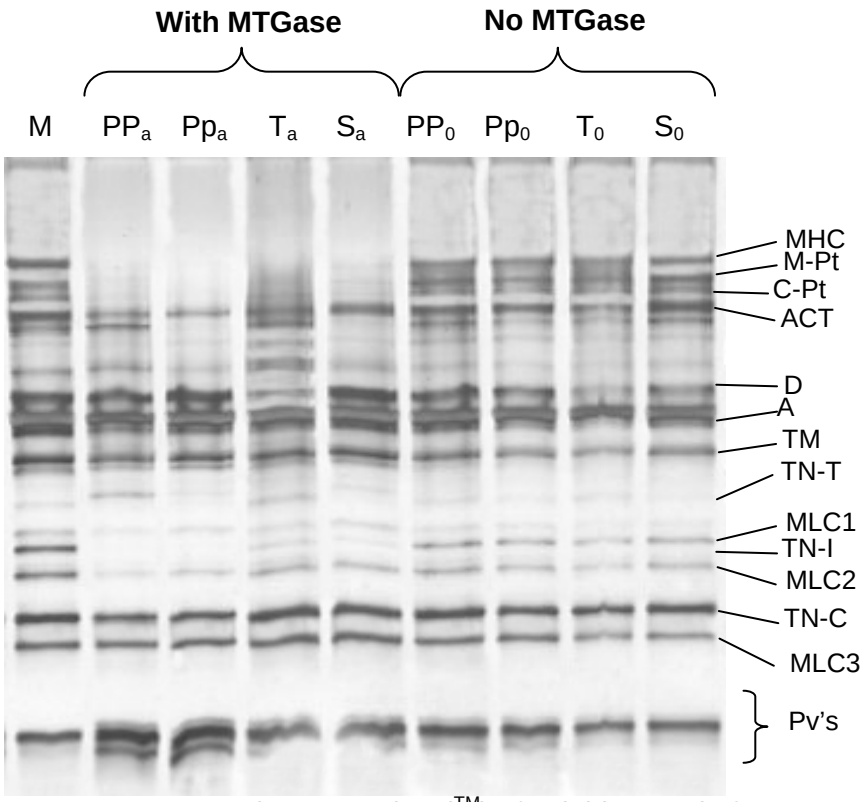

Figure 5.2.2.1 - SDS-PAGE (15% Excel Gel^{TM}) of soluble protein from raw material (M) and produced gels in SDS+DTT. 0: no MTgase; a: with MTGase; S: control (only setting); T: control (heat-induced gels); P: pressure-induced gels; Pp: 100 MPa/5 min/1 cycle (1st set); PP: 300 MPa/15 min/1 cycle (1st set). MHC: myosin heavy chain; M-Pt: M-protein; C-Pt: C-protein; ACT: actinin; D: desmin; A: actin; TM: tropomyosin; MLC1: myosin light chain 1; TNC: troponin-C; MLC3: myosin light chain 3; Pv's: parvalbumins.

MTGase caused the disappearance of HMW protein bands (assigned to MHC and M- and C-proteins). Therefore, electrophoresis showed that mainly heavy proteins were excluded from the soluble protein fraction as a result of aggregation. Moreover, actin was resistant to cross-linking, probably as a result of low exposure of Gln and Lys in its globular conformation (Ramírez-Suárez et al., 2005).

SEM was used to analyze the microstructure of the different gel products (Figures 5.2.2.2-4). MTGase addition promoted the formation of network structure. This was most notorious in S gels. MTGase had no significant effect on the heat-induced gels. The micro-structure of pressure-induced gels became more uniform, with smaller and more evenly distributed pores. Other authors (Tammatinna et al., 2007) also reported a more ordered network structure with MTGase addition. The setting step is crucial for the development of this network (Montero et al., 2005a). Moreover, SEM images (particularly for P gels) may explain the colour effect of MTGase incorporation since smaller pores increase opaqueness (see Figure 5.2.2.4, P300-5$_0$ and P300-5$_a$ for examples).

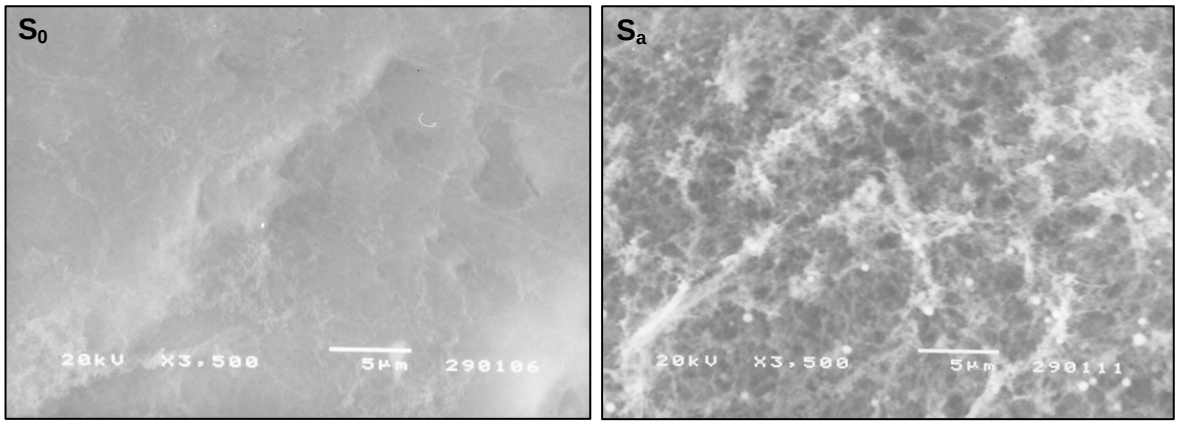

Figure 5.2.2.2 - Scanning electron microscopy (SEM) micrographs of S_0, control (set only) with no MTGase, and S_a, control (set only) with MTGase.

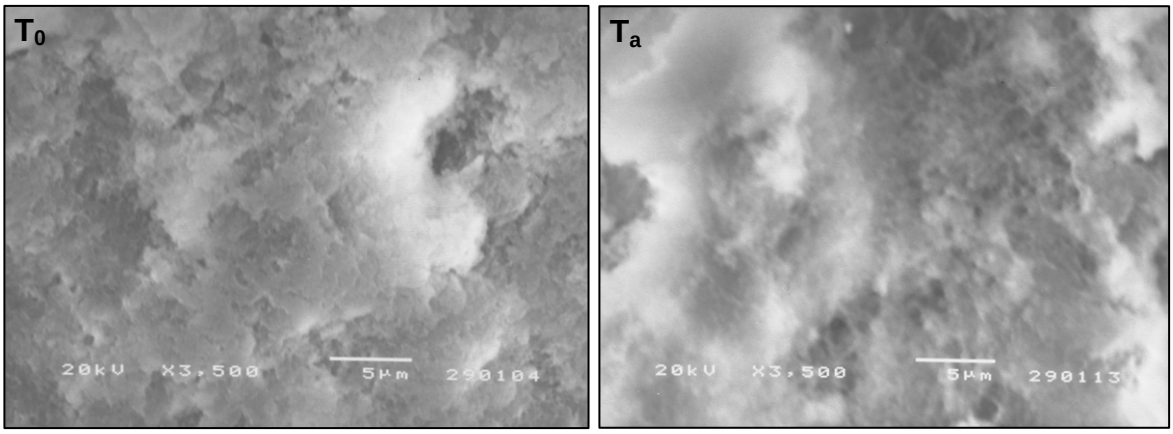

Figure 5.2.2.3 - Scanning electron microscopy (SEM) micrographs of T_0, heat-induced gel with no MTGase and T_a, heat-induced gel with MTGase.

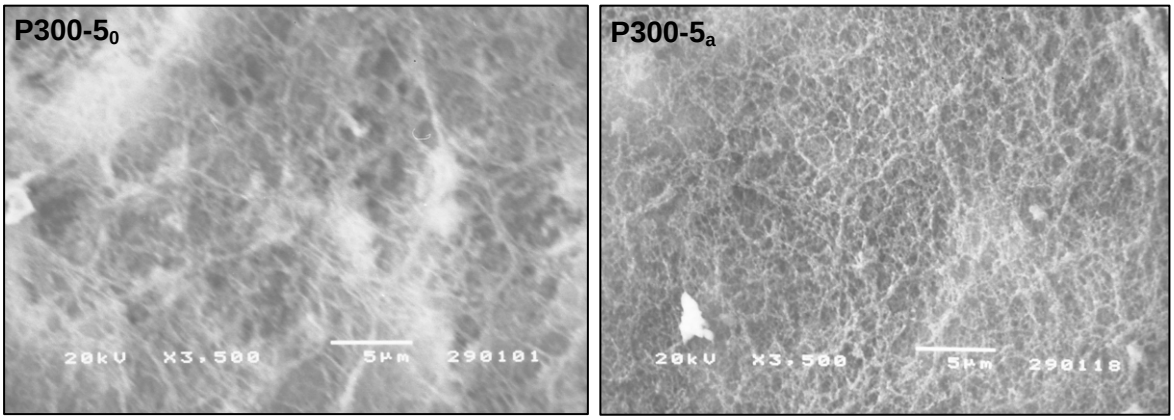

Figure 5.2.2.4 - Scanning electron microscopy (SEM) micrographs of P300-5_0, pressure-induced gel (300 MPa/15 min/1 cycle) with no MTGase and P300-5_a, pressure-induced gel (300 MPa/15 min/1 cycle) with MTGase.

Effect of HHP Treatment

Textural parameters and WHC values are shown in Tables 5.2.2.3 and 5.2.2.4.

The folding test (FT) values of heat-set gels were lower than the cold-set ones. Lowest gel strength (GS) was found in S samples and the highest in T samples, while HHP (with respect to S samples) (up to 200 MPa) increased ($p<0.05$) GS, compression time and

number of compression cycles did not affect GS. The combination of HHP and MTGase had a greater effect than the sum of HHP and MTGase, MTGase increased GS from 6.7 ± 0.2 to 13.0 ± 1.0 N.mm, HHP (100 MPa/5 min/1 cycle) to 12.4 ± 1.5 N.mm and HHP (100 MPa/5 min/1 cycle) + MTGase to 23.2 ± 5.9 N.mm (detailed data not shown in the tables). P gels presented an intermediate hardness between S (softest) and T (hardest) gels. Compression time and multi-cycles had no effect on hardness, only pressure level hardened ($p<0.05$) samples in the 2nd set. Regarding cohesiveness and springiness, heat-induced gels were of poor quality compared to pressure-induced gels. Chewiness (a global parameter that sums up textural quality) was almost identical for T and P samples. However, T samples held less water ($p<0.05$) than P samples. Therefore, application of HHP —even at low pressure level (100 MPa) and for a short time (5 min)— to hake mince yielded positive results, not only with respect to control S, but also to T. This confirms previous findings that HHP may contribute to the improvement of poor functionality proteins (Pérez-Mateos and Montero, 1997). Differently from other works (Cárlez et al., 1995), results show that —with the exception of hardness— heat-induced gels do not necessarily present better physicochemical properties than pressure-induced gels. In fact, the latter ones were more cohesive and springy. This agrees with literature (Moreno et al., 2009), since pressure-induced fish gels have been described as softer, but more elastic than heat-induced gels. These findings suggest that gelation follows different paths depending on temperature and pressure. According to Uresti et al., 2006, HHP application to fish mince at low temperature promotes a kind of protein aggregation characterized by protein-protein interactions with reduced denaturation instead of large conformational changes as with thermal gelation. Compression time and the substitution of multi-cycles for a single cycle of the same duration did not affect properties, higher pressure level brought some textural benefit. However, 100 MPa was able to produce good quality gels, at least, in samples containing MTGase. Results pointed to a synergistic effect of HHP and MTGase (the combined effect HHP+MTGase was greater than the sum of the isolated effects). Some authors oppose this view, since it has been reported that MTGase is pressure sensitive (Shoji et al., 1990). However, later studies have shown that TGase activity was unhindered by pressures between 100 and 300 MPa (Ashie and Lanier, 1999), thus suggesting that also MTGase could be active in this range. This apparent contradiction may be explained by the effect of HHP on proteins. HHP promotes the unfolding of proteins, thereby making them more susceptible to the action of any enzyme still active under the applied pressures (Gilleland et al., 1997). It must be stressed that a high activity or the effects of it correspond to a higher rate of cross-linking formation, whether it is due to a lower degree of denaturation of the enzyme or to a modified conformation of the substrate. Moreover, recent works dealing with giant squid (*Dosidicus*

gigas) (Moreno et al., 2009) or arrowtooth flounder (*Atheresthes stomias*) (Uresti et al., 2006) have also shown that the combination of MTGase and HHP may improve hardness, springiness or cohesiveness of seafood gels. A synergistic effect of added TGase and HHP in a lower pressure level range was also reported by other authors (Gilleland et al., 1997; Ashie and Lanier, 1999). Concerning WHC, the deleterious effect of thermal treatment when compared to HHP treatment seems to reinforce the idea of different action mechanisms for both kinds of gelation induction. Uresti et al. (2004) have also reported a positive effect of HHP on the water binding properties of fish proteins.

HHP had a much smaller impact on the colour of the gels than thermal treatment (Tables 5.2.2.5 and 5.2.2.6). In both experimental sets, T samples presented higher ($p<0.05$) a* (redder hue), b* (more yellow hue), chroma and whiteness than P samples. HHP parameters had also an effect on colour, namely a higher pressure level made gels whiter in both sets, though not reaching the whitish appearance of T gels. Longer compression times also augmented ($p<0.05$) whiteness (70.1 ± 1.4 *vs* 71.7 ± 1.6). For an equal compression time (15 min), replacement of a single compression by 3 or 5 compression cycles produced a slight albeit significant ($p<0.05$) darkening of gels (from 71.8 ± 1.8 to 71.0 ± 1.5). These results show that heat gelation increases opacity thus enhancing light reflexion. This may be related to the extensive muscle protein denaturation (Hwang et al., 2007). For pressure gelation, this phenomenon is less important, as already suggested by WHC results. Nonetheless, there was some opacity increase with HHP. Literature results also point to a higher opacity (Ashie and Lanier, 1999) as a result of HHP application, however these results were obtained at higher pressures. Hence, less intense HHP treatments (lower pressures and/or shorter compression times) are recommended for a raw appearance. Similar conclusions were reached for high pressure treated minced albacore (*Thunnus alalunga*) muscle (Ramírez-Suárez and Morrissey, 2006). Moreover, substituting three consecutive steps for a single cycle seems also beneficial for the maintenance of a raw appearance. Gómez-Estaca et al. (2009) working with fish 'carpaccios' (a thinly sliced product) did not find any differences as a result of applying pressure in one continuous step or in three consecutive cycles.

The effect of different processing conditions on the solubility of gel proteins is shown in Tables 5.2.2.5 and 5.2.2.6. Among P samples, only the extreme treatments (100 MPa/5 min and 300 MPa/15 min) were considered. Regarding T samples, protein solubility declined more sharply in urea than in urea+DTT, when compared with S samples. Pressure-induced gels, besides presenting a similar contrast between solubilities in SDS (about 20 % lower than S gels) and SDS+DTT (only 10 %), also showed with addition of DTT to urea a much larger increase of solubility than S gels:

whereas P proteins were less soluble in urea than S proteins, in urea+DTT, solubility of P proteins was as high (or even higher) as that observed for S proteins. Intensity of the HHP treatment seemed to matter, since P300-15 proteins were less soluble ($p<0.05$) in urea than P100-5 proteins. These results show that both HHP and heating may favor disulphide bonding. Heating left solubilities in SDS and SDS+DTT unaltered. This fact, taken together with the increase of covalent bonds (disulphide bonds) as a result of heating —which tends to decrease protein solubility in SDS—, suggests an increase of non-covalent/hydrophobic interactions in heat-induced gels. For pressure-induced gels, hydrophobic interactions are also important, since solubility values in SDS were also high and protein unfolding also occurs during compression. However, the SDS solubility data show that these interactions had a smaller importance than in heat-induced gels. The hardness of heat-induced gels (Tables 5.2.2.3 and 5.2.2.4) does not contradict this observation, given the findings of some authors, which report that gel hardness may increase with increasing hydrophobic bonding (Havea et al., 2004). A more intense HHP treatment reduced the portion of protein extractable with urea, thus pointing to a greater protein aggregation —not caused by disulphide bonding as solubility had a similar variation with DTT for both studied P samples. The hardening of pressure-induced gels with respect to S samples may be due to a greater role of hydrogen bonding. The urea solubility results may not reflect this variation because only those proteins which interact uniquely through H-bonds are extracted with urea. H-bonding may increase between proteins also aggregated by ionic and covalent bonds, thus contributing to the hardening of gels. Therefore, results point, besides S-S bonding, to an important role of H-bonding in P gels formed at low pressures (100-300 MPa).

Electrophoresis showed that desmin band lost intensity in T gels with respect to raw hake mince (lane M on Figure 5.2.2.1) and the other samples (S and P). S and P samples had similar electrophoretic profiles, suggesting that observed protein solubility differences corresponded to variations occurring with all main hake proteins. This contrasts with MTGase action, which mainly affected HMW proteins. Therefore, it seems that chemical alterations promoted by HHP were not particularly selective. However, other works claimed that HHP processing of horse mackerel (*Trachurus trachurus*) mince caused, under similar conditions (300 MPa/15 min/25 ºC and prior setting), the disappearance of HMW protein bands in the approximate range of 60-200 KDa, using an extracting solution with SDS and mercaptoethanol (Montero et al., 2005). Differences between proteins of different species may account for such different findings.

The micro-structures of the gels revealed a sharp contrast between P and T gels (Figures 5.2.2.2-4). The former, even in the absence of MTGase, exhibited a less dense structure, similar to a network. It has been reported for pressure-induced gels a better

mesh structure (Moreno et al., 2009). On the other hand, heating favors denaturation and large conformational changes in proteins, causing the loss of any previous ordered structure that might have existed, for instance, after setting with MTGase (S_a *vs* T_a images).

Conclusions

MTGase incorporation improved texture, but it did not improve WHC. The protein solubilities in all extractants were lower for those gels containing MTGase as a result of myosin heavy chain cross-linking. Heat processing of hake mince worsened some textural properties, but hardened the gels. Moreover, the protein solubility study revealed the important role of the non-covalent (particularly, hydrophobic) interactions and disulphide bonding. On the other hand, HHP processing improved texture and WHC, which seemed to correlate with an enhancement of disulphide bonding between proteins. Pressure level was the most important operative parameter of HHP. Regarding other parameters, three consecutive steps for a single pressure cycle was beneficial for the maintenance of a raw appearance, thus associating colour changes induced by HHP with the duration of constant pressure phase. Additionally, a synergistic effect of MTGase and HHP was found for some textural properties, such as gel strength. Therefore, HHP combined with MTGase may improve texture of protein gels prepared from hake trimmings having poor functionality.

Acknowledgments

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5.3 - Fish Substitution in Meat Products

5.3.1 - Development of a Healthy Low Fat Fish Sausage Containing Dietary Fibre

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Original article

Development of a healthy low-fat fish sausage containing dietary fibre

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Summary In order to develop a healthy low-fat fish sausage containing dietary fibre (DF), three fundamental changes were made to an ordinary pork sausage recipe: incorporation of 4% (w/w) of Swelite® (a DF obtained from inner peel), different levels of pork meat replacement (0%, 50% and 100%) by hake mince and the combination of varying amounts of Fibraline® (a DF obtained from chicory root) and hake mince (Fibraline: additional hake mince, 2.6:5.2, 5.2:2.6 and 7.8:0.0, % w/w) as a substitute for pork fat. It was found that the addition of Swelite to pork sausage favoured greater gel strength and hardness. On the contrary, increasing levels of pork meat replacement by hake reduced the sausages' gel strength and hardness. Finally, sausages without pork fat showed promising textural and colour parameters. High-Fibraline sausages were less cohesive and chewable than pork fat sausages (control), but also exhibited a greater gel strength. Low-Fibraline ones presented almost all textural properties similar to the control, with exception of hardness and gumminess. Therefore, regarding some key textural parameters, it was possible to produce low-fat fish sausages similar to the ordinary pork sausages.

Keywords Colour parameters, dietary fibre, fish sausage, textural properties, upgrading fish wastes.

Introduction

There is an ever-increasing consumer demand for both vegetarian products and for meat products with reduced fat (Niong *et al.*, 1999; Kühberger *et al.*, 2002). Consumer preference for alternative healthier products is promoting the research and development of different meat systems (Giese, 1996; Cofrades *et al.*, 2000). Furthermore, there is growing consumer interest in the development of meat analogues and fat substitutes using alternative sources of protein (Beggs *et al.*, 1997; Shand, 2000; Yang *et al.*, 2001). Concerning fat, it was found that low-fat Frankfurters containing soy protein or starch had sensory and texture properties similar to the high-fat products (Yang *et al.*, 2001).

The development of restructured fish products and the application of new food ingredients have been used as a way of reaching young and health-conscious consumers, but also as a means to upgrade low-value species and the waste generated by the fish processing industry (Sánchez *et al.*, 2004). Therefore, fish can be an interesting alternative source of protein and, moreover, among the various traditional meat products in the market, sausages are specially suited – given their organoleptic and technological characteristics – for experimenting meat replacement by fish. Regarding fish sausages, with total or partial replacement of live-stock meat, there are various products developed recently; however, most of them neither totally replace pork meat (Morris, 1988) nor mimic the common Frankfurter sausage (Chupeohuk *et al.*, 2001; López-Caballero *et al.*, 2005). Nonetheless, it was found that one sausage formulation with similar quantities of surimi and pork meat could maintain the hardness of commercial sausage and, furthermore, without adversely affecting its flavour, acceptability and consumer preference (Murphy *et al.*, 2004).

On account of its high nutritional value, one of the food ingredients frequently used in the design of functional foods is dietary fibre (DF) (Peuponnet-Pinnia *et al.*, 2002). The importance of DF in nutrition and health is well established (Anderson *et al.*, 1990; Kritchevsky & Bonfield, 1995). Knowledge of the beneficial effects of high-DF diets – namely, regarding prevention of cardiovascular diseases and several types of cancer – has promoted the development of a large and profitable market for products enriched in DF.

Dietary fibre can make up for some ingredients' negative effect on the texture properties and, furthermore,

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Abstract

In order to develop a healthy low fat fish sausage containing dietary fibre, three fundamental changes were made to an ordinary pork sausage recipe: incorporation of 4 % (w/w) of Swelite® (a dietary fibre obtained from inner pea), different levels of pork meat replacement (0, 50 and 100 %) by hake mince and the combination of varying amounts of Fibruline® (a dietary fibre obtained from chicory root) and hake mince (Fibruline®: additional hake mince, 2.6:5.2, 5.2:2.6 and 7.8:0.0 %, w/w) as a substitute for pork fat.

It was found that the addition of Swelite® to pork sausage favoured greater gel strength and hardness. On the other hand, increasing levels of pork meat replacement by hake reduced the sausages gel strength and hardness. Finally, sausages without pork fat showed promising textural and colour parameters. High Fibruline® sausages were less cohesive and chewable than pork fat sausages (control) but also exhibited greater gel strength. Low Fibruline® ones presented almost all textural properties similar to the control, with exception of hardness and gumminess. Therefore, regarding some key textural parameters, it was possible to produce low fat fish sausages similar to the ordinary pork sausages.

Keywords: Fish sausage, dietary fibre, textural properties, colour parameters, upgrading fish wastes

Introduction

There is an ever-increasing consumer demand for both vegetarian products and for meat products with reduced fat (Xiong et al., 1999; Kubberød et al., 2002). Consumer preference for alternative healthier products is promoting the research and development of different meat systems (Giese, 1996; Cofrades et al., 2000). Furthermore, there is growing consumer interest in the development of meat analogues and fat substitutes using alternative sources of protein (Beggs et al., 1997; Shand, 2000; Yang et al., 2001). Concerning fat, it was found that low fat Frankfurters containing soy protein or starch had similar sensory and texture properties to the high fat products (Yang et al., 2001).

The development of restructured fish products and the application of new food ingredients have been used as a way of reaching young and health-conscious consumers, but also as a means to upgrade low-value species and the waste generated by the fish processing industry (Sánchez et al., 2004). Therefore, fish can be an interesting alternative source of protein and, moreover, among the various traditional meat products in the market, sausages are specially suited —given their organoleptic and technological characteristics— for experimenting meat replacement by fish. Regarding fish sausages,

with total or partial replacement of livestock meat, there are various products developed recently, however most of them neither totally replace pork meat (Morris, 1988) nor mimic the common Frankfurter sausage (Chuapoehuk et al., 2001, López-Caballero et al., 2005). Nonetheless, it was found that one sausages formulation with similar quantities of surimi and pork meat could maintain the hardness of commercial sausage and, furthermore, without adversely affecting its flavour, acceptability and consumer preference (Murphy et al., 2004).

On account of its high nutritional value, one of the food ingredients frequently used in the design of functional foods is dietary fibre, DF (Puupponen-Pimïa et al., 2002). The importance of DF in nutrition and health is well established (Anderson et al., 1990; Kritchevsky and Bonfield, 1995). Knowledge of the beneficial effects of high dietary fibre diets —namely, regarding prevention of cardiovascular diseases and several types of cancer— has promoted the development of a large and profitable market for products enriched in DF.

DF can make up for some ingredients' negative effect on the texture properties and, furthermore, to improve them. For instance, regarding low fat chicken sausages, guar and xanthan gums were successfully used (Andrés et al., 2006). It should be mentioned that there are hardly any references on fish products with added DF. Nonetheless, some fibres such as carrageenans (Ortiz and Aguilera, 2004) or also xanthan and guar gums (Montero et al., 2000) have been used for technological purposes in fish products.

For our study two fibres were selected due to their favourable effect on the textural properties of the final products (data not published): inner pea fibre and chicory root inulin. The incorporation of the former fibre in a product can improve its textural aspects, specifically, enhancing hardness, smoothiness and water and fat binding capacity (Anonymous, 2005a). The chicory root inulin can function as a fat mimetic component, ensuring smoothiness, creaminess and an oily mouth-feel. Moreover, inulin is effectively a low caloric additive since it is not digested in the upper intestinal tract and, as such, the only energy provided by this fibre is due to bacterial fermentation in the large intestine (Anonymous, 2005b).

The authors' aim was to measure the colourimetric and textural properties of a low fat Frankfurter fish sausage (with complete replacement of pork meat by hake mince and without added pork fat) in order to assess its similarity to ordinary pork sausages.

Materials and Methods

Raw materials and additives

Frozen South African hake (*Merluccius capensis*) were bought already headed and gutted from a local frozen fish processor. Each fish batch was kept frozen at –28 ºC and processed within three to four weeks after its arrival at the laboratory.

Pork meat and fat were bought from a local supermarket and stored in a refrigerator no more than two days until processing. Traditional pork meat sausages (to be used as a reference) containing pork fat were also bought from a local supermarket and stored in a refrigerator until analysis.

Regarding dietary fibre (DF) products, two were chosen for their favourable effect on the textural properties of the final products (data not published): inner pea fibre (Swelite®) and chicory root inulin (Fibruline INSTANT), both supplied by Cosucra, S.A. (Warcoing, Belgium). Their particular composition (based on dry matter, D.M.) as well other properties are presented in Table 5.3.1.1.

Table 5.3.1.1 – Relevant properties of the used dietary fibre products.

PROPERTIES	Swelite®*	Fibruline INSTANT†
Composition (D.M.)		
Total Carbohydrates (%)	93 ± 3	min. 99.7
Of which: *Total DF (%)*	48 ± 3	min. 90
Starch (%)	min. 36	—
Protein (%)	max. 7	—
Fat (%)	max. 0.5	—
Ash (%)	max. 2	max. 0.3
Granulometry (µm)	< 400	< 700
Colour	white	white
Taste	neutral	neutral to slightly sweet

* The presented information regarding Swelite® comes from Anonymous (2005a).
† The presented information regarding Fibruline INSTANT comes from Anonymous (2005c).

The other components were all food grade materials manufactured by different companies: potato starch from Emsland-Stärke GmbH (Emlichheim, Germany); TARIPROT® 1010, emulsifying soy protein from BK Giulini (Ladenburg, Germany); SOLCON/MAICON 70, soy protein concentrate powder from Solbar Hatzor Ltd. (Ashdod, Israel); Dextropam 100, dextrose from Copam, S.A. (Loures, Portugal); TARI® K7, Di-, Tri- and Polyphosphates from BK Giulini (Ladenburg, Germany); VATEL® Salt, common salt from VATEL (Alverca, Portugal); Palatinata Cure, curing salt from BK Giulini (Ladenburg, Germany); TARIMIX® Frankfurt, sausage seasoning from BK Giulini (Ladenburg, Germany); TAROMA® Smoke, smoke aroma from BK Giulini (Ladenburg, Germany).

Experimental design

Three experiments were conducted with the purpose of studying three different effects.

In the first trial, the mean textural and colour properties of pork sausages with 4 % (w/w) Swelite or without this fibre were determined. Swelite was chosen since previous trials with this dietary fibre had produced very positive results. Afterwards, in a second trial, the mean textural and colour properties of sausages —all containing Swelite, 4 % (w/w)— with different levels of pork meat replacement by hake —encompassing three levels, 0, 50 and 100 %— were measured. Finally, a third experiment was carried out in order to assess the textural and colourimetric effect on fish sausages of substituting different combinations of Fibruline and additional hake mince to compensate the pork fat removal (see Table 5.3.1.2). Error assessment was derived from replication of the various analyses performed.

Table 5.3.1.2 – Ingredient proportions changed in the third experiment.

No Pork Meat Fish Sausage	INGREDIENTS		
	Pork fat (%, w/w)	Fibruline (%, w/w)	Additional hake mince (%, w/w)
Control	7.8	0	0
No Pork Fat	0	2.6	5.2
	0	5.2	2.6
	0	7.8	0

Production of sausages

Frozen hake were thawed overnight in a refrigerator, for the second and third trials, respectively. Afterwards, skin and bones were manually removed. The resulting fish flesh was minced once in a model 84145 meat grinder (Hobart, Troy, OH, USA), equipped with 2 cm grind blades and a metallic screen with 6 mm diameter circular holes.

The appropriate quantities of the various ingredients were weighed in order to produce 2 kg (Table 5.3.1.3).

Table 5.3.1.3 – Sausage recipes used in the different trials.

INGREDIENTS (%)	1st Trial Swelite Incorporation		2nd Trial Pork Meat Replacement (%)			3rd Trial Fibruline Level (%, w/w)			
	No	Yes	0	50	100	0	2.6	5.2	7.8
Pork meat	56.4	54.1	54.1	27.1	0.0	0.0	0.0	0.0	0.0
Hake mince	0.0	0.0	0.0	27.1	54.1	54.1	59.3	56.7	54.1
Water/Ice	26.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Pork fat	8.1	7.8	7.8	7.8	7.8	7.8	0.0	0.0	0.0
Fibruline	0.0	0.0	0.0	0.0	0.0	0.0	2.6	5.2	7.8
Swelite	0.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Potato starch	3.2	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1
Salt	2.4	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Emulsifying protein	1.3	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Soy protein	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Other	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Total (%)	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*

Regarding the preparation of the sausage batters, five sequential steps were always followed. Firstly, pork meat and/or hake mince was mixed with salt and phosphates for 1 minute at 1420 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Throughout all process, mixing was performed always under vacuum and refrigeration (temperature below 7 ºC). In a second step, ice (70 % of the total amount of ice and water), dextrose and curing salt were added and there was additional mixing for 1 minute at the same speed. Afterwards, the emulsifying protein and the soy protein concentrate were also added and further mixing at the same speed took place for 1 minute. Thereafter, pork fat or Fibruline was added and mixed for 1 minute at 2800 rpm. Meanwhile, Swelite was hydrated before mixing it with the other ingredients (chilled water was added to this dietary fibre in a ratio near 2:1). The fifth and last step involved the addition of the remaining ingredients, potato starch, the hydrated fibre (not added in the case of pork sausage without Swelite), sausage seasoning and smoke aroma and, moreover, mixing all for 2 minutes at 2800 rpm.

The batters attained were put inside a model EB-12 hydraulic filler (Mainca Equipamientos Carnicos, S.L., Granollers, Spain) and encased under pressure into cellulose sausage casings mounted over the end of the stuffing horn. Afterwards, these cellulose casings were twisted and tied, thereby, shaping sausages with a diameter of 25 mm and a length of about 20 cm. In the next step, sausages were moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and subjected to a steam cooking at 75 ºC for 15 minutes (cooking). Immediately after, sausages were taken from the oven and cooled with a mixture of water and ice (1:1, v/v). The cellulose casings were removed, sausages separated from one another and vacuum-packed in plastic bags with a model A300/52 vacuum packager (Multivac Sepp Haggenmüller GmbH & Co. KG, Wolfertschwenden, Germany). Following this operation, sausages were put in the same oven and subjected to a steam cooking at 85 ºC for 5 minutes (pasteurisation). Once again, they were immediately cooled in iced water. Afterwards, sausages were kept in a refrigerator overnight until further analysis.

Texture measurements

Folding test. The test piece was a 3 mm slice cut from the sausages middle portion. The evaluation was performed in accordance with a 5-point grade system as follows. Grade 5, no crack when folded into quadrants; Grade 4, no cracks when folded in half; Grade 3, crack develops gradually when folded in half; Grade 2, crack develops immediately when folded in half; Grade 1, crumbles when pressed by finger.

Puncture test. Prior to analysis, sausages were tempered to about 20 ℃ and cut into pieces of 25 mm diameter and 25 mm high. Each sample was penetrated to the breaking point with a metal probe equipped with a 5 mm diameter spherical head, using a model Instron 4301 texturometer (Instron Engineering Corp., Canton, MA, USA). The cross speed head was 10 mm/min and the load cell was 1000 N. Breaking force (N) and breaking deformation (mm) were measured. Gel strength (N.mm) was determined by multiplying these two parameters.

Compression tests. For the texture profile analysis, samples (diameter, 25 mm and height, 25 mm) were compressed on the flat plate of the Instron texturometer with a cylindrical plunger (50 mm diameter) adapted to a 1000 N load cell at a deformation rate of 50 mm/min. On the basis of preliminary trials to establish a compression limit that would ensure no cracking and recoverability of most samples, it was decided to compress samples to 60 % of their height. In the test, each sample was compressed twice. The following parameters were determined: hardness (N), maximum height of first peak on first compression (in terms of eating quality, food's resistance at first bite); cohesiveness (A_2/A_1), ratio of second-compression to first-compression positive areas (maintenance of food resistance during chew down); gumminess (N), product of hardness and cohesiveness (strength required in the chew down process); springiness (L_2/L_1), ratio of the detected height of the product on the second compression to the original compression distance (ability of food to reacquire its initial shape and size after a first bite); chewiness (N), product of gumminess and springiness (albeit expressed in N, a measure of the energy spent in the chew down process).

For the compression-relaxation test, the compression procedure was as for the texture profile analysis except that the sample was compressed only once for one minute and the force exerted on the sample was recorded. Relaxation (%) was calculated as $Y_T=100\times(F_0-F_1)/F_0$, where F_0 is the force registered at the onset of relaxation immediately after sample compression and F_1 is the force registered after one minute of relaxation. Thus, $(100-Y_T)$ is taken as an index of elasticity and is expressed as the percentage elasticity of the gel.

Colour measurements

After two days in chilled storage, sausages were cut and put into Petri dishes, covering the entire bottom. A model MACBETH COLOUR-EYE® 3000 colourimeter (Macbeth, New Windsor, NY, USA) was used and, prior to measurements, standardized to a specific

colour blank (CIELAB system: L*, 92.4; a*, -1.0; b*, 1.5). The attained values for L*, a* and b* of the CIELAB system were always the means of ten measurements on each Petri dish. Furthermore, for a better assessment of colour, the three mentioned coordinates were combined in order to obtain the chroma and whiteness values:

$$Chroma = \sqrt{a^{*2} + b^{*2}}$$

$$Whiteness = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

Statistical analysis

Colour and folding test determinations were performed in duplicate, the gel strength results are the average of six measurements and all other determinations were done in triplicate. A general linear model —one-way ANOVA— was used to determine significant differences ($p<0.05$) among sausages with different levels of Swelite, different degrees of pork meat replacement and, also, with diverse combinations of Fibruline and additional hake mince as substitutes for pork fat. Multiple comparisons were done by the Tukey HSD test. All statistical treatment was done with the software STATISTICA© from StatSoft, Inc. (Tulsa, OK, USA), version 6.1, 2003.

Results and Discussion

Effect of Swelite addition

Texture Evaluation. In a first trial, the consequences of 4 % (w/w) Swelite addition to pork sausages were analysed, namely, the effect on eight distinct textural parameters (folding test, gel strength, elasticity, hardness, cohesiveness, gumminess, springiness and chewiness) (Table 5.3.1.4).

Table 5.3.1.4 – Mean textural properties of pork sausages with and without Swelite*.

SWELITE LEVEL (% w/w)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
0	3.7 ± 0.6^a	21.3 ± 4.8^a	59.8 ± 0.4^a	56.7 ± 7.1^a	0.53 ± 0.02^a	32.3 ± 2.7^a	0.78 ± 0.01^a	25.0 ± 2.1^a
4	3.7 ± 0.6^a	32.5 ± 5.8^b	59.7 ± 1.2^a	89.2 ± 15.3^b	0.53 ± 0.03^a	49.2 ± 5.8^b	0.80 ± 0.01^a	39.2 ± 4.1^b

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

The presence of Swelite did not change significantly ($p \geq 0.05$) the folding test (FT), elasticity, cohesiveness and springiness of pork sausages. However, Swelite addition significantly ($p<0.05$) increased hardness (the gumminess and chewiness increases were

fundamentally a result of a greater hardness), 89.2 ± 15.3 N *vs* 56.7 ± 7.1 N and gel strength from 21.3 ± 4.8 N.mm to 32.5 ± 5.8 N.mm.

The results discussed above show that there is advantage on including Swelite in typical sausage recipes. It is worth mentioning that, besides dietary fibre, Swelite contains a substantial amount of pea starch (Table 5.3.1.1), thus, entailing an additional starch amount —besides *circa* 3 % (w/w) potato starch present in all sausages (Table 5.3.1.3)— in the final product between 1 and 2 % (w/w). Several studies involving addition of different kinds of starch to sausages have revealed, for instance, that products containing modified waxy maize starch had textural properties similar to the traditional high fat sausages (Yang et al., 2001) or that dry-addition of tapioca starch (up to 3 %) alone had a positive effect on the textural quality of low fat pork sausages (Lyons et al., 1999). Starch, in general, has the ability to improve gel-forming capacity by acting as a simple filler of the protein structure (Lee et al., 1992). Therefore, Swelite seems to be a balanced additive, ensuring an increased dietary fibre intake and better textural characteristics.

Colour Evaluation. The effect of Swelite on the colour of pork sausages was also analysed (Table 5.3.1.5).

Table 5.3.1.5 – Mean colour parameters of pork sausages with and without Swelite[*].

SWELITE LEVEL (% w/w)	a*	b*	Chroma	Whiteness
0	1.21 ± 0.14^{a}	6.83 ± 0.23^{a}	6.94 ± 0.20^{a}	75.5 ± 0.4^{a}
4	1.85 ± 0.15^{b}	6.34 ± 0.49^{a}	6.61 ± 0.51^{a}	73.1 ± 0.8^{a}

[*] Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Concerning Chroma, Whiteness and b* values, there was no statistically significant difference ($p\geq0.05$). However, Swelite presence increased a* value significantly ($p<0.05$) from 1.21 ± 0.14 to 1.85 ± 0.15, meaning a redder sausage. Therefore, Swelite did not bring about any undesirable colour for a common pork sausage.

Effect of pork meat replacement

Texture Evaluation. A second trial was conducted in order to study the influence of three levels of pork meat replacement by hake mince (0, 50 and 100 %) on the textural properties of sausages containing 4 % (w/w) Swelite. The results attained from the determination of these properties are shown in Table 5.3.1.6.

Table 5.3.1.6 – Mean textural properties of sausages containing 4 % (w/w) Swelite and presenting various levels of pork meat replacement by hake mince*.

PORK MEAT REPLACEMENT (%)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
0	3.7 ± 0.6^{a}	32.5 ± 5.8^{a}	59.7 ± 1.2^{a}	89.2 ± 15.3^{a}	0.53 ± 0.03^{a}	49.2 ± 5.8^{a}	0.80 ± 0.01^{a}	39.2 ± 4.1^{a}
50	3.0 ± 0.0^{a}	17.6 ± 0.8^{b}	55.1 ± 3.1^{a}	50.5 ± 4.0^{b}	0.52 ± 0.05^{a}	26.3 ± 2.0^{b}	0.78 ± 0.02^{a}	20.7 ± 2.1^{b}
100	3.0 ± 0.0^{a}	17.7 ± 2.2^{b}	55.9 ± 1.2^{a}	29.0 ± 2.6^{c}	0.54 ± 0.01^{a}	16.2 ± 0.2^{c}	0.79 ± 0.01^{a}	12.8 ± 0.3^{c}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

FT, elasticity, cohesiveness and springiness did not present any significant difference ($p\geq0.05$). However, the gel strength showed a significant ($p<0.05$) reduction from the pork sausage (32.5 ± 5.8 N.mm) to the 50-50 pork-hake sausage (17.6 ± 0.8 N.mm), but a total pork meat replacement did not produce any further reduction. Hardness values exhibited a significant ($p<0.05$) decreasing trend with increasing pork meat replacement by hake mince, from 89.2 ± 15.3 N to 50.5 ± 4.0 N and, with further replacement, to 29.0 ± 2.6 N. Gumminess and chewiness presented very similar variations.

The results highlighted two effects of replacing pork meat by hake mince: loss of gel strength and softening. Analysing the components of gel strength (see Material and Methods, puncture test), gel strength reduction was mainly an effect of a weaker opposition to probe penetration, that is, a lower breaking force (3.4 ± 0.3 N *vs* 5.8 ± 0.4 N). This agrees well with the hardness variation. Moreover, these results agree with other studies where the replacement of meat muscle resulted in a marked decrease in product hardness (Cofrades et al., 2000; Murphy et al., 2004). One main reason for this is the total collagen content in fish, which is lower than that found in terrestrial animals (Lluch et al., 2001). Another reason could be the observed moisture increase with growing level of pork meat replacement, from 60.5 ± 0.1 % to 71.5 ± 0.7 %. This is a consequence of the very high moisture content in hake mince. Nevertheless, a group of trained panellists (data not published) scored as acceptable the fish sausages, including its hardness. Furthermore, if needed, texture could be improved through modifications in the sausages formulation (Murphy et al., 2004), for instance, reducing water and ice addition.

Colour Evaluation. With the purpose of analysing the influence of pork meat replacement upon colour, a*, b*, Chroma and Whiteness of sausages containing 4 % (w/w) Swelite were determined (Table 5.3.1.7).

Table 5.3.1.7 – Mean colour parameters of sausages containing 4 % (w/w) Sw elite and presenting various levels of pork meat replacement by hake mince*.

PORK MEAT REPLACEMENT (%)	a*	b*	Chroma	Whiteness
0	1.85 ± 0.15^{a}	6.34 ± 0.49^{a}	6.61 ± 0.51^{a}	73.1 ± 0.8^{a}
50	0.47 ± 0.09^{b}	6.44 ± 0.30^{a}	6.46 ± 0.31^{a}	76.4 ± 2.2^{a}
100	-0.60 ± 0.07^{c}	5.42 ± 0.17^{a}	5.45 ± 0.16^{a}	76.1 ± 0.1^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Only the parameter a* showed a significant ($p<0.05$) downward trend with increasing levels of pork meat replacement (1.85 ± 0.15, 0.47 ± 0.09 and –0.60 ± 0.07). This trend towards whiter sausages is a consequence of the low heme pigment content in hake's light muscle. This pigment associated to myoglobin is very important for the product's colour since the heme iron reacts with nitric oxide present in the curing salt (Material and Methods), producing nitric oxide myoglobin and, after heating, nitrosylo-hemochrome, a stable pink pigment (Kijowski, 2001; Shahidi, 1991).

Effect of pork fat replacement

Texture Evaluation. The effect of replacing pork fat by Fibruline (7.8 %, w/w) or, alternatively, by combinations of Fibruline and extra hake mince (2.6 %:5.2 % or 5.2 %:2.6 %, w/w) upon the textural properties of fish sausages containing 4 % (w/w) Swelite was studied (Table 5.3.1.8).

Table 5.3.1.8 – Mean textural properties of fish sausages containing 4 % (w/w) Swelite and presenting various combinations of Fibruline (Fib) and additional hake mince (HM)*.

FIB/HM (% w/w)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
0.0/0.0	3.0 ± 0.0^{a}	17.2 ± 1.8^{a}	55.5 ± 1.2^{a}	41.1 ± 2.9^{a}	0.52 ± 0.01^{a}	20.7 ± 1.5^{a}	0.79 ± 0.02^{a}	16.4 ± 1.6^{a}
2.6/5.2	3.3 ± 0.6^{a}	19.9 ± 2.6^{a}	54.4 ± 0.3^{ab}	34.2 ± 1.5^{b}	0.51 ± 0.00^{a}	17.9 ± 0.6^{b}	0.78 ± 0.00^{a}	14.0 ± 0.5^{ab}
5.2/2.6	3.0 ± 0.0^{a}	18.5 ± 0.6^{a}	54.9 ± 0.5^{ab}	37.0 ± 1.4^{ab}	0.47 ± 0.01^{b}	17.3 ± 0.9^{b}	0.73 ± 0.01^{b}	12.7 ± 0.7^{bc}
7.8/0.0	2.3 ± 0.6^{a}	24.4 ± 1.5^{b}	51.5 ± 1.3^{b}	38.1 ± 3.3^{ab}	0.44 ± 0.02^{c}	16.2 ± 1.1^{b}	0.69 ± 0.01^{c}	11.2 ± 0.6^{c}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Growing levels of Fibruline significantly reduced ($p<0.05$) cohesiveness (0.52 ± 0.01 *vs* 0.44 ± 0.02), springiness (0.79 ± 0.02 *vs* 0.69 ± 0.01) and chewiness (16.4 ± 1.6 N *vs* 11.2 ± 0.6 N), whereas the gel strength for sausages richest in Fibruline (7.8 %) was significantly higher ($p\leq0.05$) than for any other sausage. Furthermore, the highest Fibruline content caused a significant elasticity decrease ($p<0.05$) from 55.5 ± 1.2 % (pork fat sausage) to 51.5 ± 1.3 %. On the other hand, low Fibruline sausages (2.6 % Fibruline and 5.2 % extra hake mince) were significantly softer ($p<0.05$) than pork fat ones (41.1 ±

2.9 N *vs* 34.2 ± 1.5 N), while their other textural properties were similar to the control sausages with pork fat.

Once more, an analysis of the gel strength components showed that breaking force increase (from 2.9 ± 0.1 N to 3.9 ± 0.2 N) was the main cause of the gel strength variation between pork fat and high Fibruline sausages. Results have shown that high Fibruline sausages albeit harder were of poorer textural quality since they have lost elasticity, cohesiveness, springiness and chewiness with regard to the control and to the other sausages. The low Fibruline (2.6 %) sausages albeit softer revealed a texture profile quite similar to control product. Therefore, an extra amount of hake protein is a better substitute of pork fat. Nevertheless, Fibruline incorporation is important in order to avoid a dry mouth-feel. Trained panellists scored as acceptable the low Fibruline fish sausages and found a mouth-feel similar to the control sausages (data not published). Furthermore, a comparison with typical commercial sausages (Table 5.3.1.9) revealed that the differences between 2.6 % (w/w) Fibruline sausages and the commercial ones albeit significant ($p<0.05$) were not very large. Moreover, experimental sausages presented a FT score and gel strength similar or superior to the commercial product. While other authors have successfully reduced fat content with isolated muscle protein (Yang et al., 2001), this study has shown that it seems possible to produce a fish sausage without pork meat and pork fat texturally resembling a commercial sausage.

Table 5.3.1.9 – Mean textural properties of fish sausages containing 4 % (w/w) Swelite and 2.6 % (w/w) Fibruline and of commercial sausages[*].

SAUSAGE	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
Experimental	3.3 ± 0.6[a]	19.9 ± 2.6[a]	54.4 ± 0.3[a]	34.2 ± 1.5[a]	0.51 ± 0.00[a]	17.9 ± 0.6[a]	0.78 ± 0.00[a]	14.0 ± 0.5[a]
Commercial	3.3 ± 0.6[a]	14.2 ± 4.5[b]	64.1 ± 1.0[b]	45.7 ± 4.5[b]	0.60 ± 0.02[b]	26.7 ± 3.0[b]	0.82 ± 0.02[b]	22.0 ± 2.4[b]

[*] Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Colour Evaluation. Regarding pork fat replacement, the colour parameters a*, b*, Chroma and Whiteness of fish sausages containing 4 % (w/w) Swelite were also measured (Table 5.3.1.10).

The attained results defined two groups of sausages, the pork fat ones, which were significantly ($p\leq0.05$) more yellow —given their higher b*— and clearer —as shown by the Whiteness parameter—, and the no pork fat ones, among which there was a significant a* increase ($p<0.05$) as a result of higher Fibruline content, from -0.15 ± 0.04 to 0.29 ± 0.01, thus making high Fibruline (7.8 %) sausages slightly red like the pork fat ones.

Table 5.3.1.10 – Mean colour parameters of fish sausages containing 4 % (w/w) Swelite and presenting various combinations of Fibruline and additional hake mince*.

Fibruline (% w/w)	Additional Hake Mince (% w/w)	a*	b*	Chroma	Whiteness
0	0	0.24 ± 0.17^{a}	6.72 ± 0.18^{a}	6.72 ± 0.19^{a}	78.7 ± 0.2^{a}
2.6	5.2	-0.15 ± 0.04^{b}	5.47 ± 0.02^{b}	5.47 ± 0.02^{b}	75.7 ± 0.5^{b}
5.2	2.6	-0.04 ± 0.06^{ab}	5.90 ± 0.01^{b}	5.90 ± 0.01^{b}	77.6 ± 0.3^{ab}
7.8	0	0.29 ± 0.01^{a}	5.74 ± 0.18^{b}	5.75 ± 0.18^{b}	75.9 ± 1.1^{b}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

The low heme pigment content in hake's light muscle and the higher amounts of hake in low Fibruline sausages explain the observed a* tendency.

Conclusions

It was found that the addition of Swelite to pork sausage favoured better textural properties and did not alter the overall colour. On the other hand, increasing levels of pork replacement by hake reduced the sausages gel strength and hardness. However, regarding no pork meat fish sausage, the values attained for these properties were deemed acceptable by a sensory panel albeit texturally inferior. Concerning colour, growing amounts of hake produced less red sausages. Finally, sausages without pork fat presented promising textural and colour parameters —high Fibruline sausages were less cohesive and chewable than pork fat sausages (control) but also presented a greater gel strength, on the other hand, low Fibruline ones (in which pork fat was largely replaced by extra hake mince) were softer than pork fat sausages, but had all other textural properties similar to the control.

On the whole, it was shown that is possible to produce a texturally acceptable white sausage (in which textural properties were most similar to high fat control) combining multiple health promoting factors, namely, higher dietary fibre intake, lower fat intake and fish nutrients, that is, a healthy low fat fish sausage containing dietary fibre.

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5.4 - Storage Stability Study

5.4.1 – Quality Changes during Storage of Fish Sausages Containing Dietary Fibre

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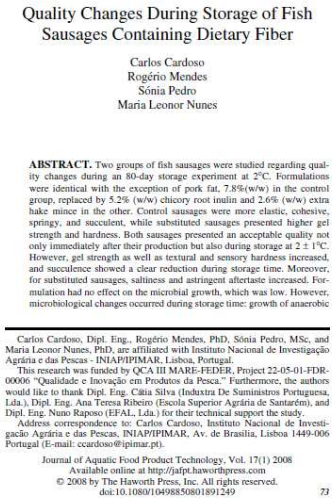

Quality Changes During Storage of Fish Sausages Containing Dietary Fiber

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ABSTRACT. Two groups of fish sausages were studied regarding quality changes during an 80-day storage experiment at 2°C. Formulations were identical with the exception of pork fat, 7.8%(w/w) in the control group, replaced by 5.2% (w/w) chicory root inulin and 2.6% (w/w) extra hake mince in the other. Control sausages were more elastic, cohesive, springy, and succulent, while substituted sausages presented higher gel strength and hardness. Both sausages presented an acceptable quality not only immediately after their production but also during storage at 2 ± 1°C. However, gel strength as well as textural and sensory hardness increased, and succulence showed a clear reduction during storage time. Moreover, for substituted sausages, saltiness and astringent aftertaste increased. Formulation had no effect on the microbial growth, which was low. However, microbiological changes occurred during storage time: growth of anaerobic

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Abstract

Two groups of fish sausages were studied regarding quality changes during an 80-day storage experiment at 2 ºC. Formulations were identical with exception of pork fat, 7.8 % (w/w) in the control group, replaced by 5.2 % (w/w) chicory root inulin and 2.6 % (w/w) extra hake mince in the other. Control sausages were more elastic, cohesive, springy and succulent, while substituted sausages presented higher gel strength and hardness. Both sausages presented an acceptable quality not only immediately after their production but also during storage at 2 ± 1 ºC. However, gel strength, textural and sensory hardness increased and succulence showed a clear reduction during storage time. Moreover, for substituted sausages, saltiness and astringent aftertaste increased. Formulation had no effect on the microbial growth, which was low. However, microbiological changes occurred during storage time: growth of anaerobic spore-formers since the 45th and 43rd day for control and substituted sausages, respectively.

Keywords: Fish sausage, storage, dietary fibre, colour, microbiological parameters, sensory analysis, textural properties

Introduction

Consumer preference for healthier products has been promoting the research and development of new foods with less or no meat (Giese, 1996; Cofrades et al., 2000). Particularly, consumer interest for meat analogues and, also, fat substitutes, has increased (Beggs et al., 1997; Shand, 2000; Yang et al., 2001). In this context, the development of restructured fish products and the application of new food ingredients have been used as a way of creating healthier meat substitutes, but also as a means to upgrade low-value species and the waste generated by the fish processing industry (Sánchez et al., 2004). Among the various traditional meat products in the market, sausages are especially convenient and present suitable sensory and technological characteristics. Therefore, this product seems adequate for studying the potential use of minced fish and the possibility of pork fat replacement by healthier ingredients.

Among such ingredients, dietary fibre (DF) is frequently used in the design of functional foods (Puupponen-Pimïa et al., 2002). The importance of DF in nutrition and health is well established (Anderson et al., 1990; Kritchevsky and Bonfield, 1995). Presently, the products containing DF available to consumers range from traditional foods (meat, dairy products, breakfast cereals, biscuits, breads, etc.) to the so-called dietary supplements. The incorporation of DF in fish products is a new research field with promising results. Some fibres obtained from algae such as carrageenans (Ortiz and Aguilera, 2004) or seeds such as Guar gum (Montero et al., 2000) have been used for technological

purposes in fish products. For instance, blue whiting gels with Guar gum presented higher water holding capacity than control gels.

In spite of this trend, there is little information with respect to fish sausages containing DF or to their stability during storage. Nevertheless, it was found that high pressure treatment can extend shelf life of chitosan-enriched sausages stored at 2 ºC. Batches containing chitosan powder (1.5 %) became harder during storage time (López-Caballero et al., 2005). Another study (Murphy et al., 2004), conducted with different surimi-pork sausages, wrapped in oxygen permeable film and held at 4 ºC showed that TBARS values increased over time, indicating a progressive lipid oxidation. Sausages with higher fat levels presented TBARS values higher than the others, probably due to the fact that lipid oxidation susceptibility is closely related to fat levels and, in particular, to the amount of unsaturated fatty acids present (Tang et al., 2001). Moreover, psychrotrophic and mesophilic counts also increased over time (Murphy et al., 2004). These findings demonstrate that important quality losses can occur in this kind of products due to ingredient replacements and storage conditions.

In this context, several quality attributes —purge loss, chemical composition, pH, TBARS, colour, textural properties, sensory appreciation and microbial counts— of two previously developed Frankfurter fish sausages (5.3.1) containing inner pea fibre —one with pork fat and other in which a combination of extra hake mince and chicory root fibre provided total fat replacement— were assessed during a 80-day storage at 2 ºC with the purpose of evaluating the effect of storage time and of formulation on products' quality.

Materials and Methods

Raw Materials and Additives

The raw materials and additives used in the manufacturing of the sausages are shown in Table 5.4.1.1. Regarding the dietary fibre (DF) products, composition (based on dry matter, D.M.) as well other properties are presented in Table 5.4.1.2.

Experimental Design

Several quality attributes of two Frankfurter fish sausages —with 7.8 % (w/w) pork fat, termed control sausage, or with a combination of 5.2 % (w/w) chicory root inulin and 2.6 % (w/w) extra hake mince and no pork fat, termed fibre-added sausage— under identical processing, packaging and storage conditions (2 ± 1 ºC) as a function of time (during 80 days) were measured. Samples were taken out every 2 weeks, except between the two last samplings, with an interval of one month. For each product and sampling date, two packages were randomly selected and independently analysed. Error assessment was derived from replication of the various analyses performed.

Table 5.4.1.1 – Raw materials and additives used in the sausages' production.

Raw material and additives	Characteristics	Supplier
Frozen South African hake (*Merluccius capensis*)	- Single batch	- Retailer
	- Caught off South African Coast	
	- Headed and gutted	
	- Immediately frozen on board	
	- Kept frozen at –28 ºC	
	- Processed within two to three weeks after its arrival at the laboratory	
Pork fat	- Single batch	- Local supermarket
	- Stored in a refrigerator no more than two days until processing	
Inner pea fibre (Swelite®)	- See Table 5.4.1.2	Cosucra, S.A. (Warcoing, Belgium)
Chicory root inulin (Fibruline INSTANT®)	- See Table 5.4.1.2	Cosucra, S.A. (Warcoing, Belgium)
Potato starch	- Food grade material	Emsland-Stärke GmbH (Emlichheim, Germany)
Emulsifying soy protein (TARIPROT® 1010)	- Food grade material	BK Giulini (Ladenburg, Germany)
Soy protein concentrate (SOLCON/MAICON 70)	- Food grade material	Solbar Hatzor Ltd. (Ashdod, Israel)
Dextrose (Dextropam 100)	- Food grade material	Copam, S.A. (Loures, Portugal)
Salt	- Food grade material	VATEL (Alverca, Portugal)
Sausage seasoning (TARIMIX® Frankfurt)	- Food grade material	BK Giulini (Ladenburg, Germany)
Smoke aroma (TAROMA® Smoke)	- Food grade material	BK Giulini (Ladenburg, Germany)
Ascorbic acid	- Food grade material	SOLGAR VITAMIN & HERB (Leonia, USA)

Table 5.4.1.2 – Source, composition and properties of the used dietary fibre products*.

PROPERTIES	Swelite®	Fibruline INSTANT®
Source	- Extracted from inner pea	- Inulin extracted from chicory root
Composition (D.M.)		
Total Carbohydrates (%)	93 ± 3	min. 99.7
Of which: *Total DF (%)*	48 ± 3	min. 90
Starch (%)	min. 36	—
Free fructose, glucose and saccharose (%)	—	Max. 10
Protein (%)	max. 7	—
Fat (%)	max. 0.5	—
Ash (%)	max. 2	max. 0.3
Granulometry (µm)	< 400	< 700
Solubility in water (at 20 ºC)	—	> 100 g/l
Colour	white	white
Taste	neutral	neutral to slightly sweet
pH	6 ± 1 (5 % in water)	5.0-7.0
Stability	—	Heat stable

* Values are manufacturer's claims (Anonymous, 2004).

Production of Sausages

Sausages were produced and stored according to a multi-barrier strategy against bacterial growth, encompassing vacuum packaging —prevention of aerobic bacteria growth—, heat treatment, 10 min at 90 ºC —according to Lund and Notermans (1993), reduction by six log cycles of the number of viable spores of *Clostridium botulinum*—, chill storage and salt addition (Cann and Taylor, 1979; Huss, 1997).

Approximately 10 kg of frozen hake were thawed over 12 hours in a refrigerator (at 2 ± 1 ºC). Afterwards, skin and bones were manually removed. All muscle portions —namely, white and red— were used. The resulting fish flesh was minced one single time in a model 84145 meat grinder (Hobart, Troy, OH, USA), equipped with 2 cm grind blades and a metallic screen with 6 mm diameter circular holes.

Five-kg batches were prepared for each formulation (Table 5.4.1.3) according to the following procedure.

Table 5.4.1.3 – Used sausage recipes.

INGREDIENTS (%)	Sausage Type	
	Control	Fibre-added
Hake mince	54.4	57.0
Water/Ice	25.1	25.1
Pork fat	7.8	0.0
Chicory root inulin	0.0	5.2
Inner pea fibre	3.9	3.9
Potato starch	3.1	3.1
Salt	2.3	2.3
Emulsifying protein	1.3	1.3
Soy protein	1.0	1.0
Ascorbic acid	0.1	0.1
Other ingredients	1.0	1.0
Total	*100*	*100*

Regarding the preparation of the sausage batters, five sequential steps were always followed:

1) Hake mince was mixed with salt for 1 minute at 1420 r.p.m. in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Throughout all process, mixing was performed always under vacuum and temperature below 7 ºC.

2) Ice (70 % of the total amount of ice/water) and dextrose were added and there was additional mixing for 1 minute at the same speed.

3) The emulsifying protein, the soy protein concentrate and ascorbic acid were also added and further mixing at the same speed took place for 1 minute.

4) Pork fat or chicory root inulin was added and mixed for 1 minute at 2800 rpm. Meanwhile, in order to hydrate inner pea fibre before mixing it with the other ingredients, chilled water was added to this dietary fibre in a ratio near 2:1.

5) Addition of the remaining ingredients, potato starch, the hydrated fibre, sausage seasoning and smoke aroma and, moreover, mixing all for 2 minutes at 2800 rpm.

The batters attained were put inside a model EB-12 hydraulic filler (Mainca Equipamientos Carnicos, S.L., Granollers, Spain) and expelled under pressure through the stuffing horn into cellulose sausage skins mounted over the end of the stuffing horn. Afterwards, these cellulose casings were twisted and tied, thereby, shaping sausages with a diameter of 25 mm and a length of about 20 cm.

In the next step, sausages were moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and subjected to a steam cooking at 75 ºC for 15 minutes (cooking). Immediately after, sausages were taken from the oven and cooled with a mixture of water and ice (1:1, v/v). The cellulose skins were removed, sausages separated from one another and vacuum-packed (two per package) in low-oxygen permeable barrier bags (O_2 transmission, <2.1 $cm^3/(m^2.day.bar)$ at 23 ºC, Colamin XX 100e, Obermühle Polymertechnik GmbH, Pössneck, Germany) with a model A300/52 vacuum packager (Multivac Sepp Haggenmüller GmbH & Co. KG, Wolfertschwenden, Germany). Following this operation, sausages were put in the same oven and subjected to a steam cooking at 90 ºC for 10 minutes (pasteurisation). In order to guarantee these conditions even in the innermost part of the sausage, the oven's digital thermometer was placed in the centre of a sausage through a hole in the bag.

After pasteurisation, packages were immediately cooled in iced water and stored in a refrigerated room at 2 ± 1 ºC in the dark during approximately 80 days. An electronic panel controlled the refrigerated room's temperature, turning on the refrigeration system whenever temperature rose above 2 ºC. Additionally, temperature was permanently monitored through a model MonoLog T temperature data logger (Digitron, Torquay, UK). Furthermore, relative humidity was in the range 90-95 %.

Quality Measurements

Purge loss. Purge loss was determined by removing two packages of each type of sausage from storage at 2 ºC. The packages were opened and the sausages were taken out, placed in funnels to drain during 5 min., dried with filter paper and weighed. As the initial (before vacuum-packaging) weight of the corresponding sausages had previously been measured, was possible to determine purge loss as a percentage content weight. The purge loss was calculated by the formula:

$$PL = \frac{IW - FW}{IW} \times 100$$

where:

PL – Purge loss (%)

IW – Initial weight (g)

FW – Final weight (g)

Proximate composition. Moisture, crude protein and ash were determined by standard procedures (AOAC, 1984), whereas crude fat was determined by a rapid method of total lipid extraction and purification (Bligh and Dyer, 1959) and total carbohydrate was determined by difference. All determinations were done in duplicate.

pH. pH was measured using a Sen-Tix 21 surface pH electrode (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) on a model pH 539 pH meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Sausages were taken from the packages, left for 1 hour at room temperature and, only then, the surface electrode was introduced in the sausages' interior and the measurements made.

TBARS. TBARS (thiobarbituric acid reactive species) were determined during storage time by a trichloroacetic acid extraction (Vyncke, 1970). For each fish sausage type, two extracts were prepared, each one from sausages of a different package. Results were expressed as mg of malonaldehyde (MA) per 1000 g of sausage.

Colour. Sausages were taken out from packages, cut and put into two Petri dishes, covering the entire bottom. A model MACBETH COLOUR-EYE® 3000 colourimeter (Macbeth, New Windsor, NY, USA) was used and, prior to measurements, standardized to a specific colour blank (CIELAB system: L*, 92.4; a*, -1.0; b*, 1.5). The attained values for L*, a* and b* of the CIELAB system were always the means of ten measurements on each Petri dish. Furthermore, for a better assessment of colour, the three mentioned coordinates were combined in order to obtain the chroma and whiteness values:

$$Chroma = \sqrt{a^{*2} + b^{*2}}$$

$$Whiteness = 100 - \sqrt{(100 - L^{*})^{2} + a^{*2} + b^{*2}}$$

Texture measurements. Sausages were taken out from packages and tempered to about 20 ℃.

Folding test. The test piece was a 3 mm slice (Ø, 25 mm) cut from the sausages middle portion. The evaluation was performed in accordance with a 5-point grade system (Lanier, 1992) as follows. Grade 5, no crack when folded into quadrants; Grade 4, no cracks when folded in half; Grade 3, crack develops gradually when folded in half; Grade 2, crack develops immediately when folded in half; Grade 1, crumbles when pressed by finger.

Puncture test. Prior to analysis, sausages were cut into pieces of 25 mm diameter and 25 mm high. Each sample was penetrated to the breaking point with a metal probe equipped with a 5 mm diameter spherical head, using a model Instron 4301 texturometer (Instron Engineering Corp., Canton, MA, USA). The cross speed head was 10 mm/min and the load cell was 1000 N. Breaking force (N) and breaking deformation (mm) was measured. Gel strength (N.mm) was determined by multiplying these two parameters.

Compression tests. For the texture profile analysis, samples (diameter, 25 mm and height, 25 mm) were compressed on the flat plate of the Instron texturometer with a cylindrical plunger (50 mm diameter) adapted to a 1000 N load cell at a deformation rate of 50 mm/min. On the basis of preliminary trials to establish a compression limit that would ensure no cracking and recoverability of most samples, it was decided to compress samples to 60 % of their height. In the test, each sample was compressed twice. The following parameters were determined: hardness (N), maximum height of first peak on first compression (in terms of eating quality, food's resistance at first bite); cohesiveness (A_2/A_1), ratio of second-compression to first-compression positive areas (maintenance of food resistance during chew down); gumminess (N), product of hardness and cohesiveness (strength required in the chew down process); springiness (L_2/L_1), ratio of the detected height of the product on the second compression to the original compression distance (ability of food to reacquire its initial shape and size after a first bite); chewiness (N), product of gumminess and springiness (albeit expressed in N, a measure of the energy spent in the chew down process).

For the compression-relaxation test, the compression procedure was as for the texture profile analysis except that the sample was compressed only once for one minute and the force exerted on the sample was recorded. Relaxation (%) was calculated as $Y_T=100\times(F_0-F_1)/F_0$, where F_0 is the force registered at the onset of relaxation immediately after sample compression and F_1 is the force registered after one minute of relaxation. Thus, $(100-Y_T)$ is taken as an index of elasticity and is expressed as the percentage elasticity of the gel.

Sensory analysis. Sensory evaluation was conducted by five panellists from INRB, I.P./IPIMAR, extensively trained with the sensory scheme for Frankfurter sausage evaluation and capable of conducting a structured scaling of products (Nielsen et al., 2002). Panellists participated in preliminary trials and in the experiments that led to the development of Frankfurter fish sausages containing inner pea fibre (5.3.1).

Sausages were taken out from the package, tempered to about 20 ℃ and cut into 2 cm long slices. These slices were distributed in white plates and presented to the panellist in random order for evaluation. Mineral water was supplied to the panellists for rinsing between samples. The evaluation was performed in a room specifically conceived for sensory analysis and with adequate lighting.

Panellists were asked to score several sensory parameters of the product, using a 0-5 scale: colour (0 – light to 5 – dark); smoke and unpleasant aroma (0 – absent to 5 - excessive); elasticity (0 – plastic to 5 – elastic), hardness (0 – soft to 5 – hard), cohesiveness (0 – scarcely cohesive to 5 – very cohesive), succulence (0 – dry to 5 - succulent) and fat mouth feel (0 – slightly oily to 5 – very oily); saltiness, smoke and unpleasant flavour (0 – absent to 5 - excessive); and astringent aftertaste (0 – absent to 5 - excessive).

Microbiological analysis. Bacterial counts were determined by pour plate method over storage. The first dilution was prepared by aseptically homogenising 10 g of sausage with 90 ml of a peptone solution (1 g/l) for 1 min in a Stomacher blender (Seward, London, UK).

Adequate serial dilutions were prepared and inoculations performed in duplicate. Plate Count Agar (PCA, Oxoid Ltd., Hampshire, UK) was used for total mesophilic aerobic count, incubated at 30 ºC for 3 days, and, for total psychrotrophic aerobic count, incubated at 6.5 ºC for 10 days. For *Enterobacteriaceae*, incubation was conducted at 37 ºC for 1 day in a Violet Red Bile Agar (Merck KGaA, Darmstadt, Germany), while, for lactic acid bacteria, incubation was done at 25 ºC for 3 days in a Man Rogosa Sharp Agar (MRS, Oxoid Ltd., Hampshire, UK). Regarding spores of sulfite-reducing clostridia, incubation was done at 37 ºC for 5 days in a Sulphadiazine Polymyxin Sulphite Agar (SPS, Merck KGaA, Darmstadt, Germany) under anaerobic conditions after treatment of serial decimal dilution volumes for 10 min. at 80 ºC. Albeit only black colonies were considered as positive result, plates were also examined with respect to presence of any other colonies. Finally, for mould and yeast, incubation was conducted at 25 ºC for 5 days in a Rose Bengal Chloramphenicol Agar (RB, Oxoid Ltd., Hampshire, UK). Results were expressed as log colony forming units (CFU)/g sample.

Statistical analysis

For each product and sampling date, sausages from two different packages were used. A general linear model —one-way ANOVA— was used to determine significant differences ($p<0.05$) among sausages with different formulation (control sausages *vs* fibre-added sausages) and, for each product, among sausages with different storage time. Multiple comparisons were done by the Tukey HSD test. All statistical treatment was done with the software STATISTICA© from StatSoft, Inc. (Tulsa, OK, USA), version 6.1, 2003.

Results and Discussion

Concerning new food products, a thorough study of stability has always been a fundamental condition for their development, encompassing an evaluation of quality changes and, also, of microbiological quality throughout storage time.

Therefore, a set of quality measurements were carried out. Proximate composition and purge loss offered a broad perspective of quality change, namely, regarding loss of moisture by the products as a result of storage time. pH and TBARS enabled the detection of spoilage compounds. Sensory analysis provided a reference for the assessment of the quality features observed as sensory changes in saltiness, succulence, hardness, astringent aftertaste, and colour. Colour and texture were also measured by instrumental analysis. Finally, microbiological analysis gave information regarding survival and growth of selected groups of microorganisms, which can produce relevant quality losses in the product.

Purge loss

For both formulations, purge loss remained low and almost constant over storage time, not being detected any significant difference (Table 5.4.1.4).

Table 5.4.1.4 – Purge loss of control and fibre-added sausages during storage time*.

Control Sausages		Fibre-added Sausages	
Storage Time (days)	Purge loss (%)	Storage Time (days)	Purge loss (%)
1	0.0 ± 0.0^{a}	1	0.5 ± 0.6^{a}
14	0.0 ± 0.0^{a}	12	0.4 ± 0.6^{a}
28	0.3 ± 0.5^{a}	26	1.7 ± 0.3^{a}
43	0.0 ± 0.0^{a}	41	1.4 ± 0.1^{a}
57	0.7 ± 0.0^{a}	55	1.8 ± 0.2^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

The observed purge loss values —not exceeding 1.8 %— were lower than those reported for beef (Candogan and Kolsarici, 2003) —from 2.13 to 8.18 %— or chicken (Andrés et al., 2006) —between 4 and 10 %— sausages. One possible reason for this

difference could be the considerable amount of inner pea fibre incorporated in both fish sausages (Table 5.4.1.3), a fibre that favours greater water retention. However, sensory results (see below, Sensory analysis) and, particularly, succulence reduction over storage time for both formulations suggests that, besides moisture loss, there are other factors determining a succulent mouth feel.

Proximate composition and pH

Proximate composition revealed higher moisture, 72.9 *vs* 71.3 % ($p<0.05$) and less crude lipid, 0.4 *vs* 6.3 % ($p< 0.05$) in fibre sausages as was expected due to the formulations. These sausages were less succulent over storage time (see below, Sensory analysis), thus, albeit, in general, succulence is closely related to moisture content, these results and those regarding purge loss suggest that, for Frankfurter fish sausages, succulence is more related to fat content and other factors. Furthermore, proximate composition showed no significant differences over time in storage for either product formulation.

No significant differences were observed for pH between formulations and during time in storage (range 6.28-6.44 for control and 6.31-6.37 for fibre-added sausages).

TBARS

No significant differences were observed for TBARS between formulations (Table 5.4.1.5).

Table 5.4.1.5 – TBARS variation of control and fibre-added sausages during storage time*.

CONTROL SAUSAGES		FIBRE-ADDED SAUSAGES	
STORAGE TIME (days)	TBARS (mg MA/1000 g)	STORAGE TIME (days)	TBARS (mg MA/1000 g)
0	0.84 ± 0.02^{a}	0	1.17 ± 0.04^{a}
14	0.85 ± 0.03^{a}	12	0.79 ± 0.02^{b}
28	1.13 ± 0.05^{bc}	26	1.86 ± 0.01^{c}
45	0.96 ± 0.02^{ab}	43	1.08 ± 0.05^{a}
57	0.88 ± 0.05^{a}	55	1.14 ± 0.00^{a}
83	1.24 ± 0.08^{c}	81	1.07 ± 0.04^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Regarding storage time evolution, the observed oscillations make any difference also not significant. Nevertheless, these results seem to agree with sensory analysis, since no panellist reported any rancid aroma or flavour, even in the control sausages after 80-day storage.

However, the observed high degree of variability, which is typical for this lipid oxidation test (Fernández et al., 1997), had unquestionably a deleterious effect on the results,

masking any underlying trend. TBARS was chosen since it is a standard industrial test. However, alternative methods should be considered in order to monitor lipid oxidation (peroxide value, specific aldehyde and ketone analysis through chromatography). Another alternative would be to combine TBARS and HPLC, thus enabling the removal of possible interfering substances (Seljeskog et al., 2006).

Colour

No significant differences were observed in colour for both formulation and time of storage (Table 5.4.1.6). Hence, there was no clear relationship between colour and fat content. Moreover, these instrumental results are in accordance with human perception of colour (see below, Sensory analysis), which, similarly, did not enable the identification of significant colour differences.

Table 5.4.1.6 – Colour variation of control and fibre-added sausages during storage time*.

CONTROL SAUSAGES					FIBRE-ADDED SAUSAGES				
STORAGE TIME (days)	a*	b*	Whit.	Chroma	STORAGE TIME (days)	a*	b*	Whit.	Chroma
1	-0.23 ± 0.02^{a}	6.35 ± 0.09^{a}	74.7 ± 0.3^{a}	6.35 ± 0.09^{a}	1	-0.25 ± 0.03^{a}	6.43 ± 0.13^{a}	74.0 ± 0.9^{a}	6.44 ± 0.13^{a}
14	-0.16 ± 0.01^{a}	6.46 ± 0.52^{a}	76.6 ± 0.9^{a}	6.46 ± 0.51^{a}	12	-0.44 ± 0.06^{b}	5.21 ± 0.20^{b}	71.2 ± 0.7^{b}	5.23 ± 0.19^{b}
29	-0.34 ± 0.14^{a}	6.30 ± 0.31^{a}	74.8 ± 0.1^{a}	6.31 ± 0.30^{a}	27	-0.15 ± 0.03^{a}	6.87 ± 0.11^{a}	76.3 ± 0.8^{a}	6.87 ± 0.11^{a}
43	-0.24 ± 0.00^{a}	6.63 ± 0.18^{a}	76.5 ± 1.1^{a}	6.64 ± 0.18^{a}	41	-0.19 ± 0.00^{a}	6.56 ± 0.05^{a}	74.1 ± 0.2^{a}	6.56 ± 0.05^{a}
57	-0.30 ± 0.02^{a}	6.38 ± 0.13^{a}	74.9 ± 0.0^{a}	6.39 ± 0.13^{a}	55	-0.27 ± 0.03^{a}	6.49 ± 0.02^{a}	75.3 ± 0.0^{a}	6.49 ± 0.02^{a}
84	-0.27 ± 0.02^{a}	6.60 ± 0.03^{a}	75.9 ± 0.0^{a}	6.61 ± 0.03^{a}	82	-0.23 ± 0.04^{a}	6.59 ± 0.08^{a}	75.1 ± 0.7^{a}	6.59 ± 0.08^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Texture measurements

Dietary fibre sausages showed, on one hand, higher ($p< 0.05$) gel strength and hardness values and, on the other, lower ($p<0.05$) elasticity, cohesiveness and springiness than controls (Tables 5.4.1.7 and 5.4.1.8). The former formulation effects are related to an increase in gel strength and hardness with growing dietary fibre incorporation and the latter were already reported in a previous study (5.3.1). Additionally, it should be mentioned that the differences in elasticity and cohesiveness found at the instrumental level were not corroborated at the human senses level (see below, Sensory analysis). This could be due to higher instrumental sensitivity and accuracy, but also to an imperfect instrumental reproduction of the human textural concepts.

Concerning storage time effect, gel strength and hardness values increased significantly ($p<0.05$) between the initial and final samplings for the two products studied, while gumminess and chewiness only increased ($p<0.05$) along storage for fibre-added sausages. The evolution of these two properties was a consequence of the strong hardness variation in fibre-added sausages, particularly, from the 1st day to the 12th day

sampling (23.9 ± 3.3 to 39.0 ± 0.9 N); afterwards there was no clear hardening effect. A similar phenomenon was observed in gel strength (17.3 ± 2.9 on 1st day to 25.5 ± 5.8 N.mm on 12th day). On the other hand, control sausages hardness oscillated over time with no clear turning point. Furthermore, control sausages gel strength exhibited a clear trend only from the 43rd day onwards (increasing from 13.8 ± 1.6 to 16.1 ± 1.1 and, then, to 19.1 ± 0.4 N.mm). The hardening of sausages over storage time was also reported in other papers, however dealing with low fat beef (Candogan and Kolsarici, 2003) or chicken (Andrés et al., 2006) Frankfurters. Some authors (Andrés et al., 2006) related similar hardness increases to water loss (purge) during refrigerated storage, however such loss was rather low in the fish sausages (see above, Purge loss). Nonetheless, it should be noted that dietary fibre sausages, which exhibited a stronger hardness increase than control ones (63 % *vs* 26 %), had also higher purge losses over time. Besides gumminess and chewiness, gel strength variation can also be explained by the hardening effect of storage time.

Table 5.4.1.7 – Textural properties variation of control sausages during storage time*.

STORAGE TIME (days)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
1	2.7 ± 0.6^{a}	12.1 ± 1.0^{a}	48.0 ± 8.5^{a}	29.4 ± 3.4^{ab}	0.51 ± 0.03^{a}	15.2 ± 1.4^{a}	0.79 ± 0.01^{a}	11.9 ± 0.9^{a}
14	3.0 ± 0.0^{a}	13.2 ± 1.5^{ab}	57.2 ± 0.0^{a}	27.7 ± 1.4^{a}	0.49 ± 0.04^{a}	14.0 ± 0.8^{a}	0.80 ± 0.02^{a}	11.1 ± 0.8^{a}
29	2.7 ± 0.6^{a}	15.4 ± 1.3^{bc}	55.7 ± 2.1^{a}	35.3 ± 4.1^{bc}	0.50 ± 0.01^{a}	16.7 ± 2.4^{a}	0.79 ± 0.01^{a}	13.3 ± 1.8^{a}
43	2.7 ± 0.6^{a}	13.8 ± 1.6^{abc}	51.5 ± 5.7^{a}	29.8 ± 3.5^{ab}	0.51 ± 0.01^{a}	15.7 ± 1.4^{a}	0.80 ± 0.01^{a}	12.6 ± 1.0^{a}
57	2.3 ± 0.6^{a}	16.1 ± 1.1^{c}	55.9 ± 0.3^{a}	29.2 ± 1.6^{ab}	0.52 ± 0.01^{a}	15.2 ± 0.0^{a}	0.80 ± 0.00^{a}	12.2 ± 0.0^{a}
84	2.7 ± 0.6^{a}	19.1 ± 0.4^{d}	58.4 ± 0.1^{a}	37.0 ± 4.0^{c}	0.53 ± 0.02^{a}	17.9 ± 0.7^{a}	0.82 ± 0.00^{a}	14.8 ± 0.6^{a}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Table 5.4.1.8 – Textural properties variation of fibre-added sausages during storage time*.

STORAGE TIME (days)	Folding test	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness	Chewiness (N)
1	3.0 ± 0.0^{a}	17.3 ± 2.9^{a}	41.4 ± 4.0^{a}	23.9 ± 3.3^{a}	0.41 ± 0.05^{a}	8.9 ± 0.7^{a}	0.72 ± 0.05^{a}	6.5 ± 1.0^{a}
12	2.7 ± 0.6^{ab}	25.5 ± 5.8^{ab}	46.4 ± 8.9^{a}	39.0 ± 0.9^{bc}	0.46 ± 0.00^{a}	17.7 ± 0.6^{b}	0.76 ± 0.01^{a}	13.4 ± 0.2^{ab}
27	3.0 ± 0.0^{a}	25.6 ± 3.1^{ab}	45.2 ± 3.4^{a}	36.5 ± 3.3^{bc}	0.45 ± 0.01^{a}	17.4 ± 1.9^{ab}	0.74 ± 0.02^{a}	12.9 ± 1.7^{ab}
41	2.3 ± 0.6^{ab}	25.9 ± 2.5^{b}	42.2 ± 3.4^{a}	36.0 ± 1.8^{b}	0.40 ± 0.01^{a}	14.4 ± 0.7^{ab}	0.75 ± 0.02^{a}	10.8 ± 0.2^{ab}
55	2.0 ± 0.0^{b}	32.9 ± 4.3^{b}	52.2 ± 5.0^{a}	44.2 ± 4.5^{c}	0.44 ± 0.04^{a}	21.0 ± 4.0^{b}	0.76 ± 0.03^{a}	16.0 ± 3.7^{b}
82	2.0 ± 0.0^{b}	26.4 ± 3.4^{b}	46.2 ± 2.3^{a}	38.9 ± 5.3^{bc}	0.45 ± 0.04^{a}	19.6 ± 2.5^{b}	0.76 ± 0.00^{a}	15.0 ± 1.9^{b}

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Sensory analysis

Fat replacement had no significant effect on the mean sensory scores, except for succulence (Figures 5.4.1.1 and 5.4.1.2). Indeed, control sausages were scored as more succulent ($p<0.05$) than fibre-added sausages.

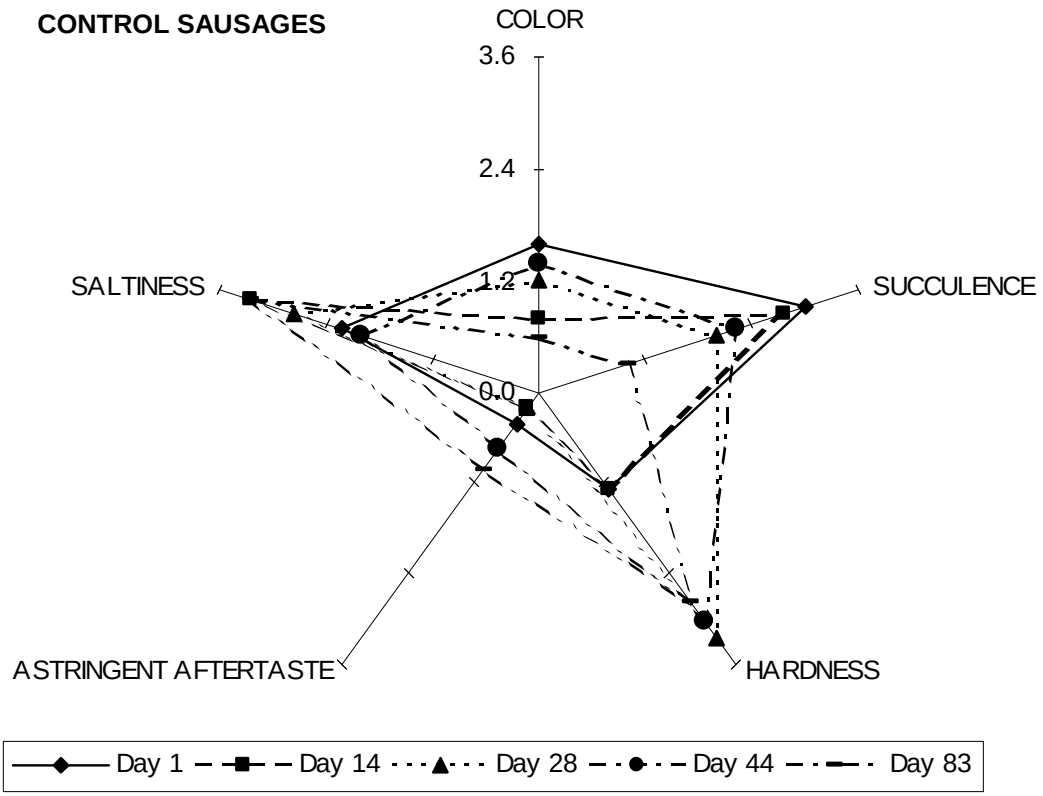

Figure 5.4.1.1 – Sensory parameters variation of control sausages during storage time.

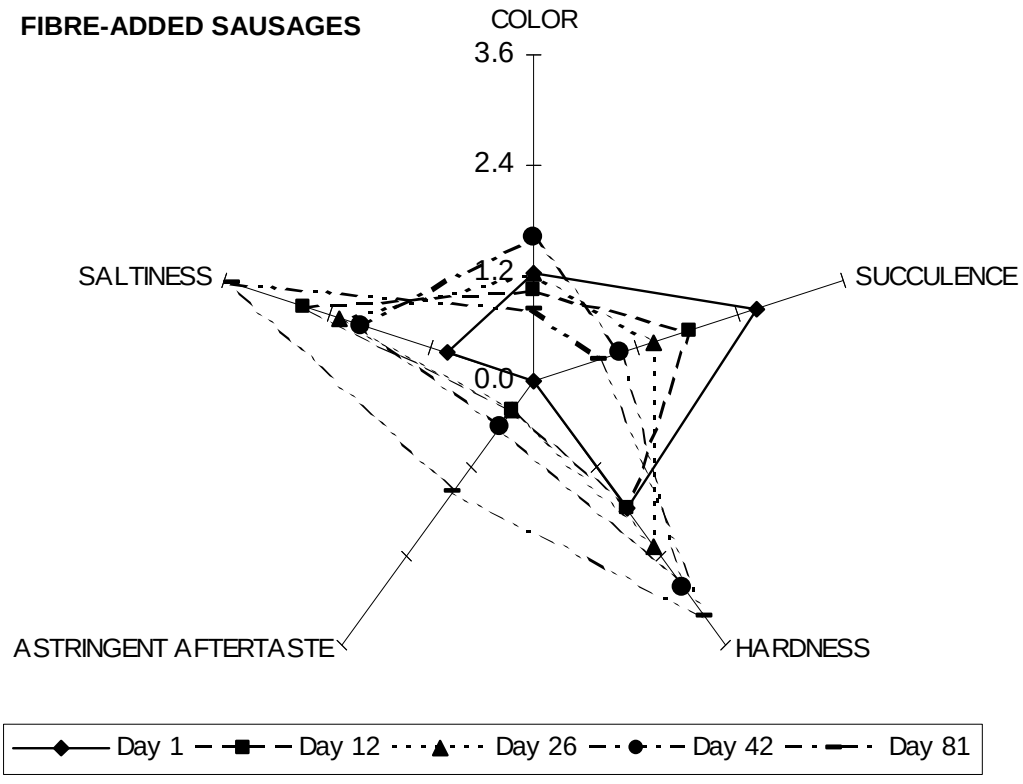

Figure 5.4.1.2 – Sensory parameters variation of fibre-added sausages during storage time.

Moreover, hardness and succulence scores changed significantly ($p<0.05$) over storage time for the two sausage types, whereas saltiness and astringent aftertaste scores only changed ($p<0.05$) for fibre-added sausages. Contrary to texture measurements, hardness increase was steeper for control sausages (115 % *vs* 77 %), being found significant differences ($p<0.05$) to the initial value from the 28th day onwards. Regarding fibre-added sausages, only the last day presented significant differences ($p<0.05$) to the initial score. On the other hand, succulence decreased to a third of initial level for both products, however, control sausages succulence was maintained longer than fibre-added sausages (44 days *vs* 26 days). Furthermore, in spite of some oscillation, the fibre-added product was increasingly saltier during storage time, being the final score, 3.5 ± 1.0 largely superior to the initial, 1.0 ± 0.8. An astringent aftertaste also became more intense, although only the last day score was significantly higher ($p<0.05$) than the initial one. The remaining sensory properties were unaltered (data not shown).

Microbiological analysis

The simultaneous application of heat treatment (10 min at 90 ºC), vacuum packaging, chill storage and salt addition enabled an initial good microbiological quality (Table 5.4.1.9).

Table 5.4.1.9 – Microbiological parameters variation of control and fibre-added sausages during storage time*.

CONTROL SAUSAGES			FIBRE-ADDED SAUSAGES		
STORAGE TIME (days)	Total mesophilic aerobic (Log CFU/g)	Mesophilic anaerobic spore-formers	STORAGE TIME (days)	Total mesophilic aerobic (Log CFU/g)	Mesophilic anaerobic spore-formers
0	2.0 ± 0.2^{a}	Absent	0	2.1 ± 0.0^{a}	Absent
13	2.0 ± 0.0^{a}	Absent	11	2.0 ± 0.0^{a}	Absent
27	2.0 ± 0.1^{a}	Absent	25	2.3 ± 0.2^{a}	Absent
45	2.0 ± 0.1^{a}	Present	43	1.9 ± 0.1^{a}	Present
56	2.1 ± 0.0^{a}	Present	54	2.2 ± 0.1^{a}	Present
80	2.1 ± 0.2^{a}	Present	78	2.1 ± 0.0^{a}	Present

* Presented values correspond to mean ± standard deviation.
Means within a column with different letters are significantly different ($p<0.05$).

Formulation seemed to have no effect on the microbial growth.

Both sausage types, exhibited growth of mesophilic aerobic microorganisms, presenting always low levels during storage period. This finding was somewhat unexpected, as the applied heat treatment (enabling 15 log cycle reduction in the number of most vegetative forms, according to Stumbo (1973)) should prevent the survival of the vegetative cells present in the raw material (which levels are low, data not shown). Nevertheless, further growth was possibly prevented by packaging conditions and storage temperature.

These microbial counts were lower than those found with low fat chicken sausages submitted to similar conditions, namely, stored at 4 ºC over a 50-day period after being pasteurised and vacuum-packed (Andrés et al., 2006). Probably, the higher pasteurisation temperature applied to fish sausages (90 ºC *vs* 74 ºC) and slightly lower storage temperature (2 ºC *vs* 4 ºC) can explain such differences.

In general, storage time had no significant effect on the above parameters. However, non-characteristic microbial growth was detected on the SPS medium for both sausage types from the 45th (control formulation) and 43rd (fibre-added formulation) day onwards, indicating the development of mesophilic anaerobic spore-formers. Although the isolated colonies in this medium were not black, as characteristic of most of the bacteria belonging to the genus *Clostridium*, some strains *C. botulinum* (particularly saccharolytic strains in group II) do not invariably form black colonies in such media (ICMSF, 1996). Hence, such microbial growth has to be considered as a relevant quality loss.

Conclusions

Both sausages presented an acceptable quality not only immediately after their production but also during storage at 2 ± 1 ºC. Particularly, the properties of the healthier substituted sausages were not very different from the control sausages, even presenting some textural advantages: higher gel strength and hardness. On the other hand, control sausages were more elastic, cohesive, springy and succulent. Regarding the effect of storage time, gel strength, textural and sensory hardness increased and succulence showed a clear reduction. Moreover, for substituted sausages, saltiness and astringent aftertaste increased. Nevertheless, many initial properties were maintained, namely, colour, most sensory and some textural parameters.

Besides, regarding microbiology, the studied parameters remained low and stable. However, growth of anaerobic spore-former bacteria was detected from the 45th (control sausages) and 43rd (fibre-added sausages) day onwards, representing a fundamental quality loss.

Future studies, namely, involving package inoculation tests, should be performed in order to have a complete evaluation of products' safety during storage.

Acknowledgments

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5.4.2 – Quality Changes during Storage of Minced Fish Products Containing Dietary Fibre and Fortified with ω3 Fatty Acids

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Quality Changes During Storage of Minced Fish Products Containing Dietary Fiber and Fortified with ω3 Fatty Acids

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Two ready-to-eat minced fish products from hake were developed, their proximate composition and fatty acid profiles determined and their quality changes followed during 3.5 months under refrigeration at 2.0 ± 1°C and 10 ± 1°C. These products contain dietary fiber and are innovative and healthy. The formulation was identical, except vegetable oil (VO), 5.6% (w/w) in one group and 2.7% in w/w plus 2.8% (w/w) cod liver oil (CLO) in the other. CLO products had a higher ω3/ω6 ratio (0.56 ± 0.02 versus 0.08 ± 0.02) and ensured, per 100g serving, the 500 mg recommended daily intake of eicosapentaenoic and docosahexaenoic acids. CLO products showed lower gel strength ($p \leq 0.05$), however, other textural properties were similar to those of the VO group. Thiobarbituric acid reactive substances values were higher in CLO products. All groups presented acceptable sensory scores and no microbiological growth. During storage products became redder and less yellow, while seafood aroma and flavor declined and saltiness perception augmented. Temperature had a negative effect on sensory elasticity and instrumental texture.

Key Words: Fish minced products, fatty acids, storage, microbiological growth, sensory analysis, texture

INTRODUCTION

The limited biological resources and the increasing demand for fish products have emphasized the need for better and value-added utilization of the underutilized fish species and the by-products from the fishing industries. Much of this waste is produced in the fish processing industry, originating mostly in fish filleting operations and frozen fish processors. Traditionally, most of underutilized species and fish wastes have been converted to powdered fish meal (Guerard et al., 2002) thus, representing a huge loss of valuable proteins and other nutrients, specially, given the world population needs regarding animal protein.

In this context, provided that such waste is carefully processed and preserved, various methods for using low-value cuts and trimmings can be applied in order to improve appearance and textural properties and enhance market value. In particular, fish mincing or restructuring combined with new food ingredients has been used as a way of creating healthier products, but also as a means to upgrade low-value species and the waste generated by the fish processing industry (Sánchez et al., 2004).

Among such ingredients, dietary fiber (DF) is frequently used in the design of functional foods (Pszczola-Pirula et al., 2002). The importance of DF in nutrition and health is well established (Anderson et al., 1990; Kritchevsky and Bonfield, 1995). Presently, the products containing DF available to consumers range from traditional foods (meat and dairy products, breakfast cereals, biscuits, breads, etc.) to the so-called dietary supplements. The incorporation of DF in fish products is a new research field with promising results. Some fibers obtained from algae such as carrageenans (Ortiz and Aguilera, 2004) or seeds such as guar gum (Montero et al., 2000) have been used for technological purposes in fish products. For instance, blue whiting gels with guar gum presented higher water holding capacity than control gels (Montero et al., 2000). Moreover, it was found that the combination of 4% (w/w) inner pea fiber and up to 2% (w/w) ι-κ carrageenan increased the hardness of restructured fish products (Cardoso et al., 2007).

Microbial transglutaminase (MTGase) is another important ingredient, widely used in the food industry with the purpose of promoting protein cross-linking (Téllez-Luis et al., 2002). MTGase has effectively

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Abstract

Two ready-to-eat minced fish products from hake were developed, their proximate composition and fatty acid profiles determined and their quality changes followed during 3.5 months under refrigeration at 2 ± 1 ºC and 10 ± 1 ºC. These products contain dietary fibre and are innovative and healthy. The formulation was identical, except vegetable oil (VO), 5.6 % (w/w) in one group and 2.7 % (w/w) plus 2.9 % (w/w) cod liver oil (CLO) in the other.

CLO products had a higher ω3/ω6 ratio (0.54 ± 0.02 *vs* 0.08 ± 0.02) and ensured, per 100 g serving, the 500 mg recommended daily intake of eicosapentaenoic and docosahexaenoic acids.

CLO products showed lower gel strength ($p<0.05$), however, other textural properties were similar to those of the VO group. Thiobarbituric acid reactive substances (TBARS) values were higher in CLO products. All groups presented acceptable sensory scores and no microbiological growth. During storage products became redder and less yellow, while seafood aroma and flavour declined and saltiness perception augmented. Temperature had a negative effect on sensory elasticity and instrumental texture.

Keywords: Fish minced products, ω3 PUFA, nutritional parameters, storage, microbiological parameters, sensory analysis, textural properties

Introduction

The limited biological resources and the increasing demand for fish products have emphasized the need for better and value-added utilization of the underutilized fish species and the by-products from the fishing industries. Much of this waste is produced in the fish processing industry, originating mostly in fish filleting operations and frozen fish processors. Traditionally, most of underutilized species and fish wastes have been converted to powdered fish meal (Guerard et al., 2002), thus, representing a huge loss of valuable proteins and other nutrients, specially, given the world population needs regarding animal protein.

In this context, provided that such waste is carefully processed and preserved, various methods for using low-value cuts and trimmings can be applied in order to improve appearance and textural properties and enhance market value. In particular, fish mincing or restructuring combined with new food ingredients has been used as a way of creating healthier products, but also as a means to upgrade low-value species and the waste generated by the fish processing industry (Sánchez et al., 2004).

Among such ingredients, dietary fibre (DF) is frequently used in the design of functional foods (Puupponen-Pimïa et al., 2002). The importance of DF in nutrition and health is well

established (Anderson et al., 1990; Kritchevsky and Bonfield, 1995). Presently, the products containing DF available to consumers range from traditional foods (meat and dairy products, breakfast cereals, biscuits, breads, etc.) to the so-called dietary supplements. The incorporation of DF in fish products is a new research field with promising results. Some fibres obtained from algae such as carrageenans (Ortiz and Aguilera, 2004) or seeds such as guar gum (Montero et al., 2000) have been used for technological purposes in fish products. For instance, blue whiting gels with guar gum presented higher water holding capacity than control gels (Montero et al., 2000). Moreover, it was found that the combination of 4 % (w/w) inner pea fibre and up to 2 % (w/w) ι+κ carrageenan increased the hardness of restructured fish products (5.1.3).

Microbial transglutaminase (MTGase) is another important ingredient, widely used in the food industry with the purpose of promoting protein cross-linking (Téllez-Luís et al., 2002). MTGase has effectively improved, in various circumstances, the textural properties of restructured fish products (Ramírez et al., 2000; Téllez-Luís et al., 2002; 5.1.3). A level of 0.1 % (w/w) MTGase improved the texture of restructured hake products with DF (5.1.3).

Fish oil rich in the ω3 fatty acids eicosapentaenoic (EPA) or docosahexaenoic (DHA) acid or, alternatively, ω3 polyunsaturated fatty acids (PUFA) concentrates have also been added to several food products —bread, milk, ice cream and others (Kolanowski et al., 1999)—, since it is thought that EPA and DHA may play an important role in the prevention of cardiovascular disease (Burr et al., 1989) as well of several inflammatory conditions (Calder, 1996). Indeed, for consumers without coronary heart disease, the American Heart Association (AHA) recommends a daily intake of 500 mg EPA+DHA (Kris-Etherton et al., 2002). Contrastingly, vegetable oil, a common food ingredient, is rich in ω6 PUFA, which, taking into account the so-called south Asian paradox (vegetarian Hindus with low consumption of saturated FA and high consumption of ω6 PUFA have a higher than expected coronary artery disease mortality), do not present a nutritional protective role as important as ω3 PUFA (Pella et al., 2003). On the other hand, fish products, given their association with marine sources and wide acceptance in most developed nations, are a logical vehicle for increasing the consumption of ω3 PUFA without the need for dietary supplements in pill or capsule form (Lanier et al., 1988). Fish oil addition to various kinds of fish products, specifically those requiring a fat component, has been tested (Park et al., 2004; Pérez-Mateos et al., 2004; Panpipat and Yongsawatdigul, 2007). However, the use of fish oil in food fortification has been limited due to the greater tendency of ω3 PUFA to oxidize, resulting in off-flavours and aromas and, consequently, substantial reduction of the products' shelf life (Pérez-Mateos et al., 2004). The formation of

hydroperoxides (primary oxidation products) from PUFA is a fundamental step of the oxidation process. This process leads to the formation of a complex mixture of secondary oxidation products, mainly, aldehydes and ketones, which are the responsible for the changes in aroma and flavour (Frankel, 1991). Oxidative deterioration is also related to the adverse changes in texture, appearance and nutritional value (Min and Boff, 2002). Regarding fish products, it was possible to avoid negative effects on the surimi gel strength with the addition of an adequate oil-in-water emulsion instead of bulk oil (Park et al., 2004). It was also found that the type of fish oil used and its antioxidant system can be critical in preventing off-flavours and aromas development in surimi seafoods over storage time (Pérez-Mateos et al., 2004).

Taking into account that the fish products developed in this study are to be chilled and not frozen, it is fundamental to follow the changes during refrigeration. For this purpose, two temperatures were selected, an optimum temperature and an abuse temperature.

Therefore, several quality attributes —chemical composition, fatty acid profile, pH, water activity, TBARS, colour, textural properties, sensory appreciation and microbial counts— of two hake (*Merluccius capensis*) ready-to-eat minced fish products (patent pending) containing inner pea fibre —one with 5.6 % (w/w) vegetable oil (VO) and other with a partial replacement of VO by cod liver oil (CLO), 2.7 % (w/w) VO + 2.9 % (w/w) CLO— were assessed during a 105-day storage at 2 ± 1 ºC and 10 ± 1 ºC with the purpose of evaluating the effect of oil source, storage temperature and time on products' quality.

Materials and Methods

Raw Materials, Ingredients, and Reagents

Taking into account the great variability associated with available frozen fish waste, it was decided to perform the present study with that portion of the raw material used for further processing instead of the waste material.

Frozen South African hake (*Merluccius capensis*) (Gelpeixe, Loures, Portugal) was caught off South African Coast, immediately frozen at ship and kept frozen at –28 ºC for two months, cut in factory into thin slices (1 cm thick) without thawing and, again, kept at -28ºC for about one month. Finally, it was processed within two to three weeks after its arrival at the laboratory. Vegetable oil, Fula® (Sovena, S.A., Oeiras, Portugal), was a mixture of soya, sunflower, peanut and corn oils, while fish oil (Winterisation, Fécamp, France) was a deodourized cod liver oil of pharmaceutical grade (see fatty acid profiles in Table 5.4.2.1) without added antioxidant. Inner pea fibre, Swelite® (Cosucra, S.A., Warcoing, Belgium), had 93 ± 3 % of total carbohydrates, of which, 48 ± 3 % were DF (Anonymous, 2004). Salt was food grade material (VATEL, Alverca, Portugal).

Carrageenan, ceamgel1830 (Ceamsa, Porriño, Spain) was a mixture of ι and κ carrageenan. Shellfish flavour (Givaudan Schweiz AG, Kemptthal, Switzerland) and sodium polyphosphate (BK Giulini, Ladenburg, Germany) were food grade materials. Sodium caseinate, EM6® (DMV International BV, Veghel, The Netherlands), had 88 % protein and less than 6 % moisture (Anonymous, 2006). Monosodium glutamate was also a food grade material (Ajinomoto Japan, Inc., Tokyo, Japan). MTGase, ACTIVA® WM, presented an activity of approximately 67 ± 10 units.g^{-1} and the enzyme itself was less than 1 % in a maltodextrine carrier (Ajinomoto Japan, Inc., Tokyo, Japan).

All chemicals used were of analytical grade and were obtained from Merck KGaA (Darmstadt, Germany).

Experimental Design

Several quality attributes of two minced fish products containing inner pea fibre —with 5.6 % (w/w) VO, termed control product, or with a combination of 2.7 % (w/w) VO + 2.9 % (w/w) CLO, termed CLO product— under identical processing, packaging and two different storage temperatures (2 ± 1 ºC and 10 ± 1 ºC) as a function of time (during 105 days) were measured. During minced fish products' production, samples were taken for microbiological analyses before and after the heat treatment at 90 ºC. Thereafter, from day 1 onwards, samples were taken out every 3 weeks. For each product, storage temperature and sampling date, two packages were randomly selected and independently analysed. Error assessment was derived from replication of the various analyses performed.

Production of Minced Fish Products

Minced fish products were produced and stored according to a multi-barrier strategy against bacterial growth, encompassing vacuum packaging —prevention of aerobic bacteria growth—, heat treatment, 1 hour at 90 ºC —according to Lund and Notermans (1993), 10 minutes at 90 ºC ensures a reduction by six log cycles of the number of viable spores of *Clostridium botulinum*—, chill storage and salt addition (Cann and Taylor, 1979; Huss, 1997).

Approximately 30 kg of frozen hake were thawed over 12 hours in a refrigerator (at 2 ± 1 ºC). Afterwards, skin and bones were manually removed. All muscle portions —white and red— were used. The resulting fish flesh was minced once in a model 84145 meat grinder (Hobart, Troy, OH, USA), equipped with 2 cm grind blades and a metallic screen with 6 mm diameter circular holes.

Table 5.4.2.1 – Fatty acid profile of the used oils and minced fish products on the 1st and 105th storage day at 2 and 10 ºC*†.

FATTY ACIDS (%)	Vegetable oil	Cod liver oil	1st storage day		105th storage day at 2 ºC		105th storage day at 10 ºC	
			Control product	CLO product	Control product	CLO product	Control product	CLO product
Myristic (14:0)	0.1 ± 0.0	3.5 ± 0.0	0.3 ± 0.0	2.0 ± 0.0	0.4 ± 0.0	2.0 ± 0.0	0.3 ± 0.0	2.0 ± 0.1
Palmitic (16:0)	6.4 ± 0.0	10.4 ± 0.2	8.2 ± 0.1	10.4 ± 0.0	8.4 ± 0.2	10.7 ± 0.2	8.5 ± 0.2	11.1 ± 0.1
Stearic (18:0)	3.4 ± 0.0	2.2 ± 0.0	3.8 ± 0.0	3.1 ± 0.0	3.8 ± 0.1	3.0 ± 0.0	3.8 ± 0.1	3.0 ± 0.0
Other saturated	0.0 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.5 ± 0.0
Total saturated	9.9 ± 0.0	16.6 ± 0.2	12.4 ± 0.1	15.8 ± 0.0	12.6 ± 0.1	16.1 ± 0.2	12.8 ± 0.3	16.6 ± 0.1
Palmitoleic (16:1 ω7)	0.1 ± 0.0	7.2 ± 0.0	0.6 ± 0.0	3.9 ± 0.0	0.6 ± 0.0	4.2 ± 0.1	0.6 ± 0.0	4.2 ± 0.1
Oleic (18:1 ω9)	24.7 ± 0.1	17.8 ± 0.0	21.9 ± 0.1	20.2 ± 0.0	22.0 ± 0.3	20.3 ± 0.1	22.7 ± 0.1	20.7 ± 0.0
Erucic (22:1 ω9)	0.0 ± 0.0	8.1 ± 0.1	0.3 ± 0.1	3.5 ± 0.0	0.0 ± 0.0	4.0 ± 0.1	0.0 ± 0.0	3.9 ± 0.0
Other monounsaturated	0.6 ± 0.0	16.4 ± 0.0	1.6 ± 0.0	8.7 ± 0.0	0.7 ± 0.0	9.2 ± 0.0	0.7 ± 0.0	9.8 ± 0.0
Total monounsaturated	25.4 ± 0.1	49.5 ± 0.1	24.5 ± 0.1	36.3 ± 0.1	23.3 ± 0.3	37.8 ± 0.4	24.0 ± 0.1	38.7 ± 0.0
Linoleic (18:2 ω6)	63.6 ± 0.2	1.2 ± 0.0	57.1 ± 0.4	28.0 ± 0.3	56.4 ± 0.6	27.2 ± 0.5	56.2 ± 1.0	25.6 ± 0.2
Other polyunsaturated ω6	0.0 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	0.7 ± 0.0
Total polyunsaturated ω6	63.6 ± 0.2	1.7 ± 0.0	57.4 ± 0.4	28.6 ± 0.3	56.7 ± 0.6	28.1 ± 0.5	56.6 ± 1.0	26.3 ± 0.3
Eicosapentaenoic (EPA- 20:5 ω3)	0.6 ± 0.0	8.7 ± 0.0	1.1 ± 0.0	5.0 ± 0.2	1.4 ± 0.1	5.2 ± 0.2	1.5 ± 0.1	5.5 ± 0.0
Docosahexaenoic (DHA- 22:6 ω3)	0.0 ± 0.0	10.9 ± 0.1	3.3 ± 0.1	8.0 ± 0.3	3.8 ± 0.2	8.2 ± 0.1	4.1 ± 0.4	8.5 ± 0.0
Other polyunsaturated ω3	0.1 ± 0.0	5.0 ± 0.0	0.2 ± 0.0	2.5 ± 0.0	0.1 ± 0.0	2.3 ± 0.0	0.2 ± 0.0	2.5 ± 0.0
Total polyunsaturated ω3	0.7 ± 0.0	24.6 ± 0.1	4.6 ± 0.2	15.5 ± 0.5	5.2 ± 0.2	15.8 ± 0.4	5.8 ± 0.5	16.5 ± 0.0
Non-identified	0.4 ± 0.2	7.6 ± 0.2	1.1 ± 0.4	3.8 ± 0.2	2.2 ± 0.5	2.2 ± 1.3	0.8 ± 0.1	1.9 ± 0.4
ω3/ω6 ratio	0.01 ± 0.00	14.73 ± 0.18	0.08 ± 0.00	0.54 ± 0.02	0.09 ± 0.00	0.56 ± 0.01	0.10 ± 0.01	0.63 ± 0.00

* Used oils values were determined by the authors.
† Presented values correspond to mean ± standard deviation.

Five kg batches were prepared for each formulation and each storage temperature according to the following procedure.

Regarding the preparation of the minced fish batters, three sequential steps were always followed:

1) Previously, an emulsion was prepared with salt (0.2 %, w/w of final product), sodium caseinate, emulsifying protein (0.5 %, w/w), water (3.7 %, w/w) and oil (5.6 % VO, w/w, or 2.7 % VO + 2.9 % CLO, w/w) (1:2:19:28, w/w): sodium caseinate was mixed with hot water (approximately at 60 ºC) in a cup with a magnetic stirrer; oil was added and mixed with the other components for 15 minutes at 40 ºC and, then, salt was added and dissolved and mixing went on for 15 more minutes at 40 ºC. Afterwards, the produced emulsion was left overnight at 2 ± 1 ºC. Finally, it was minced one single time in the model 84145 meat grinder (Hobart, Troy, OH, USA).

2) Hake mince (80.0 %, w/w) was mixed with salt (1.3 %, w/w) and sodium polyphosphate (0.5 %, w/w) for 2 minutes at 1420 rpm and 1 minute at 2800 rpm in a model UM12 refrigerated vacuum homogeniser (Stephan and Söhne, Hameln, Germany). Throughout all process, mixing was performed always under vacuum and temperature below 7 ºC.

3) The emulsion (10.0 %, w/w), inner pea fibre (2.0 %, w/w), carrageenan (1.0 %, w/w), monosodium glutamate (0.3 %, w/w), shellfish flavour (1.0 %, w/w), MTGase (0.1 %, w/w) and water (3.8 %, w/w) were added and mixed for 2 minute at 2800 rpm.

The batters attained were put inside a model EB-12 hydraulic filler (Mainca Equipamientos Carnicos, S.L., Granollers, Spain) and ‘sausages’ with a diameter of 25 mm and a length of about 20 cm produced with cellulose casings.

Minced fish products were vacuum-packed (two ‘sausages’ per package) in low-oxygen permeable barrier bags (O_2 transmission, <2.1 $cm^3/(m^2.day.bar)$ at 23 ºC, Colamin XX 100e, Obermühle Polymertechnik GmbH, Pössneck, Germany) with a model A300/52 vacuum packager (Multivac Sepp Haggenmüller GmbH & Co. KG, Wolfertschwenden, Germany). Then, these packages were immersed in water at 35 ºC for one hour (setting), moved to a model Combi-Master CM6 oven (Rational Grossküchen Technik GmbH, Landsberg am Lech, Germany) equipped with a digital thermometer and subjected to a steam cooking at 90 ºC for one hour (cooking). In order to guarantee these conditions even in the innermost part of the product, the oven’s digital thermometer was placed in the centre of a product through a hole in the bag (approximately 10 minutes until reaching 90 ºC). After, minced fish products were immediately cooled in iced water and stored in two refrigerated rooms (at 2 ± 1 ºC and at 10 ± 1 ºC, relative humidity, 90-95 %) in the dark during approximately 105 days.

Quality Measurements

Proximate composition. Moisture, crude protein and ash were determined by standard procedures (AOAC, 1984), whereas crude fat was determined by a rapid method of total lipid extraction and purification (Bligh and Dyer, 1959) and total carbohydrate was estimated by difference.

Fatty acid profile. The fatty acids profile was determined using an acid hydrolysis method according to Bandarra et al. (1999).

pH. pH was measured using a Sen-Tix 21 surface pH electrode (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) on a model pH 539 pH meter (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany).

Water activity. Water activity (a_w) was determined using a Hygropalm AW1 a_w meter (Rotronic, Bassersdorf, Switzerland). Products were taken from the packages, the cellulose skin removed, cut into small pieces, smashed inside a Rotronic a_w plate (1.5 cm depth and 5 cm diameter) and left for 1 hour closed inside the plate at room temperature. Then, the lid of the plate was taken off and the plate was immediately put inside the measuring compartment of the device. Measurements were only taken after 15 minutes stabilization.

TBARS. TBARS (thiobarbituric acid reactive substances) were determined during storage time on 7.5 % TCA extracts by a HPLC method described by Seljeskog et al. (2006). For each fish product, type and temperature, two extracts were prepared, each one from minced fish products of a different package. Results were expressed as mg of malonaldehyde per 1000 g of fish product.

Colour. L*, a* and b* were determined on a model MACBETH COLOUR-EYE® 3000 colourimeter (Macbeth, New Windsor, NY, USA) as described previously (5.4.1). Chroma and whiteness were calculated according to formulas:

$$Chroma = \sqrt{a^{*2} + b^{*2}}$$

$$Whiteness = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

Texture measurements. Folding test, gel strength, texture profile analysis, namely hardness, cohesiveness, gumminess, springiness and chewiness as well elasticity were evaluated as in chapter 5.4.1.

For the gel strength (puncture test), samples were cut into pieces of 30 mm diameter and 25 mm high. The gel was penetrated to breaking-point with a metal probe equipped with a 5 mm diameter spherical head, using a model Instron 4301 texturometer (Instron Engineering Corp., Canton, MA, USA). The cross speed head was 10 mm/min and the load cell was 1 kN.

Regarding TPA, samples (∅, 30 mm, h, 25 mm) were compressed on the flat plate of the Instron texturometer with a cylindrical plunger (50 mm diameter) adapted to a 1 kN load cell at a deformation rate of 50 mm/min. On the basis of preliminary trials to establish a compression limit that would ensure no cracking and recoverability of most samples, it was decided to compress samples to 60 % of their height. In the test, each sample was compressed twice.

Elasticity (compression-relaxation test), the compression procedure was as for the texture profile analysis except that the sample was compressed only once for one minute and the force exerted on the sample recorded.

Sensory analysis. Sensory evaluation was conducted by ten trained panellists, which participated in preliminary trials and in the experiments that led to the development of minced fish products containing inner pea fibre. Panellists were further prepared in more than five training sessions. Following evaluation of the products in individual booths with help of a preliminary sensory evaluation sheet, results were discussed by the panellists with a twofold purpose, to develop adequate sensory parameters for the sensory features of the product and to reach common concepts and standards of judgment for each parameter. In this way, panellists became completely familiar with the sensory scheme and capable of conducting a structured scaling of products (Nielsen et al., 2002).

Products were taken out from the package, their cellulose skin removed, tempered to about 20 ℃ and cut into 2 cm long slices. These slices were distributed in white plates and presented to the panellist in random order for evaluation. The evaluation was performed in a room specifically conceived for sensory analysis and with adequate lighting.

Panellists were asked to score several sensory parameters of the product, using a 0-5 scale: colour (0 – light to 5 – dark); fish aroma, shellfish aroma and unpleasant aroma (0 – absent to 5 - excessive); elasticity (0 – plastic to 5 – elastic), hardness (0 – soft to 5 – hard), cohesiveness (0 – scarcely cohesive to 5 – very cohesive), succulence (0 – dry to 5 - succulent), unpleasant texture (0 – absent to 5 - excessive); saltiness, bitterness, fish,

shellfish and unpleasant flavour (0 – absent to 5 - excessive); and salty and astringent aftertaste (0 – absent to 5 - excessive).

Microbiological analysis. Sporulated and non-sporulated psychrophilic and mesophilic bacteria counts, as well as sporulated sulfite-reducing clostridia were determined by pour plate method over storage time. The first dilution was prepared by aseptically homogenising 10 g of fish product with 90 ml of a peptone solution (1 g/l) for 1 minute in a Stomacher blender (Seward, London, UK). Adequate serial dilutions were prepared and inoculations performed in duplicate. Serial decimal dilution volumes were treated for 10 minutes at 80 ºC for sporulated bacteria. Psychrophilic and mesophilic aerobic and anaerobic bacteria counts were incubated at 6.5 ºC for 10 days and 30.0 ºC for 3 days, respectively, in Plate Count Agar (PCA, Oxoid Ltd., Hampshire, UK). Regarding spores of sulfite-reducing clostridia, incubation was done at 37.0 ºC for 5 days in a Sulphadiazine Polymyxin Sulphite Agar (SPS, Merck KGaA, Darmstadt, Germany) under anaerobic conditions. Results were expressed as logarithm of colony forming units per gram of sample (log CFU/g).

Statistical Analysis

For each product, storage temperature and sampling date, fish products from two different packages were used. A general linear model —three-dimensional factorial ANOVA, full factorial design— was used to determine significant differences ($p<0.05$) among minced fish products with different formulation (control *vs* CLO minced fish products) and, for each product, among those stored at 2 and at 10 ºC with different storage time. Multiple comparisons were done by the Tukey HSD test. All statistical treatment was done with the software STATISTICA© from StatSoft, Inc. (Tulsa, OK, USA), version 6.1, 2003.

Results and Discussion

Nutritional and Sensory Characterization of the Products

In the development of a new product, its nutritional value as well its sensory acceptability must be thoroughly studied, since, for instance, incorporation of fish oil rich in ω3 PUFA, due to the possible effects of processing operations (namely, thermal degradation), does not ensure a corresponding high ω3 PUFA content in the product. Therefore, a nutritional characterization of the product, encompassing proximate composition and fatty acid profiles has been conducted with other innovative products (Serrano et al., 2005; Ayo et al., 2007).

Nutritional profile. Proximate composition (Table 5.4.2.2) and fatty acid (Table 5.4.2.1) profiles of both products did not show significant differences. Proximate composition, particularly, fat agreed with formulation and showed that almost all fat came from the added oil and not from hake, a lean fish. Maximum caloric content (assuming total digestion and assimilation of the carbohydrates) was identical, in the range 125-130 kcal/100 g. This value enables to consider these ready-to-eat fish products as hypocaloric. Fatty acid profile of the minced fish products also reflected that of the used oils. Thus, during processing and heating, no changes occurred in the fatty acid profiles, nevertheless, some oxidation of the PUFA ω3 may have taken place (see TBARS). For CLO products (with a mixture of vegetable and cod liver oil), an acceptable ω3/ω6 ratio was achieved, 0.54 ± 0.02 and a recommended daily intake of 500 mg EPA+DHA (Kris-Etherton et al., 2002) ensured, if a 100 g serving is considered. For control, 190 mg of EPA+DHA were provided *per* 100 g serving and the ω3/ω6 ratio was low, 0.08 ± 0.00.

Table 5.4.2.2 – Proximate composition of the minced fish products[*†].

PROXIMATE COMPOSITION (%)	1st storage day		105th storage day at 2 ºC		105th storage day at 10 ºC	
	Control product	CLO product	Control product	CLO product	Control product	CLO product
Moisture	72.4 ± 0.2^{a}	71.7 ± 0.3^{ab}	72.5 ± 0.1^{a}	71.5 ± 0.5^{ab}	71.2 ± 0.2^{bc}	70.5 ± 0.1^{c}
Protein	14.4 ± 0.1^{a}	14.4 ± 0.0^{a}	14.9 ± 0.5^{ab}	14.3 ± 0.3^{a}	15.5 ± 0.1^{b}	14.8 ± 0.1^{ab}
Fat	5.7 ± 0.1^{a}	5.7 ± 0.3^{a}	5.0 ± 0.0^{a}	5.3 ± 0.2^{a}	5.0 ± 0.1^{a}	5.6 ± 0.0^{a}
Ash	3.25 ± 0.03^{ab}	3.09 ± 0.01^{a}	3.27 ± 0.15^{ab}	3.24 ± 0.06^{ab}	3.34 ± 0.03^{b}	3.32 ± 0.03^{ab}
Carbohydrate	4.2 ± 0.2^{a}	5.2 ± 0.1^{a}	4.4 ± 0.7^{a}	5.6 ± 1.1^{a}	5.0 ± 0.3^{a}	5.8 ± 0.0^{a}

[*] Presented values correspond to mean ± standard deviation.
[†] Means within a row with different letters are significantly different ($p<0.05$).

Sensory analysis. The use of CLO in the products had only a significant effect ($p<0.05$) on the mean sensory colour score, which was perceived as darker: 0.4-0.8 in CLO product *vs* 0.1-0.3 in control product (Figures 5.4.2.1 and 5.4.2.2). The remaining sensory properties were unchanged and were favourably evaluated by panellists at 2 and 10 ºC. Thus, these two ready-to-eat fish products, besides being healthy (hypocaloric, containing dietary fiber and, for CLO product, ω3 fatty acids), are potentially attractive and enjoyable for consumers.

Quality Changes during Storage

Concerning quality changes during storage, different kinds of new products have been studied with respect to their physical (colour and texture), chemical (composition and oxidation indices), sensory or microbiological properties: surimi and surimi-derived seafood (Park et al., 2004; Pérez-Mateos et al., 2004), fish sausages (Rahman et al., 2007; Panpipat and Yongsawatdigul, 2007; 5.4.1) or fish burgers (Al-bulushi et al., 2005).

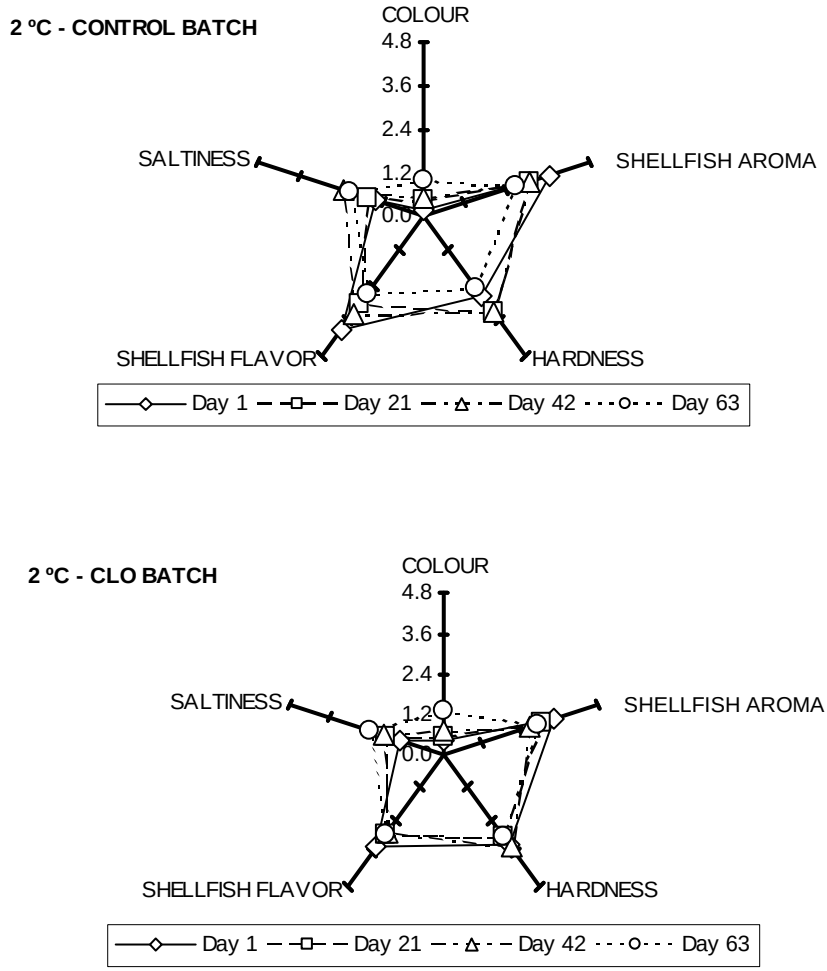

Figure 5.4.2.1 – Minced fish products' sensory parameters variation during storage time at 2 ºC.

Some of these products remained without great quality changes for considerable periods of time, for instance, fish burgers frozen at -20 ºC were acceptable for three months (Al-bulushi et al., 2005) or chilled crab analogues containing menhaden oil exhibited little change in sensory properties throughout two months of storage (Pérez-Mateos et al., 2004).

Proximate composition and fatty acid profile. For both storage temperatures, fat and carbohydrate did not change over storage time (Table 5.4.2.2). However, for moisture, protein and ash, changes were observed after 105 days at 10 ºC. These slight but significant ($p<0.05$) alterations in the chemical composition seem to indicate some water loss (< 1.5 %) during storage at the chosen abuse temperature. In fact, with increasing storage time, minced fish products became in general significantly saltier (see Sensory Analysis), thereby, suggesting a correlation between saltiness and moisture. Furthermore, storage time and temperature had no significant and plausible effect on the fatty acid contents (Table 5.4.2.1).

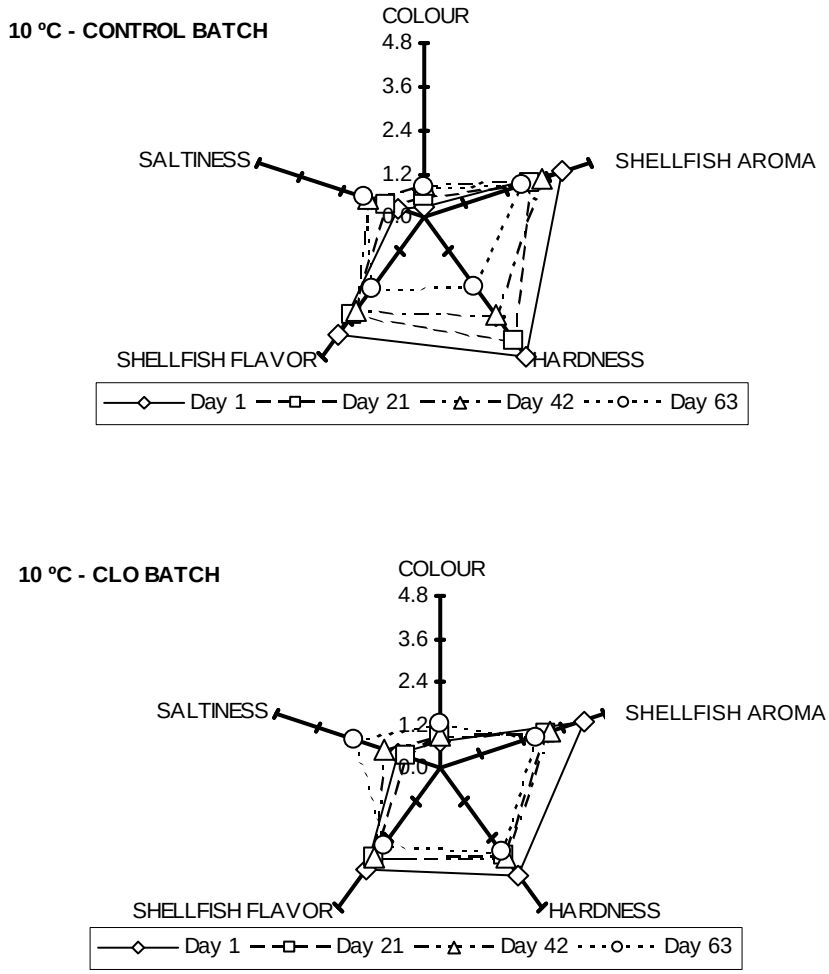

Figure 5.4.2.2 – Minced fish products' sensory parameters variation during storage time at 10 ºC.

pH and water activity. Regarding pH variation (Figure 5.4.2.3), temperature had a great and significant effect ($p<0.05$) and minced fish products held at 10 ºC presented a specific time evolution with oscillations, while at 2 ºC the values remained almost unchanged. At 10 ºC, there was an initial (until day 21) pH increase followed by a large decline from 7.06 ± 0.01 – 7.12 ± 0.01 to 6.01 ± 0.01 – 6.07 ± 0.06 (day 63) and, finally, a differentiated recovery depending on the product type, for control to 6.24 ± 0.10 whereas for CLO to 6.83 ± 0.05.

These variations suggest that higher storage temperature favoured different processes. In particular, the observed decline could be the result of biochemical or chemical reactions producing acidic compounds, for instance, hydrolytic spoilage (Cmolik and Pokorny, 2000). In this process, bonds between the fatty acids and the other components of fat, namely, glycerol, are broken, producing free fatty acids, whose OH group may have a slightly acidifying effect. Future studies should help to clarify this issue.

Water activity of the products ranged from 0.962 ± 0.017 to 0.984 ± 0.002 and no significant differences were determined in function of formulation, storage temperature

and time. In accordance with moisture reduction over storage time, a slight decline of water activity could be expected, its absence may be related to the weakening of the interactions between proteins and water molecules, thus augmenting the relative portion of unbonded water in the total.

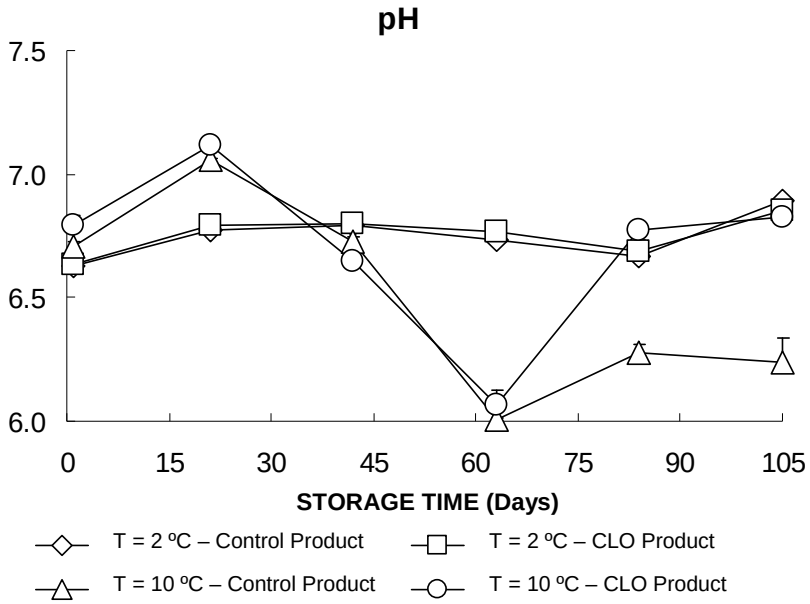

Figure 5.4.2.3 – Minced fish products' pH variation during storage time.

TBARS. Throughout storage time, malonaldehyde (MDA) was detected as TBARS in the chromatograms. Significant differences ($p<0.05$) were observed between formulations (Figure 5.4.2.4), showing CLO minced fish products higher MDA content irrespective of temperature and time. Regarding storage time, in spite of some oscillations, a common pattern was shown, a first increasing phase followed by a decline.

The higher oxidation levels found in CLO minced fish products result from the high levels of ω3 PUFA, which are more prone to oxidation (Pérez-Mateos et al., 2004), producing as secondary products aldehydes, such as malonaldehyde, and ketones. A storage stability study conducted with fortified vacuum-packed fish sausages (Panpipat and Yongsawatdigul, 2007) also showed that higher levels of vegetable oil replacement by fish (tuna) oil favoured higher TBARS results. Moreover, the greater oxidation levels at the beginning of the experiment for CLO products are probably a consequence of processing during production, namely, cooking (1 hour at 90 ºC), since ω3 PUFA are more susceptible to thermal degradation than vegetable oil. In fact, according to some authors (Montero et al., 2005b), processing for mince extraction, preparation of batter and cooking make necessary the addition of antioxidants to avoid rancidity. However, even in the CLO products, TBARS value never exceeded 0.5 mg malonaldehyde/kg and was therefore considerably lower than the maximum acceptable level (1-2 μmol malonaldehyde/g fat or

≈ 4-8 mg malonaldehyde/kg) proposed by Connell (1975) for fish products. These results seem to agree with sensory analysis (see below, Sensory Analysis), since no panellist reported any rancid aroma or flavour, even in the CLO minced fish products after 105-day storage.

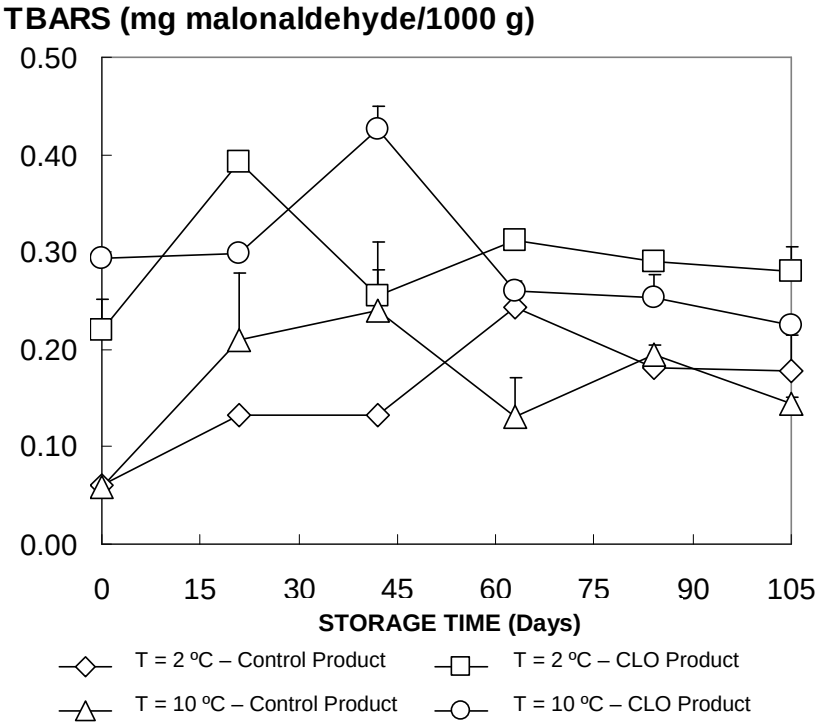

Figure 5.4.2.4 – Minced fish products' TBARS value variation during storage time.

The absence of a clear and continuous increasing trend during storage time was already observed in other vacuum packed minced fish products (5.4.1) and suggests the permanence of an anaerobic environment inside the packages over storage time. Other studies, however, reported clear correlations between TBARS and storage time under vacuum conditions (Panpipat and Yongsawatdigul, 2007). The reported increase of the TBARS values in this study over four weeks storage from 2 to 6 mg malonaldehyde/kg for a content of fish oil identical to that of CLO products points to the importance of total fat content, since more than 20 % (mostly or only soybean oil) was present in the fish sausages of this study, whereas in the minced fish products fat content was only 5.7 %. Therefore, a low content in fat, albeit much richer in ω3 PUFA and enough to guarantee 500 mg EPA+DHA *per* day and 100 g serving, may better oppose to lipid oxidation than high fat content, even when this fat is extremely poor in ω3 PUFA. Moreover, variations during storage time suggest that low rates of lipid oxidation were coupled with increased MDA degradation, due to MDA reacting with the muscle proteins (Kristinsson et al., 2006) and, thus, forcing TBARS values to decrease. Whereas, CLO products exhibited declines after 21 and 42 days for 2 and 10 ºC, respectively, control products presented the same

trend only after 42 (at 10 ºC) and 63 days (at 2 ºC). An earlier onset of oxidation in CLO products reinforces the idea that ω3 PUFA are more prone to oxidation. Furthermore, while higher temperature accelerated oxidation in the control products, the same was not found with CLO products. The higher TBARS values in CLO products could have triggered enhanced MDA degradation and, in this way, the storage temperature effect may have been attenuated.

Colour. Both formulation and storage time had effect on the minced fish products' a*, b* and chroma colour parameters (Table 5.4.2.3). On the other hand, whiteness variations did not present any clear trend. Chroma largely reflected b* values. CLO products were significantly ($p<0.05$) redder (higher a*) and more yellow (higher b*) than control minced fish products. Additionally, although only CLO products at 10 ºC produced a clear trend, minced fish products on the whole became significantly ($p<0.05$) redder and less yellow over storage time. For CLO products at 10 ºC, there was a significant variation during storage, a* increased from -1.49 ± 0.00 to -1.27 ± 0.03 and b* was reduced from 6.30 ± 0.06 to 5.55 ± 0.23.

The detected differences for a* and b* may result from pigments present in the cod liver oil, whose golden hue was much stronger than that of the vegetable oil. For both products, pigments seemed to leach out of the products (with longer storage time, a small amount of yellow liquid appeared in the packages). For CLO products (more pigmented), temperature had clearly a role in these processes. Other fish heat-induced gel products with incorporated fish oil did not present any time effect upon hue parameters during two months chilled storage (Pérez-Mateos et al., 2004). Nonetheless, the instrumentally detected differences were not perceived by the human eye (see below, Sensory analysis).

Texture measurements. Products containing cod liver oil showed lower ($p<0.05$) gel strength (GS), 20.4 ± 3.5 *vs* 22.3 ± 4.5 N.mm (global averages for both products regardless of time and temperature), however, other textural properties were similar to those of the control product (Table 5.4.2.4). Storage temperature had a significant decreasing effect ($p<0.05$) upon elasticity, hardness, cohesiveness, gumminess and chewiness. Furthermore, products containing only vegetable oil, when stored at 10 ºC, showed, particularly after two months storage, loss of textural quality (hardness, 22.3 ± 4.4 *vs* 31.5 ± 1.3 N, cohesiveness, 0.31 ± 0.06 *vs* 0.47 ± 0.02, gumminess, 7.2 ± 3.2 *vs* 15.0 ± 0.4 N and chewiness, 5.2 ± 2.8 *vs* 11.6 ± 0.4 N). Regarding folding test, 2.3 ± 0.6 *vs* 3.0 ± 0.0, and springiness, 0.69 ± 0.08 *vs* 0.77 ± 0.01, no significant effect was found.

Table 5.4.2.3 – Colour variation of minced fish products during storage time at 2 and 10 ºC.[*†]

CONTROL MINCED FISH PRODUCTS AT 2 ºC					CONTROL MINCED FISH PRODUCTS AT 10 ºC				
STORAGE TIME (days)	a*	b*	whiteness	chroma	STORAGE TIME (days)	a*	b*	whiteness	chroma
1	-1.62 ± 0.03^{a}	5.88 ± 0.13^{ab}	78.0 ± 0.8^{ab}	6.10 ± 0.14^{ab}	1	-1.62 ± 0.01^{ab}	5.91 ± 0.07^{a}	77.8 ± 0.4^{ab}	6.13 ± 0.07^{a}
21	-1.57 ± 0.02^{a}	6.00 ± 0.19^{a}	78.2 ± 0.9^{ab}	6.21 ± 0.19^{a}	21	-1.69 ± 0.02^{b}	5.79 ± 0.08^{ab}	79.1 ± 0.1^{a}	6.03 ± 0.07^{a}
42	-1.58 ± 0.02^{a}	5.31 ± 0.06^{c}	76.9 ± 0.3^{a}	5.54 ± 0.06^{c}	42	-1.57 ± 0.00^{ac}	5.50 ± 0.04^{abc}	80.0 ± 0.4^{a}	5.72 ± 0.04^{abc}
63	-1.62 ± 0.02^{a}	5.73 ± 0.01^{abc}	78.7 ± 0.2^{ab}	5.96 ± 0.01^{abc}	63	-1.51 ± 0.03^{cd}	5.07 ± 0.22^{c}	75.9 ± 1.4^{b}	5.29 ± 0.22^{c}
84	-1.61 ± 0.00^{a}	5.38 ± 0.05^{c}	78.7 ± 0.5^{ab}	5.62 ± 0.05^{c}	84	-1.48 ± 0.03^{d}	5.36 ± 0.14^{bc}	77.7 ± 0.8^{ab}	5.56 ± 0.14^{bc}
105	-1.61 ± 0.03^{a}	5.43 ± 0.02^{bc}	80.0 ± 0.1^{b}	5.66 ± 0.01^{bc}	105	-1.57 ± 0.05^{ac}	5.58 ± 0.11^{ab}	79.4 ± 0.1^{a}	5.80 ± 0.12^{ab}
CLO MINCED FISH PRODUCTS AT 2 ºC					CLO MINCED FISH PRODUCTS AT 10 ºC				
STORAGE TIME (days)	a*	b*	whiteness	chroma	STORAGE TIME (days)	a*	b*	whiteness	chroma
1	-1.46 ± 0.01^{ac}	6.38 ± 0.15^{a}	79.4 ± 0.7^{ab}	6.55 ± 0.15^{a}	1	-1.49 ± 0.00^{a}	6.30 ± 0.06^{a}	79.6 ± 0.1^{a}	6.47 ± 0.06^{a}
21	-1.38 ± 0.01^{b}	6.25 ± 0.04^{a}	78.1 ± 0.3^{ab}	6.40 ± 0.04^{a}	21	-1.43 ± 0.01^{a}	6.00 ± 0.02^{ab}	78.5 ± 0.2^{a}	6.17 ± 0.02^{ab}
42	-1.44 ± 0.00^{abc}	6.29 ± 0.04^{a}	80.4 ± 0.1^{a}	6.45 ± 0.04^{a}	42	-1.34 ± 0.00^{b}	5.81 ± 0.13^{b}	78.6 ± 0.5^{a}	5.96 ± 0.12^{b}
63	-1.48 ± 0.01^{c}	6.19 ± 0.02^{a}	79.7 ± 0.1^{ab}	6.36 ± 0.01^{a}	63	-1.33 ± 0.00^{bc}	5.92 ± 0.22^{ab}	79.6 ± 0.9^{a}	6.06 ± 0.21^{ab}
84	-1.39 ± 0.00^{ab}	5.92 ± 0.07^{a}	78.0 ± 0.1^{b}	6.08 ± 0.07^{a}	84	-1.26 ± 0.03^{c}	5.74 ± 0.05^{b}	78.6 ± 0.0^{a}	5.87 ± 0.04^{b}
105	-1.36 ± 0.00^{b}	6.01 ± 0.10^{a}	78.3 ± 0.2^{ab}	6.17 ± 0.10^{a}	105	-1.27 ± 0.03^{bc}	5.55 ± 0.23^{b}	78.3 ± 1.2^{a}	5.70 ± 0.23^{b}

[*] Presented values correspond to mean ± standard deviation.
[†] Means within a column with different letters are significantly different ($p<0.05$).

First and foremost, these results show that partial vegetable oil replacement by fish oil has, in general, no deleterious effect upon textural quality, as in other studies (Panpipat and Yongsawatdigul, 2007). The difference found for GS values, although statistically significant, is slight and, as such, may result from random factors.

Concerning storage temperature, the worse textural quality at 10 ºC results from enhanced chemical and biochemical spoilage processes (protein denaturation and aggregation) at higher temperature. Nevertheless, panellists only found a lower elasticity in minced fish products stored at 10 ºC (see below Sensory Analysis). Sensory hardness and cohesiveness presented no variation with temperature. This could be due to higher instrumental sensitivity and accuracy, but also to an imperfect instrumental reproduction of the human textural concepts.

Taking into consideration that storage time had only effect on control products at 10 ºC, it seems therefore that cod liver oil, in spite of its negative influence upon GS, may have beneficial characteristics for the preservation of the minced fish products' hardness and cohesiveness (gumminess and chewiness values essentially reflect these parameters) at higher temperatures. Nevertheless, it can be hypothesized that the lower moisture value of CLO products at 10 ºC, entailing a larger water loss, may have been able to compensate the softening effects of protein denaturation and aggregation over storage time. In fact, other authors have found that water loss can increase protein concentration in the product and thereby harden it, while protein quality and GS decline (Andrés et al., 2006). Sensory evaluation also yielded a hardness reduction for minced fish products containing vegetable oil at 10 ºC. These results also indicate that GS and its component breaking force may not correlate well with hardness, similar lack of correlation between the two types of rheological measurement was reported by other authors, who suggested that penetration test measures the density of actomyosin, while compression test reveals the overall binding property of the gel material (Lee and Chung, 1989). Regarding GS, control products at 10 ºC also presented an increase from day 1 to day 21 and, furthermore, similar increases, although not significant, were measured in the other three batches. This variation could be due to bond realignment in the first weeks (Pérez-Mateos et al., 2004). Other authors have not detected any storage time effect on texture (Panpipat and Yongsawatdigul, 2007), however, such studies were conducted always at lower temperature (4 ºC) and over shorter storage times (four weeks).

Table 5.4.2.4 – Textural properties variation of minced fish products during storage time at 2 and 10 ºC.[*†]

STORAGE TIME (days)	CONTROL MINCED FISH PRODUCTS AT 2 ºC						STORAGE TIME (days)	CONTROL MINCED FISH PRODUCTS AT 10 ºC					
	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Chewiness (N)		Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Chewiness (N)
1	21.1 ± 1.9^{a}	56.7 ± 0.1^{a}	34.9 ± 1.1^{a}	0.43 ± 0.01^{a}	14.8 ± 0.6^{a}	11.0 ± 0.5^{a}	1	16.4 ± 3.0^{a}	55.8 ± 0.5^{a}	31.5 ± 1.3^{a}	0.47 ± 0.02^{a}	15.0 ± 0.4^{ab}	11.6 ± 0.4^{ab}
21	25.1 ± 3.1^{a}	55.5 ± 1.5^{a}	34.0 ± 1.8^{a}	0.48 ± 0.01^{a}	16.8 ± 0.5^{a}	13.1 ± 0.5^{a}	21	25.0 ± 3.8^{b}	54.1 ± 0.5^{a}	34.7 ± 3.0^{a}	0.47 ± 0.04^{a}	15.8 ± 0.9^{a}	12.3 ± 0.6^{a}
42	18.4 ± 2.4^{a}	56.3 ± 0.3^{a}	35.2 ± 2.4^{a}	0.46 ± 0.04^{a}	16.7 ± 0.6^{a}	13.2 ± 0.8^{a}	42	20.5 ± 3.1^{ab}	57.9 ± 0.9^{a}	34.8 ± 2.4^{a}	0.45 ± 0.01^{ab}	16.3 ± 0.6^{a}	12.5 ± 0.5^{a}
63	20.5 ± 2.4^{a}	55.5 ± 0.0^{a}	35.0 ± 2.8^{a}	0.41 ± 0.04^{a}	14.3 ± 2.5^{a}	10.9 ± 2.0^{a}	63	24.9 ± 5.9^{b}	43.1 ± 2.9^{a}	27.5 ± 5.5^{ab}	0.36 ± 0.04^{ab}	10.4 ± 3.3^{ab}	7.9 ± 2.8^{ab}
84	24.3 ± 2.1^{a}	58.5 ± 0.2^{a}	34.8 ± 2.7^{a}	0.49 ± 0.01^{a}	18.0 ± 0.9^{a}	14.3 ± 0.7^{a}	84	26.8 ± 4.9^{b}	36.9 ± 2.0^{a}	22.3 ± 4.4^{b}	0.31 ± 0.06^{b}	7.2 ± 3.2^{b}	5.2 ± 2.8^{b}
105	21.5 ± 3.8^{a}	58.7 ± 0.3^{a}	38.6 ± 1.5^{a}	0.47 ± 0.02^{a}	17.9 ± 0.9^{a}	13.9 ± 0.8^{a}	105	23.4 ± 4.9^{b}	42.6 ± 3.6^{a}	28.6 ± 8.7^{ab}	0.33 ± 0.14^{ab}	9.5 ± 5.6^{ab}	7.1 ± 5.1^{ab}
STORAGE TIME (days)	CLO MINCED FISH PRODUCTS AT 2 ºC						STORAGE TIME (days)	CLO MINCED FISH PRODUCTS AT 10 ºC					
	Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Chewiness (N)		Gel strength (N.mm)	Elasticity (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Chewiness (N)
1	18.5 ± 1.6^{a}	54.4 ± 1.3^{a}	32.3 ± 1.5^{a}	0.45 ± 0.03^{a}	14.8 ± 1.8^{a}	11.5 ± 1.4^{a}	1	15.3 ± 4.2^{a}	44.4 ± 2.4^{a}	28.1 ± 1.6^{a}	0.40 ± 0.01^{a}	11.0 ± 0.6^{a}	8.1 ± 0.4^{a}
21	24.0 ± 0.8^{a}	58.4 ± 0.1^{a}	32.7 ± 3.1^{a}	0.46 ± 0.01^{a}	15.7 ± 0.7^{a}	12.0 ± 0.4^{a}	21	19.1 ± 1.2^{ab}	53.2 ± 0.1^{a}	28.1 ± 2.7^{a}	0.43 ± 0.05^{a}	12.1 ± 2.4^{a}	9.5 ± 2.0^{a}
42	22.3 ± 1.3^{a}	52.4 ± 0.2^{a}	34.1 ± 2.7^{a}	0.47 ± 0.09^{a}	15.2 ± 3.6^{a}	12.4 ± 3.5^{a}	42	24.1 ± 2.4^{b}	49.0 ± 0.5^{a}	28.7 ± 3.0^{a}	0.42 ± 0.06^{a}	12.5 ± 3.2^{a}	9.5 ± 2.7^{a}
63	20.5 ± 2.2^{a}	57.6 ± 0.6^{a}	35.4 ± 2.0^{a}	0.45 ± 0.03^{a}	15.6 ± 2.0^{a}	12.1 ± 1.8^{a}	63	20.4 ± 3.7^{ab}	57.8 ± 0.1^{a}	33.1 ± 2.5^{a}	0.45 ± 0.06^{a}	14.9 ± 3.3^{a}	11.8 ± 3.0^{a}
84	22.4 ± 2.6^{a}	55.6 ± 2.0^{a}	36.2 ± 1.6^{a}	0.47 ± 0.03^{a}	16.9 ± 1.2^{a}	13.3 ± 1.1^{a}	84	18.3 ± 3.5^{ab}	53.2 ± 8.2^{a}	30.4 ± 4.2^{a}	0.41 ± 0.08^{a}	12.5 ± 2.5^{a}	9.9 ± 2.5^{a}
105	20.3 ± 1.6^{a}	55.4 ± 5.5^{a}	36.3 ± 3.1^{a}	0.44 ± 0.01^{a}	15.8 ± 2.0^{a}	12.1 ± 1.6^{a}	105	20.7 ± 4.5^{ab}	47.9 ± 6.1^{a}	27.9 ± 8.7^{a}	0.38 ± 0.08^{a}	10.8 ± 6.0^{a}	8.3 ± 4.9^{a}

[*] Presented values correspond to mean ± standard deviation.
[†] Means within a column with different letters are significantly different ($p<0.05$).

Sensory analysis. Products stored at 10 ºC scored as less salty and elastic ($p<0.05$) (data not shown), being the other sensory properties unchanged by temperature. With increasing storage time, minced fish products became in general significantly darker and saltier and their shellfish aroma and flavour was progressively lost, presenting the control products at 10 ºC a pronounced hardness decline ($p<0.05$) (Figures 5.4.2.1 and 5.4.2.2).

Whereas temperature effect on elasticity corroborates instrumental determination (see above, Texture Measurements), the reduced saltiness at 10 ºC is unexpected because storage time increased saltiness, a possible consequence of water loss, making this sensory parameter a deterioration marker. Furthermore, saltiness almost doubled (from lower starting values) in CLO products at 10 ºC, precisely, the batch which presented a lower moisture value. On the whole, a higher temperature did not bring about many sensory changes over storage time: the aroma and flavour characteristics of the product were only affected by a modest decline of the shellfish component and a slight rise of the saltiness (which may well be also a result of the contrast with a reduced shellfish flavour); no bitter or unpleasant flavour was detected. The darkening had no correlation with instrumental measurements (chroma declined and no significant whiteness variation occurred). Hardness reduction, for control products at 10 ºC, exhibited a strong time effect, besides, supported by textural measurements. Autolytic processes driven by enzymes and chemical reactions, both favoured by higher temperatures, can have a major impact on the loss of textural quality, particularly during the early stages of deterioration but, generally, do not produce the characteristic off-flavours and off-odours, which are typical of microbiological activity (Truelstrup et al., 1996). As microbiological growth was not detected (see below, Microbiological Analysis), off-flavour and off-odour absence are partially explained. Moreover, the characteristics of the cod liver oil used (purified, deodourized) may have contributed for these results. According to Pérez-Mateos et al. (2004) the incorporation of fish oil concentrate in crab analogues favoured the development of fishy flavour and aroma within 30 days, refined menhaden and purified marine oils incorporation caused little change in sensory properties throughout two months of storage. Furthermore, the added shellfish flavour masked any faint fish-like flavour. This agrees with the findings of Kolanowski et al. (1999), who showed that high flavour intensity products are more effective at masking the slightly fishy taste and odour associated with the addition of fish oil. The remaining sensory properties were unchanged and products' acceptability by panellists (including control product at 10 ºC) did not change throughout two months storage (until last sensory evaluation on day 63).

Microbiological analysis. The simultaneous application of heat treatment (one hour at 90 ºC), vacuum packaging, chilled storage and salt addition enabled a good

microbiological quality throughout storage time. Heat treatment effectively eliminated all microorganisms initially present in the minced fish products' batters (Figure 5.4.2.5). Before heat treatment, no sporulated psychrophilic anaerobic bacteria and sulfite-reducing clostridia growth was detected. Regarding the other counts, though growth was observed, values were low —non-sporulated mesophilic bacteria gave the highest count, only 2.3 ± 0.1 log CFU/g. Therefore, raw material presented a good microbiological quality.

Formulation, storage temperature and time had no effect on the microbial growth, which was always absent until the end of experiment (day 105). Detection of non-sporulated bacteria in raw material followed by its absence in the heat treated product agrees with Stumbo (1973), as the applied heat treatment (enabling, at least, 15 log cycle reduction in the number of most vegetative forms) should prevent the survival of the vegetative cells present in the raw material. Nevertheless, further growth was possibly prevented by packaging conditions and storage temperature.

These microbial counts were lower than those reported in 5.4.1 in vacuum-packed and pasteurised minced fish products submitted to similar storage conditions (2 ± 1 ºC over a 80-day period). Probably, the higher pasteurisation time applied to minced fish products (one hour *vs* 10 minutes) can explain such differences.

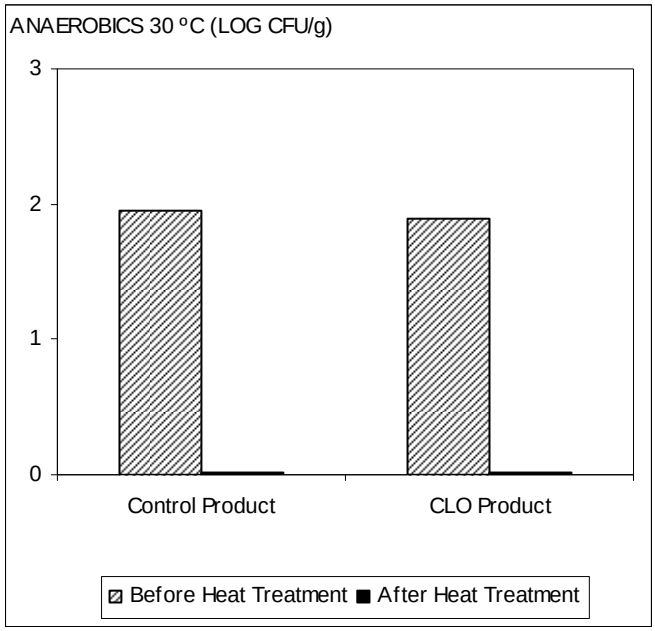

Figure 5.4.2.5 – Effect of heat treatment on the minced fish products' anaerobic bacteria counts at 30 ºC.

Conclusions

Both products were low caloric (125-130 kcal/100 g) and CLO product ensured, per 100 g serving, the 500 mg recommended daily intake of eicosapentaenoic and docosahexaenoic acids, given its higher ω3/ω6 ratio (0.54 ± 0.02 *vs* 0.08 ± 0.02). Sensory

analysis yielded a positive evaluation. Thus, a partial replacement of vegetable oil by cod liver oil did not induce any sensory quality loss.

The two groups of minced fish products presented also acceptable sensory scores as well no microbiological growth during 3.5 months. Products containing cod liver oil showed lower gel strength, however, other textural properties were similar to those of the other products. Although, throughout the experiment, TBARS values were higher in the fish products containing cod liver oil, no fishy or rancid flavour/aroma was detected.

During storage time, products became redder and less yellow, while their seafood aroma and flavour declined and their saltiness augmented. Moreover, storage at higher temperature had a negative effect on the sensory quality (reduced elasticity) and instrumental texture (lower elasticity, hardness, cohesiveness, gumminess and chewiness). Furthermore, fish products containing only vegetable oil at 10 ºC showed, particularly after two months storage, loss of textural quality (hardness, cohesiveness, gumminess and chewiness).

Therefore, these results taken together have shown that the combination of high purity of the CLO, low CLO (2.9 %, w/w) and low fat (5.6 %, w/w) contents and vacuum-packaging of the products may be enough for preventing additional quality losses in the CLO products with respect to VO products, even after a prolonged storage time at the chosen abuse temperature, 10 ºC.

Acknowledgments

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6 - General Discussion and Conclusions

Portugal has the second highest European consumption of seafood products, with a per capita value of 41.2 kg (Cardoso et al., 2010a). However, the national production is according to latest available data about 169000 tons (INE, 2008) or about 16.1 kg per capita. Therefore, most seafood consumed in Portugal comes from abroad. The main landed species in Portuguese harbours during 2007 were: sardine (36.1 %), chub mackerel (12.7 %), horse mackerel (6.4 %), common octopus (5.3 %), black scabbard fish (4.0 %), blue whiting (2.4 %), and Atlantic mackerel (1.6 %) (INE, 2008). Some of these species are underutilized, since a significant portion is discarded on board or after landing. Moreover, there are withdrawals in the fish auctions in order to ensure a minimal price level. The percentages of discards/withdrawals are highest for Atlantic mackerel (14.9 % of the total landings), followed by sardine (9.0 %), chub mackerel (3.1 %), and horse mackerel (1.0 %) (INE, 2008). This represents more than 5000 tons of fish. Particularly, mackerel species are responsible for a large quantity of discards due to low commercial value and, sometimes, to fish presenting a size below the legal limits (Cabral et al., 2003). On the other hand, frozen hake, whose imports reach 28000 tons per year (INE, 2008), are processed by Portuguese factories, thereby yielding wastes up to 10 % of the raw material weight, namely trimmings from the cutting and filleting operations (Moreno et al., 2010a). Regarding farmed species, the saturation of the market for whole fresh fish has been promoting the production of ready-to-eat foods, which involve filleting and the generation of by-products. In Portugal, the two main farmed fish species are gilthead sea bream and sea bass, which represent 20.6 % (1623 tons) and 20.1 % (1584 tons) of the total national production (INE, 2008).

The problems raised by these significant amounts of by-products and underutilized fish species in Portugal require innovative solutions. Accordingly, the experimental work was carried out with the main purpose of testing new strategies for the upgrading of underutilized fish species and by-products from the fish processing industry (Figure 6.1). Different technological solutions were applied, such as DF or MTGase incorporation and/or new processing techniques for gel induction. These solutions were applied to different raw materials, namely, hake, sea bass and gilthead sea bream trimmings (muscle joined to the bones and skin) and Atlantic and chub mackerel. The latter are underutilized fish species (particularly Atlantic mackerel) and a largely wasted animal protein resource. As explained above, these different raw materials were chosen for reasons of representativeness of both the by-products and underutilized fish species, taking into account the specific Portuguese situation.

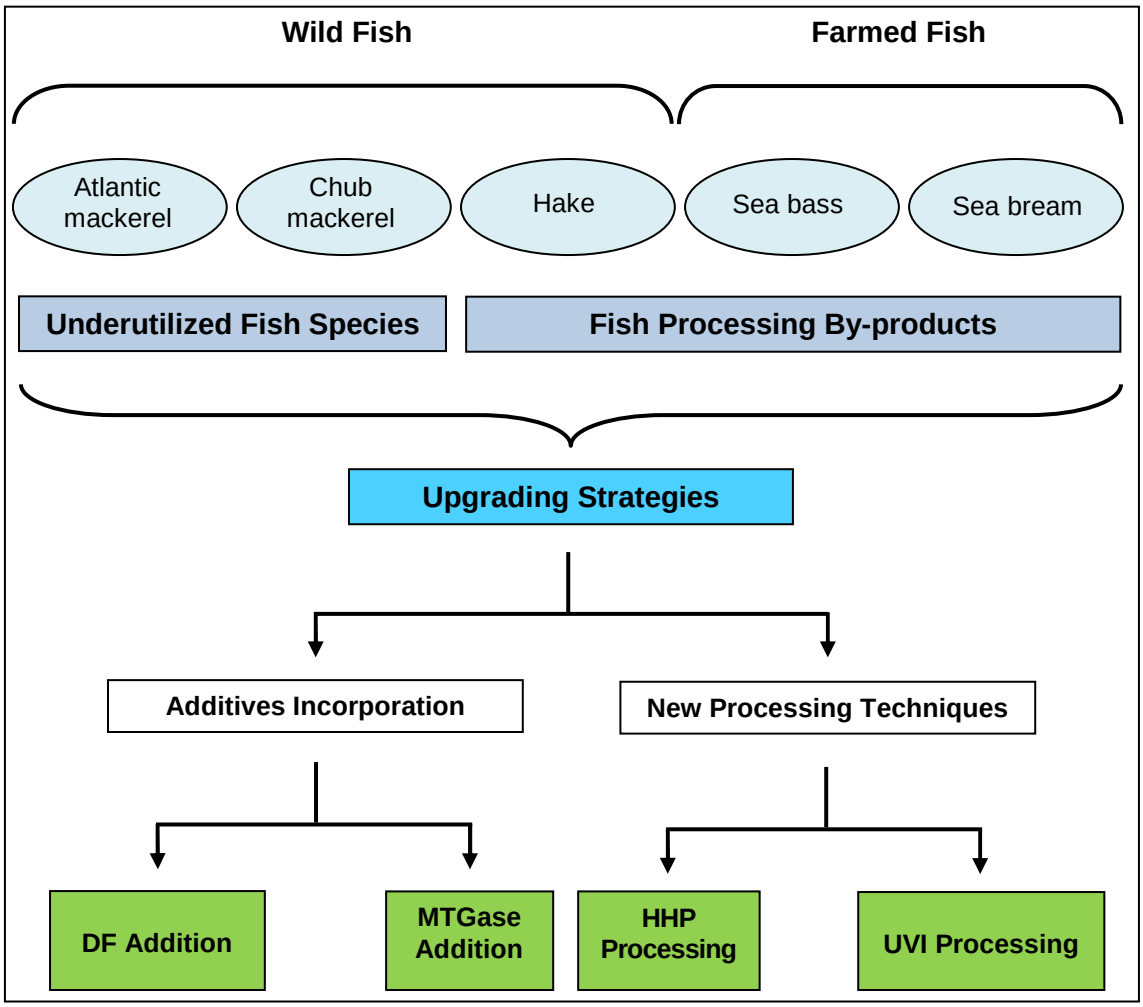

Figure 6.1 – General outline of the experimental work.

In this experimental work, different kinds of intermediate and final products, encompassing a wide range of properties and applications, were produced (Figure 6.2). The final products were all restructured products (see chapter 1.3), whether prepared from minced fish or surimi (minced fish subjected to different washing steps), and were gel or emulsion products. In the latter, their high fat content had a larger impact than any gelation phenomena (for instance, Frankfurter sausages). Whereas minced fish was produced from all studied species with exception of mackerel, surimi was produced only from mackerel.

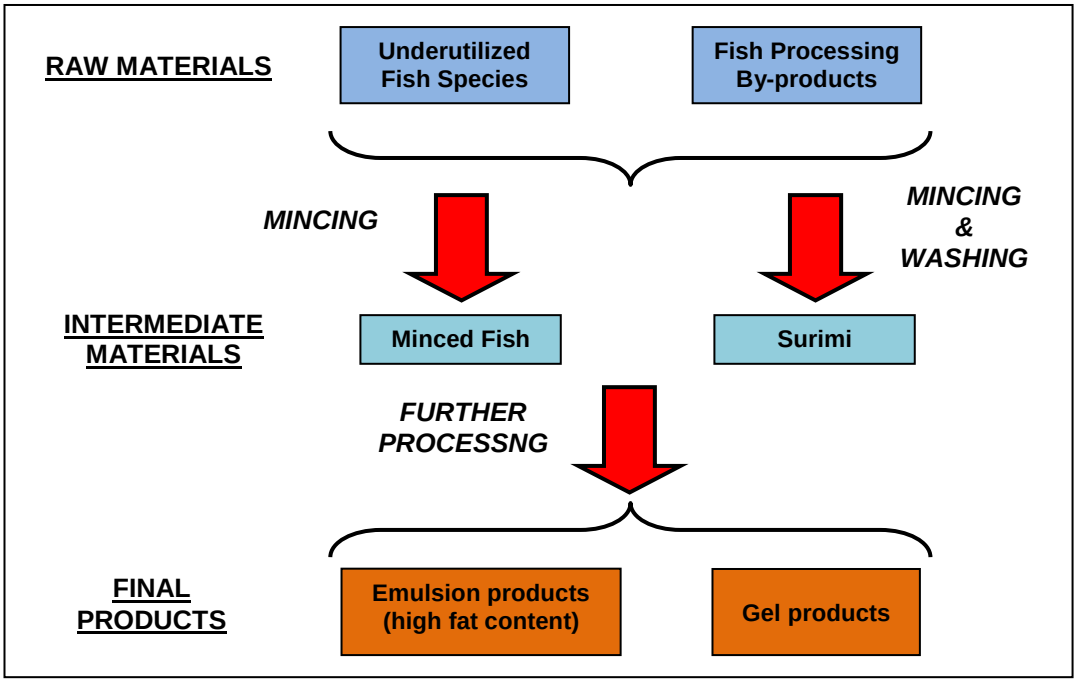

Figure 6.2 – Raw, intermediate materials, and final products used and/or produced in this experimental work.

This general discussion comprises five different areas where the main experimental work was done along the four main objectives delineated in the Ph.D. programme. For each of the first two objectives (2.1 - New fish mince products – Effect of additives and 2.2 - New processing technologies), the focus of research was directed to two different areas, corresponding to two different levels of study: a more fundamental one at the molecular level, encompassing fish protein bonding and the biochemical mechanisms of gelation and other more applied at the level of the food quality properties, such as texture, and colour. In this way, the research work strove to correlate those food properties measured at a macroscopic level with the underlying molecular phenomena, in order to better understand the effect of the incorporated additives or of the applied gelation technologies. This understanding may help in the choice of the additives and/or technologies that deserve to be tested in processed fish products. The knowledge of the biochemistry of fish proteins combined with the knowledge of the biochemistry of an additive or of the effects of a new gelation technology on proteins offers clues regarding which additives or technologies are worth to be studied, if a given product with a specific texture profile is to be achieved. For this reason, this experimental work was carried out ahead of the experiments concerning the design of innovative fish products, such as, a Frankfurt fish sausage, precisely to narrow the range of additive or technological choices to be tested in the development of such products. This development was done in accordance to the other two objectives (2.3 - Fish substitution in meat products and 2.4 - Storage stability studies). Finally and as programmed in the last objective, the

developed fish products were subjected to a storage stability study, which envisaged a thorough assessment of the quality changes brought about by time and conditions of storage.

This overall experimental design ensured that the main objective of this work —to find upgrading strategies for underutilized fish species and by-products of the fish processing industry— was fulfilled, since, previously, the chemical (additives) and technological means for the development of value-added products were tested and optimized.

Discussion follows a clear logical sequence and encompasses following chapters: 6.1 - Fish proteins and gelation mechanisms; 6.2 - Effect of additives on restructured fish products; 6.3. - Effect of new processing technologies on restructured fish products; 6.4. - Development of new fish products and 6.5 - Storage stability of developed fish products.

6.1 – Fish Proteins and Gelation Mechanisms

This chapter mainly shows the results attained under objectives 2.1 - New fish mince products – Effect of additives and 2.2 - New processing technologies, and its main purpose is to establish the main biochemical phenomena underlying fish protein gelation and the observed effects of additives and new gelation technologies.

The first major point must be the assessment of the most important biochemical aspects at the protein level which contribute decisively to a fish gel with good properties, that is, a high quality texture —taking into consideration the folding test (FT), gel strength (GS), hardness, cohesiveness or springiness—, water holding capacity, and an adequate colour. Protein bonding encompasses different chemical interactions (see 1.4) between different proteins or within each protein itself (Figure 6.3). The energy associated to each kind of interaction is different, ranging from weak bonds, such as hydrophobic bonds, to covalent bonds, whose rupture requires a large amount of energy.

One fundamental question addressed by this work was the relative importance of these bonds for the attainment of an improved product. This is important, since it can offer clues regarding the best additives and technologies to be applied to processed fish products. According to some authors (Havea *et al.*, 2004; Havea *et al.*, 2009), gel hardness may be positively correlated with hydrophobic bonding, and a more rubbery texture may be due to an enhancement of disulphide (covalent) bonding. These authors worked with whey protein concentrate (WPC) gels and incorporated chemical additives targeting selectively non-covalent or disulphide bonds in their gels, in order to assess the importance of each type of bond through the measurement of the textural properties of the gels.

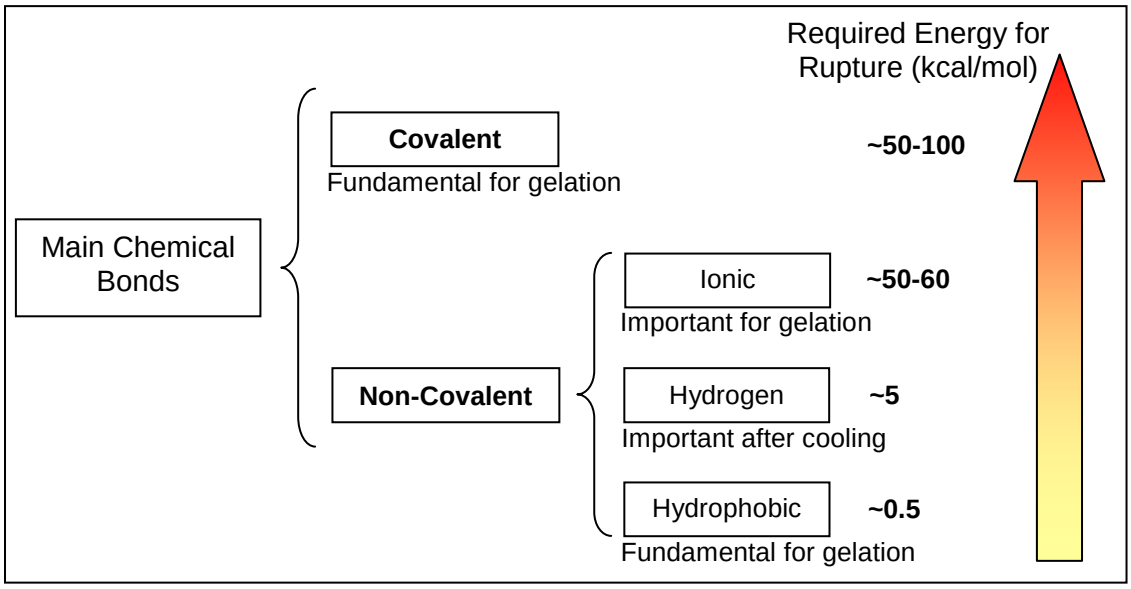

Figure 6.3 – Main chemical bonds between proteins.

These so-called informational products (products whose only purpose is to attain information at the protein bonding level), corresponding to the use of additives as chemical probes, enable a different and more straightforward way of assessing the importance of each type of bond, since, instead of attempting to evaluate protein interactions through very sophisticated techniques (such as the combination of various mixtures of extraction agents with infra-red spectroscopy), only the textural or electrophoresis analysis need to be performed. These analyses find an immediate connection with the absence of a specific type of bond, because the incorporated chemical additive disrupts that specific bond. For instance, these authors reported that those WPC gels containing DTT (additive that breaks all disulphide bonds) were much less rubbery (lower fracture strain) than all the others (control gels and gels with other additives), thus signalling the connection between disulphide bonding and a rubbery texture (Havea *et al.*, 2009). But, other authors (Pérez-Mateos *et al.*, 1997) have sustained the opposite, associating disulphide bonding to higher hardness and lower elasticity (Table 6.1).

Table 6.1 – Proposed correlations found in the literature between chemical proteins bonds and food properties and observation of the correlations in this work.

Type of protein	Chemical bond	Associated properties	Reference	Observation
Whey protein	Hydrophobic bonding	-Higher hardness	Havea *et al.*, 2004; 2009	Partially supported
Whey protein	Dissulphide bonding	-Rubbery texture	Havea *et al.*, 2004; 2009	Not supported
Fish protein (blue whiting)	Dissulphide bonding	-Higher hardness -Lower elasticity	Pérez-Mateos *et al.*, 1997	Only partially supported

For the present work, sea bass and hake mince products analogous to those mentioned above, i.e., containing specific chemical additives, were prepared with the purpose of finding connections between biochemical phenomena and the textural features of fish products (see 5.1.1). The role of MTGase was also studied in these products. However, the properties observed in these fish products did not confirm any of the two contending hypothesis concerning the connection between disulphide bonding and gel deformability. All results taken together (texture, protein solubility, electrophoresis and DRM) show the insufficiency of a simple association between stiffness and hydrophobic bonding or between deformability and S-S bonding as explanatory of the observed phenomena. The study of these same phenomena on other fish products (see 5.2.2) through protein solubility in different extraction media likewise did not yield much evidence for such relation. Whereas the hardness of heat-induced gels prepared from hake seemed to be associated to a high level of non-covalent bonding (Tables 5.2.2.3-6), thereby agreeing with Havea et al. (2004), the greater importance of S-S bonding was not matched by the development of a rubbery/deformable texture in heat-induced gels, thus disagreeing with Havea et al. (2004). Therefore, the various quality differences found between processed fish products could not be explicitly assigned or, even, only related to a higher degree of disulphide linkages, hydrogen bonds or other non-covalent associations among proteins. This finding demanded another interpretation of the results at the molecular level, which was found in the study that combined the effect of MTGase with that of different chemical additives (see below and 5.1.1).

The production of value-added products from fish by-products may require the incorporation of additives, such as MTGase, or the application of new technologies such as high hydrostatic pressure (HHP). Hence, the role of each type of chemical bond among proteins was studied in connection with the incorporation of additives (see 5.1.1, 5.1.4, 5.1.5, 5.2.1, and 5.2.2) or application of new gelation technologies (see 5.2.1 and 5.2.2). The studied additives and technologies can be found in Figure 6.4.

Regarding alternative gelation technologies, UVI and HHP were studied. Protein solubility in different extraction media (SDS, SDS+DTT, U and U+DTT) showed that UV irradiated samples presented less non-covalent (hydrogen and hydrophobic) bonding, but this did not produce any loss of hardness, once more contradicting other works (Havea et al., 2004; Havea et al., 2009). The lower cohesiveness and deformability of the UV treated gels could not be traced back to a reduction of disulphide bonds. On the contrary, their importance increased (Table 5.2.1.5). Thus, either Pérez-Mateos et al. (1997) are correct in ascribing lower deformability to S-S bonding or protein degradation by enzymes, which were present in the unwashed fish mince, was stimulated by UVI and surpassed the effect of any additionally induced cross-linking (Urbain, 1977).

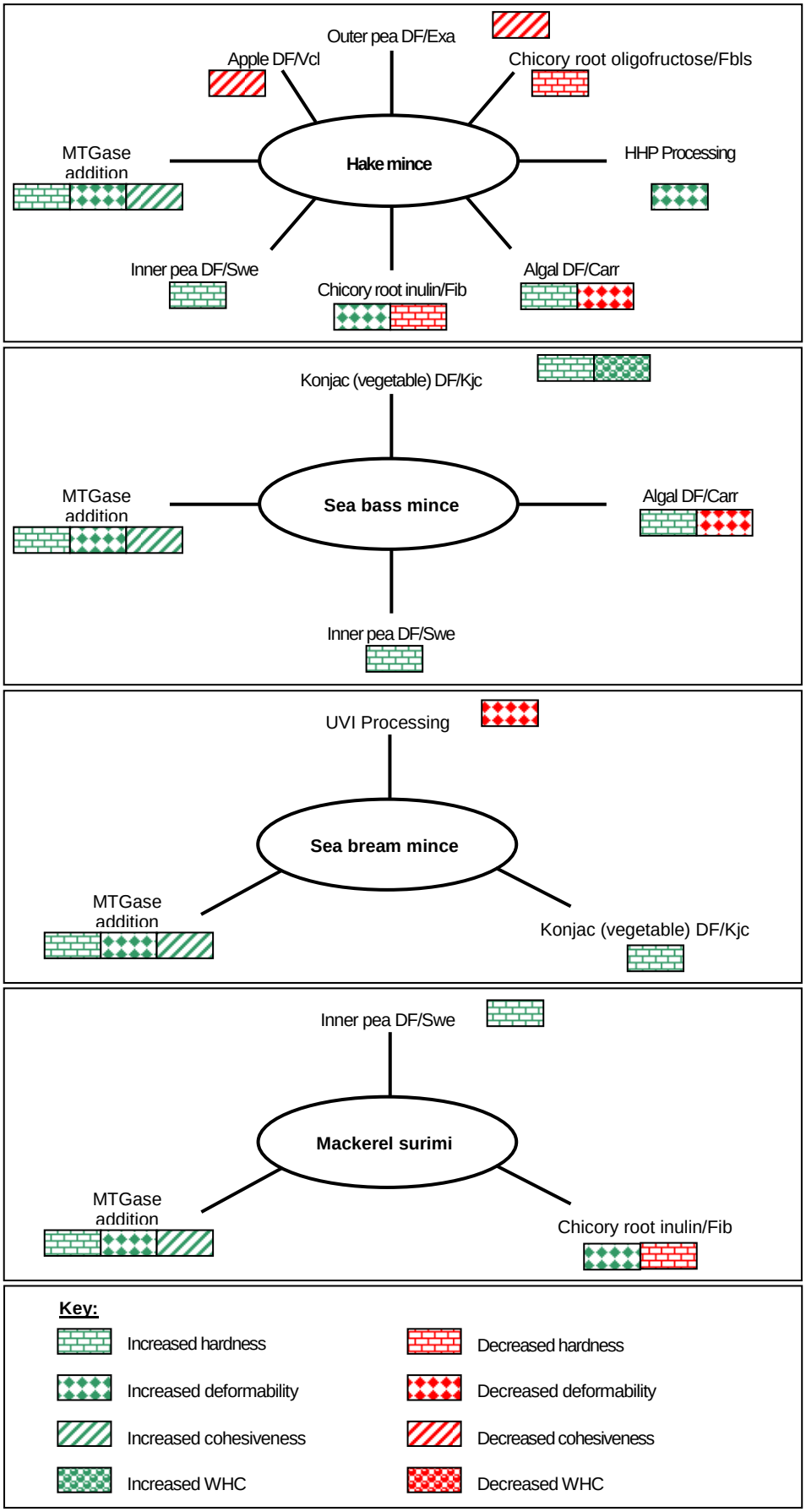

Figure 6.4 – The effect of tested additives and gelation technologies on gel quality.

The choice between the two options becomes clearer with the integration of the various experimental works, since no systematic loss of deformability with a higher level of S-S bridges was observed, thus strengthening the latter hypothesis. Above all, no gelation mechanism can be proposed for UVI, since the final product could not be considered a gel.

For pressure-induced gels and for different operational conditions (pressure level and time of compression), S-S bonding also increased (Table 5.2.2.5). Non-covalent interactions had also importance, since solubility values in SDS were also high and protein unfolding, which exposes hydrophobic areas, occurs to some extent during compression. But, the SDS solubility data showed that these interactions had a smaller importance than in heat-induced gels. Moreover, HHP was able to keep or even enhance the deformability/elasticity found in samples immediately after the setting operation. This agrees with literature (Moreno et al., 2009), since pressure-induced fish gels have been described as more elastic (and softer) than heat-induced gels. These properties of pressure-induced gels could not be assigned to S-S bonding, because the less rubbery heat-induced gels also presented an increased level of S-S bonding in comparison with the control (only set samples). So, these findings suggest that gelation follows different paths depending on the use of temperature or pressure. According to Uresti et al. (2006), it seems that HHP application to fish mince at low temperature (as that used in this work's experiments, 30 ºC) promotes a kind of protein aggregation characterized by side-to-side interactions of proteins with reduced denaturation instead of large conformational changes as with thermal gelation. Another hypothesis explaining the good texture of pressure-induced gels in comparison to heat-induced gels may be the reduction of myosin hydrolysis by proteolytic enzymes, whose activity is maximal at temperatures between 50 and 70 ºC (Uresti et al., 2006). HHP also promotes proteolysis since it renders substrates more accessible to proteases. This effect may have been limited by the lower range of pressures used (100-300 MPa) (Table 6.2).

Table 6.2 – Summary of the main biochemical results with new gelation technologies.

Technology	Intermediate material	Biochemical effects in comparison with heat-induced gels
UVI	Gilthead sea bream mince	-Less non-covalent bonding -More disulphide bonding -Proteolytic activity stimulated by UVI
HHP	Hake mince	-Less non-covalent bonding -Proteolytic activity stimulation limited by the low pressure range (100-300 MPa) -Protein aggregation characterized by side-to-side interactions with reduced denaturation instead of large conformational changes (heat-induced gels)

The functional additives, that is, those additives incorporated in the products with the purpose of ameliorating their properties and enhancing their nutritional values, also affected the relative importance of different types of bonding.

Tested DFs (Figure 6.4) had different effects upon the biochemistry of proteins, due to their different chemical groups. Four DFs were studied more extensively (see 5.1.4 and 5.1.5): inner pea fibre, Swe (composed of 36 % starch and 48 % fibre, of which 2/3 insoluble cellulose and 1/3 soluble pectic material), chicory root fibre, Fib (inulins), algal fibre, Carr (ι+κ carrageenans) and konjac (vegetable) fibre, Kjc (glucomannan). All DFs reduced the importance of hydrogen bonding between fish proteins, as can be deduced from the protein solubility in urea (Tables 5.1.4.6 and 5.1.5.8). It can be supposed that these polysaccharides with a very large number of –OH groups interacted with those protein residues involved in hydrogen bonds, thus causing conformational changes in the proteins. For carrageenan, a possible cause could be the involvement of some protonated amino acids in electrostatic interactions with the sulphate groups of this DF. This could lead to a reduction of the relative importance of hydrogen bonding. The DF impact on other non-covalent interactions between proteins was smaller. A significant impact was only detected on the proteins of mackerel surimi, when higher levels of Swe and Fib incorporation were experimented. While at 2 % (w/w) no effect was found, at 4 % (w/w) protein solubility in SDS fell significantly. Nevertheless, this fall could be due to some hydrogen bonds being targeted by SDS and, undoubtedly, the most important effect of DF was on hydrogen bonding. This effect suggests that the observed changes in food properties (see below) were not only the result of the DF presence in the products' structure, but also of a substantial degree of interaction between protein and DF. That is, DF brought about alterations in the protein matrix, which, in turn, caused relevant modifications in texture and other parameters of the products. But, the exact mechanism of this effect is not well known. Various explanations have been put forward, regarding the structures that can be formed by additives and how they can interact with the myofibrillar protein (Aguilera and Kessler, 1989; Ring and Stainsby, 1982; Tolstoguzov and Braudo, 1983; Ziegler and Foegeding, 1990). The latter authors have systemized and proposed five possible models for the spatial portioning of a gelling protein and an additive (Figure 6.5).

The first two of these models correspond to "simple" filled gel matrices, distinguishable on the basis of the phase state of the system. In the first, the additive remains soluble in the interstitial fluid of the gel matrix, while in the second the additive is present in the form of dispersed particles. For both cases, the dispersed phase does not associate or interact with the gel matrix. Such additives are termed "passive" fillers.

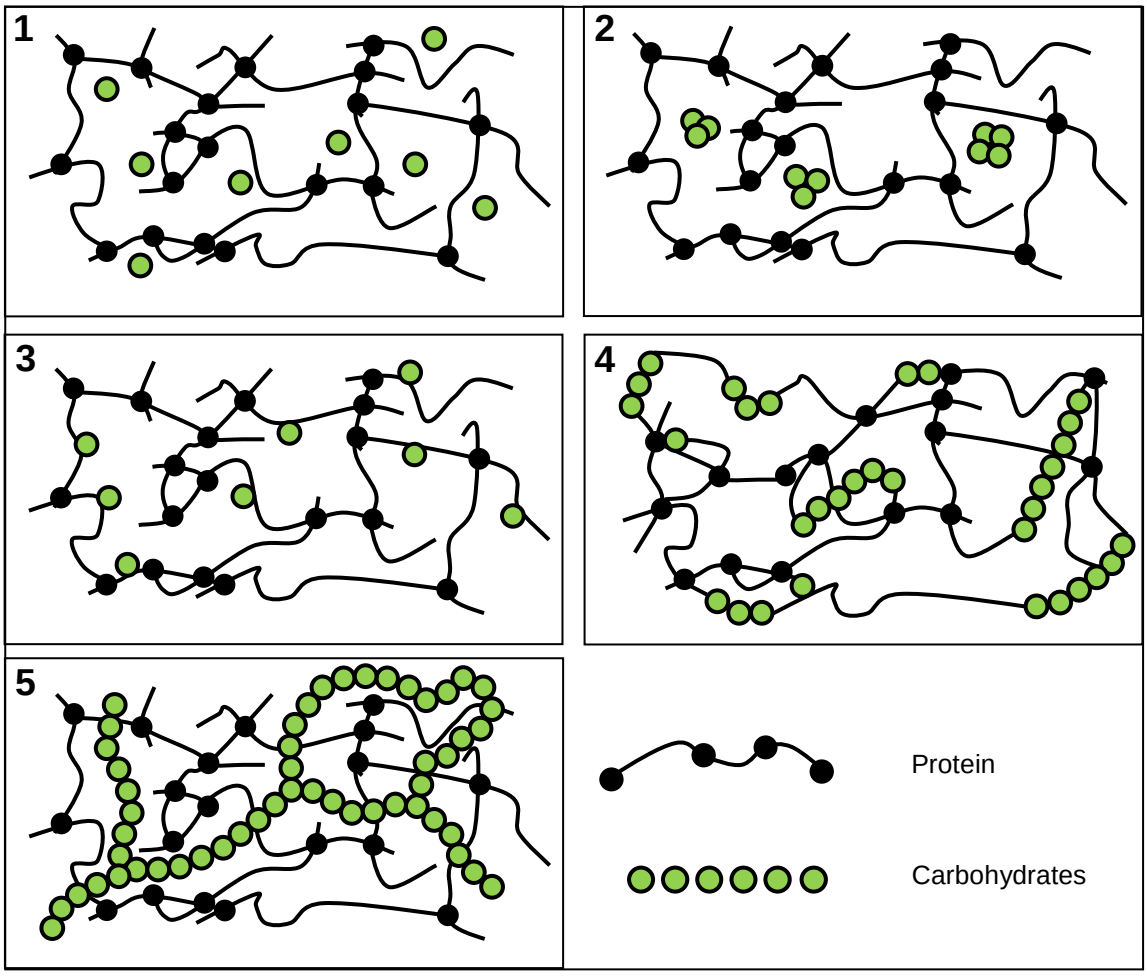

Figure 6.5 – Five possible models of protein-protein and/or protein carbohydrate interactions (adapted from: Ziegler and Foegeding, 1990).

In the other three models, the additive associates or interacts directly with the gelling protein (“active” filler). In this case, a further distinction can be drawn, the additive may lack the ability to gel (3rd model) or it may be able to gel even in pure solution (5th model). There is a fourth model, which presupposes co-polymerization. In this work with fish products, various results seem to indicate an active role of the DF. Each DF may have its own specific way for interacting with the protein matrix. Whereas, pectins, carrageenans and glucomannan can form gels, at least, under certain conditions (Park, 2005), cellulose and inulins are not gelling additives. Regarding ι-carrageenan, it was reported an independent network establishing connections between adjacent structures and supporting the principal structure formed by the fish protein (Gómez-Guillén et al., 1996). The experimental results attained in this work (mainly 5.1.4) do not preclude this conclusion, since it was found that Carr may associate directly with protein (namely, MHC) and/or may be able to form an independent network and/or may indirectly affect, through its water absorption capacity, the density of the protein. The results point to the formation of an interpenetrating gel network of protein and carrageenan (5th model). Non-gelling

additives such as cellulose may also interact with fish protein and lead to different structural arrangements. The SEM micrographs (Figure 5.1.4.2 and Figure 5.1.5.2) show that Swe's DF (whose main component is cellulose) may have formed a backbone structure, along which proteins established their interactions. These were probably not only among proteins, but also between proteins, starch and DF, thus reinforcing texturally the gels. The role of the starch present in Swe is also important. Starch gelatinizes concomitantly with the thermal gelation of fish proteins (Park, 2005). This gelatinization leads to the formation of amorphous starch granules (Couso et al., 1998). This process may have been limited by the setting treatment prior to heating, since the setting ensures a previous development of protein cross-linking, which traps water and limits its availability for hydration and gelatinization of the starch. In these conditions, starch acts as a "simple" filler of the myofibrillar protein gel (Lee et al., 1992). It has been reported that surimi gel with added starch results in the formation of a two-phase gel (Park, 2005): the second model of Ziegler and Foegeding (1990). Micrographs reveal some starch granules with a globular shape (Figure 5.1.5.2), which correspond to non-gelatinized starch (Couso et al., 1998). On the other hand, Fib incorporation to the fish gels did not cause major structural rearrangements. Nevertheless, as discussed above, inulins also altered the interaction between proteins, disrupting hydrogen bonds and, for mackerel surimi, this type of DF seemed to have a chemical effect that led to less S-S bonds and, consequently, reduced protein cross-linking (see 5.1.5). Concerning Kjc, protein solubility, electrophoresis, and microscopic information indicate an effect upon the protein bonding and gel matrix structure similar to that ascribed to Carr. There is also indirect evidence of the interaction between DF and the protein gel matrix through the detection of some hindrance of the MTGase action by some DFs, at least, in some instances: protein solubility results suggested that Swe and Fib in larger amounts (>2 %, w/w) may affect MTGase action upon fish protein configuration (see 5.1.5).

MTGase was the other major additive studied in this work. It is an enzyme that increases cross-linking of myosin heavy chains (MHC) during setting, since it catalyzes covalent ε-amino-(γ-glutamyl)-lysine bonds (Kumazawa et al., 1993; Ramírez-Suárez et al., 2001) (Figure 6.6). It creates a denser bond network, i.e., a more tightly knit web, between proteins in gels.

In addition to this added TGase of microbial origin, fish mince, especially if unwashed, also possesses its own endogenous TGase. This enzyme is removed during the washing step of the preparation of surimi (see 5.1.5) and its activity is low in the unwashed minces of some species, such as sea bass (see 5.1.1).

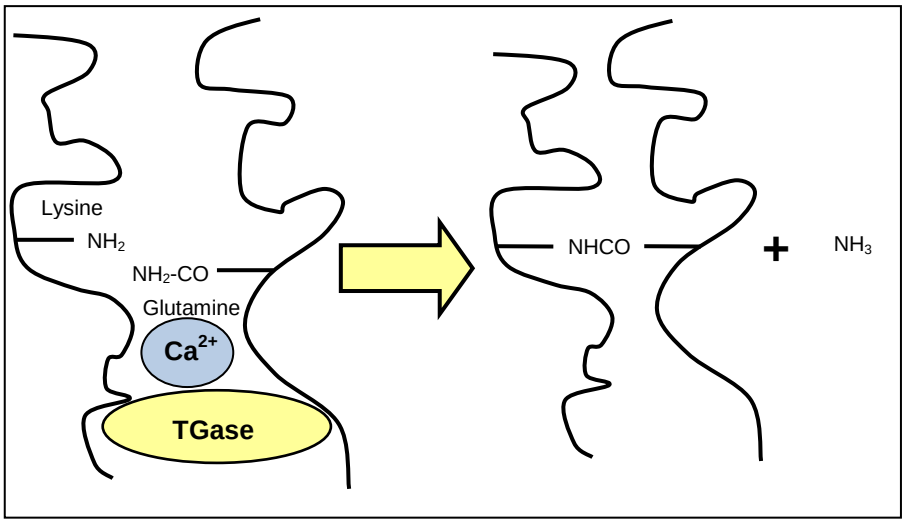

Figure 6.6 – Formation of ε-amino-(γ-glutamyl)-lysine bonds by MTGase (adapted from: Park, 2005).

On the other hand, the experiments conducted with the so-called informational products enabled to find out that hake presented significant levels of endogenous TGase activity and that this enzyme presented a catalytic cysteine residue, such as that of MTGase (Kanaji et al., 1993) and other fish TGases (Noguchi et al., 2001). This was found out because NEM —which inhibits MTGase through S-1,2-dicarboxyethylation of cysteine (Kanaji et al., 1993)— presented a negative effect on the properties of hake mince products and had a clear effect on the HMW bands of the electrophoretic profiles of hake gels (Figure 5.1.1.2). Nothing similar was observed in sea bass gels. This contrast between the two species is even more meaningful if one considers that hake was frozen stored while sea bass was fresh. Differences in the contents and/or specific activities of the endogenous TGases in fish may account for such contrast. Another possible explanation lies in the number of reactive glutamine residues on the myosin molecules. According to Maruyama et al. (1995), MHC of carp possesses less reactive glutamine residues than mackerel. As a result, only protein dimmers were formed in carp, whereas MHC of mackerel formed large polymers at a faster rate. However, the electrophoretic profile of sea bass gels and their properties were deeply modified by the addition of MTGase, which seems to dismiss the number of reactive glutamine residues as explanation and reinforces the other hypothesis. This study showed that there are important differences in the contents and/or specific activities of the endogenous TGases in fish. This fact justifies the incorporation of MTGase in those products prepared from species with low endogenous TGase activity. The addition of MTGase may also be justifiable in products from species like hake, since its addition has effect on several properties and may be useful (see below). Products from species with higher endogenous TGase activity may forgo MTGase addition or only require it at a lower level of

incorporation. Both MTGase and endogenous TGases promote cross-linking and the formation of myosin polymers with a concurrent decrease in myosin heavy chain monomers (Esturk et al., 2004). In addition to myosin, other HMW proteins were affected by MTGase addition (see 5.1.1, 5.1.4, 5.1.5, 5.2.1, and 5.2.2) or the presence of an endogenous TGase (see 5.1.1). These results agree with those of Funatsu et al. (1996), since these authors found that cross-linking seems to occur between actomyosin and the other myofibrillar protein constituents of the muscle to yield huge aggregates. Besides the direct chemical action of these enzymes, there are indirect effects upon the various types of protein bonding. It has been reported an increase in protein surface hydrophobicity during setting (Niwa, 1992; Wicker et al., 1986). The catalytic cross-linking by MTGase may lead to conformation alterations, which, in turn, entail the exposition of hydrophobic residues. The attained results (protein solubility and electrophoresis) partially support this idea. For instance, MTGase addition seemed to favour the establishment of non-covalent interactions (among them, hydrophobic interactions are important) in sea bass gels, thus, being its direct catalytic action compounded by indirect effects (Table 5.1.1.6). This hydrophobicity increase was not observed in hake gels. The indirect effect of MTGase was mainly detected on higher levels of disulphide bonding in all used species, sea bass (see 5.1.4), mackerel surimi (see 5.1.5), gilthead sea bream (see 5.2.1) or hake (see 5.2.2). This finding was mainly based in protein solubility and electrophoresis results. Although according to Smyth et al. (1998) S-S bonding is not a requirement for the gelation of actomyosin, these authors found that it contributes to the gel network formation. So, it is possible that MTGase effect on the properties of fish products (whether prepared from surimi or unwashed mince) is the net result from the formation of ε-amino-(γ-glutamyl)-lysine and disulphide bonds. The exposition of free sulfhydryl groups may be augmented by the conformation changes undergone by proteins as a response to increasing numbers of amino-(γ-glutamyl)-lysine bonds.

The main results attained with additives incorporation in this work are shown in Table 6.3.

Beyond the impact of each additive *per se*, another important point concerns the biochemical effects of the combination of different additives. The combination of MTGase with each one of those DFs more thoroughly studied (Swe, Fib, Carr and Kjc). As already mentioned above, Swe and Fib incorporated at higher levels (4 %, w/w) may account for some hindrance of the MTGase activity. The protein solubility values (Table 5.1.5.8) showed a notorious absence of variation with MTGase addition, whenever high Swe or Fib amounts were present in the mackerel surimi gels. But, the electrophoretic study revealed a similar effect of MTGase on gels with and without DF (Figure 5.1.5.1) and, for Swe, MTGase was able to improve product properties just as it did for the control

samples. So, it may be argued that among DFs, Fib had a more clear interference on the action of MTGase.

Table 6.3 – Summary of the main biochemical results attained with additives incorporation.

Additive	Intermediate material	Biochemical effects in comparison with control (no additives)
DF	All	-Lower importance of hydrogen bonding -Possibility of a substantial degree of interaction between protein and DF
DF - Carr	Sea bass mince	-Possibility of formation of an interpenetrating gel network of protein and carrageenan
DF - Swe	Hake and sea bass minces, and mackerel surimi	-Swe's main component (cellulose) may interact with protein -Swe's starch may act as a filling agent of the protein gel
DF - Fib	Mackerel surimi	-Modification of the interaction between proteins -Disruption of hydrogen bonds -Lower importance of disulphide bonding
DF - Kjc	Sea bass and sea bream minces	-Possibility of formation of an interpenetrating gel network of protein and glucomannan
MTGase	All	-Advantageous in products from species with low TGase activity -Promotion of cross-linking between HMW proteins -Possibility of conformation alterations, with exposure of hydrophobic residues (for instance, sea bass gels) -Higher importance on disulphide bonding

The reduction of disulphide bonding by Fib may also be interpreted as an additional signal of this interference, since, as concluded above, further S-S bonding is indirectly promoted by MTGase. Concerning Carr and Kjc, protein solubility results (Table 5.1.4.6), especially in SDS+DTT, U and U+DTT, also suggested that DF may hinder MTGase action. The exact mechanism for this effect of DF is unknown. It can be hypothesized the existence of steric effects and, also, a dilution effect of protein, thereby reducing the probability of an encounter between the residues of glutamine and lysine of different proteins. For Carr and Kjc, given the possibility of higher degree of chemical interaction with protein, conformational changes in the myofibrillar proteins may play a role in restraining the MTGase action. Future experiments shall help in explaining this phenomenon, namely, a comparison between products with the same protein content (which entails different moisture levels) or, for instance, the measurement of the incorporation level of radioactive [^{3}H] putrescine (Fujita et al., 1998) into myofibrillar protein (for a small substrate of the enzyme, such as [^{3}H] putrescine, any steric effects would be less important and any variations in the presence of DF could be more easily assigned to conformational changes of the myofibrillar proteins).

The effects of the combination of additives in this work are shown in Table 6.4.

Table 6.4 – Summary of the main biochemical effects of additive combination.

Additives	Intermediate material	Biochemical effects
Swe + MTGase	Sea bass mince and mackerel surimi	-Possibility of hindering MTGase activity at 4 %, w/w
Fib + MTGase	Mackerel surimi	-Possibility of hindering MTGase activity at 4 %, w/w (stronger interference than with Swe)
Carr/Kjc + MTGase	Sea bass mince	-Higher degree of chemical interaction with myofibrillar protein leads to conformational changes, which restrains MTGase action

Regarding the interaction between additives (DF and MTGase) and alternative gelation technologies such as UVI or HHP, only MTGase and Kjc were tested. For UVI, neither MTGase nor Kjc presented any synergistic interaction (see 5.2.1). The possible stimulation of proteases by UVI (Urbain, 1977) may have caused the degradation of myofibrillar proteins, rendering the MTGase useless. On the other hand, a synergistic effect of HHP treatment and MTGase incorporation was found (the combined effect of HHP+MTGase was greater than the sum of the isolated effects) (see 5.2.2). Some authors oppose this possibility, reporting that MTGase is pressure sensitive (Shoji et al., 1990). But, later studies have shown that TGase activity was unhindered by pressures between 100 and 300 MPa (Ashie and Lanier, 1999), thus suggesting that also MTGase could be active in this range (applied in this work). It must be stressed that a synergistic effect does not entail an enhanced MTGase activity under pressure: it can be explained by the establishment of MTGase-catalyzed bonds between proteins during the setting operation, which, during the following HHP treatment, make easier the encounter of specific reactive areas (such as cysteine residues) of different proteins, thereby adding to the effect of pressure itself. The apparently contradictory results regarding HHP and MTGase reported in the literature may be explained by the effect of HHP upon proteins of different species. HHP promotes the unfolding of proteins, thereby making them more susceptible to the action of any enzyme still active under the applied pressures (Gilleland et al., 1997). MTGase may be sensitive to pressure and become partially denatured, but, on the other hand, its substrate may be more accessible and this effect be more important. It must be stressed that a high activity or the effects of it correspond to a higher rate of cross-linking formation, whether it is due to a lower degree of denaturation of the enzyme or to a modified conformation of the substrate. A synergistic effect of endogenous or added TGase and HHP in a lower pressure level range was also reported by other authors (Gilleland et al., 1997; Ashie and Lanier, 1999). In order to ascertain whether

MTGase action during the HHP treatment is decisive for the observed synergistic effect, future experiments should include a pressure treated gel containing MTGase and without setting. A brief overview of the literature on the subject and the comparison with this work are presented in Table 6.5.

Table 6.5 – Brief literature review and comparison with this work's results on the subject of synergies between TGase and HHP.

Synergy	Intermediate material	Synergistic conditions	Reference
Detected with endogenous TGase	Alaska pollock surimi	-Pressure level: 100-300 MPa	Gilleland et al., 1997
Detected with MTGase	Alaska pollock surimi	-Pressure level: <300 MPa -[MTGase] ⇔ 1.5 U/g protein	Ashie and Lanier, 1999
Detected with MTGase	Hake mince	-Pressure level: 100-300 MPa -[MTGase]: 0.5 % (w/w) ⇔ 3 U/g protein*	In this work

* U - unit of MTGase activity.

Contrastingly, heat treatment and MTGase did not present a similar synergistic interaction. Heat-induced gels are likewise submitted to a setting stage however cross-linking occurring during this stage does not seem to augment the effect of the heat treatment itself. This contrast may be due to a more effective inactivation of MTGase by heat than by pressure, given that a 90 % reduction of activity has been reported at a temperature of 60 ºC (Park, 2005), which compares with a 90 ºC cooking in the preparation of gel products. Hence, though proteins are also unfolded by heat (Lanier et al., 2005), no significant levels of active MTGase remain to reinforce protein cross-linking. Alternatively, it can be hypothesized that the large conformational changes due to thermal treatment (Uresti et al., 2006) may very rapidly lead to protein aggregates and/or conformations which hinder the access of MTGase to its substrate.

This general overview and discussion of the action mechanisms of additives and gelation technologies at the protein bonding level does not allow a strict assignment of the various quality differences found between prepared fish products to one or more specific types of protein bonding. In the study on the application of additives as chemical probes (see 5.1.1), this problem was directly addressed. The effects of MTGase and of the raw material type (hake *vs* sea bass) were analysed. It was found that whereas MTGase in hake products hardens the gels without altering protein solubility, it improves the deformability of sea bass gels and reduces protein solubility, thereby indicating a greater role of non-covalent and disulphide bonding as an indirect consequence of MTGase action. This dual effect of MTGase required a closer analysis of the raw material effect. A

DRM study was conducted in order to achieve a deeper understanding of the differences between raw materials at the protein level. The rheological curves also showed differences between hake and sea bass (Figure 5.1.1.4). The initial increase of the G' up to 37 ºC, which is associated to the setting phenomenon (Gómez-Guillén *et al.*, 1997b), was much steeper in the sea bass batters. This suggests a larger contribution of the cross-linking between proteins to the viscoelasticity of the products. For hake, this contribution seemed less important, in spite of the endogenous TGase activity.

Afterwards, the reduction of G', related to the denaturation of myosin molecules as α-helices in the tail portion unfold (Romero *et al.*, 2009; Kim *et al.*, 2005b), is stronger in sea bass samples. This is very important, since it indicates a difficulty in the unfolding of hake proteins. The third phase, characterized by a relentless increase of G', is connected to protein aggregation (Romero *et al.*, 2009).

In the literature, this aggregation is considered to be dependent on the oxidation of sulfhydril groups, predominantly found in the globular head segments of myosin molecules (Acton and Dick, 1989; Sano *et al.*, 1994). This phase is also relatively stronger for sea bass gels. The proposed explanation based on the oxidation of sulfhydril groups is not corroborated by the sea bass curves, since those chemical additives (DTT, NEM) preventing formation of S-S bonds were quite ineffective in sea bass.

For hake, a considerable difference was displayed by the samples containing these additives. So, it seems that, while disulphide bonding augmented greatly during heating of hake gels, in the sea bass gels, S-S (or non-covalent bonding) had no important influence upon protein aggregation. It can be derived from all discussed results that a simple association between stiffness and non-covalent (particularly hydrophobic bonding) or between deformability and S-S bonding, as advocated by some authors (Havea *et al.*, 2004; Havea *et al.*, 2009), lacks sufficient scientific backing, thus allowing to hypothesize that MTGase operates differently in each raw material.

Whereas, for hake, it catalyzes the cross-linking between the small number of Gln and Lys residues exposed at the surface of proteins (DRM showed less unfolding of hake proteins) and, as such, the covalent ε-amino-(γ-glutamyl)-lysine bonds only link a small number of molecules and have a short distance effect, for sea bass, due to a greater unfolding of proteins, almost all Gln and Lys residues become exposed and the MTGase is able to link a large number of these residues, thereby uniting a large number of molecules and succeeding in the establishment of a vast network structure (as also shown by SEM).

These differences in the effects of MTGase activity are shown in Figure 6.7.

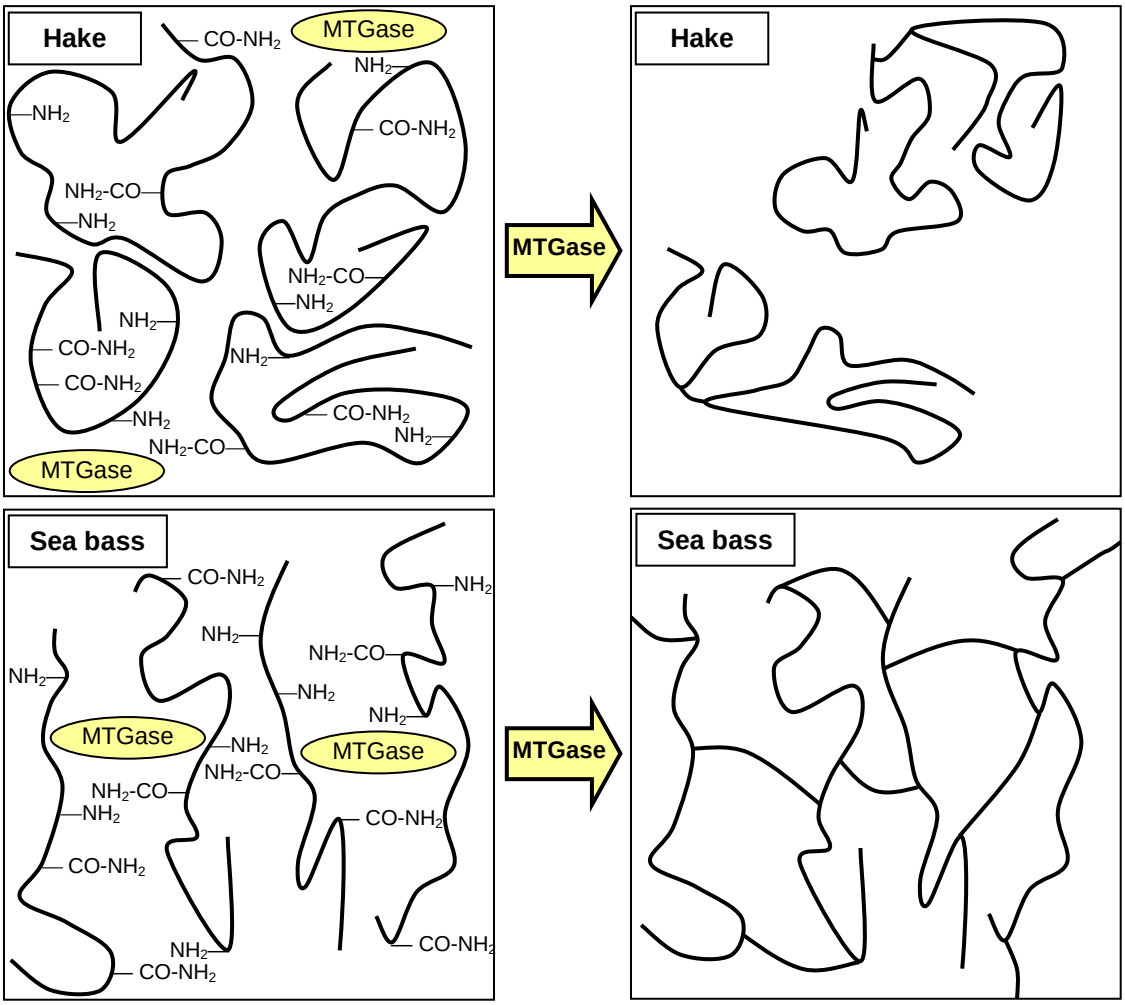

Figure 6.7 – Dependence of MTGase action on the raw material characteristics.

Concerning the reason for a lower degree of protein unfolding in hake, the denaturation of protein due to frozen storage must be pointed out. Frozen storage prior to processing is important, since hake was frozen (at least, for three or four weeks) and sea bass not. According to another study, breaking force and deformation of surimi gels produced from frozen fish decreased continuously with increasing time (mainly up to 10-12 weeks) of frozen storage (Benjakul *et al.*, 2005). The decline in gel-forming ability was associated with the decrease in Ca^{2+}-ATPase activity and mainly with formaldehyde formation. This must have contributed for the formation of intramolecular covalent bonds, which were left unscathed by any of the used chemicals. It can be concluded that raw material quality is of great importance and that the degree of denaturation of the proteins is the main factor affecting the quality of fish products, overshadowing the effect of additives and largely determining their action mechanism and effects.

Nevertheless, additives incorporation may be advisable, especially for products prepared from raw materials of poorer quality (such as trimmings of the frozen fish processing industry) because they can bring some added value and confer special

attributes to the products (see 6.2.). Even for products prepared from high quality raw materials, some additives may be advantageous also for this latter reason. Another important conclusion concerns the great usefulness of attaining a better understanding of the phenomena at the molecular level. For instance, the so-called informational products showed that there is a positive interaction between MTGase and the disruption of disulphide bonds in hake gels. This fact suggests that a combination of two additives, such as cysteine (an amino acid, generally regarded as safe substance, which breaks S-S protein bonds) and MTGase may be useful for the amelioration of poor quality hake products.

6.2 – Effect of Additives on Restructured Fish Products

The previous results and discussion provided some important information concerning the use of additives in fish products, namely those prepared from hake, sea bass, sea bream and mackerel. They supplied a significant knowledge frame concerning the molecular phenomena that underlie the effect of MTGase or DF upon restructured fish products. In order to use these additives judiciously, a thorough assessment of their effects on texture, WHC, colour and other properties of fish products was required and, effectively, was performed through the use of some additives in model experiments. These model products were not intended to be marketable products, that is, products with all sensory characteristics (for instance, flavour) meeting consumer requirements and expectations. These products were prepared with the sole purpose to allow the study of the effect on the various quality parameters of each additive *per se* and in combination with others. For instance, to know how much textural benefit can be attained from adding a given amount of DF to heat-induced gels from hake, in order to use this knowledge in the development of marketable fish products with high sensory quality, which was the subject of a later part of this work (see below).

Several DFs were tested on products prepared from various raw materials. The following incorporation experiments were carried out: apple fibre (Vcl), inner (Swe) and outer pea fibre (Exa), chicory root oligofructose (Fbls), inulin (Fib), and carrageenan (Carr) in heat-induced gels from hake mince (see 5.1.2 and 5.1.3); Swe, Carr and konjac glucomannan (Kjc) in heat-induced gels from sea bass mince (see 5.1.4); Swe and Fib in heat-induced gels from mackerel surimi (see 5.1.5), and Kjc in UV- and heat-induced gels from gilthead sea bream (see 5.2.1). These different experiments enabled to assess the interaction of DF with fish species belonging to different groups, since hake represents a typical wild lean fish, sea bass stands for farmed fatty fish and mackerel (Atlantic and chub) for wild fatty (in the studied season) fish. Atlantic mackerel is considered an underutilized fish species in Portugal and Spain and, though chub mackerel is used as a

raw material by the fish canning industry, leaner and smaller fish of this species, albeit legally marketable, are discarded by fishermen (ICES, 2006). All DFs with exception of Kjc were first tested in heat-induced hake gels. This testing enabled a first screening and revealed that some DFs had more negative effects than positive ones (Table 6.6).

Table 6.6 – General comparison of the effects of different DFs on restructured hake (sea bass, in the case of Kjc) products.

Dietary fibre	Intermediate material	Effects on food properties	Overall evaluation
Swe	Hake mince	-Unaffected hardness -Some loss of deformability	Positive
Fib	Hake mince	-Softening -Improvement of elasticity and deformability	Positive
Carr	Hake mince	-Hardening -Increase of the WHC	Positive
Kjc	Sea bass mince	-Hardening -Increase of the WHC -Some loss of deformability	Positive
Vcl	Hake mince	-Worsening of folding test -Discolouration	Negative
Exa	Hake mince	-Worsening of folding test -Lower gel strength	Negative
Fbls	Hake mince	-Extreme softening -Lower gel strength	Negative

Vcl, Exa, and Fbls imparted considerable deleterious characteristics to the gel products. The folding test of Vcl and Exa products was worse than that of control (with no added DF) products (Table 5.1.2.4). No textural parameter was improved by these three DFs. For Exa, the deleterious effect of incorporating this DF was clearly seen through the progressive loss of gel strength with higher levels of added DF (Table 5.1.2.3). Vcl affected the colour of the products, darkening them.

In what regards inulin, Fib also reduced some textural parameters, such as hardness, gumminess and chewiness. But, Fib improved other properties, such as elasticity and breaking deformation (Figure 5.1.2.1). Fib showed itself as useful for some applications, which require a similar textural profile, for instance, pâtés. The absence of colour changes and the texture profile imparted by Fib led to its application to gel products prepared from mackerel surimi. It had a negative effect on the hardness, gel strength (an effect not observed in hake products) (Table 5.1.5.4), and WHC (Table 5.1.5.6). Regarding texture, Lee and Chung (1989) proposed that while compression test (used for determining hardness) measures the overall binding property of the gel material, penetration test (used for determining gel strength) evaluates degree of compactness or density of actomyosin.

So, it seems that Fib neither improves degree of compactness nor overall binding capacity. This may be explained by inulin's short chains, which are unable to make links over long distances and to create an effective network (Figure 6.8).

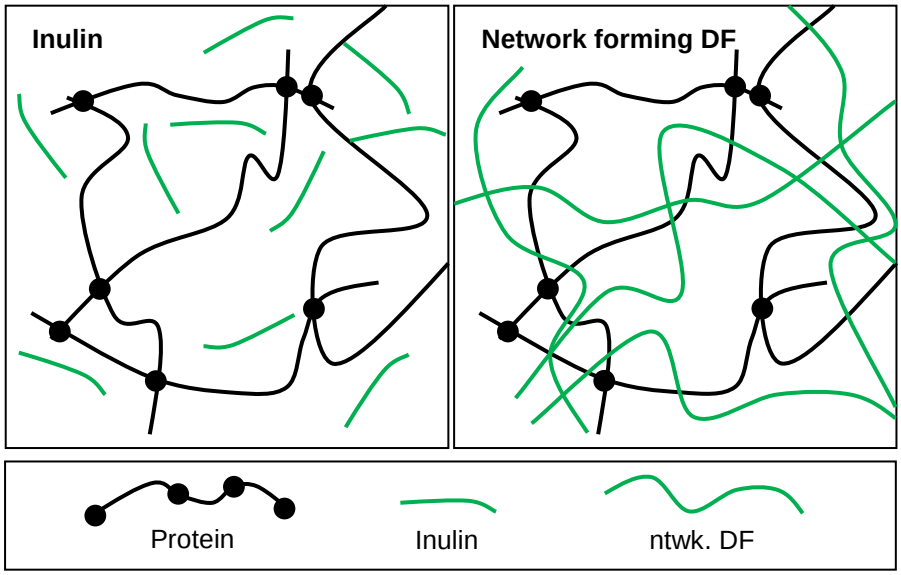

Figure 6.8 – Contrast between the effect of inulin and that of a DF able to form a network.

In accordance to biochemical phenomena discussed above (see 6.1), the single major influence of this DF was that of a diluting agent of myofibrillar protein. In the literature, hardness reduction with increasing fibre concentration has been reported (Tudorica et al., 2002) and associated to disruption of the protein matrix. The different effects on hake mince and mackerel surimi heat-induced gels may be due to the removal of sarcoplasmic proteins during the process of surimi preparation. However, it is difficult to indicate a specific cause. For instance, endogenous TGase removal during the two washing steps of the mackerel surimi preparation is not a possible explanation, since added MTGase and Fib did not present any positive interaction. Therefore any synergistic effect between Fib and the endogenous TGase in hake (whose presence was proved, see 6.1) is an implausible factor driving the increase in breaking deformation with Fib addition. Only when washing is too extensive, a removal of this water soluble enzyme is observed (Nowsad et al., 1994). Concerning WHC, the disruption of hydrogen bonding between proteins by inulin addition (see 6.1) must have been accompanied by the disturbance of the protein-water interactions, thus freeing water molecules from the protein matrix. Differently from other DFs, the inulins have no water holding ability themselves.

In what concerns inner pea fibre, Swe incorporation into restructured fish products was more advantageous than Fib. In hake products, Swe, even at 4 % (w/w) —in addition to ensuring a significant DF level with a positive health impact—, did not cause any loss of hardness or other properties, like chewiness (Table 5.1.2.3). This is noteworthy, since higher fibre contents mean lower protein contents in the final product, resulting in less firm

gels (Park, 2000). Such correlation of a progressive hardness reduction with increasing fibre concentration has been reported (Tudorica et al., 2002). Thus, Swe-containing products are unusual for retaining their hardness values. The positive results achieved with Swe could be partly due to its starch content (min. 36 % of dry weight), which is known to promote a firmer and slightly more cohesive gel matrix (Lee and Kim, 1986; Lee et al., 1992). But, inner pea fibre (mostly insoluble) must have a great role, since it represents ~50 % (w/w) of Swe and textural improvements are found in various properties other than hardness. Swe was largely able to make up for the loss of protein content and its deleterious effect (Park, 2000). Only gel strength decreased with the addition of a higher amount of this DF. An analysis of the effect of Swe content on breaking force and breaking deformation components of gel strength showed that Swe-containing gels were less deformable and, as a consequence, the force at the breaking point was necessarily lower (Figure 5.1.2.1). In this way, Swe and Fib can complement each other in restructured hake products: while Swe imparts hardness to the products, Fib imparts deformability. The results attained with Swe were better than those of Vcl-, Exa-, Fbls- or Fib-containing products. This fact led to the selection of Swe for further DF incorporation trials involving other fish species, namely, mackerel and sea bass (Table 6.7). In the former, Swe had an even more impressive effect upon the hardness —increased more than 50 % with respect to control as a consequence of adding 4 % (w/w) Swe— of heat-induced gels prepared from mackerel surimi. It hardened the gels and turned those without MTGase springier (Table 5.1.5.4). It did not increase gel strength. The whole effect of Swe in these products was positive (Tables 5.1.5.4-5) and more favourable than in hake products.

Table 6.7 – Comparison of the effects of Swe on different intermediate materials.

Intermediate material	Effects	Overall evaluation
Hake mince	-No loss of hardness and chewiness (even at 4 %, w/w) -Capacity to make up for the loss of protein content -Gel strength reduction, mainly of the deformability component	Positive
Mackerel surimi	-Hardness increased more than 50 % (at 4 %, w/w) -Springiness increased (for products without MTGase) -No effect on gel strength	Positive
Sea bass mince	-No textural positive effects -Loss of WHC	Negative

This contrasts with Fib, which was unable to enhance the deformability of mackerel surimi products, and suggests that raw material characteristics have an influence on the extent to which a given DF can alter the texture of the final products. Whereas products

prepared from hake mince gained deformability with Fib but had no hardness gains with Swe; products from mackerel surimi did not attain extra deformability with Fib and were hardened by Swe. It seems that the potential for improvement and its specificity depend on the raw material characteristics (fat level, degree of protein denaturation). Such a conclusion was already reached with MTGase and its different effects on heat-induced gels from hake and sea bass (see 6.1.).

This comparative analysis can be extended to the effect of Swe on sea bass restructured products. It was found out that Swe had no positive effects, thus, texture was quite similar to that of the control products and WHC was reduced (Tables 5.1.4.3-4). Gel strength was reduced by ~25 % with addition of 4 % (w/w) Swe to gel products containing MTGase. Swe did not significantly harden the gels and reduced their deformability, an effect more negative than that observed in hake products. This enables to propose the following order of raw material suitability to the incorporation of Swe: sea bass mince <hake mince < mackerel surimi. Although variability among species themselves may have a role, present results and those from the literature suggest also another phenomenon. Protein quantity (Table 5.1.4.2) itself is not the answer that appears to be: protein content of the sea bass products fell almost 20 % with Swe incorporation, due to the objective of producing gels with the same moisture level. There was indeed a protein dilution effect. However, for hake and mackerel surimi, the same dilution effect was present and results were different. This apparent contradiction led to conclude that if fish protein is of poor quality, replacing protein by Swe has a beneficial effect, but, if protein presents usual or good quality, Swe substitution has a negative net effect. That is, the advantages accrued by Swe addition are smaller than the disadvantages associated to a lower concentration of good quality protein in the product (Figure 6.9).

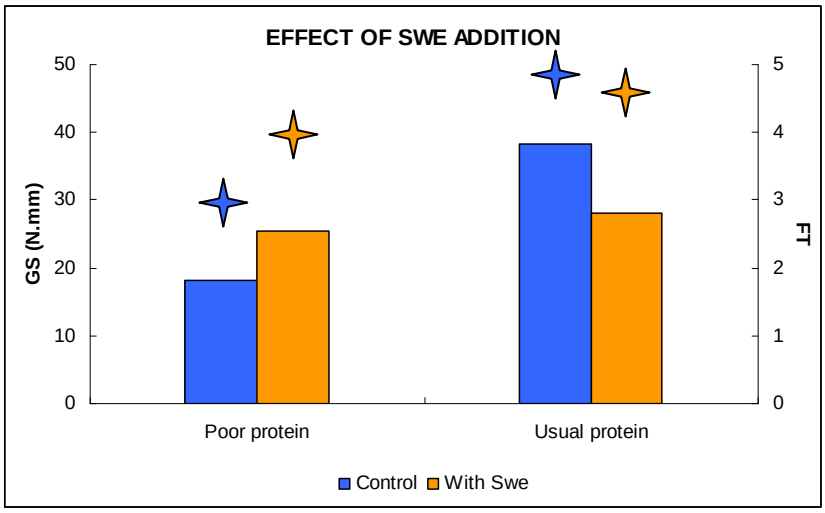

Figure 6.9 – Different effects of Swe according to fish protein quality (gel strength, GS, bars; folding test, FT, stars).

Other authors have also mentioned a deleterious effect on the textural quality of the product when the minced muscle protein is of high quality (Pérez-Mateos and Montero, 2000). Since the direct comparison between sea bass and hake (see 5.1.1) showed a higher degree of protein denaturation in the latter and given the higher degree of processing required for the manufacture of surimi and its frozen storage, it can be recommended the utilization of Swe in products whose proteins are of poor quality, that is, proteins subjected to long frozen storage periods. For instance, this makes the use of Swe advisable for restructured products prepared from hake trimmings of the frozen fish processing industry. As for Fib, it may be assumed that Swe strongly increased overall binding, but failed to produce a higher degree of compactness in the gel network (Lee and Chung, 1989). So, its main impact was observed in texture. Other important food properties, such as colour, remained unchanged, even by its incorporation at the highest level, 4 % (w/w).

Regarding carrageenan, it was tested in hake (see 5.1.3) and sea bass (see 5.1.4) heat-induced gel products. The effect of this DF was overwhelmingly positive for both products. Carr was much more effective than Swe in what concerns the hardening effect on the gels. This was most apparent in sea bass products, since both DFs were tested in this study. Whereas 4 % (w/w) level of Swe induced no significant hardness increase, only 1 % (w/w) Carr was able to augment hardness by 140 % in gels without MTGase and by one third in gels with MTGase (Table 5.1.4.3). This was a remarkable and larger effect than that attained in hake products, where Carr incorporation only increased hardness by ~20 % with a 1.2 % (w/w) level (Table 5.1.3.4). Further Carr addition up to 2.0 % (w/w) enabled some extra hardening, reaching a value ~40 % above the hardness of the control gel. But, this control had already a 4 % (w/w) Swe content and 0.1 % (w/w) MTGase level. Springiness was also improved with Carr addition to sea bass gel products. This contrasts with the poor results of Swe addition to sea bass products. Such positive results with Carr can only be achieved if substituted DF imparts more textural quality to the product than protein, even if of relatively good quality (as indicated by a lower denaturation degree, see 6.1). WHC also increased with Carr addition (Table 6.8).

Table 6.8 – Comparison of the effects of Carr on different intermediate materials.

Intermediate material	Effects	Overall evaluation
Hake mince	-Hardness augmented by 20 % with a 1.2 %, w/w, addition level -No major negative textural effect	Positive
Sea bass mince	-Hardness increased by 1/3 in gels with MTGase -Hardness augmented ~140 % in gels without MTGase -Springiness was improved -WHC was increased	Positive

Textural benefits were mainly found in those parameters measured during compression tests, which, according to Lee and Chung (1989), are connected to overall binding. Thus, Carr, just as Swe, failed to produce a higher degree of compactness in the gel network, which would be expressed by a higher level of the deformability component of the gel strength. The observed differences between the properties of products containing Swe and those of products with Carr require explanation. Previously (in 6.1), it was proposed that an interpenetrating gel network of protein and carrageenan may be formed, given the ability of Carr to gel (Figure 6.5). Carr may have an effect that goes beyond the sheer filling action and this may explain its favourable influence on the texture of sea bass gels. This is compatible with textural and WHC results, since it suggests that, additionally to the protein gel network, there is a second (polyssacharide) network enhancing hardness and related parameters. Other authors found that ι-carrageenan formed an independent network, which established connections between adjacent structures supporting the main structure formed by the seafood protein (Gómez-Guillén et al., 1996) (Figure 6.10).

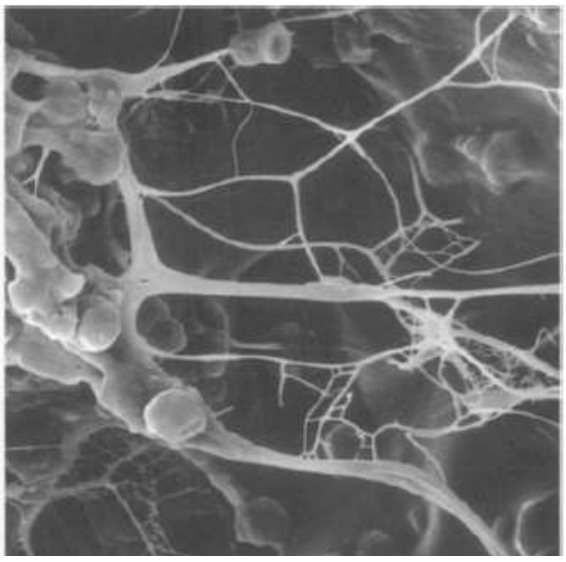

Figure 6.10 – SEM micrograph (× 6000) showing the arrangement of ι-carrageenan in seafood protein gels (from: Gómez-Guillén et al., 1996).

An adjacent network can support the main protein network, reinforce the overall binding, and enable a hardness gain, but, it cannot increase the actomyosin density and lead to a more tightly knit protein network, thus failing to produce more deformable gels. The interaction of Carr with Fib was more valuable than that between Carr and Swe (see 5.1.3). This mainly results from Carr at 2 % (w/w) making up for the softening effect of Fib, enabling a hardness level of the gels containing 4 % (w/w) Fib similar to that of gels with 4 % (w/w) Swe (Tables 5.1.3.4 and 5.1.3.6). The folding test also increased up to 4.5 (scale maximum 5) with the combination of Fib and Carr, thereby showing that gel quality can benefit much from combining the extra deformability imparted by Fib with the

mechanical firmness of Carr. Concerning other properties, Carr had no major impact, for instance, not presenting any problem whatsoever for the colour of the products.

With respect to konjac, it was tested in sea bass (see 5.1.4) and gilthead sea bream (see 5.2.1) gel products. Regarding the latter, 1 % (w/w) Kjc increased hardness (Table 5.2.1.2) and WHC (Table 5.2.1.4), but reduced deformability. The effect of Kjc was similar to that of Carr and Swe. Moreover, the combination of MTGase and Kjc was texturally advantageous, although without synergies for gel strength, since this parameter fell as a result of adding Kjc to gels containing MTGase. The combined hardening effect of Kjc and MTGase surpassed the sum of the individual effects, thereby indicating the existence of a mutual reinforcement of the hardness through MTGase and konjac. For sea bass, Kjc was added in combination with Carr, thus being required the comparison with a control containing only Carr in order to assess the effect of Kjc itself. This comparison showed that incorporation of 1 % (w/w) Kjc hardened those gels containing MTGase (Table 5.1.4.3). The effects were smaller than those displayed by Kjc addition to heat-induced gels prepared from gilthead sea bream. So, Kjc incorporation did not bring much further gain with respect to Carr incorporation. These two DFs may behave similarly at a molecular level (see 6.1), thus generating similar changes in the textural properties of restructured fish products. The hardening effect by Kjc was found by other authors working with different matrices, such as surimi gels from grass carp (Xiong et al., 2009). The amplifying effect of Kjc+MTGase on the hardness of sea bream gels seems to reduce the likelihood of any hindering effect of Kjc upon MTGase. As discussed above (see 6.1), this is a matter deserving attention in future experiments. Concerning other properties than texture, the effect of Kjc on WHC of the products was very positive (Table 6.9), which agrees with other authors' findings supporting the view that such DF may act upon the protein network through their water holding capacity, reducing the water content of the mesh and increasing the density of the surrounding protein matrix (Niwa et al., 1988; Niwa et al., 1989).

Table 6.9 – Comparison of the effects of Kjc on different intermediate materials.

Intermediate material	Effects	Overall evaluation
Sea bass mince	-Hardness augmented in gels with MTGase -No major negative textural effect	Positive
Gilthead sea bream mince	-Hardness augmented -Deformability was reduced -WHC increased between 25 and 40 %	Positive

A strong water absorptivity of glucomannan (and carrageenan) is not only verifiable in the results of this work, but also in others found in the literature (Xiong et al., 2009;

Gómez-Guillén and Montero, 1996). The other properties of the products were unaffected by Kjc. Additionally, regardless of which property is considered, the interaction between UVI and Kjc addition was not of a synergistic nature. On the other hand, it must be noticed in the comparison between both experiments, that different procedures were used for achieving the partial deacetylation of glucomannan, a necessary condition for the formation of a thermo-irreversible and highly heat-stable gel, as it is used in many traditional oriental foods (Nishinari et al., 1992; Park, 1996). Whereas, for sea bream, an alkali deacetylation with $Ca(OH)_2$ (solubilised in water and added together with the ingredients to the product's batter) was carried out (Park, 1996), for sea bass, previously to mixing with the ingredients, a Kjc-Carr gel was produced with NaOH and allowed to stand at 2 ºC (overnight) to gellify, yielding a hard and solid gum (Osburn and Keeton, 1994). This second procedure builds on a synergistic interaction between both DFs in order to achieve under milder conditions the deacetylation of glucomannan. This may be advantageous, though only an experiment directed at comparing both methodologies (with incorporation of an identical level of Carr in both cases) may ascertain which deacetylation procedure is texturally (and on other properties) more favourable.

Among all tested additives in this work, MTGase presented the most favourable effect on texture. For this reason, it was used in most studies (see 5.1.1, 5.1.2, 5.1.3, 5.1.4, 5.1.5, 5.2.1, 5.2.2, and 5.4.2), encompassing all studied fish species. From all these studies, there was one focussed on the selection of an optimal level of MTGase incorporation in hake gel products (see 5.1.3). In this study, the textural properties of these restructured hake products —with 4 % (w/w) Swe or without this DF— containing various levels of MTGase —ranging from 0.0 to 0.5 % (w/w)— were determined. Swe was chosen since previous trials with this DF had produced positive results (see above). It was found that the addition of MTGase up to 0.5 % (w/w), favoured better textural properties, particularly at 0.1 % (w/w) level or above (Table 5.1.3.3.). In the absence of DF, there was some further improvement with the increase from 0.1 to 0.5 % (w/w). For this reason, 0.1 % (w/w) MTGase was added to all hake products in that study involving the comparison between different DFs and a control without DF in order to level off conditions (see 5.1.2). The minced gel fish product containing DF produced as a marketable product (see 5.4.2) also contained 0.1 % (w/w) MTGase.

For other species and gelation technologies, there was no optimization of the MTGase level. Hence, a 0.5 % (w/w) level was chosen in order to maximize the effects of this enzyme. Regarding the textural alterations caused by MTGase, hardness and associated parameters, elasticity, folding test and gel strength were enhanced. Depending on raw material (see below), there were differences concerning which properties were improved. Sometimes deformability was increased, while in other cases only a hardening effect was

measured. According to some authors (Lee and Chung, 1989), there is an apparent lack of correlation between the two types of rheological measurement: gel strength (and its breaking deformation component)/penetration test and hardness/compression test. The first enables the assessment of the degree of compactness or density of actomyosin and the second measures the overall binding property of the gel material. Following this interpretation, MTGase sometimes increased the overall binding, sometimes promoted a higher degree of compactness in the gel network. A fundamental explanation for this phenomenon was proposed in 6.1 (Figure 6.7), where it was linked to the level of protein denaturation in the raw material. This connection with protein quality is very important for MTGase because proteins are the substrate of this enzyme. The action mode of this additive is much more influenced by protein quality than any other additive, such as the DFs tested in this study. Nevertheless, the textural benefits of MTGase have been found in many other fish products with different processing conditions, such as low-salt restructured products (Téllez-Luis et al., 2002) or surimi gels (Ramírez et al., 2000) from striped mullet (*Mugil cephalus*) (Table 6.10).

Table 6.10 – Comparison of this work with results from the literature concerning textural benefits of MTGase addition to restructured fish products.

Restructured product	Textural benefits	Reference
Heat-induced gels from striped mullet surimi	-Maximum shear stress with 0.9 % (w/w) MTGase -Maximum shear strain with 0.5 % (w/w) MTGase	Ramírez et al., 2000
Heat-induced gels from silver carp mince	-Gel strength, hardness, cohesiveness, and springiness augmented with 0.3 % (w/w) MTGase	Téllez-Luis et al., 2002
Heat-induced gels from silver carp mince	- Hardness and cohesiveness improved with combination of 0.3 % (w/w) MTGase and dairy proteins	Uresti et al., 2004
Heat-induced gels from striped mullet mince	-TPA and puncture test improved by 0.3 % (w/w) MTGase addition	Ramírez et al., 2007b
Cold gels from hake mince	-MTgase addition (0.5-1.0 %, w/w) generated more protein aggregation and better protein networks in the gels	Moreno et al., 2010a
Cold gels from hake and trout mince	-Hardness and gumminess more than doubled as a result of MTGase addition (1.0 %, w/w) to trout and hake gels	Moreno et al., 2010b
Heat-induced gels from sea bass mince	-Synergies between MTGase (0.5 %, w/w) and salt -Positive effects on gel strength, which reached a value similar to those of best quality surimi -MTGase (0.5 %, w/w) addition enabled reduction in salt content from 2.5 to 1.0 % (w/w), without loss of textural quality	Cardoso et al., 2010b and in this work

The results show that MTGase can be used as a tool to compensate protein alterations with negative textural consequences, such those alterations induced by processing,

freezing, and frozen storage, which are factors associated to important structural and physicochemical changes (Herrera et al., 2000). Not only texture, but also other properties were affected by MTGase. The WHC of mackerel surimi (Table 5.1.5.6) and sea bream mince (Table 5.2.1.4) gels was augmented by MTGase. This positive action was also reported by different studies on the effects of MTGase upon WHC or upon expressible water (Ramírez et al., 2007b). No favourable MTGase effect on WHC was observed for the other trials, such those performed with sea bass restructured products (Table 5.1.4.4). It has been reported in some products no significant effect of MTGase upon water-binding properties (Pietrasik and Jarmoluk, 2003). It seems that raw material characteristics (protein quality) may lead to different effects of the MTGase upon WHC. Just as discussed above for Swe, MTGase may only be helpful for the water-binding properties of products prepared from poor quality raw materials or whose protein is partially denatured by extensive processing, such as mackerel surimi. It may be no coincidence that Swe addition represented no advantage for sea bass products, but it improved significantly the texture of mackerel surimi gels. Concerning colour, from the four studied parameters (a*, b*, whiteness and chroma), only whiteness showed to be influence by MTGase in the various trials. The whiteness of mackerel surimi (Table 5.1.5.7) and sea bream (Table 5.2.1.4) gels was slightly reduced by MTGase addition. This slight darkening effect results from a lower level of light reflection, which, in turn, is due to a lower opaqueness of the gel, thus enabling a higher absorption of light. This lower opaqueness may be related to a more orderly network structure (Figure 6.11), which, as discussed above (see 6.1) results from the MTGase action.

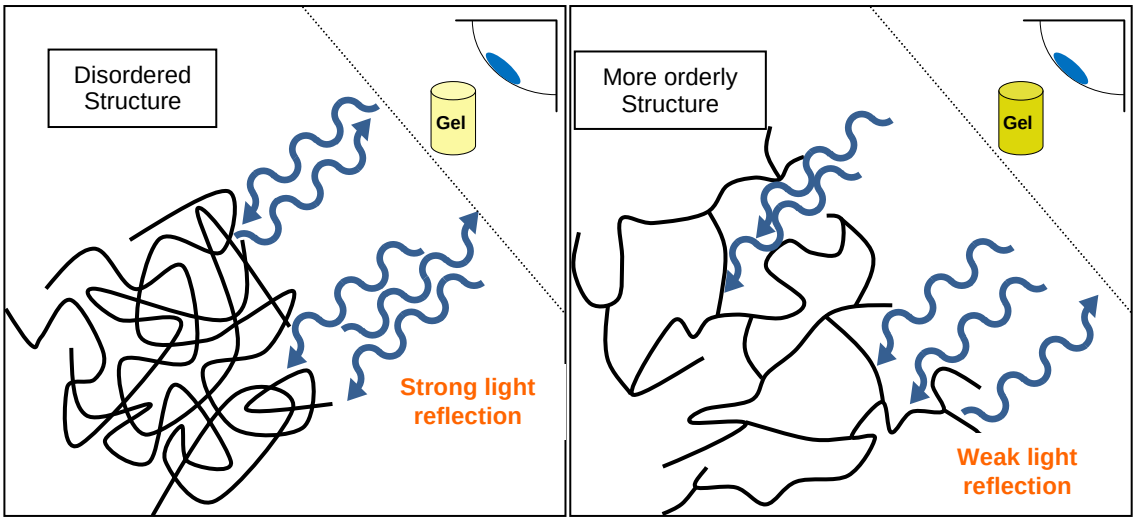

Figure 6.11 – Scheme showing the relationship between network structure and light interaction properties.

It is reported in literature (Hwang et al., 2007) that whenever protein denaturation occurs and a less orderly structure is formed, as with thermal treatment, whiteness

increases. Other authors have also observed a whiteness reduction with MTGase (Ramírez et al., 2007b). For pressure-induced hake gels, an opposite finding was made, since their whiteness was increased with MTGase incorporation (Tables 5.2.2.5-6). So, factors other than protein denaturation may be involved. A possible explanation could be the action of the endogenous TGase, whose presence was confirmed in hake (see 5.1.1 and 6.1). It has provided for an orderly net structure in the gels without MTGase, thereby lowering whiteness in these gels. This means that MTGase and TGase activities would be compounded, yielding a very finely porous micro-structure, which was observed through SEM (Figure 5.2.2.4). As a consequence of the smallness of the observed pores opaqueness may have increased, leading to lower light absorption.

Similar observations were made by authors working with gels prepared from white shrimp meat: L* (strongly correlated to whiteness, see 3) increased with higher amounts of MTGase (Tammatinna et al., 2007). Regarding pH, a slight increase of alkalinity with MTGase addition was registered for different products, namely mackerel surimi gels (Table 5.1.5.6) and sea bream gels (Table 5.2.1.4). Such an increase must be seen as an indirect effect of MTGase, probably resulting from altered protein conformations, that is, exposure of more or less acidic groups. The released ammonia as a result of the reaction catalysed by MTGase may also contribute for the effect. It is worth mentioning that mackerel surimi and sea bream gels were also the products presenting a clear effect of MTGase on WHC and colour and their textural quality, especially without MTGase, was poor. This strongly suggests a more important role of MTGase in products with denatured protein —enhancing WHC, augmenting cross-linking and generating a more orderly network structure.

The interaction of MTGase with DF encompassed different situations, namely, while no positive interaction between Fib and MTGase was observed, for mackerel surimi gels, it was shown that a combination of MTGase and Swe could improve the texture (for instance, hardness) of a poor quality surimi. For sea bream products, a synergistic effect between Kjc and MTGase upon their hardness was observed (Table 5.2.1.2). Once more, those products prepared from poor quality raw materials (such as trimmings of the frozen fish processing industry) and/or involving intense processing (and protein denaturation) may offer the best application for the additives. Similarly, it has been reported that MTGase addition has a more pronounced effect on Pacific whiting gels (Lanier and Kang, 2000), which normally exhibit lower gel strength than gels from Alaska pollock surimi. Nonetheless, a comparison of the effect of MTGase on the different tested products prepared from various raw materials and subjected to diverse processing conditions, shows that MTGase was always texturally beneficial (Table 6.11).

Table 6.11 – Comparison of the effects of MTGase on different intermediate materials.

Intermediate material	Effects	Overall evaluation
Hake mince	-Texture improved with addition up to 0.5 % (w/w) -Gel strength, elasticity, and hardness augmented by MTGase in gels without Swe -Elasticity was higher for those gels containing Swe -Whiteness of pressure-induced gels increased	Positive
Sea bass mince	-GS and force at rupture increased -No effect on the WHC -Whiteness slightly reduced	Positive
Gilthead sea bream mince	-Texture improved -Combined hardening effect of MTGase and Kjc surpassed the sum of the individual effects -WHC increased	Positive
Mackerel surimi	-Texture improved -WHC increased -Whiteness slightly reduced	Positive

For raw materials presenting better quality, such as sea bass mince, the advantages of MTGase may be not so large, but, above all, they are of a different nature. As previously mentioned and biochemically discussed (see 5.1.1 and 6.1), MTGase enhances the deformability of sea bass gels, thereby being also a valuable additive for products containing non-denatured protein. The strength of gels made with high-quality pollock surimi was also substantially increased by MTGase (Lanier et al., 2005). Differently from endogenous fish TGase, added MTGase is not calcium sensitive, thus not requiring calcium salts for the enhancement of its activity (Lanier et al., 2005). But, MTGase may have some drawbacks, particularly it has been reported that excessive cross-linking can produce a gel that is much less deformable, and therefore more brittle (Lanier et al., 2005). There are reports mentioning that added MTGase leads to a harder and less “natural” texture in surimi gels than the endogenous enzyme (Abe et al., 1996; Yasunaga et al., 1996). All these effects may be the outcome of incorporating too much MTGase to poor quality gels containing denatured and aggregated protein, as the contrast between the effects of MTGase on sea bass and hake gels illustrates (see 6.1).

6.3- Effect of New Processing Technologies on Restructured Fish Products

In the first point of this general discussion some key ideas explaining and describing the molecular phenomena that underlie the effects of new gelation technologies upon restructured fish products were expounded. In this third point these effects are thoroughly discussed with the purpose of reaching useful advice concerning the viability and suitability of the application of these technologies to restructured fish products. These

alternative gelation technologies, UVI and HHP, were tested in model products prepared with gilthead sea bream (see 5.2.1) and hake (see 5.2.2) trimmings, respectively. As with the additives, the main purpose was the assessment of the potential improvement of some properties fundamental for preparing good quality food products from fish including processing by-products and underutilized fish species. A general overview of the advantages and drawbacks of the utilized gelation technologies is shown in Figure 6.12.

Heat-Induced Gelation

ADVANTAGES:
-Well-known technology
-Low technological complexity
-Inexpensive equipment
-Gels with high levels of firmness
-High standards of microbiological safety by thermal treatment (90 ºC for 0.5-1 h)

DRAWBACKS:
-Uneven heating (overcooked areas)
-Slow heating (enabling proteolytic activity)
-Nutrient quality loss by extended thermal treatment
-Considerable energy cost
-Limited deformability of the gels

UVI-Induced Gelation

ADVANTAGES:
-Low cost technology
-Possible MTGase activation
-Successfully applied for preservation and decontamination of food products

DRAWBACKS:
-Very few studies available
-Technical difficulties regarding industrial application (thin layers, etc.)
-Possible stimulation of proteolytic activity
-Possible loss of textural quality

HHP-Induced Gelation

ADVANTAGES:
-Nutrient preservation (non-thermal treatment)
-Preservation of natural flavour and taste
-Raw/fresh appearance
-Homogeneous treatment
-Environmental friendly technology
-Possible inactivation of proteolytic enzymes
-Possible enhancement of MTGase action
-High deformability of the gels

DRAWBACKS:
-Very costly technology (hence, adequate for value-added products)
-Technological complexity
-Possible stimulation of proteolytic activity (due to proximity between enzymes and substrates)
-Softer gels
-Very high pressures (>500 MPa with a variety of times and pulse cycles) are required for ensuring pasteurization

Figure 6.12 – General overview of the advantages and drawbacks of the utilized gelation technologies.

UVI had no positive effect on textural quality (Tables 5.2.1.2-3). UVI did not alter any of the main textural parameters of the sea bream gels, with exception of cohesiveness, which declined with UVI treatment. The stress-relaxation test revealed that the elastic modules were augmented by UVI. This means that some chemical interactions were promoted by UVI treatment, enabling some toughening of gels at 10 % compression (the

degree of compression applied in the stress-relaxation test). But, these chemical interactions were insufficient for producing a beneficial effect on gel quality. This absence of textural benefits opposes the results of other authors (Jiang et al., 1998), which indicate a strengthening of the gels with exposition to UV light of MTGase-supplemented fish mince. One possible explanation may be given by the differences between myofibrillar proteins, namely myosin, of different species (Kawabata et al., 2000). It is equally possible that applied intensity, 3300 $\mu W/cm^2$, may have been quite below the required intensity, especially if compared with a recent work on fish gelatins that reported a significant improvement of gel strength with higher intensity of UVI, >10000 $\mu W/cm^2$ (Bhat and Karim, 2009). However, if the main problem was the stimulation of the proteolytic activity of enzymes present in the unwashed fish mince by UV as proposed in 6.1, a lower intensity would be necessary. Future studies must test a wide range of UVI intensities and, also, of exposure times. Similarly to texture, WHC and colour were unaltered by UVI treatment (Table 5.2.1.4). Hence, UVI does not present significant advantages as a gelation-promoting technology, at least, for sea bream minces and with applied irradiation intensity.

HHP processing ensured an acceptable level for the texture and WHC of hake mince gels (Tables 5.2.2.3-4). It was found that these pressure-induced gels presented an intermediate hardness level between only set gels (the softest) and heat-induced (the hardest) gels. Other textural parameters exhibited higher values (representing superior gel quality) for those gels treated by HHP. Pressure-induced gels were more cohesive and springy than the heat-induced gels. Chewiness (a global parameter that sums up textural quality) was almost identical for both types of gel product. Pressure-induced gels had a higher WHC than those thermally induced. Therefore, application of HHP —even at low pressure level (100 MPa) and for a short time (5 min)— had an overall advantageous effect on the mince from frozen hake trimmings, especially with respect to traditional heat treatment. This agrees with previous studies (Pérez-Mateos and Montero, 1997), according to which HHP may contribute for the improvement of poor functionality proteins. But, differently from other works (Cárlez et al., 1995), for instance with threadfin bream (*Nemipterus tabuloides*), results show that —with the exception of hardness— heat-induced gels do not necessarily present better physicochemical properties than pressure-induced gels. The latter ones were able to conserve or even enhance the deformability/elasticity found in gels immediately after the setting operation (only set gels). Pressure-induced fish gels have been described as more elastic (and softer) than heat-induced gels in the literature (Moreno et al., 2009). These findings corroborate the idea (discussed in 6.1) that gelation follows different paths depending on the use of temperature or pressure.

Regarding colour, HHP treatment had a much smaller impact on the different parameters of the gels than thermal treatment (Tables 5.2.2.5-6). For the latter, there was a significant reddening and yellowing of the products. Yet, the main colour problem of the heat-induced gels was a strong whitish appearance. This may be problematic depending on the kind of product intended to be attained (for instance, sashimi/sushi like products or new products mimicking fresh or cold smoked fillets, which could be a good outlet for frozen hake trimmings). The not so whitish appearance of pressure-induced gels may be advantageous. As explained above (see 6.2), these relatively darker gels show that pressure gelation reduces opacity, thus diminishing light reflection. This may be also related to a lower degree of muscle protein denaturation. Nonetheless, there was some opacity increase with HHP. Literature results (Table 6.12) also point a higher opacity (Ashie and Lanier, 1999) as a result of HHP application, however such results were obtained at moderate to high pressures (>300 MPa). In the HHP experiments carried out under this work, a low pressure range (100-300 MPa) was applied. This might have avoided a cooked appearance of the gel products (Moreno et al., 2009), while ensuring texture and water-binding properties comparable or superior to those of heat-induced gels.

Table 6.12 – Comparison of this work with literature on the effects of the application of new processing technologies to seafood materials.

Intermediate material	Applied technol.	Effects	Reference
Mackerel surimi	UVI	-Higher strength of MTGase-containing gels with exposure to UV light (1100 μW/cm^2)	Jiang et al., 1998
Fish gelatine	UVI	-Improvement of gel strength with higher UVI intensity (>10000 μW/cm^2)	Bhat and Karim, 2009
Sardine washed mince	HHP	-Textural improvement of poor functionality proteins with HHP (200-400 MPa)	Pérez-Mateos and Montero, 1997
Threadfin bream surimi	HHP	-Better mechanical properties (GS, breaking force) of heat-induced than with HHP-induced gels (300 MPa)	Cárlez et al., 1995
Giant squid surimi	HHP	-Higher elasticity and softness of HHP-induced gels (300 MPa) in comparison to heat-induced gels	Moreno et al., 2009
Alaska pollock surimi	HHP	-Higher opacity (and light reflection) as a result of HHP application (>300 MPa)	Ashie and Lanier, 1999
Gilthead sea bream mince	UVI	-No textural improvement with exposure to UV light (3300 μW/cm^2)	In this work
Hake mince	HHP	-Cohesiveness, springiness, and WHC improved	In this work

HHP operational parameters were also studied and found to affect product properties, being possible the selection of an optimal set of parameters.

The main operational parameter was the pressure level, which affected texture and colour of the gels. A higher pressure level made gels whiter, though not reaching the whitish appearance of thermally processed gels, and augmented gel strength.

The other two studied parameters, compression time and multi-cycles, had no major influence on texture, only on colour. Whereas longer compression times increased whiteness, the replacement of a single compression by three or five compression cycles produced a slight darkening of gels. Thus, less intense HHP treatments (lower pressures and/or shorter compression times) are recommended for fish processing with preservation of a raw appearance.

Similar conclusions were reached for high pressure treated minced albacore (*Thunnus alalunga*) muscle (Ramírez-Suárez and Morrissey, 2006). In what concerns multi-cycles, other authors did not find any differences as a result of applying pressure in one continuous step of 15 min or in three consecutive cycles of 5 min each (Gómez-Estaca et al., 2009).

For hake mince gels, a combination of 200 MPa (higher pressure level did not improve texture and led to a whitish appearance), 15 min and one single compression cycle (the impracticalities of multi-cycles were not compensated by any major improvement of the properties) may be a possible optimal set of HHP operational parameters.

Regarding the HHP operational temperature (30 ºC in this study), it may be the object of future studies. A synergistic effect of MTGase and HHP, whose underlying biochemical mechanisms were discussed previously (see 6.1), was found for some textural properties, such as gel strength.

HHP treatment showed to be an attractive gelation technology, which may be able to upgrade gel products prepared from poor quality raw materials, such as frozen hake trimmings.

However, it is still a very costly technology, especially the equipment and its maintenance, being preservation of high-valued jams and jellies in Japan commonly conducted on a commercial scale (Park, 2005) and, more recently, commercial applications have been successfully proposed, for instance, for the shucking of oysters (He et al., 2002) or to obtain high quality carpaccio-like products from fish (Gómez-Estaca et al., 2009).

A global comparison of the results attained with new processing technologies is shown in Table 6.13.

Table 6.13 – Comparison of the effects of new processing technologies on different intermediate materials.

Intermediate material	Gelation technology	Effects	Overall evaluation
Gilthead sea bream mince	UVI	-Textural quality not improved by UVI with respect to heat-induced gelation -Texture unchanged with exception of cohesiveness, which declined ~5 % with UVI -Some gel toughening	No advantage
Hake mince	HHP	-Almost all pressure-induced gels (100-300 MPa) softer than heat-induced gels -Cohesiveness or springiness improved by HHP (even after a single compression cycle of 5 min at 100 MPa) -WHC increased by 20-33 % -Colour less affected by HHP -Higher pressure levels whitened the gels -Synergistic effect between MTGase and HHP for some textural properties, such as gel strength	Positive (optimal parameters: 200 MPa, 15 min, and one compression cycle)

6.4 – Development of New Fish Products

On the basis of the attained biochemical knowledge, discussed in 6.1, and of the effects of additives (see 6.2) and alternative gelation technologies (see 6.3) on the food properties of gel products prepared from diverse raw materials, it was possible to devise new fish products. The information gathered in the previous points was used in the selection of the adequate additives (and their quantities) for the achievement of desired food traits, such as extra hardness or elasticity. The utilization of such additives was required by the characteristics of fish proteins and by the difficulties associated to the poor quality (denatured protein) of raw materials.

Two different products were developed, a low fat Frankfurter fish sausage containing DF (see 5.3.1) and a ready-to-eat minced fish product (appetizer) also containing DF (see 5.4.2). Though these new products were both prepared from hake mince, they are different restructured fish products not only concerning their nature but also their purpose. The Frankfurter sausage is not, strictly speaking, a gel product, since its various ingredients are typical of sausages, an emulsion product. This is because meat sausages present a high fat content, which requires the addition of an emulsifying protein, such as sodium caseinate. The preparation of a healthy Frankfurter fish sausage was set as a goal in order to fulfil one of the objectives of this work: investigation of the viability of substituting fish in traditional meat foods (see 2.3). So, the traditional sausage formulation used in the pork meat industry was the reference point. On the other hand, the appetizer (intended, for instance, to be an ingredient of salads) is not aimed at substituting fish in meat foods. It is based on the technology of heat-induced gel products and fulfils another

objective: creation of innovative heat-induced gel products involving fish mince as raw material and using additives capable of improving textural quality and adding value to the products (see 2.1). The use of additives, namely DF, was also essential for the development of a Frankfurter fish sausage.

Concerning fish sausage, the starting point was the traditional Frankfurter pork meat sausage with about 8 % pork fat and 56 % pork meat (Table 5.3.1.3) and the aim was to completely replace pork meat by hake mince for the achievement of a true fish sausage (Figure 6.13), thereby replacing the pork fat by new ingredients. This sausage had to be, as much as possible, a nutritionally complete and balanced product, thus requiring the addition of DF (Anderson et al., 1990; Kritchevsky and Bonfield, 1995; Puupponen-Pimïa et al., 2002). But, in order to accomplish the main objective, the properties of the final fish product had to be similar to those of the traditional product.

Figure 6.13 – Appearance of the produced low fat Frankfurter fish sausage containing dietary fibre before (A) and after culinary treatment (C). Appearance of typical white German meat sausages (B) (pictures from: IPIMAR).

This challenge was tackled in three steps and only overcome through the incorporation of some of the additives previously studied. First, DF enrichment of the pork meat sausage was assessed. Swe was chosen for this purpose because its incorporation hardened fish products (see 6.2) and from preliminary trials it was found that one of the main problems of substituting fish was the softening of the sausage. It was found that Swe increased the hardness as well as the gumminess and chewiness of the sausages, without any detrimental effect on the other main textural parameters (Table 5.3.1.4). Swe did not significantly alter the colour of the sausages (Table 5.3.1.5). Results showed that there is advantage on including Swelite in typical sausage recipes.

The second step involved a 50 % and, then, a 100 % replacement of the pork meat by hake mince. This led to a substantial softening effect, which was mitigated by the incorporation of 4 % (w/w) Swe (Table 5.3.1.6).

Other properties such as folding test, elasticity, cohesiveness and springiness were unchanged, even with a total replacement of pork meat. The results agree with other studies where the replacement of meat muscle resulted in a marked decrease of the

product's hardness (Cofrades et al., 2000; Murphy et al., 2004). This may be explained by the total collagen content in fish, which is lower than that in terrestrial animals (Lluch et al., 2001). Another explanation could be the observed moisture increase (about 11 %, w/w) with higher levels of pork meat replacement. This is due to the different moisture levels in hake and pork meat. But, a group of trained panellists was not held back by the hardness loss due to fish substitution from scoring as acceptable the 100 % fish sausages. Furthermore, if deemed necessary by the panellists, textural hardness could be improved through modifications in the sausages formulation (Murphy et al., 2004), for instance, reducing water and ice addition.

Regarding colour, fish substitution induced a progressive whitening of the product (Table 5.3.1.7). This is due to the low heme pigment content in the hake's light muscle. This pigment associated to myoglobin is very important for the product's colour since the heme iron reacts with nitric oxide present in the curing salt, producing nitric oxide myoglobin and, after heating, nitrosylo-hemochrome, a stable pink pigment (Kijowski, 2001; Shahidi, 1991).

Finally, pork fat was replaced by Fib (7.8 %, w/w) or, alternatively, by combinations of Fib and extra hake mince (2.6 %:5.2 % or 5.2 %:2.6 %, w/w) in the sausages without pork meat and containing Swe. The choice of Fib was due to its ability to act as a fat mimetic component, ensuring smoothiness, creaminess and an oily mouth-feel (Anonymous, 2005c). Fib also ensures some textural advantages (see 6.2). Moreover, Fib augments the DF content of the product and lowers its energetic content, since Fib is not digested in the upper intestinal tract and, as such, the only energy provided by this DF is due to bacterial fermentation in the large intestine (Anonymous, 2005b,c). It was found that higher levels of Fib reduced cohesiveness, springiness, and chewiness (Table 5.3.1.8). The highest Fib content also caused a significant loss of elasticity. Low Fib sausages (2.6 % Fibruline and 5.2 % extra hake mince) were texturally similar to the control sausages with pork fat, with exception of some hardness and gumminess (conditioned by hardness) reduction. So, an extra amount of hake protein can be a better substitute of pork fat.

Nevertheless, Fib incorporation is important in order to avoid a dry mouth-feel. Trained panellists scored as acceptable the low Fib fish sausages and found a mouth-feel similar to that of control sausages.

The main steps undertaken for the production of Frankfurter fish sausages and their results are summarized in Table 6.14.

Table 6.14 – Main steps in the process of development of Frankfurter fish sausages.

Main steps	Effects	Overall evaluation
1) Enrichment of a traditional Frankfurter pork sausage with Swe	-Hardness, gumminess and chewiness increased -No deleterious effect on other textural properties -No effect on colour	Positive
2) 100 % replacement of the pork meat by hake mince	-Softening effect -Whiteness increased -Folding test, elasticity, cohesiveness, and springiness unaffected - Product scored as acceptable based on sensory analysis	Acceptable change
3) Total replacement of pork fat by a combination of Fib and extra hake mince	-Best combination was 2.6 % (w/w) Fib + 5.2 % extra (w/w) hake mince -Creaminess and an oily mouth-feel ensured by Fib -Texture similar to control sausage except some softening -Redness, yellowness, and whiteness slightly decreased -The combination was considered acceptable by sensory analysis	Acceptable change

A comparison with typical commercial (meat) sausages revealed that the differences between low Fib (fish) sausages and the commercial ones were not very large, with exception of a lower hardness (~25 % less) of the experimental sausages (Table 5.3.1.9). On the whole, it was shown that is possible to produce a texturally acceptable white sausage (in which textural properties were most similar to high fat control) combining multiple health promoting factors, such as higher DF intake, lower fat intake, and fish nutrients, that is, a healthy low fat fish sausage containing dietary fibre.

The ready-to-eat minced hake product/appetizer (Figure 6.14) was developed on the basis of the knowledge collected during the study of heat-induced hake gels (see 6.1 and 6.2).

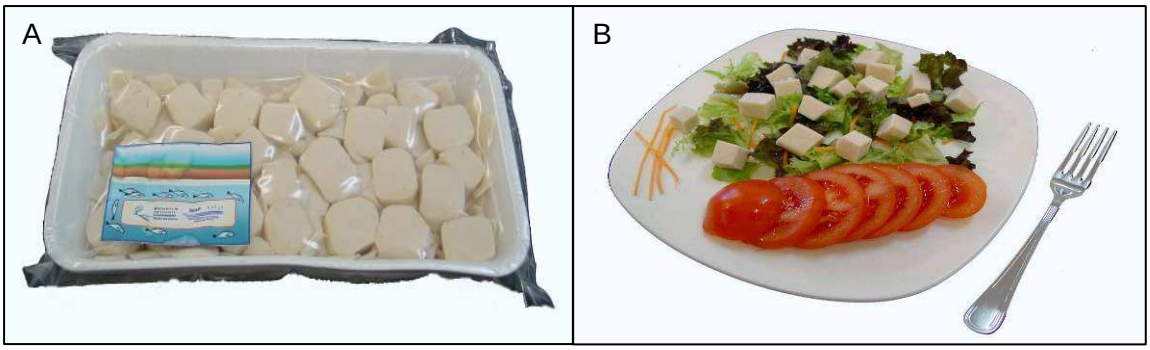

Figure 6.14 – Appearance of the produced ready-to-eat minced hake appetizer before (A) and after culinary treatment (B) (pictures from: IPIMAR).

The positive results attained with Swe, Carr and MTGase in various previous trials led to the incorporation of these additives in the product's formulation. A shellfish aroma was added to the product. The product was designed with the purpose of ensuring the recommended daily intake (RDI) of 500 mg EPA+DHA (Kris-Etherton et al., 2002). This was an important objective, since EPA and DHA are valuable nutrients, to which an important role in the prevention of cardiovascular disease (Burr et al., 1989) as well of several inflammatory conditions (Calder, 1996) has been assigned by various studies. Preliminary trials showed the sensory importance of some fat incorporation (5.6 %, w/w) (Fib substitution was not tested in this product). These various constraints led to different small alterations in the formulation of heat-induced fish gel products (Rawdkuen and Benjakul, 2008), namely, to the addition of sodium caseinate (0.5 %, w/w) to the product (see 5.4.2.).

The main identified difficulty was the incorporation of fish oil (the richest food source of ω3 fatty acids, such as EPA and DHA) in order to attain the RDI of EPA+DHA. Fish oil addition may have various repercussions (chemical, nutritional, textural and sensory) to the quality of the new product. It may impart a disagreeable fish flavour to the product. Thus, a deodourized cod liver oil (see fatty acid profile in Table 5.4.2.1) was used and only a part of the vegetable oil was replaced by cod liver oil (2.9 %, w/w). This partial replacement ensured the RDI of EPA+DHA provided that a 100 g serving is considered. For control products (with vegetable oil), only 190 mg of EPA+DHA were provided *per* 100 g serving and the ω3/ω6 ratio was very low. The fatty acid profile of the minced fish products also reflected the profile of the used oils. This fact means that during processing and heating no changes occurred in the fatty acid profiles. But, there were some chemical alterations, namely some oxidation of the ω3 fatty acids may have taken place according to the TBARS value. Although the higher oxidation levels found in substituted minced fish products may have resulted from the high levels of ω3 fatty acids —which are more prone to oxidation (Pérez-Mateos et al., 2004)— *per se*, processing during production, such as cooking (1 hour at 90 ºC) may have contributed for the outcome (Figure 6.15).

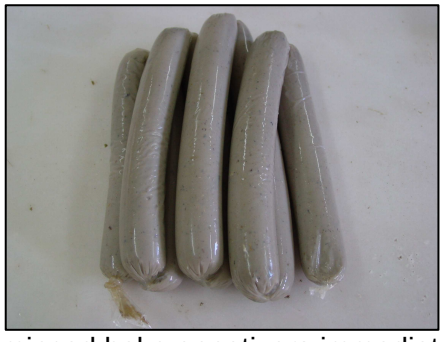

Figure 6.15 – Ready-to-eat minced hake appetizers immediately after cooking and before dicing (pictures from: IPIMAR).

A study conducted with fortified vacuum-packed fish sausages (Panpipat and Yongsawatdigul, 2008) also showed that higher levels of vegetable oil replacement by fish (tuna) oil favoured higher TBARS results. According to some authors (Montero et al., 2005b), processing for mince extraction, preparation of batter, and cooking make necessary the addition of antioxidants to avoid rancidity. However, even for the products enriched in ω3 fatty acids, TBARS value never exceeded 0.5 mg malonaldehyde/kg and was therefore considerably lower than the maximum acceptable level (1-2 μmol malonaldehyde/g fat or ≈ 4-8 mg malonaldehyde/kg) proposed by Connell (1975) for fish products. These results seem to agree with sensory analysis. The replacement of vegetable oil by cod liver oil only affected the mean sensory colour score of the products, which were perceived as darker (Figures 5.4.2.1-2). The remaining sensory properties were unchanged and were favourably evaluated by panellists. Regarding instrumental texture, measurements also showed that cod liver oil substitution has, in general, no negative effect upon textural quality (Table 5.4.2.4), as claimed in other studies (Panpipat and Yongsawatdigul, 2008). Only gel strength was slightly lower in the products containing cod liver oil. So, this ready-to-eat fish product, in addition to being healthy (hypocaloric, containing DF and ω3 fatty acids), is texturally acceptable and enjoyable for consumers. A global comparison between both ready-to-eat minced hake appetizers is shown in Table 6.15.

Table 6.15 – Comparison between produced ready-to-eat minced hake appetizers.

Minced hake appetizers	Characteristics
Control product (5.6 %, w/w, vegetable oil)	-190 mg EPA+DHA *per* 100 g serving -Very low oxidation levels -Sensory analysis panel scored the product as acceptable
ω3 enriched product (2.7 %, w/w, vegetable oil + 2.9 %, w/w, cod liver oil)	-500 mg EPA+DHA *per* 100 g serving -Higher oxidation levels (low TBARS value < 0.5 mg malonaldehyde/kg) -Texture similar to control (except lower gel strength) -Slight darkening -Product scored as acceptable based on sensory analysis
Conclusion: The ω3 enriched ready-to-eat appetizer preferable to control product, since disadvantages outweighed by a much higher nutritional value	

Though these products were prepared from frozen hake (frozen storage of about three months) and involved intense processing, their quality was acceptable for consumers and comparable to that of commercial products (in the case of fish sausages). These products may offer a potential outlet for fish industry by-products (such as trimmings of the frozen hake processing industry) and underutilized fish species, provided that adequate handling

and storage conditions are provided for these raw materials. These products may also be economically viable if prepared from more expensive raw materials, since they are healthy (in the case of sausages, much healthier than their pork meat counterparts) and ready-to-eat, and, accordingly, may target specific market niches of consumers willing to pay a premium for health and convenience. In order to assess the commercial viability of the developed fish products, it is important to know how much time they can be stored without losing quality. Thus, storage stability studies of these products were also carried out.

6.5 - Storage Stability of Developed Fish Products

Concerning new food products, a thorough study of stability is always a fundamental condition for their development, encompassing an evaluation of quality changes and, also, of microbiological quality throughout storage time. The quality of Frankfurter fish sausages (see 5.4.1.) and ready-to-eat minced fish products (see 5.4.2.) was followed during storage under refrigeration in order to estimate their shelf life. Various quality parameters were followed: chemical, nutritional, physical, textural, sensory and microbiological. Both products were vacuum-packed and stored in refrigeration (2 or 10 ºC) over various months. The products were prepared according to the usual procedures applied during their development stage, that is, a steam cooking at 90 ºC for 10 minutes (sausages) or for one hour (minced fish product).

Two groups of fish sausages were studied during an 80-day storage trial at 2 ºC. One group (control) had 7.8 % (w/w) pork fat and the other (Fib sausages) 5.2 % (w/w) Fib and 2.6 % (w/w) extra hake mince instead of pork fat. These formulations were chosen on the basis of previous results (see 6.4.). But, instead of the best combination of Fib and extra hake mince (Table 6.14), it was chosen another combination accepted by the sensory panel with the advantage of a higher DF content. For both groups, no important alterations were found during the trial. TBARS values were low (Table 5.4.1.5), not exceeding 2 mg malonaldehyde/kg. This is below the maximum level (4-8 mg malonaldehyde/kg) for fish products (Connell, 1975) and is due to vacuum-packaging of the products (Figure 6.16).

Figure 6.16 – Vacuum-pack device and vacuum-packed product (pictures from: IPIMAR).

Likewise, no significant effect of storage time on colour was detected (Table 5.4.1.6). Some textural changes were observed over storage time. Hardness increased significantly between the initial and final samplings for both studied products (Tables 5.4.1.7-8). However, gumminess and chewiness only increased along storage for Fib sausages. The evolution of these two properties was a consequence of the strong hardening in Fib sausages, particularly, from the 1st to the 12th day of storage. Afterwards, there was no clear hardening effect. The hardening of sausages over storage time has been reported in several papers, but dealing with different raw materials, such as low fat beef (Candogan and Kolsarici, 2003) or chicken (Andrés et al., 2006) Frankfurters. Some authors (Andrés et al., 2006) related similar hardness increases to water loss during refrigerated storage. But, such loss was rather low in the fish sausages. Nonetheless, it should be noted that Fib sausages, which exhibited a stronger hardness increase than control ones (63 % *vs* 26 %), had also higher purge losses over time. These textural results obtained by instrumental means were confirmed by sensory analysis. Hardness and succulence scores changed significantly over storage time for the two sausage types, whereas saltiness and astringent aftertaste scores only changed for Fib sausages (Figures 5.4.1.1-2). But, contrary to texture measurements, hardness increase was steeper for control sausages (115 % *vs* 77 %), being found significant differences to the initial value from the 28th day onwards. Succulence decreased to a third of initial level for both products, maintaining control sausages their succulence longer than fibre-added sausages (44 days *vs* 26 days).

Regarding colour, the instrumental results were also in accordance with human perception of colour, which, similarly, did not enable the identification of significant colour variations. Sensory analysis agreed with TBARS values, since no panellist reported any rancid aroma or flavour, even in the control sausages (containing pork fat) after 80-day storage.

Microbiological analysis showed growth of mesophilic aerobic microorganisms (presenting always low levels during the whole storage period) and the development of mesophilic anaerobic spore-formers (Table 5.4.1.9). Regarding aerobic microorganisms, microbial counts were lower than those found with low fat chicken sausages submitted to similar conditions, stored at 4 ºC over a 50-day period after being pasteurised and vacuum-packed (Andrés et al., 2006). Probably, the higher pasteurisation temperature applied to fish sausages (90 ºC *vs* 74 ºC) and slightly lower storage temperature (2 ºC *vs* 4 ºC) can explain such differences. But, storage time had an important effect on the development of mesophilic anaerobic spore-formers. This microbiological alteration was detected for both sausage types from the 45th (control sausages) and 43rd (Fib sausages) day onwards. Although the isolated colonies were not black, as characteristic of most of

the bacteria belonging to the genus *Clostridium*, some strains *C. botulinum* (particularly saccharolytic strains in group II) do not invariably form black colonies in such media (ICMSF, 1996). Such microbial growth is to be considered as a relevant quality loss and the storage times associated to it must be considered as maximum shelf lives for these products.

With respect to the ready-to-eat minced fish products (appetizers), the quality changes of two groups were followed during 3.5 month under refrigeration at two different temperatures, 2 ± 2 ºC (considered as optimum) and 10 ± 2 ºC (abuse). The formulation of the two groups was identical, with exception of vegetable oil (VO), 5.6 % (w/w) in one group and 2.7 % (w/w) plus 2.9 % (w/w) cod liver oil (CLO) in the other (see 6.4). Differently from fish sausages, some nutritional changes occurred over storage time. Moisture, protein, and ash showed changes after 105 days at 10 ºC (Table 5.4.2.2). These slight but significant changes in the chemical composition seemed to indicate some water loss (< 1.5 %) during storage at the chosen abuse temperature. Storage time had no significant effect on the fatty acid contents (Table 5.4.2.1). Regarding TBARS values, changes were observed (Figure 5.4.2.4): an initial increasing phase was followed by a decline phase. But, even in the CLO products, TBARS value never exceeded 0.5 mg malonaldehyde/kg and was considerably lower than the maximum acceptable level for fish products (Connell, 1975). The absence of a clear and continuous increasing trend during storage time may be related to the permanence of an anaerobic environment inside the packages over storage time (Figure 6.17).

Figure 6.17 – Vacuum-packed ready-to-eat hake appetizers (pictures from: IPIMAR).

Nevertheless, some clear correlations between TBARS and storage time under vacuum conditions have been reported for fish emulsion sausages fortified with ω3 fatty acids and stored at 4 ºC (Panpipat and Yongsawatdigul, 2008). A comparison between the experimental conditions of the cited study and this study on appetizers may elucidate the factors behind the different findings. Panpipat and Yongsawatdigul, (2008) reported an increase of the TBARS values (over four weeks storage) from 2 to 6 mg malonaldehyde/kg for a content of fish oil identical to that of CLO products. But, the total

fat content was higher in this study's product, since more than 20 % (w/w) fat was present in the sausages, which compares to a fat content of only 5.7 % (w/w) in the appetizers.

Therefore, a low content in fat, albeit much richer in ω3 fatty acids and enough to guarantee 500 mg EPA+DHA *per* day and by a serving portion of 100 g, may better oppose to lipid oxidation than high fat content, even when this fat is extremely poor in EPA and DHA. The decline phase of TBARS values suggests that low rates of lipid oxidation were coupled with increased malonaldehyde degradation, due to reaction of this compound with muscle proteins (Kristinsson et al., 2006). While higher temperature accelerated oxidation in the control products, the same was not found with CLO products. The higher TBARS values in CLO products could have triggered enhanced MA degradation and, in this way, the storage temperature effect may have been attenuated. Differently from Frankfurter fish sausages, colour of appetizers was affected by storage time (Table 5.4.2.3). Though only CLO products at 10 ºC produced a clear trend, minced fish products on the whole became redder and less yellow over storage time. This trend may have resulted from pigments present in the cod liver oil, whose golden hue was much stronger than that of the vegetable oil. For both products, pigments seemed to leach out of the products over storage time. The absence of CLO among the ingredients of the Frankfurter fish sausage as well as the higher storage temperature for part of the appetizers may be a reason for the difference between these two products. For CLO products, temperature had clearly a role in the process.

Concerning texture (Table 5.4.2.4), storage temperature had a decreasing effect upon elasticity, hardness, cohesiveness, gumminess and chewiness. This may result from enhanced chemical and biochemical degradation processes (protein denaturation and aggregation) at higher temperature. There is a clear association between textural quality and such degradation processes (see 6.1). Only VO products lost textural quality over storage time at 10 ºC (CLO appetizers were not significantly affected by storage time at this temperature). So, it seems that cod liver oil may have beneficial characteristics for the preservation of textural quality at higher temperatures.

Nevertheless, it can be hypothesized that the lower moisture value of CLO products at 10 ºC, entailing a larger water loss, may have been able to compensate the softening effects of protein denaturation and aggregation. Other authors have found that water loss can increase protein concentration in the product and thereby harden it, while protein quality and gel strength decline (Andrés et al., 2006) (Figure 6.18).

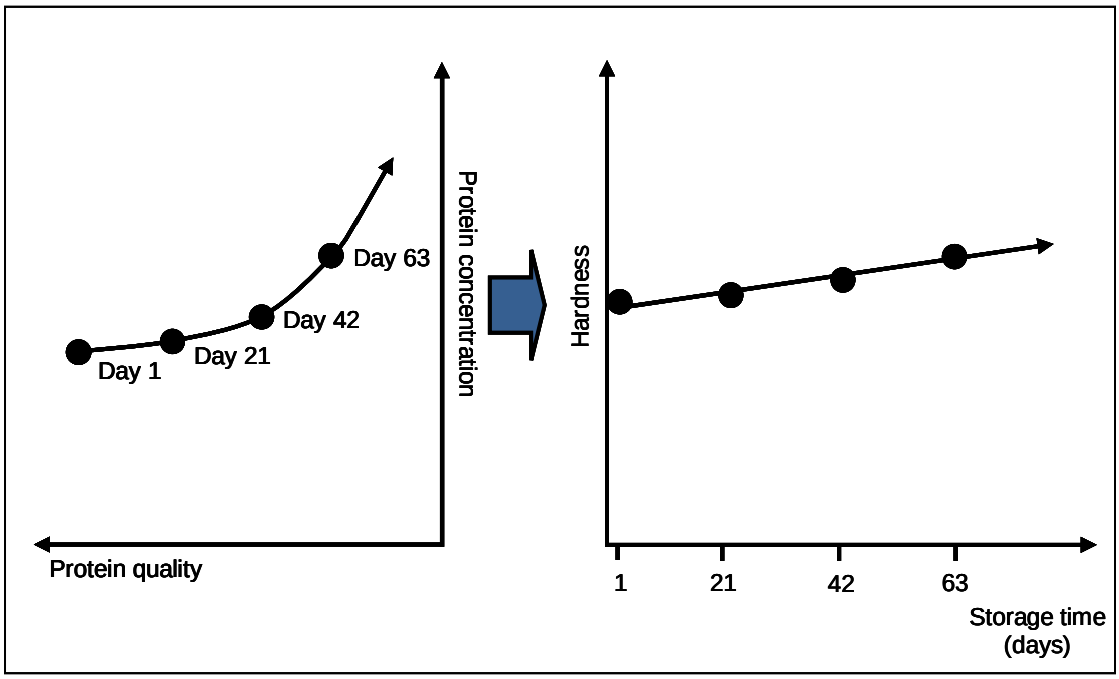

Figure 6.18 – Possible effect of the combined variation of protein concentration and protein quality on the hardness of restructured fish products over storage time.

Other authors have not detected any storage time effect on texture (Panpipat and Yongsawatdigul, 2008). But, such studies were conducted at lower temperature (4 ºC) and over shorter storage times (four weeks). The sensory analysis revealed that storage time caused a significant darkening of the appetizers and made them saltier (Figures 5.4.2.1-2). On the other hand, the appetizers lost their shellfish aroma and flavour. The VO appetizers had a pronounced hardness decline at 10 ºC. But, a higher temperature did not bring about many sensory changes over storage time. The darkening had no correlation with instrumental measurements (chroma declined and no significant whiteness variation occurred). Saltiness and moisture (see above) variations with increasing storage time, suggest a correlation between these two parameters. The observed hardness reduction is supported by textural measurements. Autolytic processes driven by enzymes and chemical reactions, both favoured by higher temperatures, can have a major impact on the loss of textural quality, particularly during the early stages of deterioration but, generally, do not produce the characteristic off-flavours and off-odours, which are specific of microbiological activity (Truelstrup et al., 1996). As microbiological growth was not detected, off-flavour and off-odour absence are partially explained. The characteristics of the cod liver oil used (purified, deodourized) may have also contributed for these results.

Furthermore, the added shellfish flavour masked any faint fish-like flavour. It has been reported that high flavour intensity products are more effective at masking the slightly fishy taste and odour associated with the addition of fish oil (Kolanowski et al., 1999). Panellists did not detect any rancid aroma or flavour (confirming TBARS results) and considered the

products as acceptable until the last sensory evaluation on day 63. Therefore, at least, a shelf life of two months for both groups of appetizers under refrigerated storage may be admitted.

A final comparison between the storage stability of Frankfurter fish sausages and that of appetizers delivers some insight (Table 6.16).

Table 6.16 – Comparison of estimated shelf life.

Fish product	Quality changes	Estimated shelf life (days)
Frankfurter fish sausage	-No important chemical or nutritional alterations -TBARS values < max. acceptable level (4-8 mg malonaldehyde/kg) -No colour variation -Some textural changes, hardness increased -Texture results confirmed by sensory assessment -Saltiness and astringent aftertaste scores increased in sausages with no pork fat -Development of mesophilic anaerobic spore-formers after 45 (with pork fat) and 43 days (with no pork fat)	45 *(pork fat sausage at 2 ºC)* 43 *(no pork fat sausage at 2 ºC)*
Ready-to-eat appetizer	-Some water loss (< 1.5 %) at 10 ºC storage -Fatty acid profile unaffected -TBARS values < max. acceptable level -Redder and less yellow -Texture quality lost by VO appetizers at 10 ºC -Saltiness increased according to sensory analysis -Shellfish flavour decreased according to sensory assessment -Acceptable quality until 63 days (last sensory evaluation) based on sensory assessment -No microbiological growth	63 *(VO & CLO appetizers at 2 & 10 ºC)*

The latter seem to present a more prolonged shelf life even at a higher storage temperature. This is most apparent for the microbiological data. The microbial counts concerning the appetizers were lower than those observed in the microbiological analysis of the fish sausages (also vacuum-packed). The higher cooking time applied to the appetizers (one hour *vs* 10 minutes) may explain such differences. Non-sporulated bacteria were detected in the raw material of the appetizers, but were absent in the heat treated product (Figure 5.4.2.5). This finding agrees with Stumbo (1973), as the applied heat treatment (enabling, at least, a reduction of 15 log cycle in the number of most vegetative forms) should prevent the survival of the vegetative cells present in the raw material. The storage stability shown by sausages and appetizers is comparable to other results found in the literature. For instance, chilled crab analogues containing menhaden

oil exhibited little change in sensory properties throughout two months of storage (Pérez-Mateos et al., 2004).

6.6 - Conclusions

This work was carried out in accordance with the proposed objectives and with the ultimate goal of finding new strategies (additives, alternative processing technologies or fish substitution in traditional meat products) for the upgrading of fish by-products and underutilized fish species. For achieving such purpose, various technical problems had to be overcome and their knowledge was much improved. This was a thoroughly integrated approach, ranging from the biochemical phenomena at the protein bonding level up to the practical problems of creating products tailored to the consumer taste and with storage stability suitable for their marketing to consumer niches concerned with health and convenience.

At the biochemical level, several important conclusions were achieved:

- The use of additives as chemical probes in products prepared for this single purpose (the so-called informational products) is a valuable tool for the understanding of the molecular mechanism underlying various macroscopic variations in properties;
- The superior and different textural quality of heat-induced gel products prepared from fresh fish (sea bass) with respect to those from frozen fish (hake) is traced back to the protein denaturation and aggregation due to freezing and frozen storage;
- The different levels of protein denaturation and aggregation and their different propensity to unfolding leads to different MTGase action modes;
- The MTGase catalyzes the cross-linking between the small number of Gln and Lys residues exposed at the surface of frozen fish proteins, thus linking a small number of molecules and exhibiting a short distance effect, which is texturally expressed by a substantial hardening effect;
- The MTGase, due to a greater unfolding of proteins, catalyzes the cross-linking between almost all Gln and Lys residues of the fresh fish proteins, thereby uniting a large number of molecules and succeeding in the establishment of a vast network structure, which is texturally expressed by a greater deformability of the products;
- The different MTGase actions seem to be more determinant for observed properties than any variation in the relative importance of non-covalent or disulphide bonding;

- The presence and important activity of an endogenous cysteine TGase in hake (but not in sea bass) products was detected;
- Each DF has its own specific way for interacting with the protein matrix, thus favouring different outcomes for the textural, colour and general quality of the fish products;
- Regarding alternative gelation technologies, whereas UVI seems to stimulate protein degradation by enzymes without any positive biochemical action upon MTGase or myofibrillar proteins, HHP may promote a kind of protein aggregation characterized by side-to-side interactions of proteins with reduced denaturation instead of large conformational changes as with thermal gelation.

These main conclusions for all their provided insight were influential on the selection of the most suitable additives and technologies for the other subjects of this work.

Concerning the various properties associated to food quality, the additives and technologies were thoroughly tested and their incorporation levels or optimal operational parameters were identified with the purpose of maximizing nutritional, textural and overall quality of the fish products.

The following results were drawn:

- From tested DFs, inner pea fibre, chicory root inulin, carrageenan and konjac glucomannan are the most advantageous, since they increase the DF content in food without any major deleterious effect on texture (or, sometimes, with some textural improvement for the product);
- Whereas inner pea fibre (4 %, w/w) and carrageenan (1 %, w/w) are particularly efficient in the hardening of various fish products prepared from different raw materials, chicory root inulin (2 or 4 %, w/w) is more efficient in the enhancement of the products' deformability;
- The beneficial effects of DF addition may depend on the quality of the raw material, since, for inner pea fibre, it was found that replacing protein by DF has only a beneficial effect if protein is of poor quality (i.e., denatured and aggregated), otherwise DF addition has a negative net effect;
- MTGase (from 0.1 up to 0.5 %, w/w) has a positive effect on the different tested products prepared from various raw materials and subjected to diverse processing conditions, especially at the textural level. For raw materials of better quality, the advantages of MTGase addition may be not so large, nonetheless, they are significant and are able to impart new and advantageous properties to the fish products;

- Differently from UVI, HHP treatment (200 MPa, 15 min and one single compression cycle) seems to be an advantageous gelation technology, which may be able to upgrade gel products prepared from poor quality raw materials, such as frozen hake trimmings. There is a strong possibility of a synergistic effect between MTGase and HHP. However, HHP is still a very costly technology.

These results showed that it is possible to attain good quality restructured fish products from poor quality raw materials through new gelation technologies and/or additive incorporation. These raw materials may be trimmings and other edible by-products from some specific steps of fish processing, such as filleting (for instance, gutting and viscera were not considered), which may present some acceptability problems regarding their textural and sensory quality. In order to fulfil the objectives 2.3 and 2.4, all these results were applied to the development of new fish products with commercial potential, yielding the following results:

- A sensory acceptable and healthy Frankfurter fish sausage (comparable to commercial pork sausages) was produced through the combination of inner pea fibre (hardening additive), chicory root inulin (mimicking fat from a sensory point of view), low fat level and fish nutrients;
- This Frankfurter fish sausage presented an acceptable storage stability, since the first significant quality loss (of a microbiological nature) was detected after 1.5 month under refrigeration at 2 ºC;
- Likewise, a ready-to-eat fish appetizer was produced with incorporation of DF and ω3 fatty acids, thereby addressing the health and convenience concerns of many consumers;
- This appetizer presented no significant quality loss over two months of storage at 2 or 10 ºC, since panellists did not detect any rancid aroma or flavour and considered the products as acceptable until the 63rd day.

Therefore, this work was able to test different technological strategies suitable for the upgrading of underutilized fish species and fish processing by-products and to prove the potential of some of them.

Future experimental work should provide a deeper knowledge of the effect of MTGase on different proteins in order to achieve a greater level of control of the changes undergone by proteins and, as a result, of the quality of processed fish products. This knowledge could be applied to other functional fish products besides those prepared in this work, such as a cooked fish ham enriched in DF.

7 - References

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Appendix

General Information about Dietary Fibre

Dietary fibre (DF) is usually defined as the plants' components that resist to digestive enzymes. It includes polyssacharides, oligossacharides, lignin and other associated substances (Figure A.1). Carrageenans and alginates from algae are also comprised in this definition.

In this appendix, more detailed information regarding DFs (especially those used in this work) is presented.

It must be emphasized that DF, unlike other food components, is not attacked by the enzymes of the stomach and small intestine and, as such, it reaches the colon undegraded. DF has been defined as that fraction of the edible part of plants (from roots, seeds, fruits, etc.) or their extracts (and also synthetic analogues) that is resistant to the digestion and absorption in the small intestine, usually with partial or total fermentation in the large intestine (Prosky, 2001).

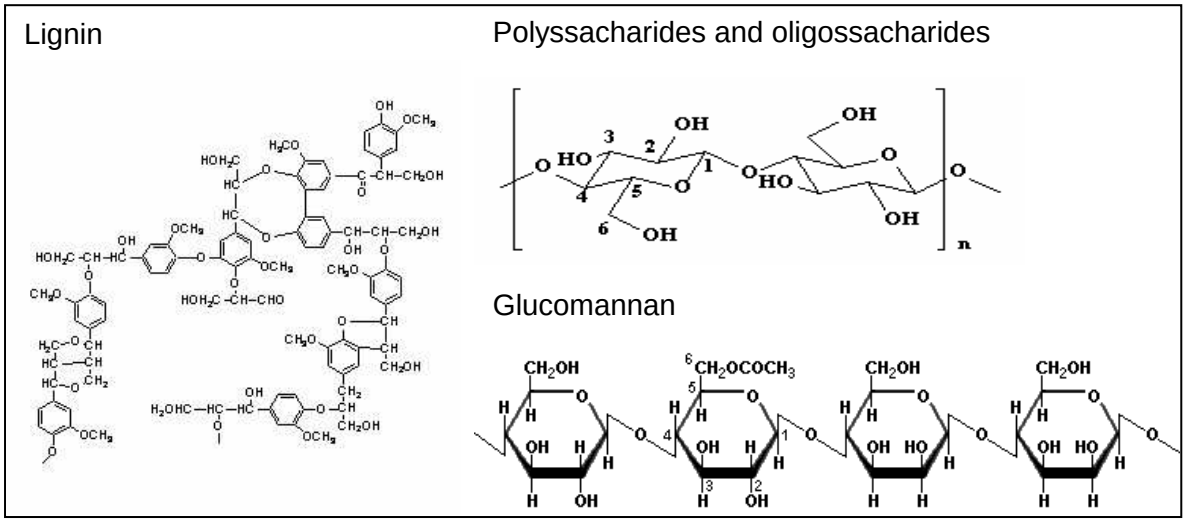

Figure A.1 – Examples of food components most commonly defined as DF.

More recently the definition of DF has been made more inclusive, comprising not only non-edible parts of vegetables, but also fibre of animal origin, such as chitosans, which are derived from the chitin contained in the exoskeletons of crustaceans and whose molecular structure is similar to that of plant cellulose (Borderías et al., 2005), and of microbial origin such as xanthan gum or curdlan.

Properties of DF Products

In this work, seven DF products were used. Four were supplied by Cosucra (Warcoing, Belgium): inner pea fibre-Swelite®, outer pea fibre-Exafine® 250, chicory root oligofructose-Fibrulose 97, and chicory root inulin-Fibruline INSTANT. Another DF product, apple fibre-Vitacel AF 401, was supplied by Rettenmaier (Holzmühle, Germany). Carrageenan-Ceamgel 1830 was provided by Ceamsa (Porriño, Spain) and glucomannan-konjac flour/Nutricol® GP 312 provided by FMC Biopolymer (Philadelphia, USA). The composition (based on dry matter, D.M.) and other properties are shown in Table A.1.

Table A.1 – Properties of the seven dietary fibre products used in this work*.

PROPERTIES	Swelite	Exafine	Fibrulose	Fibruline	Vitacel	Ceamgel	Nutricol
Composition (D.M.)							
Total Carbohydrates (%)	93 ± 3	min. 85	min. 99.7	min. 99.7	†	†	†
Total DF (%)	48 ± 3	min. 85	97 ± 2	min. 90	55 ± 5	min. 80	min. 80
Starch (%)	min. 36	max. 5	—	—	—	—	—
Protein (%)	max. 7	max. 6.5	—	—	4.6	—	—
Fat (%)	max. 0.5	max. 0.5	—	—	2.5	—	—
Ash (%)	max. 2	max. 3	max. 0.3	max. 0.3	max. 3	—	—
Granulometry (µm)	< 400	85 %< 300	< 700	< 700	90 %< 300	98 %< 250	< 250
Colour	white	cream	white	white	red brown	pale yellow	tan
Taste	neutral	slight vegetable	neutral to slightly sweet	neutral to slightly sweet	fruity	neutral	neutral
Water solubility (at 20 ºC)	mostly insoluble	mostly insoluble	soluble	soluble	mostly insoluble	soluble	soluble

* Values are manufacturer's claims (Anonymous, 2004a; Anonymous, 2004b; Anonymous, 2006; Anonymous, 2008).
† not indicated.

DF Composition of the Products

Inner pea DF (Swelite) is composed of nearly 2/3 insoluble cellulose and 1/3 soluble pectic material (Anderson and Berry, 2001). Outer pea DF (Exafine) is composed of about 55 % cellulose, 25 % hemicellulose, and 20 % pectins (Anonymous, 2010). Chicory root DFs, Fibrulose and Fibruline, are mainly oligofructose and inulin, respectively (Anonymous, 2004a). Apple DF (Vitacel) is composed of about 70 % cellulose and 24 % hemicellulose (Soukoulis et al., 2009). Algal DF (Ceamgel) is a mixture of iota and kappa carrageenans (each approximately 50 %, w/w) from red seaweeds (Anonymous, 2006). Konjac DF (Nutricol) is solely glucomannan from the konjac plant (Anonymous, 2008).

DF Chemical Structures and Characteristics

Cellulose is the major structural material of plants (Figure A.2). Cellulose is a polyssacharide consisting of a linear chain of several hundred to over ten thousand β(1→4) linked D-glucose units (Updegraff, 1969) (Figure A.3). It is a straight chain polymer with no coiling or branching. The multiple hydroxyl groups on the glucose from one chain form hydrogen bonds with oxygen on the same or on a neighbour chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength. Cellulose is insoluble water and most organic solvents.

Figure A.2 – Appearance of cellulose DF (picture from: IPIMAR).

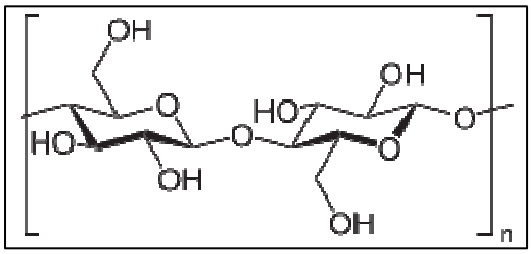

Figure A.3 – Chemical structure of cellulose (picture from: Nishiyama et al., 2002).

Hemicellulose (Figure A.4) encompasses a heterogeneous class of polyssacharides, which may contain pentoses (D-xylose and L-arabinose), hexoses (D-mannose, D-glucose, and D-galactose) and/or uronic acids (such as D-glucuronic or D-galacturonic acids). Other sugars such as L-rhamnose and L-fructose may also be present in small amounts and the hydroxyl groups of sugars can be partially substituted with acetyl groups (Gírio et al., 2010). D-xylose is always the sugar monomer present in the largest amount. Unlike cellulose, hemicellulose consists of shorter chains (from 500 to 3000 sugar units per polymer). It is a branched polymer (also unlike cellulose). Xylans are the most abundant hemicelluloses (Figure A.5). They belong to the relevant sub-group of the pentosans, consisting of D-xylose units with β(1→4) linkages. As cellulose, hemicelluloses have an important structural role in vegetable tissues (represent, in general, 15-35 % of plant biomass) and are insoluble in water.

Figure A.4 – Appearance of hemicellulose DF (picture from: IPIMAR).

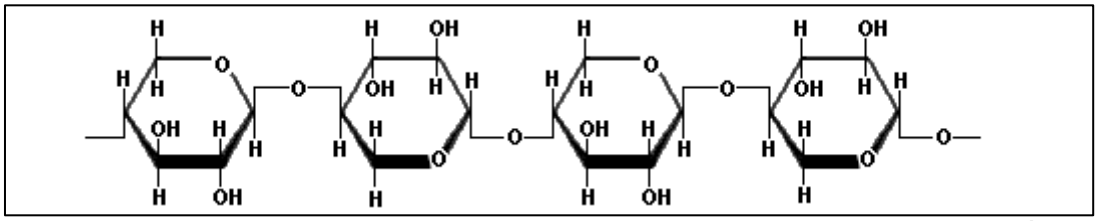

Figure A.5 – Chemical structure of xylan, a common hemicellulose (picture from: Gírio et al., 2010).

Pectin (Figure A.6) comprises a large group of polyssacharides that act as a cementing material in the cell walls of all plant tissues (Willats et al., 2006). In some fruit tissues (such as the white portion of the rind of lemons and oranges), pectin may account for as much as 30 % of the total. Pectin is the methylated ester of polygalacturonic acid, comprising its chains 300 to 1000 D-galacturonic acid units joined with with $\alpha(1\rightarrow4)$ linkages (Figure A.7). Unlike cellulose and hemicellulose, pectin is soluble in water. Pectin polymers have gelling properties, which depend on the degree of esterification. As such, pectin finds useful application in fruit preserves, jellies, jams, confectionary products, and bakery fillings (May, 1997).

Figure A.6 – Appearance of pectin DF (picture from: IPIMAR).

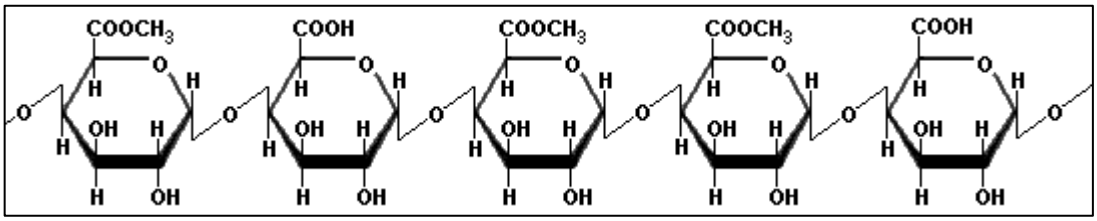

Figure A.7 – Chemical structure of pectin with a 60 % degree of esterification.

Oligofructoses encompass a group of oligossacharides, which can be obtained from raw materials such as fruits and vegetables, for instance, chicory (*Cichorium intybus*) root (Figure A.8). Oligofructoses are mainly produced from inulins that exist naturally in these raw materials. Namely, inulins can be degraded enzymatically or chemically to a mixture of oligossacharides (with less than 10 monomer units) (Kaur and Gupta, 2002). These oligossacharides consist of fructose units with β(2→1) linkages and may have a glucose-$(fructose)_n$ (Figure A.9) or $fructose_m$ structure. Oligofructoses are a group of DFs soluble in water. They have been used as alternative sweeteners and, recently, have also gained acceptance due to their prebiotic activity.

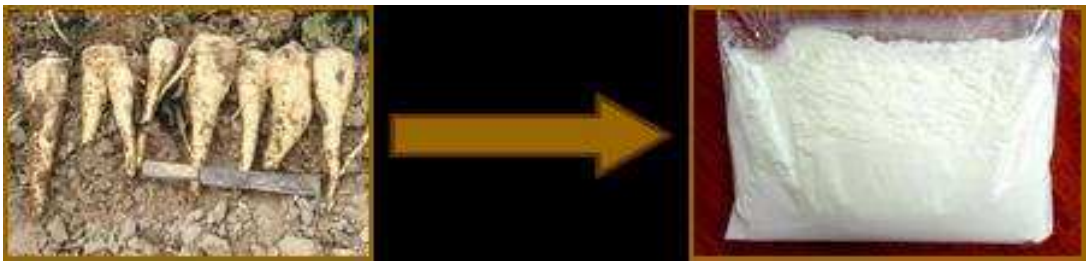

Figure A.8 – Appearance of oligofructose/inulin DF obtained from chicory root (pictures from: IPIMAR).

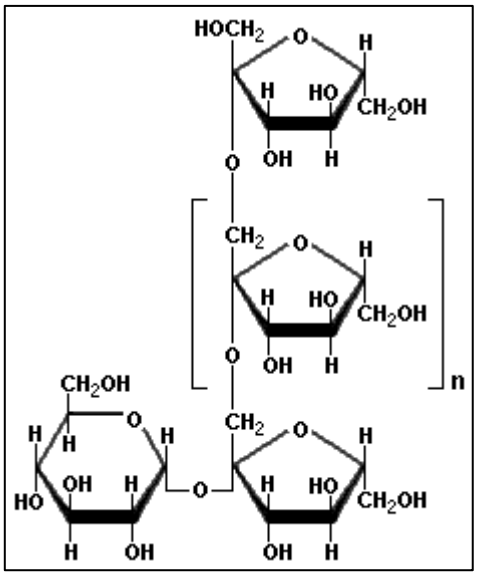

Figure A.9 – Chemical structure of oligofructose (a glucose-$(fructose)_n$ structure)/inulin (n<7 for oligofructose and n>18 for inulin).

Inulins (Figure A.8) are constituents of chicory (*Cichorium intybus*) root and other plants, such as onion, garlic or Jerusalem artichoke. Inulins function as reserve carbohydrates, present in roots and tubers of many Compositae and other plant families (De Gennaro et al., 2000). These polymers are composed mainly of fructose monomers, and typically have a terminal glucose. As in oligofructose, the fructose units are joined by β(2→1) linkages (Figure A.9). In general, inulins contain between 20 and several thousand fructose units. As pectin, inulins are soluble in water. In addition to common DF

properties, inulins of low molecular weight have prebiotic activity. Inulins are less soluble than oligofructoses and have a smooth creamy texture that provides a fat-like mouthfeel.

Carrageenan (Figure A.10) is a generic name for several polyssacharides extracted from red seaweeds (De Ruiter and Rudolph, 1997). These polyssacharides are linear, partially sulphated galactans that are mainly composed of repeating dimers of an α(1→4)-linked D-galactopyranose (D) or 3,6-anhydro-D-galactopyranose (DA) and a β(1→3) linked D-galactopyranose (G). The sulphate groups (S) are covalently bonded via ester linkages to the carbon atoms C-2, C-4 or C-6 of individual galactose residues. The amount of $–O\text{-}SO_3^-$ in sulphated polyssacharides can be considerable and varies in the range of 0-41 % (w/w), resulting in highly negative polymers. The three main repeating dimmer structures of carrageenans are G4S-DA (main dimeric structure of κ-carrageenan), G4S-DA2S (ι-carrageenan), and G2S-D2S,6S (λ-carrageenan) (Figure A.11). The κ-, ι-, and λ-carrageenan dimers have one, two, and three sulphate ester groups, respectively, resulting in calculated sulphate contents of 20, 33, and 41 % (w/w). There may be some divergence from these 'ideal' structures. This group of polyssacharides is water-soluble and has been used in food and cosmetics. Typically, κ-carrageenan forms firm and brittle gels that can undergo syneresis (exudation of water), whereas ι-carrageenan forms elastic and soft gels that usually do not undergo syneresis; the non-gelling λ-carrageenans are used as thickening agents.

Figure A.10 – Appearance of carrageenan DF (picture from: IPIMAR).

Glucomannan (Figure A.12) is a polyssacharide obtained predominantly from tubers of *Amorphophallus konjac* (Figure A.13) cultivated in Asia (Alonso-Sande et al., 2009). Traditionally, flour from konjac tubers has been used to make Japanese 'shirataki' noodles. This polyssacharide is composed of β(1→4) linked D-mannose (M) and D-glucose (G) monomers (Figure A.14). The mannose/glucose monomer ratio may vary depending on the original source of glucomannan. For example, it has been reported that konjac glucomannan has a molar ratio of around 8:5.

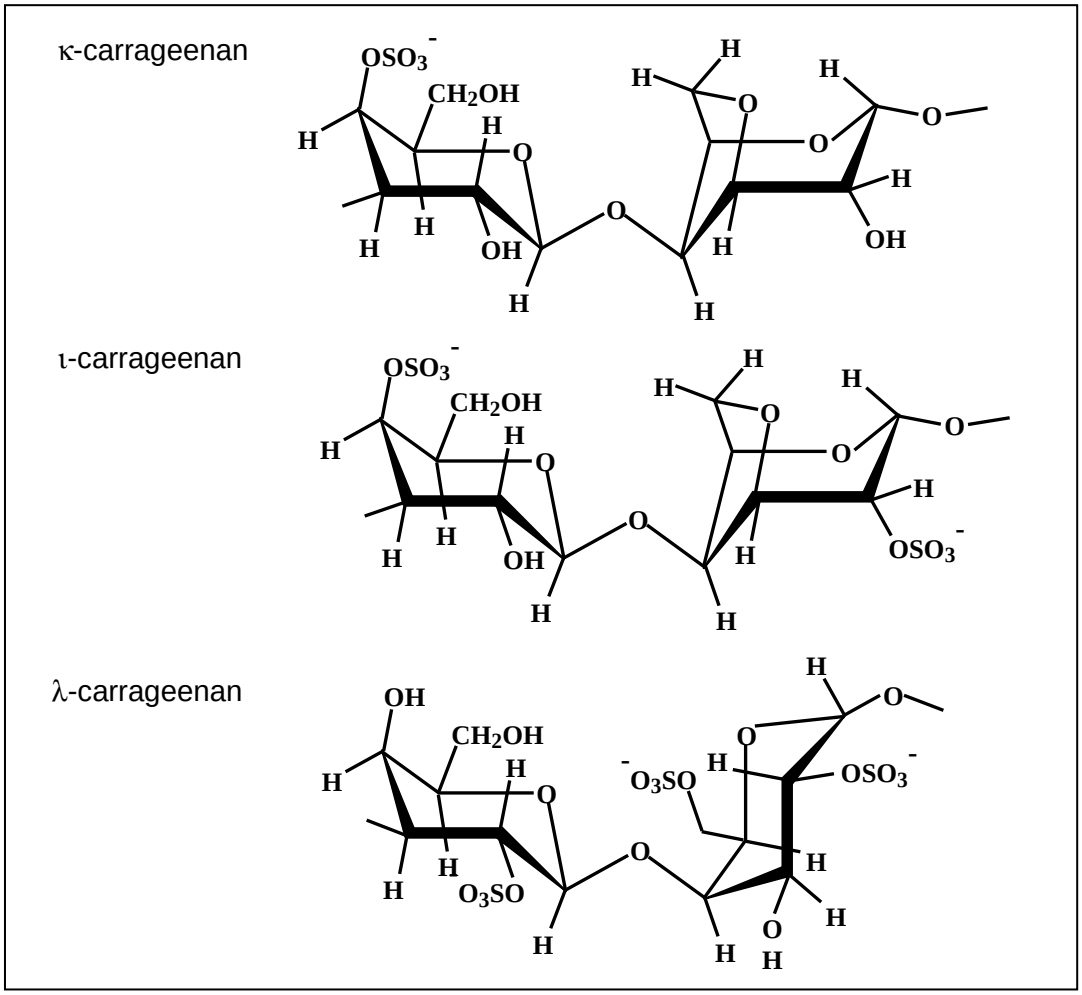

Figure A.11 – Chemical structures of κ-, ι- and λ-carrageenan (adapted from: De Ruiter and Rudolph, 1997).

Additionally, glucomannan may differ in the acetylation degree. The typical acetylation degree values are 5-10 %. This polyssacharide is soluble in water and is used as a hunger suppressant because it produces a feeling of fullness by creating very viscous solutions that retard nutrient absorption of foods. One gram of this polyssacharide can absorb up to 200 ml of water. Hydrolysis of the acetate groups favours the formation of intermolecular hydrogen bonds that are responsible for a strong gelling effect.

Figure A.12 – Appearance of glucomannan DF (picture from: IPIMAR).

Figure A.13 – The konjac plant and its tuber (picture from: IPIMAR).

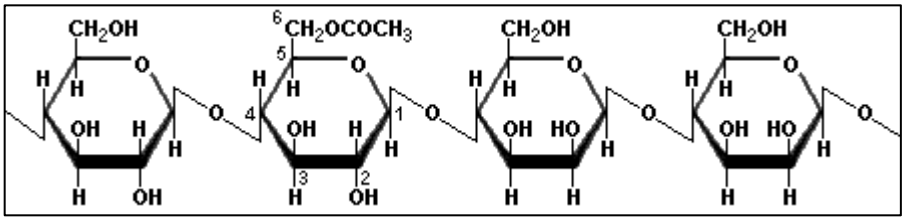

Figure A.14 – Chemical structure of glucomannan (a portion GGMM of it, with acetylation of the second glucose).

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