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Valorisation of codfish (*Gadus morhua* L.) salting processing wastewater through the extraction of high added value compounds

Thesis submitted to the *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology – with specialisation in Environmental Engineering

By

VINCENZA FERRARO

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Under the academic supervision of Prof. *Paula Maria Lima Castro*

Under the co-supervision of Prof. *Maria Manuela Estevez Pintado*

September 2011

To my Family and Faustino

Without whose encouragement,
understanding and support,
I could not have finished my PhD course.

Thank you so much

Preface

The research work published in this PhD dissertation has been developed at *WeDoTech -Companhia de Ideias e Tecnologias, Lda*., spin-off enterprise of the College of Biotechnology of Catholic University of Portugal, in Porto, through an Early Stage Research grant inside the *InSolEx-RTN* Programme (*Innovative Solution for Extracting high value natural compounds-Research and Training Network*) of Marie Curie Actions, under the 6th Frame Programme of the European Research Area.

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Scope of the Thesis

The studies reported in this PhD thesis aimed at the valorisation of the wastewater arising from the dry salting processing of codfish (*Gadus morhua* L.). The residual water object of this research has been collected at *Pascoal & Filhos*, S.A., a cod fishing and processing company based in Aveiro (Portugal).

Salting processing wastewater has been selected among all liquid waste effluents – salting, drying and re-hydrating water – based on its composition. Codfish salting processing wastewater is currently treated as an ecotoxic effluent, due to the high load of chloride, and according to the Portuguese environmental regulation.

Extensive characterisation has shown that 10 g/L of valuable organic compounds, such as free amino acids and proteins, are present in codfish salting wastewater along with ca. 250 g/L of sodium chloride. The nutritional, medical and also technical value of both essential and not essential free amino acids and of proteins found in codfish salting processing wastewater corroborated the potential of valorisation of the effluent.

The release of free amino acids and proteins has been studied in order to understand the mechanisms of their liberation from codfish muscle tissue along time. Free amino acids and proteins released at the end of salting have been selectively extracted from the wastewater by sorption using a commercial resin. Free amino acids extract has shown to have high intestinal bioavailability, antioxidant capacity and protection against oxidation of DNA, supporting the possibility of application as a nutritional or medical supplement.

Resumo

Apesar do desenvolvimento de outros métodos de preservação, a salga do bacalhau (*Gadus morhua* L.) continua a ser uma realidade devido a um conjunto de factores, entre os quais a simplicidade e o baixo custo do processo bem como as características sensoriais do produto final, muito apreciadas pelos consumidores.

Ao longo do processo de salga o bacalhau incorpora sal até 20% do seu peso e liberta concomitantemente cerca de 22% da sua água fisiológica; assim, aproximadamente 200 litros de água residual salgada são gerados para cada tonelada de bacalhau fresco. Esse efluente é actualmente tratado como resíduo tóxico devido ao alto teor de cloro, que pode atingir valores de concentração de cerca de 160 g/L.

A libertação desta água trás como consequência alterações significativas na composição e na estrutura do tecido muscular do bacalhau, levando à perda de compostos bioativos importantes, entre os quais aminoácidos livres, peptídeos e proteínas, nutrientes que, embora não essenciais, podem ser benéficos em certas circunstâncias. Assim, a recuperação de compostos orgánicos e do sal marinho de grau alimentar utilizado no processo de salga pode contribuir para a gestão integrada da água residual em questão. Com este objetivo, o perfil químico da água libertada foi avaliado. No final do período de salga o conteúdo de matéria seca na água libertada atinge o valor de ca. 10 g/L. A concentração de aminoácidos livres aumentou de 3.5 g/L a 6.5 g/L em 6 dias, devido a fenómenos de proteólise. Creatina, ácidos aspártico e glutâmico, arginina, glicina, metionina, lisina, taurina e triptofano, foram os aminoácidos livres predominantes e cuja libertação demonstrou-se obedecer a uma cinética monomolecular ou de pseudo-segunda ordem, dependendo do aminoácido. Embora em menor escala, a concentração de proteínas miofibrilares também aumentou com o tempo – de 3 para 3.7 g/L – fenómeno este ainda atribuível à proteólise.

A análise do azoto total e as suas fracções mostraram que, no final do processo de salga, 36.6% (w/w) de azoto total corresponde a peptídeos curtos com até 20 residuos e a aminoácidos livres, 14.7% (w/w) aos peptídeos com mais de 20 residuos e os restantes 48.7% (w/w) às proteínas. A concentração total de aminas biogénicas na água no final do processo de salga foi de ca. 100 mg/kg.

Proteínas, peptídeos a aminoácidos foram recuperados com sucesso por meio de um processo de sorção em *batch* e após um pré-tratamento com etanol de grau alimentar de forma a reduzir a concentração de sal da água, cuja elevada concentração, ca. 4.3 M, demonstrou afectar negativamente o mecanismo de adsorção dos aminoácidos. A presença do sal em certas concentrações demonstrou-se no entanto ser positiva, devido ao efeito da força iónica sobre a adsorção de aminoácidos.

A resina polimérica comercial *Amberlite XAD16*, uma resina neutra e não-polar, foi seleccionada para prosseguir com estudos de adsorção. Aminoácidos livres, proteínas e peptideos foram adsorvidos no mesmo estágio. O processo de recuperação foi efectuado recorrendo a solventes de grau alimentar.

Foi efectuada uma análise paramétrica do processo de adsorção examinando o efeito de diferentes parâmetros, nomeadamente temperatura, pH, percentagem de etanol adicionado a água residual, agitação, força iónica da solução, quantidade de adsorvente. O volume de solvente eluente e a temperatura foram os parâmetros avaliados na etapa de desadsorção. Os resultados mostraram que o processo de adsorção é controlado pela temperatura e pela força iónica, a qual neutraliza os efeitos do pH, e que a desadsorção é controlada pela temperatura e pela natureza do solvente eluente. A acetona resultou como o melhor solvente para desadsorção de aminoácidos livres, com um rendimento de recuperação de aminoácidos hidrofobicos e neutros de 100 %. As proteínas foram desadsorvidas por uma solução básica de hidróxido de sódio em água ao 4% (w/v), com um rendimento de recuperação de 100 %.

Os aminoácidos livres extraídos da água residual mostraram actividade antioxidante em geral e de proteção do DNA contra a oxidação em particular. Para os mesmos aminoácidos, a biodisponibilidade *in-vitro* foi estudada usando celulas Caco-2, medindo a taxa de transporte paracelular e a resistência elétrica transepitelial para verificar a integridade das células do epitelio intestinal. Os resultados mostraram que todos os aminoácidos livres extraídos permearam através da monocamada celular intestinal, embora em taxas diferentes e dependendo da sua concentração inicial; o transporte foi superior a 90 % para todos os aminoácidos livres, excepto a creatina, cujo transporte não passou de 6 %. A presença de sal na solução teve um papel positivo sendo o cloreto de sódio entre os mais importantes osmólitos de aminoácidos em seres humanos.

Abstract

Despite the development of other means of preservation, salt-cured codfish (*Gadus morhua* L.) continues to be widely produced due to the simplicity of processing, low operating costs, and the highly appreciated sensory characteristics promoted by salt. Codfish takes salt up to ca. 20 % (w/w) during the dry-salting and drains concomitantly ca. 22 % (w/w) of its physiological water. Approximately 200 L of heavy salted wastewater are generated for each ton of fresh codfish undergoing the dry-salting process, which drives important changes in composition and structure of the muscle tissue. Water drained away through the salting process is currently treated as an ecotoxic waste due to the high content of chlorine, which can reach values as high as 160 g/L, representing a strong impact to the environment.

This residual water carries important bioactive compounds, ca. 10 g/L, such as free amino acids, peptides and proteins, which although not regarded as essential nutrients, can be considered beneficial under certain circumstances. As such, the recovery of organic compounds and of food-grade marine salt used in the salting process can be considered as valuable management options for that wastewater.

In this study, the chemical profile of the salting process residual water has been thoroughly examined. During the salting period the content of dry matter in wastewater increased with time. Concentration of free amino acids increased from 3.5 g/L to 6.5 g/L within 6 days probably due to proteolysis. Myofibrillar proteins concentration also increased however at a lower extent, from 3 to 3.7 g/L, a phenomenon still related to proteolysis. Creatine, aspartic and glutamic acids, arginine, glycine, methionine, lysine, taurine and tryptophan were the dominant free amino acids, which release was successfully modelled through a monomolecular or pseudo-second order kinetic, depending on the amino acid. When total nitrogen and relative fractions have been scrutinised, results showed that, by the end of the salting process, 36.6 % (w/w) of total nitrogen corresponded to small peptides (up to 20 residues) and free amino acids, 14.7 % (w/w) corresponded to peptides and the remaining 48.8 % (w/w) represented proteins. Total biogenic amines concentration in wastewater at the end of the salting process was ca. 100 mg/kg.

The organic load was successfully recovered by batch sorption on a polymeric resin after a pre-treatment aimed at reducing salt concentration in wastewater and whose elevated concentration – 4.3 M – negatively affected the mechanism of adsorption of amino acids. Food-grade ethanol was used for salt precipitation from wastewater. Investigation carried out revealed however the positive effect of ionic strength – up to certain molarities – on amino acids adsorption onto resin up to certain molarities.

Amberlite XAD16, a commercial macroreticular polymeric resin, neutral and non-polar, has been selected amongst all resins tested. Free amino acids and proteins were adsorbed in the same stage but desorption has been performed selectively. Parametric analysis of the adsorption process has been carried out by studying the effect of six entities, namely temperature, pH, mixing rate, ionic strength, amount of ethanol in solution and adsorbent dose. Effect of temperature and solvent nature were examined for the desorption step. Results showed that the adsorption process is controlled by temperature and ionic strength, which neutralize pH effects, and desorption is controlled by temperature and by the nature of the regenerant solvent. Acetone resulted as the best solvent for free amino acids desorption with a yield of recovery of hydrophobic and neutral amino acids of 100 %. Proteins were desorbed by a strong basic solution of sodium hydroxide in water at a rate of 4 % (w/v), with a yield of recovery of 100 %.

Free amino acids extracted from wastewater showed chemical antioxidant activity; they also demonstrated biological activity by preventing DNA oxidation. *In vitro* bioavailability of extracted amino acids was studied using Caco-2 cell line by measuring the paracellular transport of free amino acids extracted and the transepithelial electrical resistance to verify intestinal cell monolayer integrity. Results showed that all free amino acids were transported through the intestinal monolayer, however at different rates depending on initial concentration; transport has been higher than 90 % for all free amino acids except for creatine, whose transport has been not higher than 6 %. The presence of salt in solution contributed to the paracellular transport of free amino acids being sodium chloride among the most important amino acids osmolytes in human beings.

Keywords
(By alphabetic order)

Adsorption

Amberlite *XAD16*

Antioxidant activity

Bioavailability

By-products and waste valorisation

Chromatography

Codfish salting process

Design of experiments

Desorption

DNA protection

Equilibrium

Factorial design

Food-grade

Free amino acids

High added value compounds

Inorganic salt

Kinetic

Marine by-products

Microscopy

Modelling of release

Muscle proteins

Nitrogen fractions

Pelagic fish

Precipitation processes

Protic and aprotic solvents

Regenerant

Salting-out

Solvent extraction

List of Abbreviations

HAVC – High Added Value Compounds

WSN – Water Soluble Nitrogen

FAA – Free Amino Acids

TCASN – Trichloroacetic Acid Soluble Nitrogen

PTASN – Phosphotungstic Acid Soluble Nitrogen

$ABTS^{\bullet+}$ – 2,2'-Azino-Bis(3-ethylbenzThiazoline-6-Sulphonic acid) Cationic Radical

ORAC – Oxygen Radical Absorbance Capacity

HPLC-UV/Vis – High Performance Liquid Chromatography interfaced to Ultra Violet and Visible field detector

SDS-Page – Sodium Dodecyl Sulphate Polyacrilamide gel electrophoresis

SEM – Scanning Electronic Microscope

DNA – Deoxyribonucleic Acid

TEER – Trans-Epithelial Electrical Resistance

MW – Molecular Weight

IP – pH at the isoelectric point

HI_2 and HI_7 – Hydrophobicity index at pH 2 and pH 7, respectively

R – Aliphatic or aromatic lateral chain of amino acid

COD – Chemical Oxygen Demand

BOD – Biochemical Oxygen Demand

SS – Suspended Solids

ROS – Reactive Oxygen Species

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Keynotes and Outlines

This thesis is organized in five parts.

In **Part I**, a general introduction to the valorisation of marine by-products and waste produced worldwide has been delineated. This part comprises one chapter, **Chapter 1,** which describes important high added value compounds found in marine by-products and waste, with a special attention to those arising from pelagic fish processing for food purposes.

Part II focuses on residues generated along the salting process of codfish and comprises two chapters. In **Chapter 2** by-products and waste generated though the salting process of codfish at a Portuguese company operating inside the fishing and fish processing industry are presented. **Chapter 3** delineates the characterisation of residue selected for valorisation – codfish salting processing wastewater – from a general description of the main classes of organic compounds found in the residue to the specific components of each of those. Qualitative and quantitative analysis is reported along with modelling of release of most important class of substances – free amino acids and proteins.

Part III focuses on the extraction of free amino acids and proteins in codfish salting processing wastewater by sorption, and includes three chapters. In **Chapter 4** the study of adsorption equilibrium and thermodynamics of selected amino acids upon *Amberlite XAD16* resin, as well as the investigation on optimal conditions for adsorption is reported. In **Chapter 5** a wastewater pre-treatment step, consisting of partial precipitation of salt, which allowed for a higher recovery of amino acids and proteins, is described. Adsorption kinetics and desorption are finally discussed in **Chapter 6**.

Part IV is dedicated to the characterization and bioavailability of the free amino acids mixture recovered from codfish salting wastewater by the sorption process. That part includes two chapters. **Chapter 7** is dedicated to the description of chemical antioxidant activity of amino acids mixture evaluated by ABTS and ORAC methodology, and also a description of protection against oxidation of DNA.

Bioavailability of extracted free amino acids, based on Caco-2 cell line test, has been reported in **Chapter 8**.

A synthesis of the research work carried out during the PhD course is traced in **Part V**, with a general conclusion on the main results achieved and a note on the future work, both covered by **Chapter 9**.

List of Publications

Publications in peer-reviewed international journals

Published articles

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PART I

GENERAL INTRODUCTION

Abstract

The wide chemical and biological diversity observed in the marine environment makes the Ocean an extraordinary source of high added value compounds (HAVC), which can be employed in many applications. Minerals, lipids, amino acids, polysaccharides and proteins from marine sources have unique features and, surprisingly, their highest concentration is often found in parts of marine organisms that are commonly discarded. Fish heads, viscera, skin, tails, offal and blood, as well as seafood shells possess several HAVC suitable for human health applications, yet most end up as residues throughout the raw material processing.

Part I updates information on marine by-products and waste valorization and conveys critical analysis of the chief methodologies to carry out extraction, purification and eventual transformation, with a focus on their actual and potential applications.

Chapter 1 has been published as "Ferraro, V., B. Cruz, I., Ferreira Jorge, R., Malcata, X.F., E. Pintado, M. M., Castro L., P. M. *Valorisation of natural extracts from marine source focused on marine by-products – A Review*. Food Research International 43, (2010) 2221-2233."

CHAPTER 1

HIGH ADDED VALUE COMPOUNDS IN MARINE BY-PRODUCTS AND WASTE

1.1 Introduction

Fishing is a rather ancient human activity and has played an important role in many human societies. Since the early 1960s, the state of fisheries has been consistently monitored by the Food and Agriculture Organization (FAO) Fisheries Resources Division. According to FAO (2000), the world fish production from marine catches has been increasing at an yearly rate of 6%, from 1950 (ca. 19.3 million tons) up to 1970 (ca. 60 million tons) (Blanco et al., 2007); however, more recent data point at more than 91 million tons of fish and shellfish caught each year (Rustad, 2003). In the 25 Member States European Union, the annual fish captures (from fishing and aquaculture) added up to 6.9 million ton in 2003 (Fishery Statistic Data, Eurostat, 2006) and the fish processing sector has generated an added value of EUR 3.8 billion (Eurostat, NACE, 2008).

Nowadays, an integrated and sustainable exploitation of fisheries resources is a must as only 50% of the catch is used for actual human consumption. Fisheries worldwide annual discards are estimated to be ca. 20 million tons, which represent 25% of the catch (Rustad, 2003) and include "non-target" species, and processing waste and by-products. In the European Union those discards represent a total of ca. 5.2 million tons per year (Mahro & Timm, 2007; AWARENET, 2004). Fish filleting, salting and smoking generate the major amount of solid waste and by-products (50-75 % of the fish) with a total of 3.17 million tons per year; fish canning is regarded as the second source of solid waste and by-products (30-65 % of the fish) with an amount of 1.5 million tons per year; crustaceans and mollusk processing also generate significant amounts of solid residues (20-50 %), ca. 0.5 million tons per year (AWARENET, 2004). In the UK it is estimated that ca. 313.000 tons of seafood processing residues are produced every year, so only 43% of the catches end up as products for human consumption (Archer et al., 2005).

Waste and by-products discharged by fisheries are currently rising, driven by both a net increase in fisheries products consumption and the changing consumer trend towards ready-to-use products. The seafood consumption has consistently increased during recent years as seafood has been progressively recognized as an important source of nutrients for human health. Proteins, lipids and polysaccharides and minerals from seafood possess unique features some of which are a consequence of specific factors

prevailing in the marine environment, such as temperature, pressure, salt concentration and oxygen availability (Shahidi, 1997). Seafood by-products and waste constitute at present a serious environmental problem (Arvanitoiannis & Kassaveti, 2008); those by-products and waste require appropriate management, especially because they are highly perishable, owing chiefly to the action of microorganisms that there find an excellent growth medium. Furthermore, in recent years more stringent quality and hygienic standards enforced to industries have caused a significant increase in the amounts of waste and wastewater generated from seafood processing. Major efforts have been focused on how to deal with the aforementioned by-products and waste in efficient ways, including useful applications; at present, most are taken advantage of as animal feed and plant fertilizers as well as ingredients for adhesive manufacture (AWARENET, 2004). Nutraceutical, functional food formulation or pharmaceutical applications can also be hypothesized as they already exist in such inexpensive marine feedstocks as head, viscera, skin, tails, offal, blood and shells (Kim & Mendis, 2005). Extraction of those high added value compounds (HAVC) - which can be profitable owing to their beneficial role upon human health - joined to the development of new technologies for recovery and purification, will run along with concomitant benefits towards long term sustainability of marine activities.

1.2 High added value compounds in marine by-products and waste

By-products generated during seafood processing typically range between 20 and 60% of starting raw material. For pelagic fish, such as tuna, cod, mackerel, anchovy and herring, major amounts of residues are represented by offal, head and tail (27% of the fish) collected through eviscerating, cutting and filleting processes. Skin, bones, blood and frames are the second major residues (25% of the fish) collected along skinning and cutting processes (AWARENET, 2004).

At present, the majority of these by-products are sent to fish meal plants, where fish meal and fish oil are produced. Fish meal is by far the most valuable non-edible commodity produced from marine by-products; its global production ranges between 5.5 and 7.5 million tons/year (Hardy & Tacon, 2002). It is a relatively dry product, used either as animal feed or as plant fertilizer, and is composed of protein (70%), minerals

(10%), fat (10%) and water (10%), on a weight basis (Blanco et al., 2007). The first stage of fish meal production includes mincing, cooking and pressing of solid fish by-products, from which wastewaters and a solid cake are generated. At a second stage, those wastewaters are desludged to remove any remaining solid particles, which are then added to the solid cake, and then centrifuged to separate the oil fraction; this crude fish oil is further processed or sold as such. The water is fed back to the process line where the solid cake, along with the material from the wastewater, is dried and grounded to produce fish meal (SEAFISH, 2008). Fish oil can have edible and non-edible applications depending on its composition. Production of soap, glycerol, varnishes, drying and hydraulic oils, fertilizers and substrates for fermentation, are the most common uses, whilst its edible applications are essentially accounted for the production of margarine and shortenings (AWARENET, 2004).

Fish meal can exhibit very diverse specifications, in terms of amino acid profile, digestibility and palatability, depending on the raw material and production process employed (Blanco et al., 2007). Fish meal from pelagic fish is the most widely used, and may generate an income up to 108 Euro/ton depending on its specific composition; currently its average market value is 46 Euro/ton (SEAFISH, 2008; AWARENET, 2004). The main HAVC that can be found in marine by-products are presented in Table 1.1. The highest concentration of these bioactive compounds is generally found in the parts of the marine organisms that are discarded (AWARENET, 2004). Note that present concentrations can attain up to 80% of the by-product in question – especially in the case of lipids in cod liver. Table 1 also shows the mean market values for selected HAVC that, as expected, are strongly dependent upon purity. It must be highlighted that the least expensive HAVC – gelatin/collagen – hold a value 30-fold higher than fish meal. Finally, for HAVC such as natural free amino acids and hydroxyapatite, a market value has not been established yet, since only synthetic forms have been used until now.

In the following sections, the current and potential uses of the most important types of HAVC will be described in detail together with reference to state-of-the-art technologies for extraction and purification.

Table 1.1. Content and typical market value of high added value compounds found in marine residues

High added value compounds	Marine residues	Content (% w/w)	Market value (Euro/kg)	References
Polyunsaturated fatty acids (ω-3 and ω-6)	Algae, cod liver, oil of mackerel flesh residues	50-80% in cod liver, 23% are ω-3 PUFA	24 (as purified cod liver oil)	*Bimbo, 2007; Falch et al., 2006; Mondello et al., 2006; Ward & Singh, 2005; AWARENET, 2004*
Free amino acids	Mussels, fresh clams, white fish flesh residues, crustacean shells	0.8-2% of taurine, 2.7% of creatine (on dry matter)	Not available	*Larsen et al., 2007; Leuchtenberger et al., 2005; Heu et al.,2003*
Chitin and chitosan	Shrimp and crab shells	15-40%	15-750	*Abdou et al., 2008; Sini et al., 2007; Kurita, 2006; AWARENET, 2004; Healy et al., 2003; Kumar 2000;*
Collagen and gelatin	Pelagic fish skin, scales and bones	Up to 80% in skin, up to 50% in scales	9-14	*Wang et al., 2008; Kim et al., 2007; Wasswa et al., 2007; AWARENET, 2004*
Hydroxyapatite	Pelagic fish scales and bones	60-70% in bones, up to 50% in scales	Not available	*Barakat et al., 2008; Aronov et al., 2007; Haberko et al., 2006*
Antifreeze proteins	Cold water pelagic fish blood and skin	5-35 mg/ml in cold water fish blood	5000	*Jin & de Vries, 2006; AWARENET, 2004; Evans & Fletcher, 2004; Crevel et al., 2002;*
Astaxanthin	Algae, crustacean shells	2.3-33%	3000-12000	*Babu et al., 2008b; Yang, 2007; Lopez-Cervantes et al., 2006; AWARENET, 2004*
Enzymes	Algae, pelagic fish viscera	–	14400 (cod proteases)	*SEAFISH, 2008; Rasmussen & Morrissey, 2007; Shahidi & JanakKamil, 2001*

1.2.1 Polyunsaturated fatty acids ω-3

ω-3 fatty acids concentrates remains a topic of general interest for the pharmaceutical and food industries, for the production of drugs with enhanced performance and for the production of nutritional supplements. As known, fat is a source of energy but is also a vital structural component of cellular membranes and is involved in many important cell-signaling pathways (Ma et al., 2004). Since the early studies on long-chain ω-3 polyunsaturated fatty acids (PUFA) carried out by Burr back in 1929, the health benefits of these compounds have been thoroughly investigated, so their market is expected to grow further in the future (Bimbo, 2007). Such HAVC are considered as "essential fatty acids" due to their key role in many metabolic processes and because they cannot be synthesized *de novo* by mammalian cells (Harris et al., 2007). Dietary ω-3 PUFA ameliorates numerous biological and physiological functions in human body, being particularly valuable in treating some diseases (Ma et al., 2004).

In cell culture, ω-3 and ω-6 PUFA are generated from the precursor α-linolenic acid and linoleic acid, respectively (Tapiero et al., 2002); the former leads to eicosapentaenoic acid (EPA, 20:5ω3) and docoesahexaenoic acid (DHA, 22:6ω3), whereas the latter leads to arachidonic acid (AA, 20:4ω6) and other long-chain ω-6 fatty acids (Harris et al., 2007; Tapiero et al. 2002). After absorption in the human body, PUFA are incorporated at cellular level into triglycerides (i.e., 3 fatty acids molecules on a glycerol backbone), phospholipids (i.e., 2 fatty acids molecules on a phosphatidic acid backbone) and cholesterol esters (i.e., 1 fatty acid molecule on free cholesterol). Some studies indicate that ω-3 PUFA are most promptly absorbed from the intestine when taken orally in the form of free fatty acids, moderately absorbed if in the triglyceride form and poorly absorbed if in ethyl esters form. However, ω-3 PUFA in the form of triglycerides are the most stable and desirable for food formulation, unlike as free fatty acids, which are easily oxidized, and as ethyl esters, which continue to be unacceptable for food purposes in terms of safety (Liu et al., 2007). The major reported effects of ω-3 PUFA - EPA and DHA - on human tissues are described in Table 1.2.

PUFA can be recovered from a number of marine by-products. Marine protists and microalgae have been referred as the major sources of EPA and DHA (*Schizochytrium* spp. for the specific recovery of DHA; *Phaeodactylum* and *Monodus* spp. for the specific recovery of EPA; *Isochrysis galbana* spp. for the recovery of both EPA and

DHA) (Ward & Sing, 2005; Robles et al., 1998). Antarctic krill (Ju & Harvey, 2004) and by-products from edible fish (e.g. sardine, anchovy, salmon and cod) are the second most important sources of PUFA (Mondello et al., 2006), followed by fish caught especially for fish meal and oil production (Bimbo, 2007).

Table 1.2. Effects of ω-3 polyunsaturated fatty acids EPA and DHA on human being

Claimed function of EPA and DHA	References
Improvement of vision acuity, field of vision and adaptation to light. Alteration of permeability, fluidity, thickness and lipid phase properties of retinal photoreceptor membrane *(DHA only)*	*Harris et al., 2007; SanGiovanni & Chew, 2005*
Reduction of risk of myocardium lesion and coronary heart disease via lowering blood pressure, triglyceride levels and platelet aggregation, and prevention of arrhythmias	*Harris et al., 2007; Moore et al., 2006*
Protective effects against some common cancers (breast and colon) via inhibition of eicosanoids synthesis	*Goldberg & Katz, 2007; Rose & Connoly, 1999*
Increase of level of insulin	*Patel et al., 2007*
Beneficial effects in sclerosis treatments via affecting functions of central nervous system	*Weinstock-Guttman et al., 2005*
Preventive and curative effects upon non-alcoholic fatty liver disease via lowering liver fat content	*Spadaro et al., 2008*
Increase of toleration to noxious therapies (e.g. chemotherapy)	*Morisco et al., 2007*
Decrease of heart rate beating	*Svensson et al., 2007*
Improvement of emotional state (e.g. in depression and anxiety conditions)	*Raeder et al., 2007*
Beneficial effects on autoimmune diseases (e.g. rheumatoid arthritis, psoriasis, systemic lupus, Crohn's disease).	*Curtis et al., 2004*
Reduction of joint pain associated with inflammatory conditions, via inhibition of central regulators of inflammation.	*Fedacko et al., 2007; Goldberg & Katz, 2007*

The most important natural sources of ω-3 PUFA are indeed fish oils of common species such as sardine, mackerel, cod, shark and menhaden with PUFA levels of ca. 30%, which makes them commercially interesting raw materials to prepare ω-3 PUFA concentrates (Chakraborty & Raj, 2007). In particular, cod liver has for long been the most suitable marine by-product for recovery of ω-3 PUFA, for extended use as nutraceutical, having a high content of vitamins A, D and E (Mondello et al., 2006); this oil contains high amounts of lipids (ca. 50-80%), of which 23% is accounted for EPA (Falch et al., 2006; Kolakowska et al., 2002). Anchovy and bluefin possess

exceptionally high DHA levels. Salmon head, which represents the main salmon by-product, is also regarded as a good source of PUFA due to a significant content of lipids, ca. 15-18% (Huang & Sathivel, 2008). Among fish flesh, mackerel is accordingly the main source of EPA/DHA: it yields ca. 15% (w/w) of oil, with 9% (w/w) of PUFAs and 4% (w/w) of DHA in particular, as triglycerides (Linko & Hayakawa, 1996). Mackerel flesh content of PUFA is in order of 1810 mg per 100 g, followed by salmon with 1800 mg per 100 g, tuna with 1500 mg per 100 g, herring with 1200 mg per 100 g), salmon trout with 1060 mg per 100 g and cod with 240 mg per 100 g (Fedacko et al., 2007).

Microcapsules containing ω-3 PUFA concentrates are a useful form of PUFA supply to the human body, as they convey an extended stability and allow for the release of the active compound in the intestine only (Kantor et al., 1990). Microencapsulation also eliminates the well-known unpleasant taste and smell of fish oil, as well as its aftertaste (particularly when ingested in relatively large quantities), and effectively prevents oxidation of PUFA. When microencapsulation is not applied, lipid peroxidation is normally difficult to avoid and addition of antioxidants to PUFA is required. For preventing oxidation several combinations have been tested: the most successful result has been achieved with a ternary antioxidant system containing α-, γ- and δ- tocopherol concentrates (in the range of 0.2-2.0 %), ascorbic acid (or ascorbyl palmitate), soy lecithin, quercetin, morin or catechin (Kamal-Eldin & Yanishlieva, 2002). Other alternative solutions have also been proposed: histidine, for instance, shows a pronounced effect towards preservation of herring oil, whereas catechin, morin and quercetin show strong antioxidant effects upon sardine oil up to 60 °C (Kamal-Eldin & Yanishlieva, 2002).

Numerous methods are currently employed for ω-3 PUFA concentration; however, only few are suitable for large-scale production. A single separation/purification method is not often feasible, because the raw material is usually too heterogeneous with relatively high contents of undesirable compounds. Nowadays, PUFA are mainly recovered as free fatty acids, after chemical or enzymatic hydrolysis of marine oil, followed by purification. Depending on the desired yield, available methods include distillation (Shahidi & Wanasundara, 1998b; Chang et al. 1989), enzymatic splitting (Halldorsson et al. 2004; Camacho-Páez et al., 2002), low-temperature crystallization (Harris et al.,

2007; Shahidi & Wanasundara, 1998b), supercritical fluid extraction (Catchpole et al., 2000) coupled or not with nanofiltration (Sarrade et al., 1998), urea complexation (Chakraborty & Raj, 2007; Gámez-Meza et al., 2003), and argentation chromatography (Chakraborty & Raj, 2007; Mondello et al.,2006). Each technique has its own advantages and drawbacks and leads to ω-3 PUFA concentrates in different forms. Chromatography, crystallization and urea complexation are useful techniques for collecting PUFA as free fatty acids (Chakraborty & Raj, 2007; Gámez-Meza et al., 2003) whereas supercritical fluid extraction, distillation and also urea complexation, are suitable techniques for the recovery of PUFA as fatty acids esters (Perretti et al., 2007; Shahidi & Wanasundara, 1998b). PUFA as acylglycerols can be obtained by enzymatic methods (Halldorsson et al., 2004; Shimada et al. 2001).

Distillation has been mentioned (Shahidi & Wanasundara, 1998b; Chang et al., 1989) to produce odorless and flavorless oils, with improved flavor and oxidative stability, which contain only insignificant amounts of undesirable minor constituents, such as thermal and oxidative polymers of unsaturated glycerides, trans-isomers, conjugated dienes and trienes, cholesterols, pesticides and heavy metals. Two distillation methods currently in use are: vacuum steam distillation followed by silica gel treatment, and short-path molecular distillation. The former cannot improve significantly the ω-3 PUFA concentration, however the resulting oil is highly pure and can already be subjected to specific techniques such as microencapsulation (Chang et al., 1989). Short-path molecular distillation has been used for partial separation of fatty acid esters; it is a rather old technique, performed at very high temperatures (250 ºC) and for very short heating intervals, in the order of seconds. This method leads to increases in EPA and DHA concentrations up to the ranges of 16-28.4% and 9-43% respectively, in the case of menhaden oil (Shahidi & Wanasundara, 1998b). However, the ω-3 PUFA alkyl esters form cannot be directly used for food or pharmaceutical purposes, so a further step of conversion to fatty acids or acylglycerols is required (Shahidi & Wanasundara, 1998b).

To recover concentrated PUFA as acylglycerols, subcritical and supercritical fluid extraction has been performed with carbon dioxide as solvent and at various conditions depending on the fish oil initial composition. The most interesting result was obtained at 28 ºC and 7.8 MPa, with EPA showing the greatest resistance to fractionation (Perretti et al., 2007). Note that in fish oils, EPA and DHA are preferentially located in the

middle carbon atom of the glycerol backbone, so it has not been possible to date to achieve very high PUFA concentration if the oil is fractionated as triacylglycerols by physical methods (Corrêa et al., 2008). In this case, as also happened with distillation, the best performance is obtained only if oil is previously subjected to random hydrolysis and esterification (Perretti et al., 2007), where simultaneous hydrolysis and esterification are carried out via an alkaline catalyst (KOH or NaOH) and an alcohol (methanol or ethanol).

Some authors (Halldorsson et al., 2004; Gámez-Meza et al., 2003) observed that chemical methods partially destroy the natural all-*cis* ω-3 PUFA structure, whilst enzymatic hydrolysis provides milder conditions in terms of temperature, pH and pressure, and protects ω-3 PUFA against oxidation, *cis-trans* isomerization and double bond migrations. When an enzymatic process is chosen, it is brought about by lipases, i.e., glycerol ester hydrolases that catalyzes the hydrolysis of triacylglycerols into fatty acids, partial acylglycerols and glycerol. PUFA are firstly enriched in the free fatty acids by hydrolyzing PUFA-containing oil with a microbial lipase (e.g., *Aspergillus niger, Candida cylindracea, Chromobacterium viscosum* or *Pseudomonas spp.*), and such free fatty acids are then converted to esterified forms with a simple alcohol reaction using a lipase (Shimada et al., 2001). Due to potential benefit of PUFA as acylglycerols, enzymatic esterification of glycerol with individual free fatty acids EPA and DHA recovered by hydrolysis, and purification of PUFA-containing oils is gaining momentum; considerable attention is being given to microbial lipases since EPA and DHA are resistant to hydrolysis by commercial lipases (Gámez-Meza et al., 2003).

1.2.2 Amino acids taurine and creatine

The food and pharmaceutical industries use free amino acids extensively for the production of food supplements, infant and adult formulae, drugs (European Commission, May, 2003; Stapleton et al., 1997), and nutraceutical energy formulations (Bagchi, 2005). Two amino acids widely used in the free form - taurine and creatine - will be covered by this review paper.

1.2.2.1 Taurine

Taurine (or 2-aminoethanesulfonic acid, NH_2-CH_2-CH_2-SO_3H) is a neutral β-amino acid, which is not utilized in protein synthesis but found mostly in the free form in the human body (Redmond et al., 1998); a small amount of taurine is present in small peptides in the brain (Franconi et al., 2004). Taurine is the dominant free amino acid in many living species; in terms of content, in the human body taurine is second only to glutamic acid. Both the amine and sulphonic groups of taurine can undergo ionization, and their dissociation constant contributes to the biological and physiological activities showed by taurine (Petrosian & Haourutounian, 2000). Osmotic regulation (Chiarla & Giovannini, 2004), cell membrane stabilization (Han et al., 2000), body detoxification (Lourenço & Camilo, 2002), antioxidant protection (Métayer et al., 2008), immune defense enhancement (Redmond et al., 1998) and intracellular calcium homeostasis regulation (Takahashi et al., 1997) are the biological functions reported for taurine. Central nervous system neuromodulation (Rose, 1996), regulation of renal development and renal function (Han et al., 2000), anti-inflammatory activity (Chiarla & Giovannini, 2004), bile acids conjugation and choleostasis prevention (Lourenço & Camilo, 2002), antiarrhythmic, anti-inotropic and anti-chronotropic effects (McCarty, 2001), endocrine and metabolic effects (Rose, 1996) have been referred as physiological roles of taurine in human body. As reported by Petrosian and Haroutonian (2000), taurine can also convert lipid and lipid soluble compounds into a water-soluble state, a characteristic that makes it an interesting emulsifier in various food and pharmaceutical formulations.

Among water-soluble amino acids, taurine is the most heat-stable and after glycine, is the most water-soluble among all the heat-stable (Petrosian & Haourutounian, 2000). Although taurine is a conditional essential amino acid for adults (Lourenço & Camilo, 2002), it is essential for newborns (Chiarla & Giovannini, 2004), for whom the availability of taurine is critical to assure the development of their central nervous system and muscles (Stapleton et al., 1997). In newborns, the normal level of taurine is provided by their mother's milk, as it is the predominant free amino acid in human milk. It is particularly important for preterm newborns of gestational age not above 32 weeks, as they possess a limited capacity to convert methionine into cysteine and hence to taurine, due to immaturity of the liver enzymes responsible for taurine synthesis (Rose, 1996). Since taurine is the predominant free amino acid in human milk, it is added in

milk-based infant formulae, aimed at substituting mother's milk, at 5 mg/100 ml. On this regard, the European Scientific Committee of Food has proposed that taurine addition to cow's milk and any type of infant formula should not exceed 12 mg/100 ml (European Commission, May, 2003).

In adult human body, taurine is synthesized from methionine and cysteine, in the presence of vitamin B_6 and it is excreted through urine and bile at daily rates of ca. 0.23 g although varying with age, sex, renal function and clinical condition (Lourenço & Camilo, 2002). Adult individuals are expected to exhibit an average of 1 g/kg of taurine of body weight. However, the synthesis ability varies widely amongst individuals: under stress conditions, such abilities may be impaired, hence making an external supply necessary; for this reason some authors consider taurine as a conditionally essential amino acid for adult population.

High concentrations of taurine have been found in animal sources; conversely, it is essentially undetectable in vegetable matrices. Marine organisms in particular exhibit high levels of taurine, as it is the main organic osmolite in marine species (Omura & Inagaki, 2000). Within seafood, raw mussel is the main source of taurine with typical levels of 655 mg/100g, followed by fresh clams and raw white fish flesh with 240 mg and 151 mg per 100 g, respectively. Cooking causes no adverse effects on taurine levels in food, due to its thermal stability (Stapleton et al., 1997). In recent years the use of taurine has increased especially in the so called "energy drinks", due to particularly successful marketing strategies (Babu et al., 2008a). In the case of nutraceutical formulations, taurine is added to 400-600 mg/l (European Commission, March, 2003) often in combination with chromium, D-ribose and withanolides, i.e. steroidal lactones with anti-inflammatory, anti-arthritic and anti-cancer activities (Bagchi, 2005), and also with potassium bicarbonate or ascorbate complexes (Boynton, 2005). Specifically in the case of drug formulation with antihypertensive action, taurine is present as calcium taurate (NH_2-CH_2-CH_2-SO_3)-Ca^{2+} in the form of an aqueous suspension (McCarty, 2001).

1.2.2.2 Creatine

Creatine (or methyl guanidine-acetic acid, NH_2–C[=NH_2^+]–NH–CH_2–C[= O]–O^-), is an α-amino acid synthesized from glycine, arginine and methionine (Clark, 1998) and can

exist in the free or phoshorylated forms. It is stored in muscle tissues, mainly skeletal, followed by heart and smooth muscles, in which 60-70% thereof exists in the form of phosphocreatine (Clark, 1998). It is also present in the brain, liver, kidney and testes, but in smaller amounts. For a typical 70 kg-man, the total creatine in his body pool amounts to ca. 120 g, of which 95% is stored in the muscle (Greenhaff, 1997). In the human body, the average rate of creatine synthesis is 1-2 g/day (Clark, 1998), which is consumed by the normal physical activity as this amino acid is responsible of skeletal muscle contraction and regeneration (Bishoff & Heitz, 1994). Particularly, it provides energy during intense muscle exercise (Bigard, 1998) and as for taurine, it shows important biological and physiological functions, even in the presence of some diseases. Main claimed biological roles of creatine are neuroprotection against ischemic and oxidative insults, even in Parkinson's disease (Bender et al., 2008; Sullivan et al., 2000); slowing down neurodegeneration in Huntington's disease (Bender et al., 2005) and therapeutics effects in mitochondrial encephalomiopathies disease (Komura et al., 2003). Physiological functions of creatine, other than skeletal muscle regeneration and contraction, have been referred as cardiac muscle performance maintenance (Allard et al., 2006; Gordon et al.,1995), energy provision during intense muscular exercise (Bigard, 1998), and fat-free mass promotion of human body (Sullivan et al., 2000).

The amino acid creatine is largely used in various food formulae, drugs and food supplements production. At present, the latter is the most profitable due to a widespread demand in sports at almost every level of athletic performance (Sullivan et al., 2000).

The main marine source of creatine is found in herring with ca. 6.5 g/kg. For comparison beef and pork meat possess levels of 5.5 and 5 g/kg, respectively. Salmon and cod come second and third highest in creatine content with ca. 4.5 and 3 g/kg on dry tissue, respectively (Newsholme & Hardy, 1998). As a food supplement or in drug formulation, creatine has been originally brought onto the market as a water-soluble powder, first in the form of creatine monohydrate, (till now the cheapest and the most widespread form), and later in more expensive versions of creatine phosphate, citrate and malate, even in powder form; tablets, gels, liquids, chewing gums and candies variants are also commercialized, but are less used and less efficient as a delivery method for creatine supplementation and are more expensive than powder (Bigard, 1998; Green & Green, 1998). In milk-based infant formula, for instance, creatine is

added as creatine monohydrate powder up to 0.8-2 mg/100 ml (European Commission, March, 2003). In the recent years, a different form of creatine has been introduced into the market: research studies reported in fact that creatine supplements are poorly absorbed by muscle cells when administered in its polar form, leading to the need of larger dosages. Creatine ethyl esters, the esterified lipophilic form of creatine monohydrate, has been reported to increase the bio-availability of creatine since it is able to cross the muscle cells membrane easier, not requiring the presence of creatine transporters in the human body. However, creatine ethyl ester does not show any additional benefit to increase muscle strength or performance than creatine monohydrate; also, this ester form has been demonstrated to undergo an extended hydrolysis in the gastrointestinal tract after ingestion, with a rapid conversion in the derivative product creatinine (Spillane et al., 2009; Fons et al., 2010).

Currently, commercial creatine and taurine are widely produced via chemical synthesis in Europe, Asia and North America, since natural extraction from animal and fish flesh usually incurs in higher processing costs. Taurine is currently produced from monoethanolamine, sulfuric anhydride and sodium sulfate, using a relatively expensive purification process, because taurine is insoluble in most organic solvents commonly used in extraction and purification (Gu et al., 2004). Creatine is usually produced in the monohydrate form, starting from sarcosine and cyanamide; however, the final product always contains non-reacted sarcosine, for which there is a low tolerance level by the human body, in the order of ppm, enforced by health authorities agencies, and some other contaminant by-products such as dicyandiamide, dihydrotriazines, creatinine, and several ions, resulting in a formulation with a poor nutrition value.

Extraction from cold water fish protein hydrolysis appears to be a cost-effective technology. The enzymes provided by fish offal and viscera themselves are mixed with fish by-products in water and the resulting enzymatic protein hydrolysate may contain up to 90% of free amino acids, high molecular weight peptides, calcium salt and phosphorus (Pyntikov & Salerno, 2004). The stream can be processed by nanofiltration using inorganic membranes allowing separation of peptides and amino acids based on membrane/solute charge interaction (Martin-Oure et al., 1997). So far, commercial developments have been reported encompassing animal sources for free amino acids, but which resort to fermentation processes using a highly performance strain of

Escherichia coli in the presence of sugar sources such as molasses, sucrose, or glucose (Leuchtenberger et al., 2005). Nevertheless, marine by-products possess great potential, as they are less affected by virus transmissible to humans from higher warm-blooded animals.

1.2.3 Chitin and chitosan

Chitin and chitosan are ubiquitous marine polysaccharides; over the years they have attracted a great deal of attention in food, pharmaceutical and health applications due to their distinctive biological and physicochemical characteristics (Kurita, 2006; Shahidi et al., 1999). In fact, the last two decades have witnessed a great amount of work on these biopolymers, which highlighted wide potential uses (Dodane & Vilivalam, 1998). The current trend towards the use of natural products has strongly contributed to the observed increase in demand, which has turned chitin and chitosan production processes into highly profitable ones. The annual production of chitin is estimated to approach that of cellulose (Kumar, 2000), which is ca. 2.6 million tons/year (Davies, 2009).

The name 'chitin' was firstly used by Bradconnot, back in 1811; it is derived from the Greek word 'chiton', that means tunic or 'coat of mail', because it was first found in the exoskeletons of insects and crustaceans. Chitin is an odorless, white to cream-colored solid compound with a structure similar to that of cellulose, and built from *n*-acetyl-glucosamine monomers. It can exhibit various degrees of acetylation but pure chitin with full acetylation does not exist in nature (Meyers et al., 2008).

Depending on its source, chitin can occur as either of two allomorphs, namely α and β forms (Rinaudo, 2006). The α type is the most abundant and also the preferred form for industrial applications; amongst crustaceans it is found in shrimps and crabs. The relatively rarer β form is found in squid pen and is apparently more reactive than the α form, yet β-chitin can not be biosynthesized *in vitro*. Both α and β types are insoluble in water and are stable when exposed to acids, bases or organic solvents (Dodane & Vilivalam, 1998; Malinowska & Rozylo, 1997); this water insolubility hampers most applications of chitin (Rinaudo, 2006). Conversely, its deacetylated form named chitosan is soluble in several solvents, depending on its degree of deacetylation.

Chitosan is rather soluble in acidic solutions and slightly soluble in weak alkaline solutions (Dodane & Vilivalam, 1998); the degree of deacetylation is once again the key

parameter that dictates solubility of chitosan in aqueous solutions. This polymer is indeed an alkaline or neutral polysaccharide as opposed to other commercial polysaccharides, such as cellulose, dextran, pectin, alginic acid, agar-agar, starch, carrageenan, and heparin, which are all neutral or acidic (Sing & Ray, 2000); this unique feature offers a range of unique applications, as summarized in Table 3. Chitosan possess three types of reactive functional groups: an amino group, and primary and secondary hydroxyl groups at C-2, C-3 and C-6 positions, respectively. Deliberate chemical modifications of these groups have produced several chitosan oligomers (Kurita, 2006; Shahidi et al., 1999) suitable in various fields of applications like normal chitosan, but with tailored features (Table 1.3).

Chitosan is commercially obtained mainly from chitin isolated from shell waste of crabs, shrimp and krill (Kurita, 2006). In practice, chitin is used almost exclusively as raw material for production of chitosan, oligosaccharides and glucosamine, to levels that amount worldwide to ca. 37300 tons yearly (Chang et al., 2007). Shrimp cuticle exhibits the higher amount of chitin, i.e. 30-40%, followed by crab, i.e. 15-30%; in both cases the remaining fraction is made of proteins and minerals (Qin & Agboh, 1997).

In extraction process on the industrial level, shells are first treated with dilute hydrochloric acid at room temperature in order to remove metals and salts, primarily calcium carbonate. Decalcified shells are then exposed to an alkaline media to hydrolyse proteins and pigments (Kurita, 2006). Final polishing – via drying – eventually leads to powdered α-chitin. Demineralization and deproteination of shrimp waste can also be carried out via enzymatic fermentation (Sini et al., 2007; Kurita, 2006); e.g., proteases derived from *Bacillus subtilis* are used for chitin extraction from shrimp shells (Sini et al., 2007), whereas *Pseudomonas maltophilia* seems to be particularly effective in removing proteins from decalcified shrimp cuticle chips (Qin & Agboh, 1997). Based on the final dried form, the yield of this process is of the order of 30-35%, with the higher values observed on crab shells due to the higher content of calcium carbonate in crab cuticle.

Production of chitosan from crustacean is economically feasible, especially if it also includes recovery of pigments such as carotenoids (Abdou et al., 2008). To produce 1 kg of 70% deacetylated chitosan from shrimp shells, 6.3 kg of HCl and 1.8 kg of NaOH are required, in addition to nitrogen and water (Kumar, 2000).

Table 1.3. Applications of chitin, chitosan and their oligomers

Field of application	Claimed applications	References
Nutrition	Dietary fiber Lipid absorption reduction Antigastritis agent Infant feed ingredient Hypocholesterolemic effect	*Liao et al., 2007;* *Muzzarelli et al., 2007*
Food Additive	Beverage clarification and deacidification Natural flavour extender Texture controlling agent Emulsifying agent Food mimetic Colour stabilizer	*Kim & Thomas, 2007;* *Bautista-Baños et al., 2006*
Antimicrobial agent	Bactericidal Fungicidal Mold contamination indicator	*Bautista-Baños et al., 2006;* *Kurita, 2006*
Pharmaceutical	Wound healing Drug delivery system Regenerative medicine (bone, skin, liver and cartilage) Hemostatic	*Kim et al., 2008;* *Muzzarelli et al., 2007;* *Salgado et al., 2004*
Edible film	Respiration rate control Antimicrobial substances controlled release Antioxidant controlled release Oxygen partial pressure reduction Temperature control Moisture transfer control	*Maher et al., 2008;* *No et al., 2007*
Water purification	Dyes removal Pesticides, phenols and PCB recovery.	*Gamage & Shahidi, 2007;* *Wan Ngah et al., 2006*
Other	Enzymes immobilization Nutraceutical encapsulation Chromatographic separation Analytical reagent	*Grenha et al., 2008;* *Qiu & Bae, 2006*

Chitosan is largely produced in India, Japan, Poland, Norway and Australia from crustacean shells, and traded under such various forms as powder, paste, film and fiber (Sing & Ray, 2000).

Since chitin and chitosan have peculiar structures and properties that are quite different from synthetic polymers, they have often been considered as promising for development of desirable properties (Kurita, 2006); however, their large molecular weight and high viscosity restricts the *in vivo* use to dietary fiber. Conversely, modified chitin and chitosan have low viscosity and are characterized by low and short-chain length, which make them easily soluble in neutral aqueous solutions, thus readily absorbable *in vivo* (Jeon et al., 2000). Various techniques are suitable to derivatize chitin and chitosan; chemical or enzymatic substitution, chain elongation and depolymerisation are the preferred methods (Senel & McClure, 2004). Chitin and chitosan-based materials find application usually in the form of powders and flakes, but mainly as gels for membrane, coating, capsule, fiber, sponge and scaffold formulations. The methods used for chitosan and chitosan oligomers gel preparation can be tentatively divided into five groups: solvent evaporation, neutralization, crosslinking, ionotropic gelation and freeze-drying (Krajewska, 2004). For all the applications, expect for use as food fibers, chitin and chitosan are accordingly used as their oligomers, owing to the versatility imparted by specific functional groups (Venugopal, 2009).

1.2.4 Collagen and gelatin

The worldwide industrial demand for collagen and gelatin has undergone an increasing trend and currently accounts to 326000 tons (Karim & Bath, 2009). Collagen is the major structural protein present in skins and bones of all animals where it accounts for ca. 30% of the total protein content (Wasswa et al., 2007). Collagen is mainly sought for the production of gelatin, a high value functional protein due to its unique gel-forming capacity; of all hydrocolloids in use today, none is as popular as gelatin. Despite its low biological value, gelatin, like collagen, is commonly used in pharmaceutical and medical applications, because of its biodegradability and biocompatibility in the physiological environments, and also for technical applications (Young et al., 2005). Gelatin is hence utilized, for instance, as ingredient to improve elasticity, consistency and stability of food, as well as for encapsulation and film formation in the pharmaceutical, cosmetic and photographic industries (Gómez–Guillén et al., 2000).

Gelatin is the hydrolyzed form of collagen, and is a heterogeneous mixture of fibrous, denatured, biodegradable and water-soluble protein, with molecular weights typically

ranging between 80 and 250 kDa: it contains ca. 88% of protein, as well as 10% of moisture and 1-2% of salt, and is able to retain more than 50 times its weight of water within its gel structure (Ying Liu et al., 2008). Inside gelatin molecule, glycine is the dominant amino acid, constituting ca. 27% of the total amino acid pool (Tabata & Ikada, 1998). Proline and hydroxyproline come second in abundance and play a key role towards thermal stability of collagen. The total amount of those two imino acids is higher in mammalian (20-24%) than in fish (16-20%), which also leads to different rheological properties. Gelatin is amphoteric, so it possesses both acidic and alkaline properties depending on the nature of the solution. It is relatively economical to manufacture gelatin in large amounts since suitable raw materials are easily available (Baziwane & He, 2003). Most gelatin in the market is obtained from mammalian sources, typically bovine and pig skins, which account for 46% of the world gelatin output, followed by bones and hooves, representing 23% and 29% of the total gelatin production, respectively; only the remaining percentage, i.e. 1%, comes from marine sources (Karim & Bath, 2009; Wang et al., 2008). However, a few factors recognized as a serious risk for human health, e.g., the outbreak of bovine spongiform encephalopathy (BSE) and hoof-and-mouth disease (FMD) (*Aphtae epizooticae*), which are transmissible via food, have severely hampered the use of by-products from bovine and porcine origin (Wang et al., 2008). Additionally, the use of land animals as sources of gelatin is restricted by socio-cultural reasons in some countries.

Fish by-products appear then to be a good alternative, and furthermore show peculiar functional properties, which are dependent on the fish environment which is obviously rather different from traditional mammalian sources (Baziwane & He, 2003). Due to similar rheological behaviors, gelatin from warm water fish can be a good alternative to that from pork (Baziwane & He, 2003), which has been considered to be the best among all gelatins due to its higher proline and hydrxyproline contents (Wasswa et al., 2007). Note that collagens derived from species living in cold environments (e.g. cod), have lower contents of proline and hydroxyproline, so they present a lower melting point and inferior thermal stability than those from fish that live in warmer environments (e.g. tuna); 10% of either mammalian or warm water fish gelatins are able to form gel at room temperature, but 10% of cold water fish gelatin only gels at ca. -2 ºC (Gildberg et al., 2002).

The final characteristics and behavior of gelatin, depends not only on their animal origin, but also on the technology used in the extraction process. Gelatins can be recovered from raw material through acid, basic or enzymatic treatments, all of which are assisted by heat and water. The acid process is preferentially used with fish and pig skins, and is carried out with hydrochloric, sulfuric and phosphoric acids (Ying Liu et al., 2008). In case of high number of cross links, due to the high content of proline and hydroxyproline, lactic acid entertains the best performance upon swelling the collagen (Giménez et al., 2005). High pressures (i.e., above 200 MPa) facilitate swelling by destabilizing acid-soluble cross links and accelerating hydrolysis of collagen (Gómez–Guillén et al., 2005). The acid process takes ca. 3 days and the result is a type A gelatin with a pH between 3.5 and 6, which is the most widely used. Conversely, the alkaline process (which is employed exclusively for bovine bonds) may take up to 20 weeks and generates a type B gelatin which is harder and more viscous than type A, and holds a pH between 5 and 7; its main application is in stabilizing other food hydrocolloids (Wasswa et al., 2007). After either acidic or alkaline extraction, the final gelatin is colorless, transparent, brittle and tasteless, and is thus shapeable onto various forms, such as sheets, flakes or powder. Despite solvent extraction being currently the method of choice, the final gelatin product lacks a few functional properties, so enzymatic hydrolysis has been regarded more and more often as the best way to obtain gelatin with given specifications. In fact, high added value mammalian and warm water fish gelatin can be treated with proteolytic enzymes, to produce gelatin with a very soft color, yet with high gel strength and a wide range of viscosities (Wasswa et al., 2007).

Although collagen and gelatin from fish origin have in general a few drawbacks when compared to their mammalian counterparts, e.g. low stability, fishy odor and dark color, these products are well suited for many industrial applications. Gelatin from warm water fish (e.g. tuna, sardine and anchovy) is currently exploited as ingredient for food and drugs coating (Pérez-Mateos et al., 2007), in particular when odor and taste of drugs are unpleasant (Vandervoot & Ludwing, 2004). Gelatin from warm water fish also find application in ophthalmological formulations (Supavititpatana et al., 2008); in production of photographic films (Surch et al., 2006) and low-set time glues (Wasswa et al., 2007). Cold water fish (e.g. cod, hallock and pollock) gelatin is used as

structuring agent in non-gelling formulations (Wasswa et al., 2007) and as active ingredient in cosmetic preparations (Baziwane & He, 2003).

Gelatin possesses a characteristic melt-in-the-mouth property, which make it suitable to a wide range of applications in food and pharmaceutical industries; in particular, fish gelatin releases aroma better and shows a higher digestibility than animal one (Gómez–Guillén et al., 2000). Because of its solubility at room temperature, fish gelatin permits relatively low temperature coacervation, which is not feasible with other gelatins (Gómez–Guillén et al., 2005). Fish collagen is widely used in beauty and cosmetic product formulation (Bae et al., 2008), mainly intended for soft tissues (e.g. skin) as well as cartilage and bone repair (Salgado et al., 2004). In the latter application, and as happens with other biopolymers, e.g. chitosan, starch and hyaluronic acid, natural collagen triggers low immune responses, so it is a good substrate for cell adhesion, with good capacities to interact with host tissues, owing to good chemical versatility and chemotactic properties (Salgado et al., 2004). This biopolymer is nowadays used in the manufacture of biocompatible glues which consist of a mixture of collagen with citric acid as a cross linking agent; particularly powerful in binding tissue-tissue interfaces, yielding less toxicity than such semi-synthetic and synthetic formulations as gelatin-aldehyde and cyanoacrylate-based glues (Taguchi et al., 2006). Its intrinsically high water retention capacity it is also taken advantage of in rough skin repairing. Finally, fish and pig collagens exhibit anti-radical action (characterized by $IPOX_{50}$ of 0.18 and 0.45 mg/mol, respectively) and a potential in decreasing blood pressure (characterized by IC_{50} of 0.16 and 0.41 mg/mol, respectively) (Morimura et al., 2002).

The intrinsic biological and physicochemical features of collagen and gelatin make them useful in various fields. The current market demand is surging more reliable less expensive sources, and fish by-products clearly fit into this status.

1.2.5 Hydroxyapatite

Research in the biomedical area has been focusing on the identification of biomaterials with long term physiological compatibility, so current development of advanced materials with biomimetic features is one of the most promising trends in biotechnology. The Institute of Materials (London, UK) estimated a world market of ca.

$12000 million per year, with an average global growth between 7 and 12% per annum (Aronov et al., 2007).

Materials obtained from natural sources obviously show the best biocompatibility with the human body. In many circumstances, synthetic materials are hardly accepted because the organism fights against the invasion by foreign matter. Naturally occurring hydroxyapatite, $Ca_5(PO_4)_3OH$, is a form of bioceramics with the highly desirable physicochemical attributes of stability, inertness and biocompatibility. This mineral is the primary constituent of bones, teeth and calcified cartilage tissues in human and higher animals, as well as fish; it represents ca. 43% of human bone, 65-70% of higher animal bone and 60-70% of fish bone (Barakat et al., 2008; Aronov et al., 2007). It has an elastic modulus that is ca. two orders of magnitude greater than collagen, coupled with a density that is three-fold that of most biological materials, which makes it suitable for several biomedical applications.

Nowadays, polymeric composites involving hydroxyapatite are particularly demanded because of their easy tailorable manufacturing processes; bioinert composites for permanent applications, biodegradable composites for temporary applications and injectable composites include hydroxyapatite among the main compounds, both in pure form or as part of hybrid materials (Mano et al., 2004). Bone cement for craniofacial, oral-maxillofacial and orthopedic defect repair, and coating for femoral components are the most common applications of hydroxyapatite so far reported (Woodard et al., 2007; Mano et al., 2004; Salgado et al., 2004). Its beneficial osteoconductivity permits binding to human hard tissues and thus allows implementation of very stable scaffolds in bone as well as in tooth repair and substitution (Moshaverinia et al., 2008; Deville et al., 2006). Hydroxyapatite has also been reported in combination with other specific materials; for instance, biodegradable metal matrix composites based on magnesium and hydroxyapatite, have been demonstrated to be cytocompatible biomaterials with adjustable mechanical and corrosive properties, which justify their high demand for biodegradable metal implants (Witte et al., 2007; Deville et al., 2006; Monkawa et al., 2006). Hydroxyapatite powder has also been successfully impregnated with cellulose sponges in attempts to obtain porous bodies with adequate porous dimension and distribution that mimic the morphology of a spongy bone and favor osteoconduction (Landi et al., 2003). Other applications include substrate for adhesion of proteins,

peptides, lipids, bacteria and strains, useful for drug delivery (Aronov et al., 2007); cartilage regeneration (Woodard et al., 2007; Mano et al., 2004); wastewater heavy-metal and dyes decontamination (Aronov et al., 2007). Hydroxyapatite has classically been chemically synthesized to tailored properties using techniques such as the hydrothermal and sol-gel synthesis (Fathi et al., 2007). However, hydroxyapatite obtained from natural source, as fish and animal bones, inherits such structural properties of the original raw material, besides the intended chemical composition, so it is a better alternative for numerous devices based on synthetic hydroxyapatite (Haberko et al., 2006; Kim & Mendis, 2005). Hydrothermal alkaline hydrolysis is the most common route for hydroxyapatite extraction from fish and animal bones (Barakat et al., 2008). A method recently proposed for hydroxyapatite extraction from natural sources consists of subcritical water extraction, which has the further advantage of producing carbonated hydroxyapatite, which is highly required in biomedical applications. The former and more usual process also keeps the carbonate ions of the natural hydroxyapatite source, although to a lower extent than the subcritical water one. On the other hand, alkaline hydrothermal process produces relatively better nanoparticles (Barakat et al., 2008), which is highly desirable to human bone applications (Li et al., 2007).

Therefore, there is a continuing interest in exploring features offered by hydroxyapatite composites, so commercial applications are expected to rely on more accessible and less expensive raw materials as is typically the case of fish by-products.

1.2.6 Antifreeze proteins

Antifreeze proteins, also known as thermal hysteresis proteins, are ice-structuring proteins able to influence the growth of ice crystals and inhibit ice recrystallization. Those unique molecules were first identified by de Vries back in 1969 in the blood of fish living in frozen sea areas (Crevel et al., 2002); they apparently serve to lower fish blood freezing point below seawater freezing point, thus avoiding the increase in plasma osmotic pressure and without altering the blood melting point (Barrett, 2001). Proteins with similar features have meanwhile been found, although to lesser levels, in plants and insects that survive at very low temperatures (Cheng, 1998).

There are two major types of antifreeze proteins: glycoproteins and non-glycoproteins, based on presence or absence of carbohydrate, respectively (Harding et al., 2003). However, both types can lower the freezing point of an aqueous solution via a non-colligative mechanism, by binding to the ice crystal surfaces and inhibiting further crystal growth (Evans & Fletcher, 2004). Since antifreeze proteins prevent fish from freezing, a significant percent is present only in species adapted to very cold sea water; the greatest amount has been found in the blood of Antarctic fish, particularly in cod (Harding et al., 2003). Their antifreeze proteins are mainly glycoproteins, the blood concentration of which varies from 5 to 35 mg/ml (Jin & de Vries, 2006). In both glycol- and non-glycoproteins, alanine makes up two-thirds of the total number of amino acid residues, with the remaining third being accounted for threonine and cysteine.

Antifreeze proteins find large application in frozen food technology, low-fat content food manufacture, transplanted organs cryopreservation, cryosurgery and aquaculture (Venketesh & Dayananda, 2008; Feeney & Yeh, 1998). Two applications arise as particularly important in terms of frozen foods: ice-cream manufacturing and frozen meat technology. Antifreeze proteins may improve quality of frozen foods, hence allowing for the maintenance of their natural texture, reduction of cellular damage and loss of nutrients, all of which contributes to preserve their nutritional value (Li & Sun, 2002). It is illustrative that meat (bovine and ovine muscle) soaked in solutions of up to 1 mg/ml of antifreeze glycoproteins prior to freezing at -20 ºC showed evidence of reduced ice crystal size, and conveyed a better nutritional value after thawing. Furthermore, antifreeze proteins have been used in ice-cream manufacturing to reduce the overall fat content down to 1%, conferring a good texture thanks to their nanostructuring ability (Feeney & Yeh, 1998). A few companies have recently invested in this feature and consequently earned substantial economic benefits, by designing and launching a new range of low-fat products with pleasing texture, which are becoming even more popular among consumers around the world due to the low caloric content (Sigman-Grant et al., 2003).

Although yeasts and some other types of microorganisms are a suitable source of antifreeze proteins, the most promising one is fish. For the recovery of such unique proteins, fish skin or blood are usually frozen in nitrogen, powered and eventually

homogenized in an alkaline medium. After centrifugation, the lyophilized supernatant is dissolved in the same alkaline medium, and finally is subjected to preparative chromatography, using a specific silica gel column (Evans & Fletcher, 2004). An alternative isolation/purification method is adsorption on ice, using a cold finger apparatus; depending on the intended final purity, a further chromatographic step may also be required (Kuiper et al., 2003).

More performing and less expensive purification technologies are now urged, as the final cost of 1 g of antifreeze proteins is very high, with reported values of ca. $50 (Feeney & Yeh, 1998) to ca. $10.000 (A/F Proteins, Walthma, USA), currently accounting for the purification steps mainly. If that constraint is successfully addressed, a wide diversification of the novel food portfolio employing those specialty proteins is likely.

1.2.7 Enzymes

Nowadays, enzyme-based technology represents an important contribution (and, in some cases, essential one) for many industrial applications (Rasmussen & Morrissey, 2007). The boom in life sciences has led worldwide industries to a sustained rate of annual growth of ca. 7.5% for enzymes, with an estimated volume of up to US$ 7000 million in 2013 (Market Research Report, 2009). This is so because enzymes possess a high selectivity, and a very high activity even at very low concentrations and mild conditions of pH and temperature, which leads to fewer unwanted side-effects and by-products (Shahidi & JanakKamil, 2001).

Aquatic invertebrates at large, as well as the internal organs of fish and the shells of crustaceans, constitute natural sources of enzymes with huge interest. Due to the prevailing environmental conditions, marine enzymes can effectively operate at low temperatures, below 4 ºC, and within a neutral to alkaline pH values (SEAFISH, 2008). As emphasized elsewhere (Shahidi & JanakKamil, 2001), most enzymes from fish and aquatic invertebrates also occur in higher animals, yet they possess unique characteristics, in terms of molecular weight, amino acid composition, pH and temperature stability, inhibitory behavior and kinetic properties.

Proteases constitute at present the dominant group of marine enzymes with a commercial expression; recall that proteases bring about hydrolysis of peptide bonds,

and have been termed as proteinases or peptidases, depending on whether they act on proteins or polypeptides, respectively. Gastric, intestinal and hepatopancreas proteinases have been scrutinized more thoroughly (Beynon & Bond, 2001); pepsin from polar cod is one of the most extracted gastric proteases, followed by collagenase, elastase, trypsin and chymotrypsin, as well as non proteolytic enzymes, such as transglutaminase, lipases and chitinolytic enzymes (chitinases) to lower extents (Rasmussen & Morrissey, 2007). The main enzymes of fish, aquatic invertebrates and marine mammals that may be potentially recovered for practical uses are presented in Table 1.4.

Table 1.4. Sources of enzymes from fish, aquatic invertebrates, marine mammals and their by-products (adapted from Shahidi & JanakKamil, 2001)

Group	Enzyme	Source
Gastric proteases (aspartic protease family)	Pepsin	Atlantic cod, Greenland cod, salmon, mackerel, sardine, capelin, American smelt, tuna (bluefin), orange roughy
	Pepsinogen	shark, tuna (bluefin), rainbow trout
	Chymosin	harp seal
	Gastricsin	Atlantic cod, hake
Intestinal proteinases (serine protease family)	Trypsin	Atlantic cod, Greenland cod, sardine, capelin, cunner, chum salmon, Atlantic salmon, Coho salmon, anchovy, carp, Atlantic white croaker, palometa, hybrid tilapia, krill, crayfihs, oyster
	Chymotrypsin	capelin, herring, Atlantic cod, spiny dogfish, rainbow trout, scallop, abalone, white shrimp, grass carp
	Collagenases	fiddler crab, freshwater prawn, crayfish, Atlantic cod, king crab
	Elastases	carp, catfish, Atlantic cod
Chitinolytic enzymes	Chitinases	crustaceans, squid, octopus,
	Lysozymes	Arctic scallop
Transglutaminases		red sea bream, rainbow trout, Atka mackerel, walleye pollok liver, scallop muscle, Botan shrimp, Squid
Lipases		shark, Atlantic cod

Even though no reliable market data are presently available, recovery of marine enzymes is potentially profitable; well established commercial applications already exist (Table 1.5) but there are major opportunities for developing new niche markets.

Table 1.5. Current and potential applications of enzymes obtained from marine products and by-products (adapted from Esposito et al., 2009; Shahidi & JanakKamil, 2001)

Field of application	Claimed applications
Fish curing and fermentation	Fish sauce and silage production
Hydrolysed products production	Fish protein hydrolysate
	Flavour compound
Pigment extraction	Pigments recovery from shellfish waste
Protein coagulation	Rennet substitute in cheese manufacturing
	Removal in milk oxidized flavor
Selective tissue degradation	Fish and aquatic invertebrates deskining
	Fish roe purification
	Cod liver membrane removal
	Shellfish exoskeleton removal
	Salted cod swim bladder production
Wastewater treatment	Viscosity reduction of sticky water
Detergents industry	Laundry detergent production
Other potential applications	Meat tenderizing
	Fish oil enzymatic extraction from raw material
	ω-3 fatty acids concentrates production
	Antibacterial enzymes
	Antioxidant enzymes
	Gene cloning technology

There is an outstanding profitable market for cod transglutaminase in surimi manufacture (Shahidi & JanakKamil, 2001) as well as for lipases in the detergent industry (Esposito et al., 2009). A recent addition to this list is the use of lipases in polyester production (Schmid et al., 2002). A great number of research studies are currently focusing on novel purification methods and immobilization methodologies

that can improve both stability and enantioselectivity of the enzymes and thus render those processes more competitive (Mohapatra et al., 2003).

1.**2.8 Astaxanthin**

Astaxanthin (3,3-dihydroxy-β,β-carotene-4,4-dione) is a ketocarotenoid oxidized from β-carotene, which plays biological roles and possesses a number of desired features for food applications, such as natural origin, nil toxicity, high versatility, and both hydro and liposolubility (Delgado-Vargas & Peredes-López, 2003). Its attractive pink color, its biological functions as vitamin A precursor and antioxidant, make astaxanthin widely sought for food and medical applications (López-Cervantes et al., 2006). Its superior antioxidant power has even coined his label of "superior vitamin E"; in fact, it possesses an antioxidant effect greater than β-carotene, as well as vitamins C and E (Stepnowski et al., 2004), especially in quenching singlet oxygen and scavenging free radicals. Moreover, astaxanthin is active in protecting against chemically-induced cancers and age-related macular degeneration, and also in enhancing the immune system and in preventing damages arising from ultraviolet radiation (Yang, 2007).

This carotenoid occurs naturally in a wide variety of marine and aquatic organisms (López-Cervantes et al., 2006) with algal cells and crustacean shells being the major sources, followed by plants, bacteria and egg yolk. Astaxanthin represents between 74 and 98% of the total pigments in crustacean shells, which contains from 2.3 to 33.1 g/100g of carotenoids (Shahidi et al., 1998a). Due to these high contents, crustacean shells are used not only for recovery of chitin but also for recovery of carotenoids.

The methods currently available for the extraction of astaxanthin from shell matrices employ different elements such as edible oils, hydrochloric acid and organic solvents, besides proteolytic enzymes (Babu et al., 2008b). Such plant oils as sunflower oil, groundnut oil, ginger oil, mustard oil, soybean oil, coconut oil, rice bran oil and palm oil have accordingly been used to extract astaxanthin from crustaceans and fish by-products (Handayani et al., 2008). Some countries, such as India, use proteolytic enzymes, such as trypsin, papain and pepsin, dissolved in citrate phosphate, to precipitate carotenoids from the shells. At present, a feasible technique for partial concentration of astaxanthin from crustacean shells is via lactic acid fermentation, which also has the advantage of protecting the biomass from bacterial decomposition.

The silage formed contains insoluble chitin, a protein-rich fraction, and a lipid-rich fraction composed of astaxanthin, sterols, and vitamins A and E (Babu et al., 2008b).
Owing to its useful properties, astaxanthin from natural sources is increasingly being marketed as a functional food ingredient in many countries (Yang, 2007) with prices that range between 3000 and 12000 US$/kg (AWARENET, 2004) and fish by-products are thus expected to become increasingly important as industrial feedstock.

1.3 Conclusions

The dual problem arising from the surplus of several commodities, which bear low economic returns for the farmers, while entertaining especially in the view of increasingly tighter environmental regulations, has been promoting the commercial exploitation of many products that were so far discarded, and eventually leading to better manufacturing practices and more sophisticated processing technologies. Valorisation of by-products arising from fish catching and processing is a must order, since it constitutes a central issue for the long-term sustainability of the fish industry at large. Moreover, a few high value added compounds recovered from such type of by-products are economically more attractive than the target products themselves. Development of novel and clean technologies aimed at more efficient recovery of bioactive nutraceutical compounds from marine by-products will lead to the development of more profitable processes, thus giving rise to many great opportunities to the marine industry.

PART II

HIGH ADDED VALUE COMPOUNDS FROM THE CODFISH (*Gadus morhua* L.) SALTING PROCESSING RESIDUES

Abstract

Among all food, fish is the main source of waste and by-products when is processed for human consumption and the residues have to be discharged or valorized/upgraded depending on their characteristics and market possibility. In Portugal, fishing and fish processing are a major economic activity and the Country generates ca. 173000 ton of solid residues every year. Salting process, along with filleting, is one of the main source of by-products and waste, accounting for ca. 63 % of the overall generation.

Cod (*Gadus morhua* L.) is one of the white fish most processed in Europe, and in Portugal is usually consumed salt-cured. Along the salting process 50-75 % of solid by-products and ca. 18 m^3 of liquid waste are generated for each ton of salted and re-hydrated codfish. Among liquid waste, the water drained away from codfish during the salting process showed the highest potential for upgrading, carrying important compounds that can be valorised for pharmaceutical, cosmetic and food applications. The chemical profile of such a residual water, composed with various soluble nitrogen fractions relative to free amino acids, peptides and proteins, has been thoroughly determined. Around 10 g/L of nitrogen containing compounds have been found in the wastewater at the end of the typical 6 days salting process. Concentration of free amino acids increased from 3.5 g/L to 6.5 g/L within 6 days, and concentration of proteins increses from 3 g/L to 3.7 g/L. Aspartic and glutamic acids, arginine, creatine, glycine, lysine, methionine, phenylalanine, taurine and tryptophan were the dominant free amino acids, the release of which was successfully modelled. Total biogenic amines concentration in wastewater at the end of the salting process was ca. 100 mg/kg.

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CHAPTER 2

BY-PRODUCTS AND WASTE THROUGH THE SALTING PROCESS OF CODFISH

2.1 Introduction

The fish processing industry is widespread and quite varied in terms of type of operations, scale of production and outputs. Among all food, fish is the main source of waste and by-products when is processed for human consumption, and the residues have to be managed or upgraded depending on their characteristics and market possibility (Arvanitoyannis & Kassaveti, 2008). According to European legislation a by-product is a secondary product of a manufacturing chain which can be further processed to obtain commodities having, in general, a lower market price than the main product. A waste is a secondary product of a manufacturing chain which cannot be further processed, and then has to be treated according the national environmental regulations before discharged (Bontox & Leone, 1997; European Council, 1991). Waste and by-products generation prevention and minimisation are a major item within the agro-food industries in Europe. Large amounts of wastewater and solid residues are generated leading to important costs for their treatment and disposal. Prevention or minimisation of waste ranges from simple to low-cost practices that can decrease the environmental impact significantly (such as cleaning procedures optimisation, adequate equipment maintenance, monitor of water and energy consumption), to technological renewal of machinery and more recently to valorisation (AWARENET, 2004).

By the *Growth Programme* the European Commission (2000) has started defining new strategies in the field of agro-food waste and by-products valorisation. Identification of new technologies and methodology that allow for upgrading, identification of market demand for new products from valorised agro-food waste, and definition of new research and development lines for improving actual agro-food valorisation, have been the major concerns and can represent an important opportunity for countries where the fish processing sector is a reality.

In Portugal fishing and fish processing comprise one of the major economic activity. The country has a long tradition in the sector and is among the countries in the world with the highest fish consumption per capita. Fish has been an important staple for the entire Portuguese population, at least since the *Portuguese Age of Discovery*. It has to be highlighted that Portugal's Exclusive Economic Zone – a sea zone over which the Portuguese have special rights over the exploration and use of marine resources – has 1.727.408 km². This is the 3rd largest Exclusive Economic Zone of the European Union,

and the 11th in the World. Around 173000 ton of solid by-products and waste are generated each year in Portugal, of which ca. 63 % is originated from the salting and filleting processes, ca. 31 % is originated along canning, ca. 2.5 % is originated from fish preservation and ca. 3.5 % arises from drying and thawing (AWARENET, 2004). However, in Portugal, by-products and waste from fishing and fish processing are not yet valorised for human consumption but just for fishmeal production.

2.2 By products and waste generated through the codfish salting process at a processing company

Codfish ("*bacalhau*") is one of the fish species most processed in Europe, along with tuna, herring, mackerel, pollack, hake, haddock, salmon, anchovy and pilchard (AWARENT, 2004). Salted codfish has been produced for at least 500 years, since the time of the European discoveries of the *New World*. Salting preservation allows for a longer shelf-life of fish products, because it absorbs much of the water in the food, making microbial growth and survival difficult. Salting is a quite inexpensive technique when salt is cheap, it does not require low temperatures for storage, and the final product has adequate quality and nutritional values (Ismail & Wootton, 1982; Andrés et al. 2006).

Fish salting, along with canning, filleting, curing and smoking, is a process which is important in terms of production and economic significance, and at same time for waste and by-product generation. Salting, like filleting, curing and smoking operations, originates 50-75 % of solid residues; however, major environmental impact associated with codfish salting processing operations are the high consumption of water, consumption of energy and discharge of effluent with a high organic concentration due to the presence of lipids, oil, proteins and suspended solids. They may also contain phosphates, nitrates and chloride and unpleasant odour mainly generated from organic matter decarboxylation. Around 18 m^3 of liquid waste are generated for salting and re-hydrating each ton of codfish (AWARENTE, 2004).

Codfish salting is accomplished by a number of stages (Table 2.1). Like other white fish with low oil content, cod is eviscerated, cleaned and beheaded on board just after caught. Then the fish are kept on ice or frozen until arriving at the processing plant.

Upon arrival, codfish are thawed or re-iced and stored until processing. Codfish salting pre-treatment is a source of residues as well. This stage involves removal of ice, removal of blood by aspiration, deboning and screening, which sorts the fish by size. In Figures 2.1 to 2.4 (kindly supplied by the cod fishing and codfish processing company *Pascoal & Filhos* S.A., Aveiro, Portugal) by-products and waste arising from the codfish salting process are shown. In Table 2.1 by-products and waste amount generated throughout the codfish salting processing are described.

Figure 2.1 Codfish skin

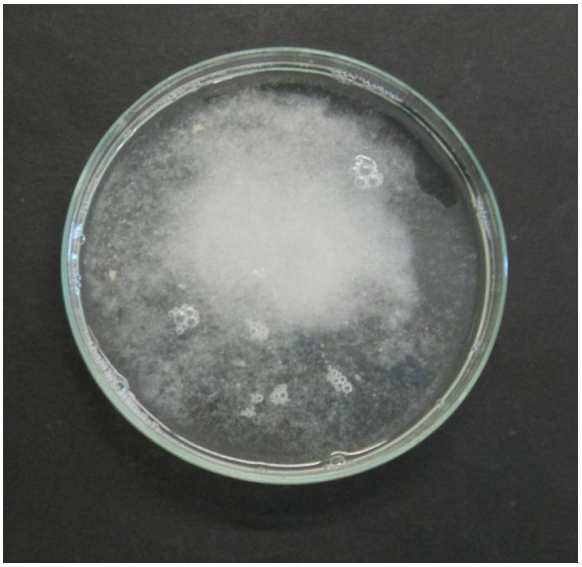


Figure 2.2 Supernatant of codfish salting and drying processing wastewater

Table 2.1 By-products and waste obtained through the codfish salting process (adapted from AWARENET, 2004)

LIQUID WASTE				**CODFISH PROCESSING**	**SOLID WASTE and BY-PRODUCT**		
Current management	Liquid waste	Polluting load composition	Wastewater	salting/drying/desalting	Solid waste/ by-product	% solid waste in weight	Current management
Anaerobic treatment	5 m^3/t	Blood COD : 1000-7000 mg/L	Thawing water	**Thawing** (if the fish is frozen)			
	1.4 m^3/t	COD: 1000–5000 mg/L	Washing water	**Washing and Grading**	Rejected fish	1 – 5	Production of foodstuff and animal feed
Screening and filtration/ Aerobic treatment	1 m^3/t	BOD : 3.4-45.5 Kg/t SS : 2-2.4 Kg/t COD: 2000-4000 mg/l Blood	Washing water Blood water	**De-heading and evisceration**	Offal, Heads	25 – 30	Production of foodstuff (fish meal, food ingredients, surimi, polyunsaturated fatty acids, gelatin, collagen). Production of animal feed (fish protein, fish silage, fish protein hidrolysate, fish oil). Production of fertilizer (fish solubles, fish protein hydrolyzsate). Production of pharmaceuticals (gelatin, collagen). Production of coating (fish oil, pearl essence) and adhesives (fish glue).
					Skin if skining occurs	4	
Sedimentation/ Aerobic/ Anaerobic treatments	1 – 3 m^3/t	COD: 4000-12000 mg/L	Wastewater	**Filleting**			
	0.2 – 1 m^3/t	COD: 2000-5000 mg/L	Wastewater	**Washing**	Bones, Frames	20 – 30	
	0.2 – 0.5 m^3/t	250-800 g/Kg Ash High Cl (100 – 500 g/L)	Wastewater	**Salting/Drying**			
Screening and filtration	6 – 9 m^3/t	0.1 g/Kg Ash Low Cl (300 mg/L)	Wastewater	**Desalting/Re-hydration**	**Total solid by- product and waste: 50 – 75%**		

Figure 2.3 Sediment of codfish salting and drying wastewater stream

Figure 2.4 Codfish salting processing wastewater

By-products generated at the Portuguese codfish processing industries are currently valorised for animal feeding, while liquid waste is discharged after treatment according to national environmental regulations, representing a cost without any economic return for producers.

Liquid waste from codfish salting processing generated at the company *Pascoal & Filhos S.A.*, located in Aveiro (Portugal), have been collected for a screening of their composition with the aim to estimate their potential of valorisation.

Based on 30 ton of codfish, which represents the amount processed in one day when the fish caught reaches the processing plant, the company produces the residues listed in Table 2.2.

Table 2.2 Amount of by-products and waste from salting process of codfish generated at *Pascoal & Filhos S.A.* and based on 30 ton/day of codfish

Residue	Amount generated from 30 ton
Solid by-products:	
Skin	300 Kg/day
Bones	3000 Kg/day
Liquid waste:	
Wastewater with blood	300 L/day
Salting and drying wastewater	7600 ton/day
Re-hydration wastewater*	100.000 L/day*

*Re-hydration wastewater is based on a daily quantity of 10 ton of codfish

Codfish skin and scale residues are currently managed for fishmeal production, whereas liquid waste needs an appropriate treatment before disposal. Regarding the latter, salting and drying wastewater are sent to the same reservoir and managed as a unique slurry stream which spontaneously separates in two phases, supernatant and sediment (Figures 2.2 and 2.3 respectively). Samples of each phase have been collected for a preliminary screening of composition (Table 2.3). Samples of the salting wastewater (Figure 2.4) and of re-hydrating wastewater have been also collected and results for composition are shown in Table 2.3. Preliminary screening of composition has been performed with collaboration of *CINATE – Centro de Inovação e Apoio Tecnologico e Empresarial* (Centre for Innovation and Industrial Technological Support) at College of Biotechnology of Catholic University of Porto, Portugal.

Table 2.3 Screening of composition of codfish salting processing liquid waste generated at *Pascoal & Filhos, S.A.*(Analysis were performed by an external accredited laboratory)

Parameter (g/100g)	**Sample**				**Method of determination**
	Supernatant	Sediment	Salting wastewater	Re-hydration water	
Dry matter	25.6	83.7	26	0.1	EN 12880 (2000)
Ash	22.4	81.2	23	< 0.01	Incineration at 550 ºC (AOAC, 1990a)
Total Lipids	< 0.1	< 0.1	< 0.1	< 0.1	Soxhlet (AOAC, 1990b)
Total Protein	1.4	1.9	1.9	< 0.1	Kjeldhal (ISO, 1978)
Saturated fatty acids	< 0.1	< 0.1	< 0.1	< 0.1	AOAC (1990b)
Monounsaturated fatty acids	< 0.1	< 0.1	< 0.1	< 0.1	AOAC (1990b)
Polyunsaturated fatty acids	< 0.1	< 0.1	< 0.1	< 0.1	AOAC (1990b)
Sodium	8.5	32.6	8.5	21 mg/100 g	AES flame (de Oliveira et al., 2009)
Salt	22	78	22	50 mg/100 g	Calculated from the sodium amount

As can be observed in Table 2.3, the sediment phase is almost exclusively composed of salt and carries the same amount of total proteins than codfish salting wastewater. This finding suggested that the organic load in the sediment phase might be carried by the salting wastewater itself, which shows almost the same dry matter content of the supernatant. Re-hydration water did not show significant organic matter content.

Codfish salting processing wastewater has thus been identified as the waste with the greatest potential of valorisation among all liquid residues scrutinised and was the object of study of this thesis. Extensive characterisation of codfish salting wastewater is reported in the following chapter.

CHAPTER 3

CHARACTERISATION OF WASTEWATER THROUGH THE SALTING PROCESS OF CODFISH

3.1 Introduction

Despite the development of other means of preservation, salt-cured codfish continues to be widely produced due to low operating costs, simplicity of processing and highly appreciated sensory characteristics promoted by salt (Martínez-Alvarez & Gómez-Guillén, 2006). Codfish takes salt up to ca. 20 % (w/w) during dry salting, but drains concomitantly ca. 22 % (w/w) of physiological water — which drives important changes in the composition and structure of the muscle tissue (Andrés et al., 2005). The major effects imparted by salt are: modifications in muscle protein conformation, changes in water–holding capacity (with subsequent protein denaturation), and losses of nutrients (e.g. peptides and amino acids). As reported by Kinsella (1982), the pH of the salt itself also plays an important role upon protein denaturation and loss: the soluble protein fraction of codfish muscle salted with basic salt or unsalted are largely similar to each other, whereas acidic pH favors actin and myosin release (Martínez-Alvarez & Gómez-Guillén, 2005).

Water drained from codfish salting is currently treated as an ecotoxic waste due to the high content of chloride (Meneses et al., 2010) which can reach values as high as 160 g/L, representing a strong impact to the environment. However, this residual water carries important compounds, which, although not regarded as essential nutrients, can be considered beneficial under certain circumstances. Organic load of codfish salting processing wastewater accounts mainly for proteins of the muscle system and for some free amino, which are well known micronutrients and active compounds (Larsen et al., 2007; Sikorski et al., 1990). Aspartic and glutamic acid, arginine, creatine, glycine, lysine, methionine, phenylalanine, taurine and tryptophan have been the major amino acids detected in codfish salting processing wastewater.

Properties of amino acids creatine and taurine have been extensively discussed in Chapter 1 so that a brief introduction to other amino acids will follow.

Glutamic acid, together with γ-amino butyric acid, is the major excitatory neurotransmitter in the mammalian central nervous system; it plays an important role in the learning processes and memory and also in taste perception, although excessive doses can induce neurodegeneration (Platt, 2007; Garattini, 2000). The most important practical application seems to be related to taste enhancement where it is provided in the form of sodium salt. Applications of glutamic acid as chemical intermediate and

constituent of enteral/parenteral nutrition solutions, in synergy with aspartic acid, have also been mentioned (Arsenian, 1998). Aspartic acid is widely used to manufacture the sweetener aspartame; it is the second most important neurotransmitter in higher living organisms together with glycine (D'Aniello, 2006; Butchko & Stargel, 2001).

Arginine (L-form), a conditional essential amino acid, is an essential precursor of amino acids creatine and γ-amino butyric acid, as well as of nitric oxide which is necessary for the normal regulation of vascular tone in human body; arginine is also responsible for the secretion of growth hormone, insulin and glucagon. Assists in wound healing, helps removing excess ammonia from the body and stimulate immune function (Tapiero et al., 2002). Oral administration of arginine have been proven to have positive effects in decreasing blood pressure and fatigue, enhancing growth of short children, preventing congestive heart failure and promoting gut health; however, this amino acid should be always balanced with lysine (Loche et al., 1993; Wang et al., 2009).

Glycine, a non-essential amino acid, is a biosynthetic intermediate involved in synthesis of glycogen and proteins, particularly collagen. Glycine inhibits sugar craving and a recent study has also demonstrated a protective effect on the gut (Wang et al., 2009).

Lysine (L-form) is an essential amino acid for human and a basic building block of proteins. It is essential in collagen formation, calcium absorption, inhibition of viral growth, and critical for bone formation in children. Lysine can lower triglycerides and LDL cholesterol levels in blood serum, and may have a potential role in treating osteoporosis in combination with arginine (Torricelli et al., 2002).

Methionine is one of the nine essential amino acids, and the only sulphur containing essential amino acid; it is a precursor of amino acid taurine, and is the most important source of sulphur for human and animals. Methionine regulates proteins synthesis and breakdown (and probably fats breakdown as well), and is considered a strong antioxidant since it destroys reactive oxygen species (ROS) in human body (Métayer et al., 2008).

Phenylalanine is an essential amino acid that plays an important role in mood, memory, and mental alertness and is used to relieve chronic pain and to treat depression. It is essential for protein synthesis and for synthesis of tyrosine, aromatic amino acid as phenylalanine (Matthews, 2007).

Amino acid tryptophan, which behaves as a hormone, is a crucial compound in humans, animals and marine organisms owing to its involvement in protein and serotonin synthesis (Lin, Smith & Bayley, 1988).

Finally, myosin and actin are two proteins currently applied for food rheology improvement, due to their emulsifying properties and to their ability to develop structuring gels (Venugopal, 2009); one of the best illustrative example is surimi manufacture (Carjal et al., 2005).

This Chapter describes composition of water drained away during the codfish salting process, in terms of total nitrogen and total lipid content, free amino acids and myofibrillar proteins, and salt load. A kinetic study has been carried out in an attempt to model the release of free amino acids and myofibrillar proteins throughout the duration of codfish salting. Biogenic amines — a product of free amino acids decarboxylation — have been also characterized.

3.2 Materials and methods

3.2.1 Wastewater sampling

Wastewater samples have been supplied by *Pascoal & Filhos, S.A.* (Aveiro, Portugal). Atlantic codfish (*Gadus morhua*) has been caught in the Northern Sea, at a depth of 20 m and 200 m away from the Southern coast of Norway; it has been immediately gutted and beheaded, and kept cold on board (at −20 ºC) until inshore uploading. Upon arrival, codfish has been thawed, deboned and washed; then it has been "butterfly-split" and salted by dry (or "kench") salting, i.e. mixed with alternating layers of dry and alkaline food–grade marine salt (pH 8.5), and stacked in a tank for six days at ca. 17 ºC.

Each tank contains approximately 800 kg of codfish. Throughout salting treatment, wastewater samples have been collected every day at the same time, in duplicate and from the same tank, and frozen at −20 ºC until analysis. The volume of wastewater drained away from the codfish to the tank has been also measured every day at the same time of sampling. Before analysis, wastewater samples have been centrifugated at 5000 rpm and 4º C for 10 min in order to precipitate suspended solids, such as fish flesh, skin and bond resides.

3.2.2 HPLC-UV/Vis analysis

Chromatographic analysis have been performed using a Beckman&Coulter 168 series HPLC system interfaced with a Photo Diode Array UV/Vis detector (PDA 190–600 nm) (Beckman&Coulter; Fullerton, CA, USA). Data acquisition and analysis has been accomplished using Karat32 software. All eluents have been filtered through a 0.45 μm cellulose membrane (Millipore-Interface; Amadora, Portugal) and degassed in an ultrasound bath (Millipore-Interface) for 15 min prior to use as mobile phases. All determinations have been carried out in triplicate.

3.2.2.1 Taurine and creatine

Taurine and creatine have been analysed using a Waters Nova-Pack RP-HPLC, C18 column (150 × 3.9 mm, 4 μm) (ViaAthena; Lisbon, Portugal). Taurine analysis has been executed using the method described by Orth (2001), with a slight modification in gradient elution and in column. Standard solutions for calibration, covering the range of 10-80 μg/mL, have been prepared from a stock solution containing 100 μg/mL taurine (Sigma-Aldrich; Sintra, Portugal) dissolved in ultrapure water. The gradient of the mobile phases was as follows: 100% of acetonitrile (Frilabo; Porto, Portugal) from 0 to 50 % in 15 min, and constant at 50 % for the subsequent 5 min. The other mobile phase consisted of 1.298 g $NaHPO_4 \cdot H_2O$ and 0.106 g $NaHPO_4 \cdot 2H_2O$ in 1 L ultrapure water at pH 6.0. The elution flow rate was 1 mL/min. Taurine peak appeared at 360 nm, with a retention time of 9.20 min.

Creatine analysis has been performed as described by Smith-Palmer (2002), specifically for fish tissues. Standard solutions for calibration, in the range 10–100 μg/mL, were prepared from a stock solution containing 100 μg/mL creatine (Sigma-Aldrich) dissolved in ultrapure water. Creatine peak has been detected at 210 nm, with a retention time of 2.10 min.

3.2.2.2 Other free amino acids

For the remaining free amino acids, the method reported by Alonso, Alvarez and Zapico (1994) has been selected, using a RP-HPLC, C18 Ultrasphere 5-ODS (25 × 4.6, 5 μm) column (Beckman&Coulter). The external standards used for the calibration (Sigma-

Aldrich) were 12.5 mM tyrosine, and 25 mM aspartic acid, glutamic acid, glutamine, serine, asparagine, glycine, threonine, histidine, arginine, alanine, proline, valine, methionine, cysteine, leucine, phenylalanine, tryptophan, lysine and isoleucine, all dissolved in ultrapure water. Amino acids were detected at 254 nm and retention times were 3.41 min for aspartic acid, 3.62 min for glutamic acid, 6.91 min for serine, 7.05 min for asparagine, 7.19 min for alanine, 8.10 min for glutamine, 8.93 min for glycine, 10.42 min for histidine, 13.11 min for cysteine, 13.35 min for threonine, 14.38 min for arginine, 18.29 min for proline, 21.82 min for tyrosine, 24.60 min for valine, 25.82 min for methionine, 30.01 min for isoleucine, 30.82 min for leucine, 33.31 min for phenylalanine, 33.49 min for tryptophan and 36.94 min for lysine.

3.2.2.3 Actin

This protein has been analysed using an ion exchange DAEA-5-PW column (75×7.5 mm) (Bio-Rad; Lisbon, Portugal). A stock solution was prepared by dissolving pure actin (Sigma-Aldrich) in a mixture of 90:10 (v/v) ultrapure water and acetone (Frilabo). In order to enhance solubility of the actin standard (Xiong, 1997), 5g/100ml of NaCl (Merck; Hamburg, Germany) was also added to the mixture. Standard solutions used for calibration were in the range of 5–50 μg/mL. Samples were eluted with a mobile phase containing 20 mM of triethanolamine (Merck) in 1 L ultrapure water, with a flow rate of 0.250 mL/min. The actin peak was detected at 280 nm with a retention time of 2.90 min.

3.2.2.4 Biogenic amines

Biogenic amines content has been assessed in a sample of wastewater collected at the end of the salting process with the method reported by Moret and Conte (1996) for liquid samples, using a Waters Symmetry RP-HPLC C18 (25 × 4.6, 5 μm) column. Derivatising agent dansyl chloride, internal standard 1,7-diamminoeptane and biogenic amines standards — tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, spermine — were purchased from Sigma-Aldrich. Biogenic amines were detected at 254 nm with retention times of 10.12 min for tryptamine, 11.35 min 2-phenylethylamine, 11.87 min for putrescine, 12.28 min for cadaverine, 12.87 for

histamine, 15.32 for internal standard, 18.11 min for tyramine, 19.21 min for spermidine and 21.31 min for spermine.

3.2.3 Assessment of total nitrogen and nitrogen fractions

The nitrogen content has been quantified using a Kjeltec system 1002 distilling unit (Tecator; Höganäs, Sweden). Total nitrogen was quantified by micro-Kjeldahl (ISO R-973:1978) and expressed as water-soluble nitrogen (WSN). Trichloroacetic acid-soluble nitrogen (TCASN), accounting for small peptides (2-20 residues) and free amino acids, and phosphotungstic acid-soluble nitrogen (PTASN), accounting for free amino acids, were determined with the following procedure. The TCASN fraction was quantified by adding 5 ml of an aqueous solution of 48 %(w/v) TCA (Merck) to 15 mL of the water-soluble extract; the mixture was allowed to stand for 30 min at room temperature, and then was filtered through a Whatman No. 42 filter paper (Millipore-Interface). The PTASN fraction was determined by adding 7 ml of 3.95 M sulfuric acid (Pronalab; Lisbon; Portugal) and 3 mL of 33.3 %(w/v) PTA (Merck) to 10 mL of water-soluble extract. The mixture was allowed to stand overnight at 4 °C, and subsequently was filtered through a Whatman No. 42 filter paper. Aliquots of 5 mL from both filtered TCA and PTA–soluble extracts were then analysed by the micro-Kjeldahl. All determinations have been made in triplicate.

3.2.4 Assessment of proteins

The concentration of water–soluble proteins was determined by the Biuret method (Copeland, 1994; Chance & Redfearn, 1961), using an UV mini 1240 spectrophotometer (Shimatzu; Carnaxide, Portugal). The Biuret color yield was standardised against an aqueous solution of analytical bovine serum albumin (Sigma-Aldrich) covering the range 1-10 mg/ml. A volume of sample of 0.5 mL was used for the aliquots. All determinations have been performed in triplicate.

3.2.5 Electrophoresis of proteins

Prior to electrophoresis (SDS-Page), a sample of wastewater collected at the end of the salting process was heated at 80 ºC for 15 min, and then mixed with an equal volume of

sample buffer. This buffer was prepared by adding 6 g of glycerol (Merck) and 1.6 g of sodium dodecyl sulphate (Sigma-Aldrich) to 4 ml of Tris-SDS solution (Sigma-Aldrih). SDS-PAGE was performed in a Protean II, XL Cell (Bio-Rad), using 4% stacking gel, 10% spacer gel and 16.5% running gel - as described in detail by Schägger and von Jagow (1987).

The protein bands were stained with 0.25% Coomassie brilliant blue solution (Merck) for 1 h and then washed with a destaining solution of 2.5:1 (v/v) acetic acid and methanol (Frilabo), until protein bands became clearly visible in a colourless gel matrix. Band analysis has been performed using a GS-700 Densitometer equipped with Molecular Analyst v.1.0 Software (Bio-Rad).

3.2.6 Total lipid determination

Quantitative determination of total fat content was performed by solvent extraction. Total fat is the sum of free-fats, which can be quantified by direct extraction, and bound-fats, which must be hydrolysed by acid digestion prior to quantification. The Weilbull-Stoldt method (AOAC, 1990c) has been implemented for the hydrolysis of bound-lipid by using the Hydrolysis Unit B-411 equipment (Rotoquimica; Maia, Portugal) and the extract has been subjected to the Soxhlet procedure to determine lipid content (AOAC, 1990b; Campbell-Platt, 2009) by using the Extraction Unit E-812 SOX (BÜCHI). According to the Weilbull-Stoldt method, the amount of wastewater used for lipid determination was 50 g, for an expected lipid content of 1–5 % (w/w). Solvent used for lipid extraction was diethyl ether (Frilabo; Porto, Portugal). Soxhlet procedure has taken ca. 2 h.

3.2.7 Assessment of salt and dry matter

Total salt content, as NaCl, has been determined by titration according to the Mohr method (AOAC, 1990d). Dry matter was quantified as described in EN 12880:2000. All determinations have been made in triplicate.

3.2.8 Experimental data modelling and statistical analysis

Modelling of the experimental data, pertaining to the ten dominant free amino acids, to total proteins and to actin, and analysis of variance (one-way ANOVA) have been performed with the software STATISTICA v.9.0. The ANOVA test has been carried out to asses the variation with time of the all parameters determined.

Kinetic parameters in reaction rate expressions have been determined by *non-linear regression* via the method of "*non-linear least square"*. Such a method is based on the "*linear"* (also called "*ordinary*") equivalent and successive iterations for parameters refining. Least square method minimizes the sum of squared vertical distances (also called "*residual sum of squares*") between experimental and the predicted data. A confidence level of 95% has been set for regression.

Adequacy of kinetic models proposed has been evaluated by three *goodness-of-fit* criteria, namely coefficient of determination (R^2), coefficient of determination adjusted to the numbers of predictors and to the degrees of freedom (adjusted R^2), and standard error of the regression (SER) (Shumaker, 1992).

3.3 Results and Discussion

3.3.1 Composition of codfish salting processing wastewater along time

After 6 days of salting of 800 kg of codfish, 155 L of water have been drained away. Almost half of the volume (ca. 75 L) has been released from codfish tissue within the first 9 h. According to Xiong (1997) water release occurs due to myofibrillar protein modifications arising from changes in ionic strength, polarity and capillary forces associated with the high salt concentration. Therefore, the water confined to thick filaments (myosin) and thin filaments (actin), has been prone to release, and eventually accounted for a fish weight loss of about 22 %(w/w) throughout the whole process.

Composition of wastewater generated along the codfish salting process is shown in Table 3.1, in terms of dry matter and salt content, total nitrogen (WSN), non-protein nitrogen (TCASN and PTASN), ratios TCASN/WSN and PTASN/WSN, and total lipids.

Table 3.1 Wastewater composition (mean ± standard deviation) throughout the salting process of codfish

Entity concentration	Processing time (h)					
	9	33	57	81	105	129
Water released (L)	75	102	123	135	147	155
Dry matter (%)	23.27 ± 0.24^{a}	24.21 ± 0.10^{b}	24.57 ± 0.51^{bc}	25.26 ± 0.09^{c}	25.50 ± 0.08^{d}	25.63 ± 0.11^{d}
Salt (g/L)	245.31 ± 0.22^{a}	246.55 ± 0.17^{a}	246.74 ± 0.28^{a}	245.43 ± 0.32^{a}	245.86 ± 0.18^{a}	245.61 ± 0.25^{a}
WSN (g/L)	1.19 ± 0.13^{a}	1.79 ± 0.12^{b}	2.40 ± 0.23^{cd}	2.79 ± 0.21^{de}	2.92 ± 0.12^{e}	3.17 ± 0.13^{e}
TCASN (g/L)	0.79 ± 0.19^{a}	0.88 ± 0.06^{a}	1.35 ± 0.17^{b}	1.44 ± 0.23^{b}	1.50 ± 0.20^{b}	1.62 ± 0.20^{b}
PTASN (g/L)	0.45 ± 0.08^{a}	0.46 ± 0.11^{a}	0.63 ± 0.10^{ab}	0.79 ± 0.91^{b}	1.05 ± 0.11^{c}	1.16 ± 0.21^{c}
TCASN/WSN (%)	67.21 ± 15.69^{a}	49.30 ± 4.05^{bc}	56.51 ± 8.23^{ad}	51.91 ± 8.04^{cd}	51.52 ± 6.16^{d}	51.25 ± 5.79^{d}
PTASN/WSN (%)	37.93 ± 0.06^{a}	25.54 ± 0.03^{b}	26.36 ± 0.04^{b}	28.39 ± 0.06^{b}	36.14 ± 0.04^{c}	36.55 ± 0.06^{c}
Water released/WSN (L^2/g)	63.80 ± 7.38^{a}	57.10 ± 4.08^{b}	51.56 ± 5.08^{b}	48.64 ± 3.72^{b}	50.38 ± 2.09^{b}	48.96 ± 2.00^{b}
Water released/TCASN (L^2/g)	98.85 ± 24.54^{a}	116.13 ± 7.36^{a}	92.16 ± 11.12^{a}	95.36 ± 15.36^{a}	98.94 ± 13.26^{a}	96.59 ± 12.63^{a}
Water released/PTASN (L^2/g)	171.55 ± 29.02^{a}	226.37 ± 31.94^{a}	199.21 ± 33.73^{a}	172.86 ± 19.85^{a}	140.44 ± 14.51^{b}	137.21 ± 26.60^{b}
Total Lipids (g/L)	$< 0.1^{a}$	$< 0.1^{a}$	$< 0.1^{a}$	$< 0.1^{a}$	$< 0.1^{a}$	$< 0.1^{a}$

Mean values in the same line that are not followed by the same superscript letter are significantly different ($p \leq 0.05$)

Total (WSN) and fractional (TCASN and PTASN) nitrogen evolution trends are displayed in Figure 3.1; the ratios water released/WSN, water released/TCASN and water released/PTASN along processing time are plotted in Figure 3.2, and progression of ratios TCASN/WSN and PTASN/WSN is shown in Figure 3.3.

By the end of the process, the WSN concentration was 3.1 g/L, which is equivalent to 18.1 g/L of total protein, assuming a nitrogen-to-protein Kjeldahl conversion factor of 5.82 (Sosulski & Imafidon, 1990). As expected, the non-protein nitrogen TCASN concentration has been higher than its PTASN counterpart, and both fractions were lower than the WSN; recall that TCASN accounts for mainly small peptides (2–20 residues) and free amino acids, whereas PTASN corresponds only to free amino acids (Kuchroo & Fox, 1982).

Total lipids content was considered not relevant, < 0.1 g/L.

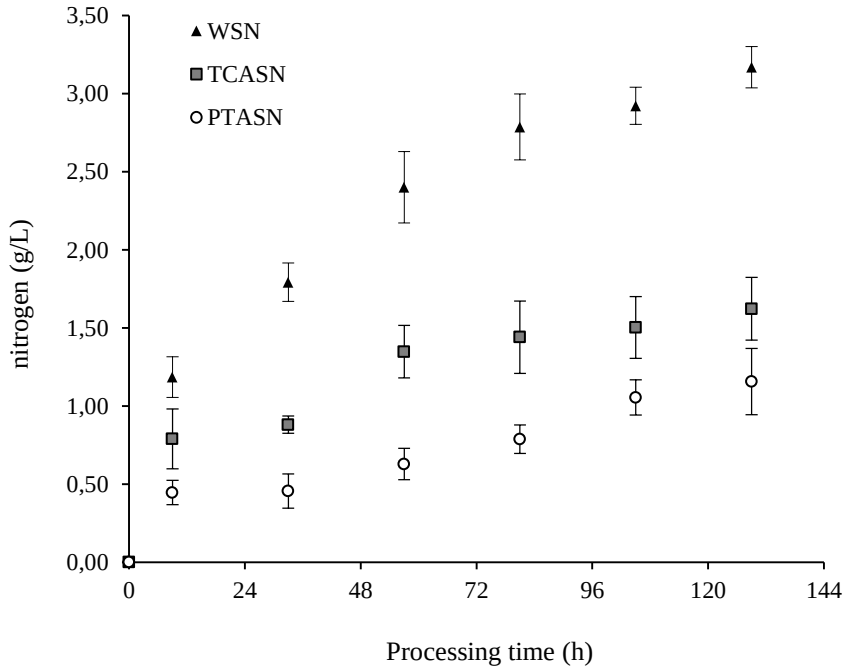


Figure 3.1 Nitrogen (mean ± standard deviation) evolution trend throughout the codfish salting process

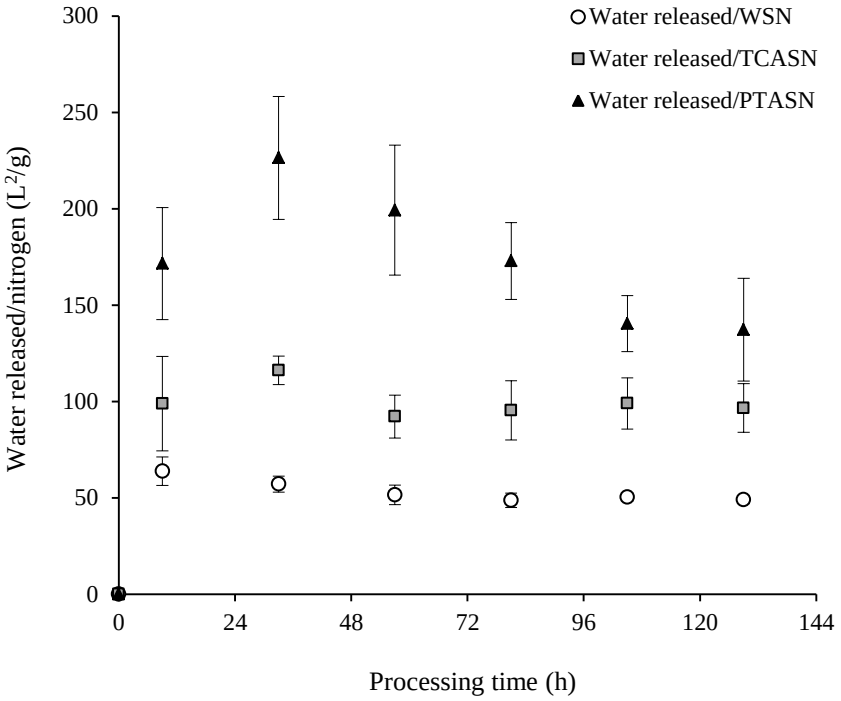


Figure 3.2 Ratios of volume of water released to total and fractional nitrogen (mean ± standard deviation) throughout the codfish salting process

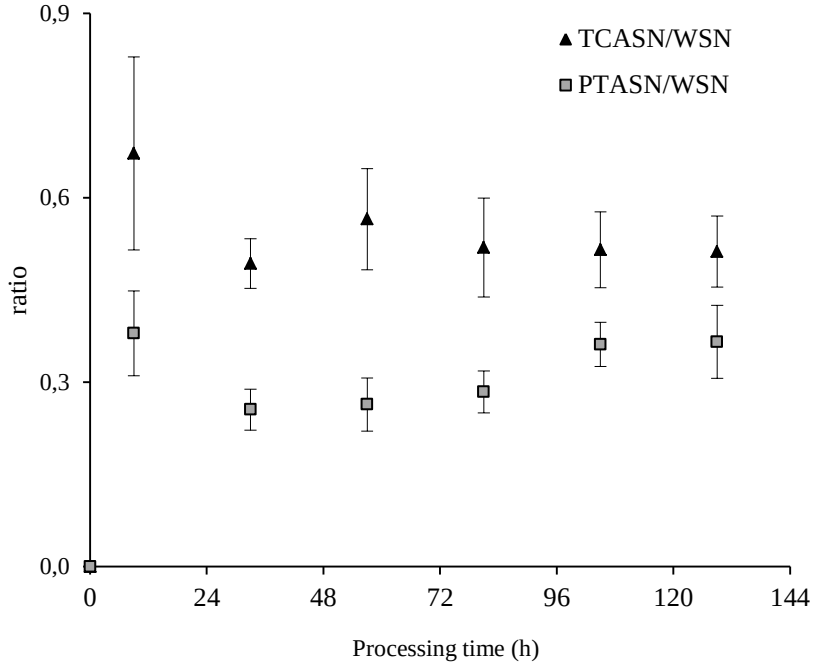


Figure 3.3 Evolution trend of ratios TCASN/WSN and PTASN/WSN (mean ± standard deviation) throughout the codfish salting process

Analysis of ratios PTASN/WSN and TCASN/WSN (see Table 3.1) indicates that about 54.62 % (with a range 49.30–67.21 %) of total nitrogen corresponded to small peptides and free amino acids, so the remaining nitrogen, 45.38 % (with a range 32.79–50.70 %), represented soluble proteins. ANOVA results pertaining to the ratio TCASN/WSN have revealed no significant changes throughout the salting treatment, after the first 9 h (Figure 3.3). Conversely, the ratio PTASN/WNS has shown significant changes ($p \leq 0.05$) with time (Figure 3.3), mainly because of the free amino acids (except for taurine) released from muscle proteins and peptides, due to hydrolysis throughout the heavy salting (Martín et al., 1998). During the process, the content of WSN increased because of the water released and the proportion of water released to WSN has been constant throughout salting ($p>0.05$) after the first 9 h, with a average value of 53.41 L^2/g and a range 63.80-48.96 L^2/g (see Table 3.1 and Figure 3.2). The proportion of water release to TCASN has been also constant with time, with a mean value of 99.67 L^2/g and a range 92.16-116.13 L^2/g, as the changes were found to be not significant ($p>0.05$) (Figure 3.2). Conversely, the proportion of water released to PTASN decreased from an initial 226 down to a final 137.21 L^2/g, after first 9 h, as proteins and peptides became hydrolysed (see also Table 3.1 and Figure 3.2).

Hence, it can be hypothesized that proteins and peptides have been released from codfish muscle promptly from the starting period of processing, whereas amino acids release —and probably the decarboxylation of some of them into biogenic amines — has taken place gradually as a consequence of proteolysis; this has been apparently not the case of taurine, since it is naturally in its free form in muscle tissue.

Wastewater composition in terms of total protein, actin and free amino acids is reported in Table 3.2. Among the amino acids lost by codfish, creatine accounted for the higher amount, along with aspartic and glutamic acids, arginine, glycine, lysine, methionine, phenylalanine, taurine and tryptophan. Throughout the salting process, creatine has been apparently expelled from muscle tissue via leaching, as claimed elsewhere by Larsen et al. (2007), while aspartic acid and glutamic acid, arginine, lysine, methionine, phenylalanine and tryptophan have been liberated from myosin residues as a consequence of proteolysis, as reported by Venugopal (2009) and Bonet et al. (1988).

Table 3.2 Concentration of total muscle protein, actin and free amino acids throughout the codfish salting process (mean ± standard deviation)

Entity (g/L)	Processing time (h)					
	9	33	57	81	105	129
Total muscle proteins	3.015±0.002^{a}	3.104±0.016^{b}	3.235±0.036^{c}	3.344±0.067^{cd}	3.517±0.089^{d}	3.668±0.201^{e}
Actin	0.068±0.003^{a}	0.070±0.002^{a}	0.072±0.001^{a}	0.073±0.003^{ab}	0.075±0.002^{b}	0.079±0.002^{c}
Aspartic acid	0.354±0.003^{a}	0.499±0.001^{b}	0.496±0.003^{b}	0.656±0.005^{c}	0.674±0.001^{d}	0.765±0.004^{e}
Glutamic acid	0.867±0.006^{a}	0.912±0.001^{b}	0.869±0.001^{a}	1.045±0.002^{c}	1.097±0.004^{d}	1.171±0.021^{e}
Alanine	0.008±0.003^{a}	0.015±0.002^{b}	0.016±0.004^{b}	0.019±0.003^{b}	0.021±0.004^{b}	0.023±0.007^{b}
Arginine	0.043±0.013^{a}	0.083±0.012^{b}	0.109±0.023^{b}	0.105±0.018^{b}	0.107±0.014^{b}	0.108±0.073^{b}
Creatine	1.598±0.101^{a}	1.632±0.048^{a}	2.301±0.204^{b}	2.449±0.194^{b}	2.576±0.222^{b}	2.697±0.205^{b}
Glutamine	0.006±0.001^{a}	0.007±0.002^{a}	0.009±0.002^{a}	0.009±0.001^{a}	0.010±0.003^{a}	0.009±0.002^{a}
Glycine	0.100±0.012^{a}	0.106±0.025^{a}	0.103±0.036^{a}	0.092±0.065^{a}	0.090±0.074^{a}	0.097±0.080^{a}
Histidine	0.004±0.001^{a}	0.004±0.001^{a}	0.007±0.001^{b}	0.007±0.003^{b}	0.007±0.002^{b}	0.008±0.003^{b}
Lysine	0.104±0.002^{a}	0.269±0.002^{b}	0.408±0.004^{c}	0.437±0.003^{d}	0.472±0.003^{e}	0.486±0.005^{f}
Methionine	0.057±0.002^{a}	0.134±0.003^{b}	0.189±0.003^{c}	0.206±0.004^{d}	0.218±0.003^{e}	0.230±0.004^{f}
Phenylalanine	0.097±0.002^{a}	0.233±0.003^{b}	0.300±0.001^{c}	0.331±0.004^{d}	0.357±0.002^{e}	0.370±0.002^{f}
Proline	0.005±0.001^{a}	0.009±0.003^{b}	0.005±0.002^{a}	0.006±0.001^{a}	0.006±0.001^{a}	0.006±0.002^{a}
Threonine	0.019±0.002^{a}	0.029±0.001^{b}	0.035±0.002^{b}	0.032±0.003^{b}	0.034±0.002^{b}	0.037±0.001^{c}
Tryptophan	0.090±0.006^{a}	0.161±0.010^{b}	0.154±0.013^{b}	0.181±0.007^{c}	0.201±0.005^{d}	0.184±0.006^{c}
Taurine	0.123±0.063^{a}	0.180±0.025^{b}	0.206±0.085^{b}	0.213±0.084^{b}	0.224±0.093^{b}	0.228±0.076^{b}

Mean values in the same line that are not followed by the same superscript letter are significantly different ($p \leq 0.05$)

Lambert et al. (2001) additionally demonstrated that taurine was drained away from the aqueous phase of codfish muscle cells, where that free amino acid naturally exists, along with cell water. Free glycine has been likely released from proteins of the connective tissue (which are insoluble in water) as a consequence of denaturation due to high salt concentration, as shown by Karatzas and Zarcadas (1988). Other amino acids released in minor amount (≤0.05 g/L) were alanine, glutamine, histidine, proline and threonine (Table 3.2). Based on the characterization of the unprocessed codfish flesh, it can be concluded that, by the end of the salting process, it has lost ca. 0.45 % (w/w) of muscle proteins and mainly actin, 1.24 % (w/w) of aspartic acid, 1.4 % (w/w) of glutamic acid, 2 % (w/w) of arginine, 11 % (w/w) of creatine, 0.2 % (w/w) of glycine

0.5 % (w/w) of lysine, 0.4 % (w/w) of methionine, 0.3 % (w/w) of phenylalanine, 3.4 % (w/w) of taurine, 3.02 % (w/w) of tryptophan (Beach et al., 1943).

Throughout the salting process, the concentration of total muscle proteins and actin increased with time ($p \leq 0.05$), as well as the ratios of water released to total muscle proteins and water released/actin ($p < 0.05$) (Table 3.2). However the proportion of actin to total muscle proteins released remained approximately constant throughout the whole process, at ca. 2.2 % (w/w). These results corroborate the findings of Martinez-Alvarez & Gómen-Guillén, 2006, who have reported that actin is, in general, more prone to release than other muscle proteins (viz. myosin, tropomyosin and troponin): actin accounted indeed for ca. 32.85 % (mol/mol) of the total proteins released from codfish muscle, which compares with ca. 6.67% (mol/mol) in the unprocessed codfish postmortem actomyosin. Recall that in postmortem muscle, myosin and actin exist as an actomyosin complex, i.e. long filaments of actin and myosin, bound by tropomyosin and troponin (Venugopal, 2009). Myosin heavy chain has been the most vulnerable to denaturation as induced by the heavy salting (Figure 3.4); its constitutive helix underwent denaturation to yield two light chains, represented by the 205 kDa band. Actin appeared to be more resistant and showed a clearly visible band at ca. 45 kDa. Bands between 205 and 97.4 kDa accounted for heavy meromyosin (and its subunits) and light meromyosin, all originated from myosin fragmentation. The band appearing at 78 kDa may be either tropomyosin or a remaining of light meromyosin, as reported elsewhere (Thorarinsdottir et al., 2002). The peptide of ca. 8.2 kDa, could also be a product of myosin degradation as also found by Ball, Krus and Alizadeh (1987).

Among biogenic amines detected in the wastewater at the end of the salting process, cadaverine has been the most abundant, with a concentration of 51 mg/kg, followed by putrescine, 26 mg/kg, histamine, 15 mg/kg, 2-phenylethylamine, 13 mg/kg, and tryptamine, 1 mg/kg. The presence of biogenic amines in wastewater could not be related to lysis of liberated free amino acids only, but also to the release from codfish muscle where biogenic amines could be present even before the salting process. This conclusion can be supported by literature reporting that, in general, cadaverine is frequently present in fish in higher amount than histamine and putrescine (Taylor & Summer, 1997).

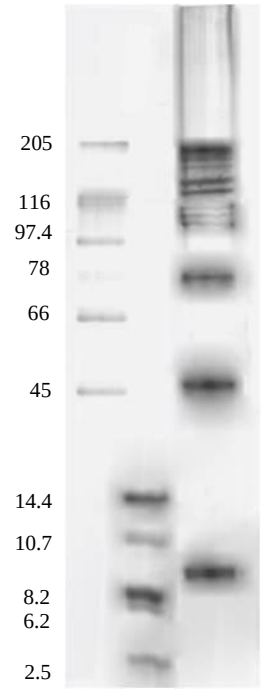


Figure 3.4 SDS-Page profile of codfish salting process wastewater. First lane: high molecular weight standard; second lane: low molecular weight standard; third lane: wastewater sample. Molecular weights are expressed in kDa

Also, the presence of glycine and the high concentration of sodium chloride indicate that there could have been some inhibitory effects upon decarboxylase activity of biogenic amines producing strains (except for halotolerant and halophilic strains) in wastewater (Mah & Hwang, 2009). Histamine and 2-phenylalanine are the more hazardous biogenic amines — together with tyramine — however, their content in wastewater is quite below the toxicity limit fixed at 50 mg/kg for histamine and 30 mg/kg for 2-phenylethylamine (Silva, 2005), whereas putrescine and cadaverine, although not having a direct toxicity effect, can increase the toxic effects of histamine (Al Bulushi et al., 2009).

Wastewater, total muscle protein, actin and free amino acids have been found released from codfish tissues following different kinetics. Experimental data have been successfully fitted with kinetic models, which generally describe a large number of biochemical reactions and physiological interaction occurring in living systems (Brouers & Sotolongo-Costa, 2006; Branson, 1952). These kinetic are described in details in the following paragraphs.

3.3.2 Kinetics of release of water from codfish tissues

Experimental data for wastewater have been well fitted by power-law kinetics (Figure 3.4), which models non-ideal reactions, according to the following rate expression:

(**Eq.** 3.1) $$r = \frac{dV}{dt} = k_n \cdot V^n \ , \quad V_{(t=0)} = 0.$$

Integration of Equation 3.1 by separation of variables leads to:

(**Eq.** 3.2) $$V = \sqrt[\frac{1}{1-n}]{(1-n) \cdot k_n \cdot t}$$

where V (expressed in L) is the instantaneous volume of wastewater released from codfish, k_n represents the time-independent kinetic constant (expressed in $h^{-1} \cdot L^{1-n}$), n represents the order of releasing with respect to the wastewater, and t represents time (expressed in h). Values for the kinetic parameters k_n and n were 119756 h^{-1} L^{1-n}, and - 2.53, respectively.

As reported by Shnell & Turner (2004), power-law kinetic model has been demonstrated to be the best approach for describing non-ideal biochemical phenomena occurring in intracellular environment under molecular crowding within cells – as is the case of codfish muscle system. Fitting model curve is reported in Figure 3.5 along with experimental data.

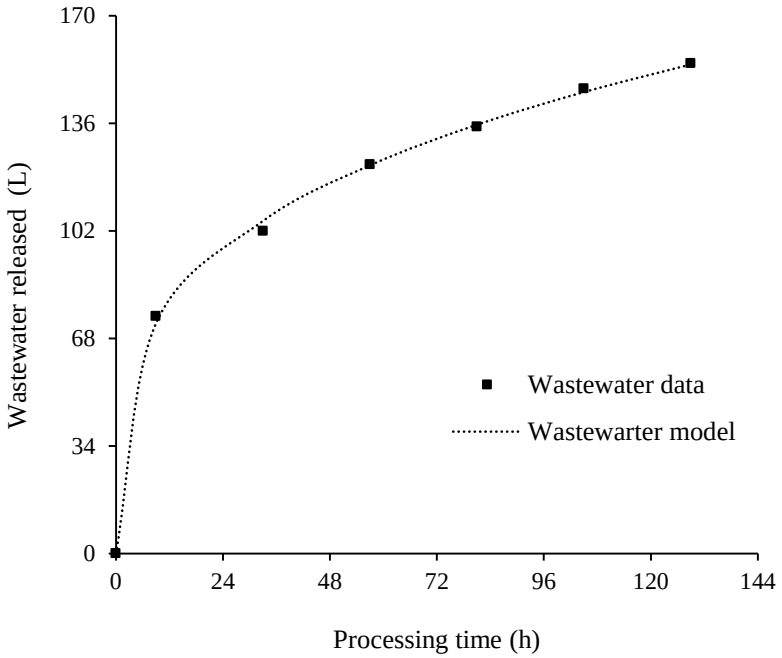


Figure 3.5 Experimental and model dataset for wastewater released according to a power-law kinetics

3.3.3 Kinetics of release of free amino acids from codfish tissues

Free amino acids were found to be released according to two kinds of kinetic models, depending on amino acids themselves. Glycine, taurine and tryptophan have been drained away from codfish muscle according to a pseudo-first order model, also called Lagergren model, with the steady-state approximation. The mass balance to these amino is based on a unimolecular rate according to

(**Eq.** 3.3) $$r = \frac{dC}{dt} = k \cdot (C_{\infty} - C), \quad C_{(t=0)} = 0.$$

Integration of Equation 3.3 by separation of variables leads to

(**Eq.** 3.4) $$C = C_{\infty} \cdot (1 - e^{-k \cdot t})$$

where C, C_{∞} and C_0 (all expressed in $g \cdot L^{-1}$), denote the instantaneous concentration of amino acid in the solution at time t, the concentration of amino acid in the solution at equilibrium, and the concentration of amino acid in the solution at the initial time,

respectively; k is the time-independent first order constant of release (expressed in h^{-1}), and t is the time of salting (expressed in h). Kinetic parameters are reported in Table 3.3 and model results are overlaid to experimental dataset in Figure 3.6.

For aspartic acid, glutamic acid, arginine, creatine, lysine, methionine and phenylalanine, a pseudo-second order kinetic, with steady-state approximation, has been found as the best model for experimental data fitting. Kinetics is expressed as:

(**Eq.** 3.5) $$r = \frac{dC}{dt} = k_2 \cdot (C_\infty - C)^2, \quad C_{(t=0)} = 0.$$

Integration of Equation 3.5 by separation of variables leads to

(**Eq.** 3.6) $$C = \frac{k_2 \cdot t \cdot C_\infty^2}{1 + k_2 \cdot t \cdot C_\infty}$$

Kinetic parameters C_∞, k_1 and k_2, are tabulated in Table 3.3. Model results are overlaid to experimental dataset in Figure 3.7.

In reactions involving proteins, pseudo-first and pseudo-second order kinetics generally describe the hydrolysis of proteins, either due to enzymes or to denaturing agents, like heavy salting. Pseudo-second order kinetic accounts for a non-cooperative (or "*zipper"*) proteolysis, while pseudo-first order kinetics describes a co-operative (or one-by-one) lysis. The faster stage of non-cooperative hydrolysis leads to the formation of high molecular weight protein fragments along with a number of short peptides and free amino acids, while the cooperative process leads to the degradation of proteins in TCA-soluble peptides and free amino acids as well (Vaintraub, 1998). According with experimental data finding, it can be concluded that free amino acids have been mainly released by a non-cooperative hydrolysis, exception than for glycine, tryptophan and taurine.

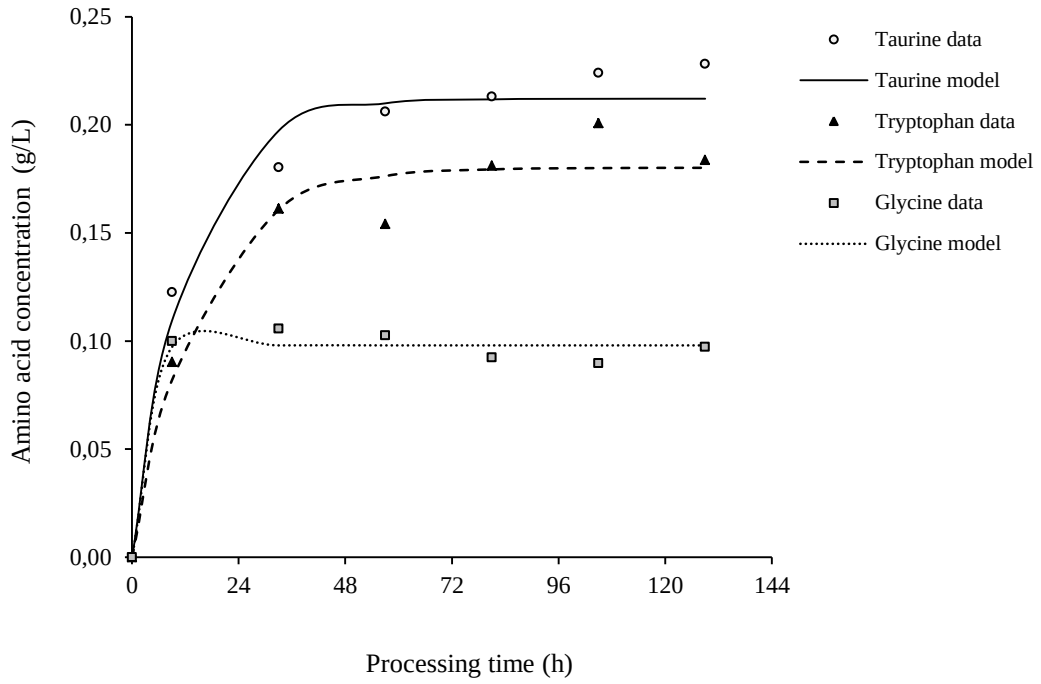


Figure 3.6 Experimental and model dataset for free amino acids released according to a pseudo-first order kinetics

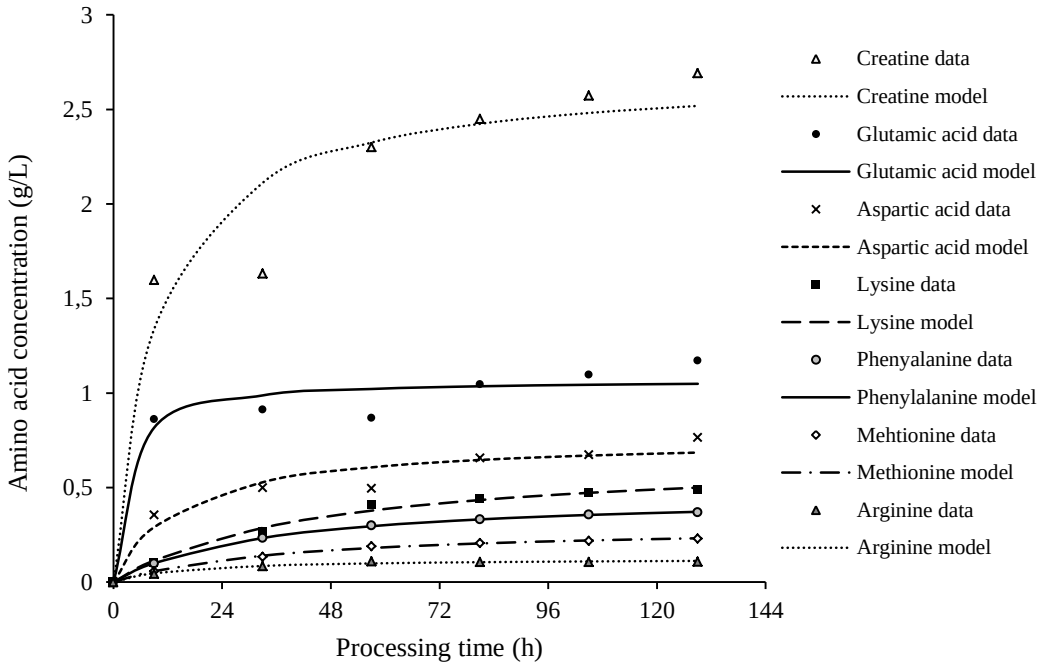


Figure 3.7 Experimental and model dataset for free amino acids released according to a pseudo-second order kinetics

3.3.4 Kinetics of release of muscle protein from codfish tissues

Total muscle proteins were found to be released according to pseudo-second order kinetics (Eq. 3.5) while actin was liberated with a first-order kinetic (Eq. 3.3). Kinetic parameters are reported in Table 3.3. Model curve is overlaid to experimental data in Figure 3.8.

The stage of protein aggregation – with subsequent protein precipitation and releasing – is a bi(or multi)-molecular reaction (Kurganov, 2002), which corroborates the finding of a pseudo-second order kinetics for experimental data modelling. Regarding actin, the co-operative proteolysis of actomyosin system can be hypothesised as the mechanism of release, since a pseudo-first order kinetic model has been found to describe experimental data (Vaintraub, 1998).

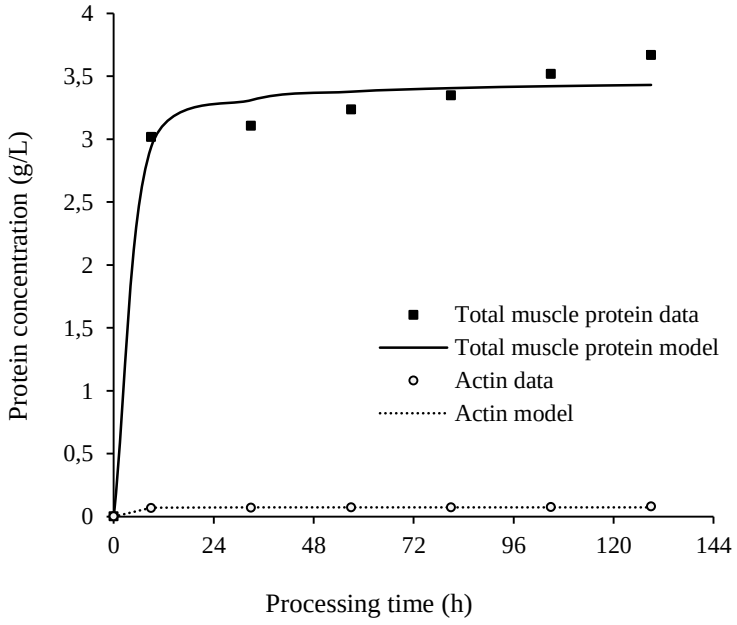


Figure 3.8 Experimental and model dataset for muscle proteins released according to a pseudo-second order kinetics

Table 3.3 Kinetic parameters (C_∞, n, k_1, k_2 and k_n) and statistic parameters (R^2, adjusted R^2 and SER) total muscle protein and actin, and for major free amino acids released (confidence level of 95%)

Entity	Parameter					
	C_∞ ($g \cdot L^{-1}$)	k_1 (h^{-1})	k_2 ($L \cdot h^{-1} \cdot g^{-1}$)	R^2	Adjusted R^2	SER
Total muscle proteins	3.474	–	0.176	0.99	0.98	0.200
Actin	0.0725	0.619	–	0.99	0.98	0.004
Aspartic acid	0.763	–	0.090	0.98	0.97	0.047
Glutamic acid	1.071	–	0.333	0.97	0.93	0.101
Arginine	0.125	–	0.512	0.99	0.99	0.005
Creatine	2.697	–	0.041	0.97	0.94	0.222
Glycine	0.098	0.540	–	0.99	0.97	0.006
Lysine	0.671	–	0.034	1	0.99	0.017
Methionine	0.300	–	0.089	1	1	0.006
Phenylalanine	0.467	–	0.065	1	1	0.002
Tryptophan	0.180	0.067	–	0.99	0.98	0.010
Taurine	0.212	0.081	–	0.99	0.99	0.008

3.4 Conclusion

Wastewater generated by the codfish salting process possesses a sufficiently high nutritional value to justify its upgrade: ca. 1000 g of free amino acids and ca. 570 g of muscle proteins have been found in 155 L of wastewater after the 6 days process, with a concentration of biogenic amines of 106 mg/kg (or 124 mg/L).

PART III

EXTRACTION OF FREE AMINO ACIDS AND PROTEINS FROM CODFISH SALTING PROCESSING WASTEWATER BY SORPTION

Abstract

Sorption is a separation process in which certain components of a fluid phase are selectively transferred to the surface of a solid by a preferential partioning, and are successively eluted by the same mechanism. Technological, environmental, and biological importance of sorption can hardly be overestimated. The impact of such phenomena on our everyday lives is evident in many areas like food science, agriculture, cosmetics, pharmaceuticals, cleaning, lubrication, surface protection, paints and inks. Each one of these applications would be difficult, if not impossible, in the absence of solid–liquid interface phenomena.

The capability of an adsorbent to retain organic molecules is primarily determined by its physicochemical characteristics and porous structure, and secondly by the environmental conditions at which sorption is carried out. A slight change in one of these factors can result in a significant change in the adsorption characteristics of the system.

Mesoporous materials, such as the *Amberlite XAD16* resin used in this research, have been identified as promising adsorbents for biochemical molecules such as amino acids, proteins and peptides, assured by the large pore volume, the high surface area and the ease of regeneration when compared with other commercial resins.

When considering an adsorption process two aspects must be addressed: the study of equilibrium of adsorption by which it is possible to determine the affinity of a specific compound towards a resin, and the kinetics of the adsorption process by which it is possible to indentify the mechanism of adsorption and also optimizing the recovery of target compounds.

Adsorption of free amino acids and proteins contained in the codfish salting processing wastewater has been carried out in the same stage, while desorption has been accomplished selectively. Maximum recovery for amino acids and proteins (100 % of hydrophobic amino acids and 100% of proteins), has been obtained by two adsorptions and desorption steps.

CHAPTER 4

EQUILIBRIUM AND THERMODYNAMICS OF ADSORPTION OF SELECTED AMINO ACIDS UPON *AMBERLITE XAD16* RESIN

4.1 Introduction

Adsorption is a separation process in which certain components of a fluid phase are selectively transferred to the surface of a solid by preferential partitioning (Keller et al., 1987). Adsorption is always followed by the inverse operation called "*desorption*" whenever the objective of a separation is the recovery of a solute carried out by a fluid medium. In that case, the term "*sorption*" in generally used since encompasses both processes. Over the past thirty years adsorption separation technology has been developed from a relatively minor niche process to a major unit operation. Dramatic increase in adsorption research has led to important findings ranging from new microporous adsorbent materials to new theoretical approaches yielding improved understanding of adsorption and diffusion in porous materials. Based on the observation of many natural physics, biological and chemical systems, the early applications of bone char for decolorisation of sugar solutions back in the 19^{th} century, have evolved to the present adsorption unit operation, useful tool for purification of fluid streams, bulk separations and recovery of solutes (Sirasankar, 2008).

Adsorption can occur by various mechanisms such as electrostatic attraction, covalent bonding, hydrogen bonding or nonpolar interactions. In general, it is a complex process since the nature and extent depends on properties of solid, solution ionic strength, fluid phase composition, nature of solvent species, pH and temperature of the system (Shrotri, 1998). Adsorption is a surface-related phenomenon so that surface characteristics and pore structure of adsorbent are key properties in determining adsorption success (Sirasankar, 2008). During adsorption, a film formed by the compounds attracted and retained, called "*adsorbate*", is created on the outer and inner surface of the solid, called "*adsorbent*", as a consequence of the solid surface energy. Most important surface features are surface specific area and surface functionality (i.e. polarity, charge and type of functional group) from which depend the adsorbent selectivity. Pore structure and dimension are important since they control diffusion of adsorbate through adsorbent body, hence controlling the adsorption rate (Suzuki, 1990).

When efficiency, selectivity and costs are considered, the sorption separation technique can be considered the most promising method for extraction of amino acids and proteins from aqueous solutions. Several studies have been reported in literature for amino acids and proteins adsorption upon various materials including activated carbon, silica, ion-

exchange, alumina and polymeric resins. Of these, polymeric sorbents are the more attractive because of the ease of regeneration characteristic, so that have been extensively used for the recovery of compounds from diluted liquid solutions and also for removal of organic contaminants from diluted aqueous and air streams (Shim et al., 2004).

In this research study *Amberlite XAD16* resins has been selected among all polymeric adsorbents. *Amberlite XADs* are commercial macroreticular synthetic polymeric adsorbents, based on styrene-divinylbenzene copolymer, which can have a variety of surface areas and surface polarities, and diversified average pore-size distributions. Synthesis of polymeric adsorbents has been driven by the need to overcome some restrictions associated with the use of ion-exchange resins, typically employed for the recovery of same classes of compounds of *Amberlite XAD*. Ion-exchange resins show in fact worse diffusion kinetics and hydraulic properties, and lower chemical stability when compared with *Amberlite XAD* analogues. For both ion-exchange and *Amberlite XAD* resins, particle size of spherical beads is approximately 0.5 mm; however each 0.5 mm bead of *Amberlite XAD* consists of many small microspheres whose diameter is as small as 10^{-4} mm, a feature which allows to speed up the adsorbate diffusion through each beads, and also to improve hydraulic properties when column equipments are employed (Kennedy, 1973). Furthermore, because of the highly cross-linked nature, *Amberlite XAD* resins have excellent physics durability (Rhom & Haas, 2010). Regeneration of ion-exchange resins is more costly since more energy consuming and leading to the undesirable use of acids and bases, while less expensive organic solvents and/or water are required for regenerating synthetic polymeric adsorbents (Grzegorczyk & Carta, 1996). The last mentioned feature can be considered the most important characteristic of *Amberlite XAD* resins since for an economical point of view, the success of a sorption system usually depends on the regeneration of the sorbent (Yang et al., 2003).

Physicochemical properties of compounds to be extracted are the most important parameter to consider when adsorptive separation must be carried out, and when adsorption is accomplished by polymeric resins, hydrophobic/hydrophilic nature of substances is the most important feature. Hydrophobic/hydrophilic behaviour of a chemical compounds is determined by different factors. A substance easily dissolves in

water and other polar solvents, i.e. is hydrophilic, when contains ionic or polar covalent bonds which can hydrogen bond with solvent molecules. Polar covalent bonds result when electrons are not shared equally between atoms so that molecules results strongly electronegative or electropositive. For instance, compounds which contain O–H, N–H and CO–OH groups are hydrophilic, while compounds which contains C–H group can be considered nonpolar or less polar (Lide, 2004). However, when a molecule contains both a polar and a nonpolar group, the dominant behaviour will depends on the relative size of the specific functional groups: if the ratio of the size of the hydrophilic portion to the hydrophobic one is much larger than the unit value, then hydrophilic nature will prevail and *vice versa* (Mancera, 1996). Amino acid hydrophobicity/hydrophilicity essentially depends on the nature of the R group, since both amino group (NH_3^+) and carboxylic group ($COOH^-$) are hydrophilic, while the middle –CH– group is hydrophobic (Tandford, 1962). Thereby, the more the number of $–CH_2–$ groups in the R chain, the more the hydrophobicity of the amino acid, which furthermore increases when R contains aromatic ring, due to the presence of conjugated π electrons (Doulia et al., 2001; Monera et al. 1995; Sereda et al., 1994). Amino acids glycine and taurine – where R group is just a hydrogen atom – have pure amphiphilic behaviour, which means they are hydrophilic and hydrophobic in an equal manner (Tandford, 1962).

Based on hydrophobicity index value, free amino acids found in the codfish salting process wastewater can be ordered as follow: tryptophan > phenylalanine > methionine > glycine > taurine > lysine > arginine > creatine > glutamic acid > aspartic acid. Taurine and glycine are neutral and amphiphilic acids and taurine is most soluble than glycine (Table 4.2), however the characteristics of sulphonic acid group SO_2OH (Yang et al., 2005) strongly contributes to the interaction of taurine with non-polar *Amberlite XAD* resins, as will be discussed in paragraph 4.3. Hydrophobicicy/hydrophilicity of amino acids in water does not vary with the pH except at the isoelectric point when they are in "*zwitterionic*" form (from German "*zwitter*" meaning "*hydrid*") and their solubility is maximum (Tandford, 1962). However, hydrophobicity of amino acids decreases by increasing ionic strength (Ni & Yalkowsky, 2003) and temperature (Jeliǹska-Kazimierczuk & Szydlowski, 1996). Regarding muscle proteins, it is well known that these are hydrophobic in the native state, and this feature largely contributes to their stability, structural conformation and biological functions. Indeed, hydrophobic

nature is responsible of their functionality such as gelation ability, fat retention and emulsification (Boyer et al., 1996). Myosin shows higher hydrophobicity than actin since a higher number of hydrophobic amino acids is present in myosin helix surface (Pinaev et al. 1982). Actin filaments have hydrophobic loops which stabilize the filaments structure and interact with myosin head by hydrophobic interactions, but also have hydrophilic NH_2–terminal peptides (Scoville et al., 2006).

In this chapter, equilibrium and thermodynamics study of batch adsorption of selected amino acids is reported. Adsorption data have been collected for four amino acids: tryptophan which is the most hydrophobic, taurine and glycine that are amphiphilic and borderline line (i.e. neutral) amino acids with different acidic groups, and lysine which is the most water soluble although it is not the most hydrophilic. These amino acids have been selected to study the influence of the physical nature (hydrophobicity/hydrophilicity) on the amount adsorbed upon *Amberlite XAD16* and also to mimic real sample solvent, which is water. Adsorption has been optimised for taurine recovery. Effects of environmental conditions such as temperature, pH, ionic strength, agitation speed, initial concentration and adsorbent dose upon adsorption have been studied by one-variable-at-a-time approach for each amino acid, while a fractional factorial design has been further applied for taurine.

Adsorption has been carried out batchwise. A solution-adsorbent system can be characterised by either batch or column technique and is possible to achieve the same results since the physical and/or chemical forces applicable in each case must be the same (Suzuky, 1990; Turton et al., 2009). Discontinuous operations are more advantageous than continuous when some circumstances coexist such as when small quantity of products must be delivered; when product quality and yield must be verified frequently; when high operational flexibility is required; when strictly scheduling and control is necessary; when feedstock availability is limited, for example, seasonally; when significant equipment fouling is present, as generated for example from high inorganic salts content (Turton et al., 2009).

4.2 Materials and Methods

4.2.1 Adsorbent

Adsorption equilibrium and thermodynamics study and thus extraction of free amino acids and muscle proteins present in codfish salting process wastewater has been accomplished by the *Amberlite XAD16 (Amberlite* is a registered trademark of the Rohm & Haas Company, Philadelphia, USA) resin whose chemical structure is reported in Figure 4.1; selection has been based on physicochemical properties of compounds to be adsorbed and on properties of the medium where are dissolved. *Amberlite XAD16* is a non polar, uncharged, non functional and high surface area (at least 800 m^2/g) resin useful for adsorbing neutral and hydrophobic compounds of low and medium molecular weight (100 – 350000 Da) from aqueous (i.e. polar) solutions, and suitable for batch or column operation (Rohm & Haas, 2010). Detailed features of that resin are summarised in Table 4.1.

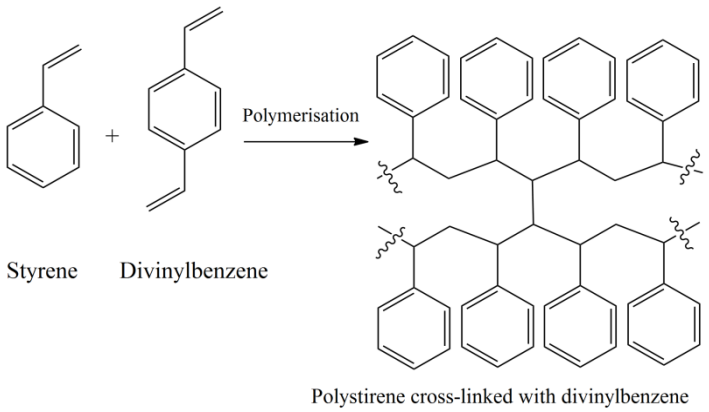


Figure 4.1 Chemical structure of *Amberlite XAD16* polymeric adsorbent

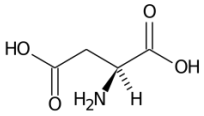
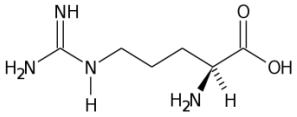
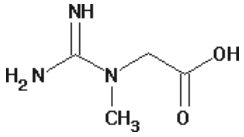
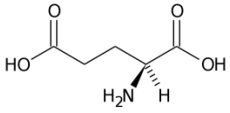
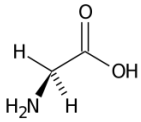
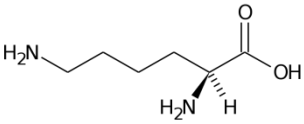
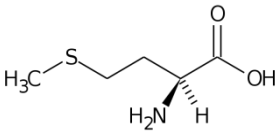
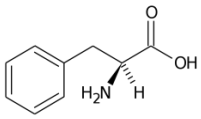
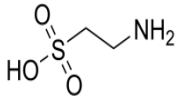
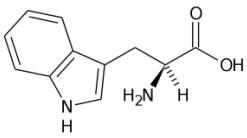
Table 4.1 Properties of *Amberlite XAD16* resin (Rohm & Haas, 2010)

PROPERTIES of *Amberlite XAD16* resin	
Matrix	Polystyrene cross-linked with divinylbenzene. Apolar (hydrophobic) and neutral (uncharged)
Physical form	White translucent beads
Retention mechanism	van der Waals' forces, hydrophobic interactions, hydrogen bonding
Moisture holding capacity	62 to 70 %
Specific gravity	1.015 to 1.025
Harmonic mean size	0.50 – 0.70 mm
Porosity	$\geq$ 0.55 ml/ml
Average pore diameter	100 Å
Surface area	$\geq$ 800 m^2/g
Dipole moment	0.3 D
Operating pH range	1 to 14
Temperature limit	150 ºC (maximum value)

4.2.2 Free amino acids and muscle proteins to be adsorbed

Physicochemical characteristics of free amino acids found in the codfish salting processing wastewater, and crucial for a sorption process, are listed in Table 4.2. Hydrophobicity index values reported have been extrapolated to amino acids which are more hydrophilic than glycine, and are normalised so that to the most hydrophobic amino acid is given a value of 100 relative to glycine which is amphiphilic (0 value), i.e. hydrophilic and hydrophilic in equal manner (Tandford, 1962; Sereda et al. 1994; Monera et al, 1995). Regarding muscle proteins, it is well known that these are hydrophobic in the native state, and this feature largely contributes to their stability, structural conformation and biological functions. Myosin can be splitted into two fragments, namely the water-soluble region and the water-insoluble rod region which is the most important structural part of myosin filament; however, in aqueous solutions of physiological ionic strength and pH, myosin molecules spontaneously assemble into insoluble filaments (Tanabe & Saeki, 2001).

Table 4.2 Physicochemical properties of amino acids

Amino acid	Molecular structure (non-ionised form)	Parameters
Aspartic acid		MW = 133.10, IP = 2.85, $S_{w,\ 25^oC}$ = 4 g/L HI_2 = -18 (neutral), HI_7 = -55 (hydrophilic) R = ACID, negatively charged at neutral pH
Arginine		MW = 174.20, IP = 10.76, $S_{w,\ 25^oC}$ = 15 g/L HI_2 = -26 (neutral) HI_7 = -14 (neutral) R = BASIC, positively charged at neutral pH
Creatine		MW = 131.13, IP = 8.45, $S_{w,\ 25^oC}$ = 15 g/L HI_2 = n.a (neutral) HI_7 = n.a. (hydrophilic) R = NEUTRAL at neutral pH
Glutamic acid		MW = 147.13, IP = 3.22, $S_{w,\ 25^oC}$ = 11 g/L HI_2 = -8 (neutral) HI_7 = -31 (hydrophilic) R = ACID, negatively charged at neutral pH
Glycine		MW = 75.07 , IP = 5.97, $S_{w,\ 25^oC}$ = 25 g/L HI_2 = 0 (neutral) HI_7 = 0 (neutral) R group is Hydrogen; AMPHIPHILIC amino acid
Lysine		MW = 146.19 , IP = 9.74, $S_{w,\ 25^oC}$ = 300 g/L HI_2 = -37 (hydrophilic) HI_7 = -23 (hydrophilic) R = BASIC, positively charged at neutral pH
Methionine		MW = 149.21, IP = 5.74, $S_{w,\ 25^oC}$ = 48 g/L HI_2 = 74 (hydrophobic) HI_7 = 74 (hydrophobic) R = NEUTRAL at neutral pH
Phenylalanine		MW = 165.19, IP = 5.48, $S_{w,\ 25^oC}$ = 27 g/L HI_2 = 84 (hydrophobic) HI_7 = 97 (hydrophobic) R = NEUTRAL at neutral pH
Taurine		MW = 125.15, IP = 5.12, $S_{w,\ 25^oC}$ = 65 g/L HI_2 = n.a. (neutral) HI_7 = n.a. (neutral) R group is Hydrogen; AMPHIPHILIC sulfonic acid
Tryptophan		MW = 204.23 , IP = 5.89, $S_{w,\ 25^oC}$ = 10 g/L HI_2 = 92 (hydrophobic) HI_7 = 100 (hydrophobic) R = NEUTRAL at neutral pH

"n.a": not available

Myosin shows higher hydrophobicity than actin since a higher number of hydrophobic amino acids is present in myosin helix surface (Pinaev et al. 1982). Actin filaments have hydrophobic loops which stabilize the filaments structure and interact with myosin head by hydrophobic interactions, but also have hydrophilic NH_2–terminal peptides (Scoville et al., 2006). Muscle proteins hydrophobicity decrease significantly with an increase in ionic strength above 1.2 M NaCl, with a decrease in temperature below 20 ºC, and with an increase of pH above 7 (Pinaev et al., 1982).

4.2.3 *Amberlite XAD16* pretreatment and recovery of resin washing solvents

4.2.3.1 *Amberlite XAD16* pretreatment

Commercial *Amberlite* resins were rinsed with a sodium chloride and sodium carbonate solution before packaging to control bacteria and mould growth during storage. These preservative agents, along with the residual monomeric compounds still present in the pores, must be removed in order to allow adsorption. In particular, residual monomers must be eliminated from the resin because of the wide band ultraviolet absorbance due to the π-electrons of conjugated bond structure (Cockell & Knowland, 1999), which interferes with the spectrophotometric determination of compounds to be adsorbed. Thereby, *Amberlite XAD16* was washed according to the resin manufacture (Rohm & Haas, 2010). For each adsorption experiments, 1 volume of a fixed amount of resin was placed in an Erlenmeyer flask with 10 volumes of distilled water and rinsed under continuous stirring for 30 minutes at ambient temperature. Then, the resin was filtered with a paper filter, put back in the Erlenmeyer flask with 10 volumes of acetone and rinsed under the same conditions. Finally, the same operation was carried out with distilled water to displace any residual acetone. After washing, an increase of 39% in resin weight was observed due to water retention, and this phenomenon has been taken into account in adsorption calculation.
Resin pre-treatment has been carried out before each adsorption experiment.

4.2.3.2 .Washing solvents recovery

Washing water and acetone have been recovered by evaporation using the lab-scale BÜCHI Rotavapor R-215 (Rotoquimica, Lda.; Maia, Portugal). Operating conditions

for evaporation were selected so as to reduce as much as possible the energy requirements and then the costs, the boiling point of the compound and to ensure complete condensation to avoid vapour emission in the atmosphere. These conditions can be conjugated with the general rule of 20/40/60 ºC (or ΔT=20 ºC), which states that for a temperature of the refrigerant water of 20 ºC, the boiling temperature must be adjusted by the vacuum pressure to 40 ºC, and bath must have a temperature of 60 ºC (Hoegger, 1997). Boiling pressures at 40 ºC have been then determined by the *Antoine* equation (Liley et al., 1997) which gives a value of 578 mbar for acetone and 72 mbar for water. At the optimum operating conditions and optimum rotation speed of 150 rpm, evaporation rates have been ca. 10 g/min for water and 120 g/min for acetone. No significant increases in evaporation rates have been observed by increasing rotation speed over 150 rpm. Dry matter collected has been ca. 0.7 % (w/w) of unwashed resin.
Solvents recovered by evaporation have been used in the succesive washing operations.

4.2.4 Adsorption operating condition

Adsorption equilibrium and thermodynamic study and thus extraction of amino acids and proteins from codfish salting processing wastewater has been carried out batchwise.

4.2.5 Study of equilibrium of adsorption upon *Amberlite XAD16*

4.2.5.1 Theoretical background of equilibrium of physical adsorption

In order to estimate the practical adsorption capacity it is essential to have information on adsorption equilibrium, i.e. to know the number of species adsorbed under a given set of conditions (Suzuki, 1990). In practical operations, maximum capacity of an adsorbent cannot be fully utilized because of mass transfer effects involved in actual fluid-solid contacting processes.
When an adsorbent is in contact with the surrounding fluid of a certain composition, adsorption takes place and after a sufficient long time, the adsorbent and the surrounding fluid reach the equilibrium. Equilibrium relationship between compound concentration in the fluid phase, C_{eq}, and compound concentration in the adsorbent particles, C_{ad}, at a given temperature, T, is known as adsorption isotherm and is expressed by the general function (McCabe et al., 1993):

(**Eq.** 4.1) $C_{ad} = C_{ad}(C_{eq})$ at T

The analysis of the isotherm data by fitting them with different isotherms models is an important step for the design of adsorption systems (Smith, 1987; McCabe, et al., 1993). Adsorption isotherms are described in many mathematical forms, some of which are based on a simplified physical picture of adsorption and desorption, while other are purely empirical and intended to correlate experimental data in simple equations with two or at least three empirical parameters: the more the number of empirical parameters the better the fit between experimental data and the empirical operations. Typical isotherms are shown in Figure 4.2.

Linear isotherm goes through the origin, and the amount of compound adsorbed is proportional to the equilibrium concentration in the fluid phase. Linear isotherm, described by the *Henry*'s equation (4.2), fits situations in which the amount of compound adsorbed is far smaller compared with the adsorbent capacity, a behaviour observed for processes carried out at relatively high temperatures and/or very low solute concentrations (Marczewski & Marczewska, 1988).

(**Eq.** 4.2) $C_{ad} = K_H \cdot C_{eq}$ *Henry's Isotherm*

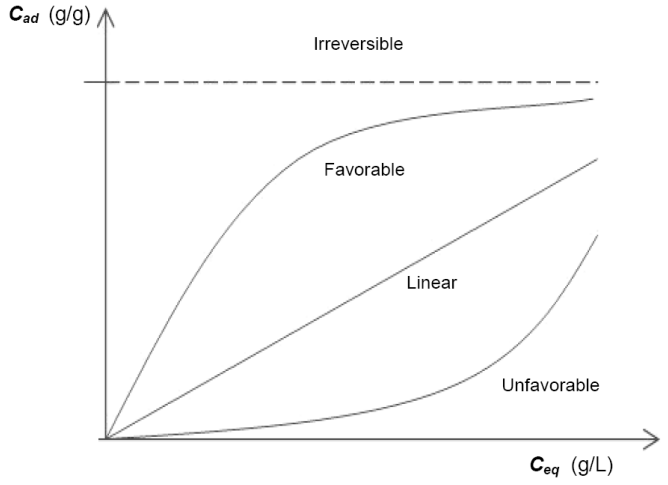


Figure 4.2 Adsorption isotherms at a constant temperature

Parameter K_H in *Henry*'s model represents the adsorption equilibrium constant and can be evaluated from the slope of the graphical form of *Henry*'s equation.

Isotherms that are convex upward are called favourable, because a relatively high adsorbent loading can be obtained for a low concentration of the solute in the fluid (McCabe et al., 1993). Favourable equilibrium relationships have been studied by several authors but *Langmuir* and *Freundlich* models are the most commonly used since they can be applied to a wide range of adsorbate concentrations (Milonjić, 2007). *Langmuir* isotherms, developed in 1916, is a theoretical model described by equation 4.3, based on few assumptions: adsorption takes place on an energetically uniform surface and without any interaction between adsorbed molecules, which implies that adsorption cannot proceed beyond a monolayer and that trans-migration of the adsorbate in the plane of the surface is precluded (Coulson & Richardos, 1991).

(**Eq.** 4.3) $$C_{ad} = C_{adMAX} \frac{K_L \cdot C_{eq}}{1 + K_L \cdot C_{eq}}$$ *Langmuir's Isotherm*

Parameter C_{adMax} in the *Langmuir*'s equation represents the maximum adsorption capacity corresponding to complete monolayer coverage of the adsorbent surface, while K_L is the *Langmuir*'s constant. The values of C_{adMax} and K_L can be evaluated from the slope and the intercept of the linear form of *Langmuir*'s equation:

(**Eq.** 4.4) $$\frac{C_{eq}}{C_{ad}} = \frac{1}{C_{adMAX} \cdot K_L} + \frac{C_{eq}}{C_{adMAX}}$$ *Langmuir's Isotherm linear form*

Langmuir isotherm model is not adequate to perform description of real systems since it is based on not real assumption; however, the relation works fairly well for gases that are weakly adsorbed (McCabe et al., 1993).

For energetically heterogeneous surfaces, the equation of *Freundlich* (Smith, 1987) is the most useful model for describing adsorption isotherms. This model, expressed as in Equation 4.5, has been derived empirically in 1912, describes real systems, which are heterogeneous, and is often the better fit for adsorption from liquids.

(**Eq.** 4.5) $C_{ad} = K_F \cdot C_{eq}^{1/n}$ *Freundlich's Isotherm*

The parameter K_F in Equation 4.5 is the constant related to the overall adsorption capacity and *1/n* is the constant related to surface heterogeneity and is dimensionless. Value of *1/n* range from 0 to 1 and the closer this value to zero, the more heterogeneous the adsorbent surface and the more favourable the adsorption (Zawani et al., 2009). The value of K_F and *1/n* can be evaluated from the linear form of *Freundlich* equation:

(**Eq.** 4.6) $\ln C_{ad} = \ln K_F + \frac{1}{n} \cdot \ln C_{eq}$ *Freundlich's Isotherm linear form*

The limit of a very favourable adsorption isotherm is the case of irreversible adsorption, where the amount of adsorbed compounds in independent of the equilibrium concentration in the fluid. An isotherm that is concave upward is called unfavourable because relatively low adsorbent loadings are obtained and because it leads to quite long mass-transfer zone. Isotherms of this shape are rare and describe the regeneration process, i.e., if the adsorption is favourable, they describe mass transfer from the adsorbent back to the fluid phase. All systems show a decrease in the amount adsorbed with an increase in temperature since physical adsorption is an exothermic process (Figure 4.2) (McCabe, 1993).

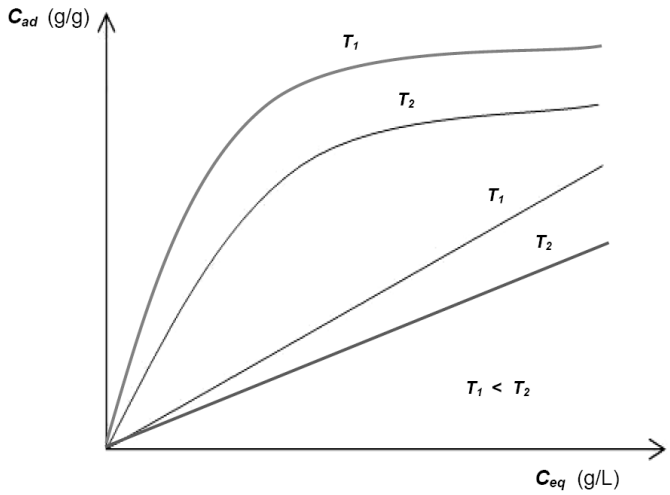


Figure 4.3 Examples of effect of temperature on physical adsorption isotherms

4.2.5.2 Theoretical background of thermodynamics of physical adsorption

Physical adsorption is an exothermic process and heat is always released when adsorption occurs. However, this process may be justified thermodynamically. When any spontaneous process occurs there is a decrease in the *Gibbs* free energy ($\Delta G < 0$); further there must also be a decrease in entropy ($\Delta S < 0$) because, after adsorption, molecules lost at least one degree of freedom (of translation). It follows from the thermodynamic expression

(**Eq.** 4.7) $\Delta G = \Delta H - T\Delta S$

that enthalpy also decreases ($\Delta H < 0$), which means that heat is released (Thomas & Crittendem, 1998). Thermodynamic parameters ΔG, ΔS and ΔH can be determined by equation 4.7 and *Van't Hoff* equation

(**Eq.** 4.8) $$\left(\frac{\partial \ln K_{eq}}{\partial \frac{1}{T}} \right) = -\frac{\Delta H}{R}$$

where R is the gas constant (8.314 J/mol K), T is the absolute temperature (K), and K_{eq} is the dimensionless thermodynamic adsorption equilibrium constant expressed as $K_{eq} = (C_0 - C_{eq})/C_{eq}$, where C_0 is the initial concentration of compound in solution, ($C_0 - C_{eq}$) represents the solid-phase equilibrium compound concentration (g/L), and C_{eq} represents equilibrium compound concentration in solution (g/L). Integration of equation 4.8 by separating variables and combination with equation 4.7 leads to the following expression

(**Eq.** 4.9) $$\ln K_{eq} = \frac{\Delta S}{R} - \frac{\Delta H}{RT} \quad \text{or} \quad K_{eq} = e^{\frac{-\Delta G}{RT}}$$

Therefore, a plotting of the natural logarithmic of the equilibrium constant versus the reciprocal temperature gives a straight line whose slope is the minus enthalpy variation divided by the R constant ($-\Delta H$/R), and the intercept is equal to the entropy variation

divided by the R constant (ΔS/R). A decrease in temperature favours adsorption since it is always followed by a decrease in ΔG (Myers, 2006).

4.2.5.3 Determination of the adsorption isotherms

Experimental equilibrium adsorption data (adsorption isotherms) have been obtained with the following procedure. A fixed amount of pretreated *Amberlite XAD16* resin has been added to an 250 ml Erlenmeyer flask containing 100 ml of distilled water at a known initial concentration of amino acid, C_0 (g/L), and placed onto a stirring hot plate (model MR 2002, Heidolph; Moreira da Costa e Santos, Porto, Portugal), where mixing has been achieved by a magnetic bar. Continuous sampling has shown that approximately 3 hours of continuous stirring are required for obtaining equilibrium.
Equilibrium concentration of amino acid in solution, C_{eq} (g/L), has been determined by HPLC-UV/Vis, using the methods reported in Chapter 3, paragraph 3.2.2 at pages 54 and 55. The amount of amino acid retained on the resins at equilibrium, C_{ad} (g/g), has been calculated as follow:

(**Eq.** 4.9) $$C_{ad} = \frac{C_0 \cdot V_0 - C_{eq} \cdot (V_0 + \Delta V)}{1000 \cdot W_d}$$

where V_0 is the initial volume of amino acid solution (ml), ΔV is the dilution brought into amino acid solution by addition of conditioned wet resin, and expressed as $\Delta V = (W_w - W_d)/\rho_{H2O}$, where W_w is the wet resin weight (g), W_d is the dry resin weight (g) and ρ_{H2O} is the density of water (g/ml) at the experimental temperature.
Adsorption equilibrium data have been collected for four synthetic amino acids (Sigma-Aldrich; Sintra, Portugal): tryptophan which is the most hydrophobic, taurine and glycine that are amphiphilic and borderline line amino acids with different acidic groups, and lysine which is the most water soluble although it is not the most hydrophilic (Table 4.2). These amino acids have been selected to study the influence of the physical nature (hydrophobicity/hydrophilicity) on the amount adsorbed upon *Amberlite XAD16* and also to mimic real sample solvent, which is water.
For each of the four amino acids, the effect of six parameters on adsorption equilibrium has been evaluated, namely: the effect of temperature, the effect of ionic strength, the

effect of ethanol added to the wastewater, the effect of pH, the effect of amino acid initial concentration and the effect of adsorbent dose. Ten solutions of each amino acid have been used to study each parameter, and experiments have been carried out by modifying only one variable at each run. Initial concentrations, C_0, have been in the range 0.05-0.5 g/L (that mimics real amino acids concentration in wastewater) and with the following values: 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 g/L. The effect of temperature on adsorption equilibrium has been tested for the values 10 ºC, 20 ºC and 30 ºC. The effect of pH has been tested for the actual codfish wastewater value of 6, for a lower value of 2, and for a higher value of 11, at 20 ºC; the buffering agents sodium hydroxide NaOH (Sigma-Aldrich) and chloridric acid HCl (Sigma-Aldrich) have been used for alkalizing and acidifying, respectively, the amino acids solutions to the desired value. Sodium chloride, NaCl, (Sigma-Aldrich) has been added to the amino acids solutions to study the effect of ionic strengths where the real wastewater value of 4.3 M NaCl, the lower value of 1.5 M and the null value of 0 M have been investigated at 20ºC. The effect of ethanol on adsorption equilibrium has been tested at 20 ºC by comparing the results for the normal (non ethanolic) solutions of amino acids with two mixtures composed of 25% (v/v) and 50 % (v/v) food-grade ethanol 99.5% purity (Aga; Porto, Portugal). Adsorbent dose has been tested at 20 ºC for the values 5 g, 10 g and 20 g of *Amberlite XAD16* resin added to 100 ml of normal (without ethanol and without salt) amino acid solution. All experiments executed to study adsorption equilibrium by one-variable-at-a-time approach are summarised in Table 4.3. Percentage of amino acid retained on the resin has been calculated as follow:

(**Eq.** 4.10) $$R(\%) = \frac{C_{ad} \cdot W_d}{(C_0/1000) \cdot V} \cdot 100$$

Table 4.3 Experiments for adsorption equilibrium study

Parameter	Value tested		
pH	2	6	11
Temperature (ºC)	10	20	30
Ethanol (% v/v)	0	25	50
Ionic Strength (M NaCl)	0	1.5	4.3
Adsorbent dose (g_{resin}/100 $ml_{Solution}$)	5	10	20
Initial concentration (g/L) (range)		0.05 – 0.5	

4.2.6 Modelling of experimental data and design of experiments

Experimental isotherms have been compared with the theoretical isotherm model proposed by *Freundlich* (Smith, 1987). For each adsorption isotherm, parameters *n* and K_F have been determined by the linear form of *Freundlich* equation (Eq. 4.6), where K_F represents intercept and *1/n* the slope. Adequacy of the model proposed has been evaluated by two goodness-of-fit criteria, namely coefficient of determination (R^2) and standard error of the regression (SER) (Shumaker 1992).

A design of experiment for taurine has been performed by a 3^2 fractional factorial design (2 variables with 3 levels) which requires 9 experiments. According to factorial design symbology, the symbol "+" indicates a parameters combination whose response (R(%)) is known from the one-variable-at-a-time approach, while symbol "–" indicates a parameters combination whose response must be tested (Jiju, 2003).

The software StatSoft *STATISTICA v.9* has been used for data analysis.

4.3 Results and Discussion

4.3.1 Adsorption of selected free amino acids

Results obtained provide evidence of amino acids adsorption, as well as differences in adsorption extent depending on operating conditions (temperature, pH, ionic strength, ethanol, initial concentration and adsorbent dose), and differences in amino acids recovery depending on the specific physical nature, i.e. hydrophobic or hydrophilic.

The effect of chemical composition and structure of each amino acids on the extent of adsorption is illustrated by the adsorption isotherms of tryptophan, taurine, glycine and lysine (Figure 4.4), relative to C_0=0.5 g/L, a temperature of 20 ºC and an adsorbent dose of 10 g of dry resin for 100 ml of solution, with null ionic strength and without any ethanol addition.

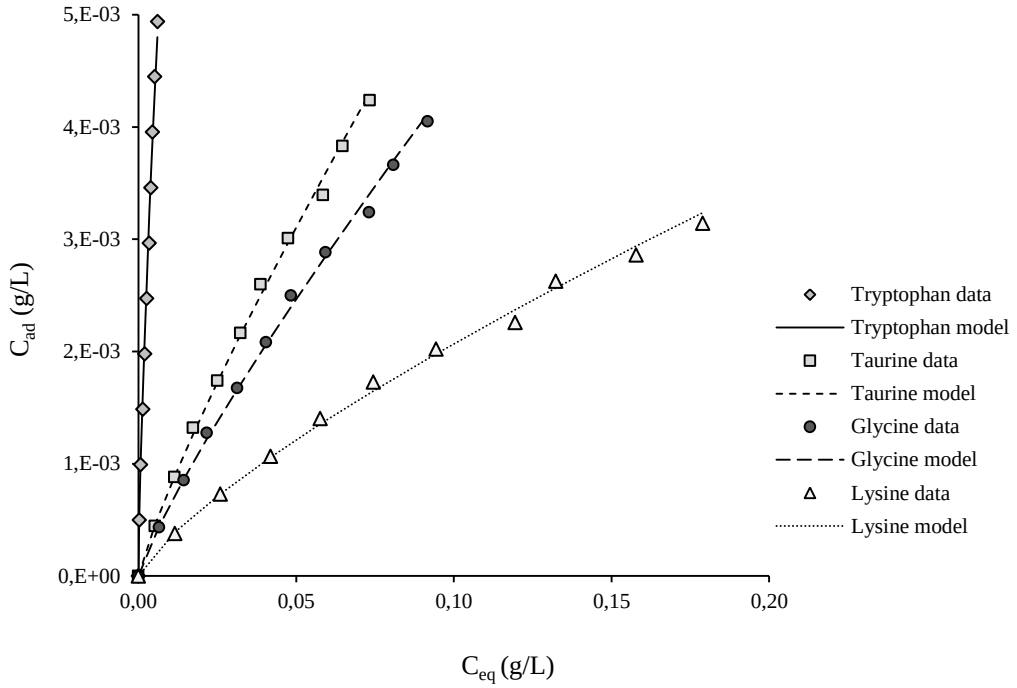


Figure 4.4 Amino acids adsorption isotherms at 20 ºC (mean values ± standard deviation bars, which are not visible) with 10 g of resin for 100 ml of solution, and theoretical model data set

It is observed that adsorption increased by increasing hydrophobicity and/or decreasing the hydrophilicity of the amino acid molecules. Adsorption decreases in the order tryptophan > taurine > glycine > lysine, which is also the order either of a decrease in the carbon number of the amino acid hydrocarbon chain (in the case of taurine and glycine) or a decrease in the hydrophobicity of amino acid (in the case of lysine, recall Table 4.2). At the higher initial concentration C_0=0.5 g/L, amino acids recovery has been 98% for tryptophan, 85 % for taurine, 81 % for glycine and 63 % for lysine.

This behaviour was expected since the forces responsible for adsorption interactions between the hydrophobic tail of the amino acid and the non-ionic adsorbent *XAD16*

resin. The phenomenon of a compound capture onto *Amberlite XAD16* resin, and its following release, is a physisorption process characterised by *van der Waals* forces, hydrophobic interactions and the low energy hydrogen bonding by CH group (Gao et a., 2003; Rhom & Haas, 2010). As known, *van der Waals* forces consist of attraction between two permanent dipoles (named *Keeson* forces), attraction between a permanent dipole and a corresponding induced dipole, named *Debye* forces, and attraction between two instantaneous induced dipoles, named *London* dispersion forces (Mancera 1996).

Results confirmed that orientation of adsorption has been with the hydrophobic tail of the amino acids on the resin surface and the hydrophile directed toward the aqueous solution. In the case of the amino acid tryptophan, in addition to the above mentioned mechanisms, adsorption by polarisation of π electrons should occur due to the electron-rich aromatic nuclei of its molecule. Thus, the interaction developed between the longer hydrophobic and aromatic chain of the tryptophan molecule, consisting of 10 carbons atoms, and the *XAD16* surface has been stronger than those developed between the shorter taurine, glycine and lysine molecule and the resin. It must be highlighted that the addition of an aromatic group to the hydrocarbon chain should have an effect on the hydrophobicity equivalent to about three and one-half methylene groups (Rosen, 1989).

Taurine and glycine are both amphiphilic and neutral amino acids, and taurine is more soluble in water than glycine; nevertheless, a higher extent of adsorption occurred for taurine. Taurine and glycine molecules have the same terminal groups OH and NH_2 that are hydrophilic; however the acidic group SO_2OH of taurine exhibits a higher inductive effect and electron-acceptor character than the acidic group COOH of glycine, which *per contra* shows both electron-donor and acceptor behaviour. This resulted in a higher affinity of taurine, with respect to glycine, with *Amberlite XAD16* since this resin exhibits strong electron donor properties due to the rich π electrons nuclei (Doulia et al., 2001). Also, since the SO_2OH group is bigger than the COOH group of glycine, the electron-acceptor behaviour of taurine has been still more evident on adsorption (Kolesnik & Kozlov, 1968; Yang et al., 2005).

Regarding lysine, the lower extent of adsorption is attributable mainly to the presence of the hydrophilic terminal groups OH and NH_2, and in a lower amount to the middle NH_2 group. It should be noticed that strong negative effects in adsorption from aqueous solutions on hydrophobic adsorbents could be attributed to either the presence of an

additional hydrophilic group or to its position. When the hydrophilic group is positioned at the end of the hydrocarbon chain the negative effect is more pronounced in comparison with a hydrophilic groups present at a central position (Dutta et al., 1997).
When environmental conditions of adsorption are changed, the extent of the process itself is modified, however the order of adsorption of the tested amino acids remained the same, as discussed in the following paragraphs.
Equilibrium parameters n and K_F, and statistic parameter R^2 and SER for each amino acid and for each variable studied are reported in Table 4.3. Values for statistics parameters allowed concluding that *Freundlich* model is suitable since coefficients of determination are very near to the perfect fit (R^2 near to 1, and in some case equal to 1), and the standard error of the regression is close to zero and in any case not greater than 10% of the value estimated. Parameters n and K_F varied according to environmental conditions. Heterogeneity factor, n has been always > 1 indicating that adsorption has been favourable. The capacity factor K_F showed a higher variability that n being dependent on the amino acid nature and, as expected, the higher the capacity factor the higher the adsorption (Doulia et al., 2001).

Table 4.4 Kinetic parameters *n* and K_F, and statistic parameters R^2 and SER for tested amino acids

Parameter	Amino acid															
	Lysine				Glycine				Taurine				Tryptophan			
pH	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*
2	1.27	0.016	0.96	$2{\cdot}10^{-5}$	1.16	0.038	0.97	$3{\cdot}10^{-5}$	1.17	0.041	0.98	$4{\cdot}10^{-5}$	1.36	0.244	0.98	$2{\cdot}10^{-5}$
6	1.30	0.012	0.98	$1{\cdot}10^{-5}$	1.19	0.031	0.95	$3{\cdot}10^{-5}$	1.18	0.040	0.99	$7{\cdot}10^{-6}$	1.37	0.197	0.99	$8{\cdot}10^{-6}$
11	1.33	0.009	0.98	$8{\cdot}10^{-6}$	1.19	0.026	0.98	$2{\cdot}10^{-5}$	1.09	0.036	0.97	$5{\cdot}10^{-5}$	1.34	0.194	0.97	$3{\cdot}10^{-5}$
Temp. (ºC)	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*
10	1.28	0.015	0.98	$3{\cdot}10^{-5}$	1.20	0.034	0.99	$7{\cdot}10^{-6}$	1.20	0.045	0.95	$6{\cdot}10^{-5}$	1.40	0.204	0.99	$7{\cdot}10^{-6}$
20	1.32	0.012	0.99	$9{\cdot}10^{-6}$	1.19	0.031	0.98	$8{\cdot}10^{-6}$	1.18	0.040	0.99	$3{\cdot}10^{-6}$	1.37	0.197	0.98	$6{\cdot}10^{-6}$
30	1.36	0.008	0.98	$2{\cdot}10^{-5}$	1.25	0.021	0.96	$5{\cdot}10^{-5}$	1.24	0.025	0.99	$7{\cdot}10^{-6}$	1.30	0.179	1	$3{\cdot}10^{-6}$
EtOH (% v/v)	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*
0	1.30	0.012	0.98	$9{\cdot}10^{-6}$	1.19	0.031	0.99	$9{\cdot}10^{-6}$	1.18	0.040	0.97	$5{\cdot}10^{-5}$	1.37	0.197	0.98	$4{\cdot}10^{-5}$
25	1.28	0.013	0.98	$1{\cdot}10^{-5}$	1.18	0.038	1	$5{\cdot}10^{-6}$	1.15	0.047	0.98	$9{\cdot}10^{-6}$	1.38	0.222	0.98	$4{\cdot}10^{-5}$
50	1.29	0.013	0.99	$7{\cdot}10^{-6}$	1.17	0.046	0.98	$2{\cdot}10^{-5}$	1.21	0.048	0.99	$9{\cdot}10^{-6}$	1.38	0.269	0.99	$1{\cdot}10^{-5}$
Ionic Strength (M)	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*	*n*	K_F	R^2	*SER*
0	1.30	0.012	0.99	$9{\cdot}10^{-6}$	1.19	0.031	0.98	$8{\cdot}10^{-5}$	1.18	0.040	0.99	$3{\cdot}10^{-5}$	1.37	0.197	0.98	$4{\cdot}10^{-5}$
1.5	1.31	0.011	0.98	$8{\cdot}10^{-6}$	1.18	0.038	0.99	$5{\cdot}10^{-6}$	1.23	0.043	0.97	$3{\cdot}10^{-5}$	1.41	0.241	0.99	$6{\cdot}10^{-6}$
4.3	1.40	0.006	0.98	$8{\cdot}10^{-6}$	1.21	0.019	0.96	$5{\cdot}10^{-5}$	1.26	0.023	0.98	$3{\cdot}10^{-5}$	1.36	0.089	1	$5{\cdot}10^{-6}$

4.3.2 Effects of temperature upon adsorption equilibrium

An increase in temperature corresponded to a decrease in adsorption. In Table 4.5 thermodynamics parameters for each amino acid investigated, at three different temperatures, are reported. Parameters have been calculated taking into account the mean K_{eq} value over the range of amino acid initial concentration 0.05-0.5 g/L. Value for ΔG and ΔH < |− 5 Kcal/mol| (absolute value) confirmed the mechanism of physical adsorption.

Table 4.5 Thermodynamic parameters of adsorption ΔG, ΔH and ΔS for each amino acid and for each temperature

Amino acid	ΔG (Kcal/mol)			ΔH (Kcal/mol)	ΔS (Kcal/mol·K)
	10 ºC	20 ºC	30 ºC		
Tryptophan	− 2.85	− 2.62	− 2.31	− 4.92	− 0.0103
Taurine	− 1.25	− 1.12	− 0.93	− 4.95	− 0.0133
Glycine	− 1.03	− 0.96	− 0.81	− 4.17	− 0.0110
Lysine	− 0.63	− 0.51	− 0.30	− 4.82	− 0.0169

In the range studied, optimal adsorption temperature was 10ºC. In Table 4.6 amino acids recovery yields are reported, while in Figure 4.5 taurine isotherms are shown as an example of the effect of temperature upon adsorption.

Table 4.6 Recovery of amino acids depending on temperature, at 0 M NaCl, C_0=0.5 g/L and 10 g of resin for 100 ml of solution

Temperature (ºC)	Recovery of amino acid (%)			
	Tryptophan	Taurine	Glycine	Lysine
10	100	87	83	69
20	98	85	81	63
30	96	79	76	52

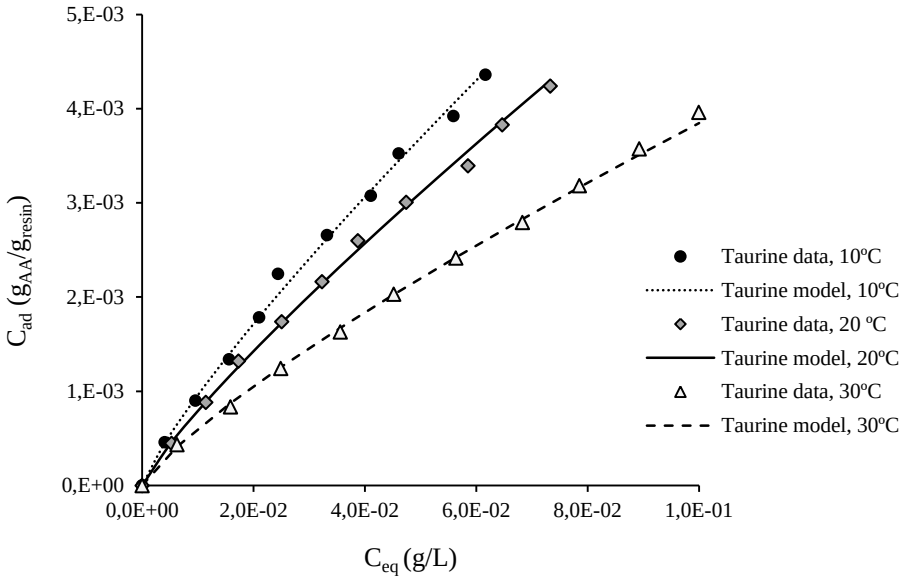


Figure 4.5 Adsorption isotherms of taurine at 10, 20 and 30 ºC (mean values ± standard deviation bars, which are not visible) with 10 g of resin for 100 ml of solution, and theoretical model data set

As known from literature, the adhesion and release of an atom, ion and molecule from a liquid onto a generic solid surface can occur by two types of interactions named physisorption and chemysorption. Chemysorption is the basis of the catalytic activity of some solids for reaction between molecules, and involve covalent bonding (Smith, 1981). In physical adsorption, forces attracting the molecules of the fluid to the solid surface are weak when compared to chemical adsorption and ion-exchange (Smith, 1981). Energy of activation in adsorption involving *van der Waals*'s forces, hydrogen bonding by CH group and hydrophobic interactions, is usually not higher than 5 kcal/mol, and the binding process is exothermic (i.e. favoured), where heat evolves with same order of magnitude of condensation process, 0.5 to 5 kcal/mol. Conversely, chemical adsorption process is endothermic, often irreversible and ca. 90-200 kcal/mole are required for covalent bonding onto adsorbent, while ca. 12-15 kcal/mol (by hydrogen or sodium ions, common binding atoms) are needed for ion-exchange based capture/release, and the process can be either exothermic or endothermic. For the aforementioned differences, equilibrium between the solid surface and the fluid molecules is usually rapidly attained and easy reversible during physisorption, because the energy requirements are small. Process reversibility, which stands for easy and

economic regeneration of adsorbent, is the main advantage of *Amberlite XAD16* compared with natural activated carbon and synthetic ion-exchange resins.

4.3.3 Effects of ionic strength upon adsorption equilibrium

An increase in amino acids adsorption occurred by increasing ionic strength of solution up to ca. 1.5 M NaCl. A decrease in adsorption has been noticed when ionic strength has been increased to 1.5 M up to the actual codfish salting wastewater value of 4.3 M. In Table 4.7 amino acids recoveries depending on ionic strength are reported. In Figure 4.6 taurine isotherms, as an example of the effect of NaCl concentration upon adsorption, are shown. Optimal ionic strength for recovery of taurine and other hydrophobic amino acids was 1.5 M.

Table 4.7 Recovery of amino acids depending on ionic strength, at 20 ºC, C_0=0.5 g/L and 10 g of resin for 100 ml of solution

Ionic strength (M of NaCl)	Recovery of amino acid (%)			
	Tryptophan	Taurine	Glycine	Lysine
0	98	85	81	63
1.5	100	89	84	60
4.3	94	75	71	47

Results are in agreement with the relevant literature and confirmed that ionic strength is indeed one of the major factors affecting adsorption (Myers, 2006; Rosen, 1988). Generally, it is observed that by increasing ionic strength, adsorption from aqueous solutions onto non-polar hydrophobic adsorbent, and probably onto polar adsorbents without strongly charged sites, increases as well (Kroeff & Pietrzyk, 1978).

The influence of salt on adsorption is attributable to an increasing of the hydrophobic effect, which leads to segregation of water molecules and non-polar and amphiphilic substances, a phenomenon also known as “salting-out” (Fujita et al., 2007). Apart affecting solvent power (i.e. modification in H-bonding between water molecules and amino acids) ionic strength also influences interactions at the amino acid-resin interface, such as electrostatic forces, H-bonding forces and repulsive forces between electron-

rich aromatic nuclei of adsorbate and adsorbent as well as interactions between amino acids themselves (Kyriakopoulos et al., 2006).

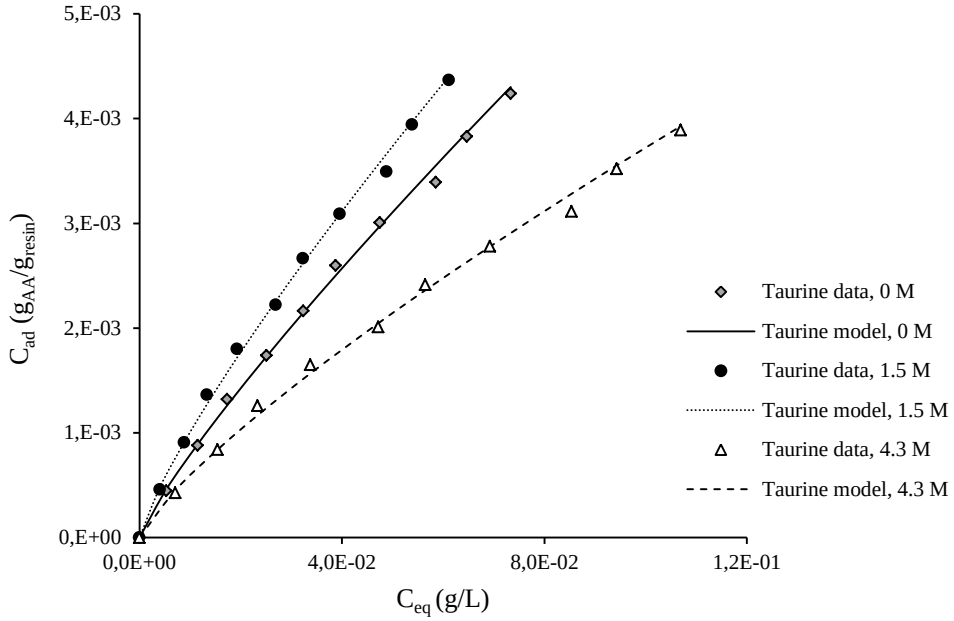


Figure 4.6 Adsorption isotherms of taurine at 20ºC from 0, 1.5 and 4.3 M NaCl aqueous solution (mean values ± standard deviation bars, which are not visible) with 10 g of resin for 100 ml of solution, and theoretical model data set

A reinforcement of some types of attractive interactions should occur, determined by the resin, by the amino acids structure and by the environmental conditions. Since *XAD16* resin molecule contains an electronic aromatic nuclei (styrene-divinylbenzene copolymer), it has a pronounced ability to interact by polarisation of π electrons with oppositely charged solute molecules. The presence of aromatic rings at both sides of the amino acids-resin interface causes repulsive forces to be developed. Sodium chloride ions, interacting by induced dipolar forces with the aromatic π electrons, should decrease the repulsive forces at the amino acid-resin interface, consequently resulting in an increase in adsorption. The more the electronic density in aromatic ring of an adsorbate the more pronounced the effect of salt, since the reduction in repulsive forces is higher (Rosen, 1989).

At the higher ionic strength 4.3 M, actual wastewater content, adsorption diminished, most probably due to the excessive Na^+ and Cl^- ions concentration, which reduce

mobility of amino acids in solution as well as the access of amino acid to resin surface (Kyriakopoulos et al., 2006).

4.3.4 Effects of ethanol upon adsorption equilibrium

Addition of ethanol into solution resulted in an increase of adsorption for the hydrophobic amino acid tryptophan and for the neutral amino acids taurine and glycine, while no significant differences have been observed for lysine. Results are reported in Table 4.8, while in Figure 4.7 and Figure 4.8 are reported taurine and lysine isotherms, as an example of the effect of ethanol upon adsorption depending on amino acid nature.

Table 4.8 Recovery of amino acids depending on percentage (v/v) of ethanol in the amino acids solutions, at 20 ºC, C_0=0.5 g/L and 10 g of resin for 100 ml of solution

Ethanol (% v/v)	Recovery of amino acid (%)			
	Tryptophan	Taurine	Glycine	Lysine
0	98	85	81	63
25	99	87	84	64
50	100	88	85	64

Based on experimental data obtained, it can be concluded that optimum percentage of ethanol in solution is 50% by volume at which corresponds the higher recovery of amino acids.

Addition of ethanol to an aqueous solution exerts a similar “salting-out” effect of neutral electrolytes onto hydrophobic and neutral species, i.e. reduces their solubility, while solubility of hydrophilic amino acids remains substantially constant (Ji et al., 2009). This phenomenon resulted in a higher affinity of tryptophan, taurine and glycine for the *XAD16* resin, while result for lysine remained essentially unchanged, as can be seen in Table 4.8.

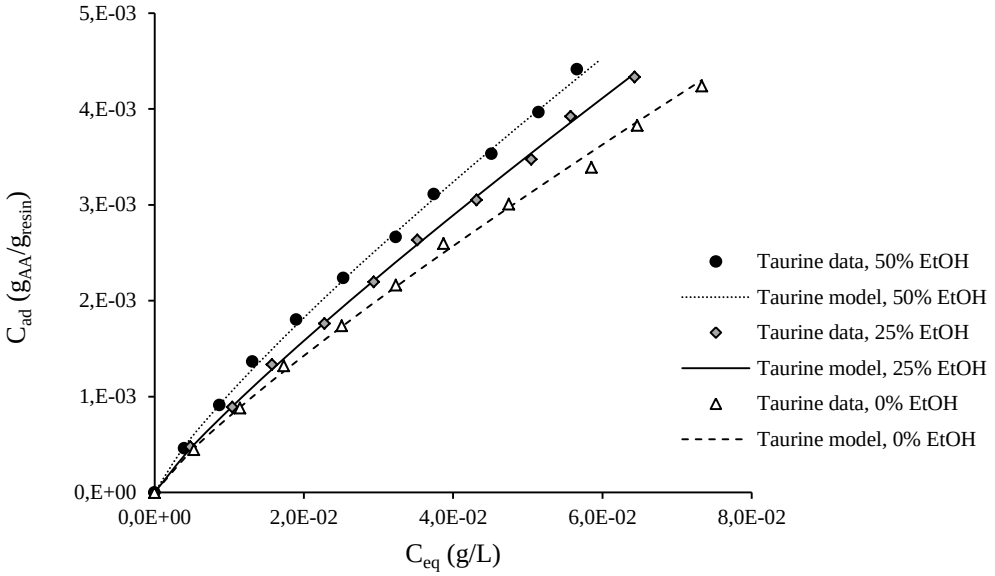


Figure 4.7 Adsorption isotherms of taurine at 20 ºC from 0, 25 and 50 % of ethanol (v/v) in 100 ml of solution of amino acids with 10 g of resin (mean values ± standard deviation bars, which are not visible), and theoretical model data set

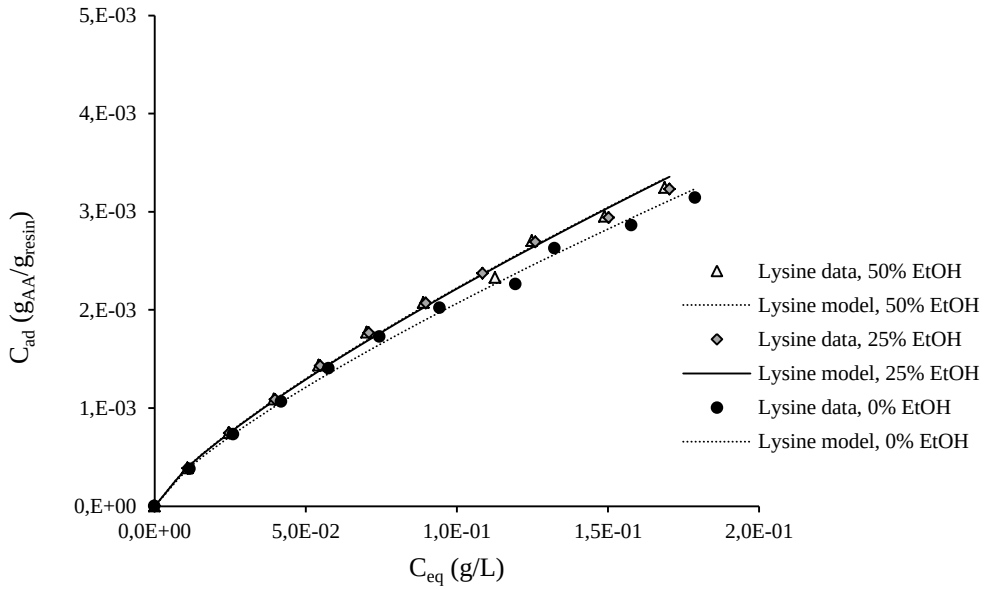


Figure 4.8 Adsorption isotherms of lysine at 20 ºC from 0, 25 and 50 % of ethanol (v/v) in 100 ml of solution of amino acids with 10 g of resin (mean values ± standard deviation bars, which are not visible), and theoretical model data set

4.3.5 Effects of pH upon adsorption equilibrium

Changes in solution pH affected mechanisms and extent of adsorption of tryptophan, glycine and lysine. Regarding taurine, no changes have been observed by decreasing pH, but a decrease in adsorption occurred at alkaline pH values as in the case of other amino acids. In Table 4.8 are reported amino acids recovery yields depending on solution pH, while in Figure 4.8 are reported taurine isotherms, as an example of pH effects upon adsorption.

Table 4.9 Recovery of amino acids depending on pH of solution, at 20 ºC, C_0=0.5 g/L and 10 g of resin for 100 ml of solution

pH	Recovery of amino acid (%)			
	Tryptophan	Taurine	Glycine	Lysine
2	100	85	84	69
6	98	85	81	63
11	94	81	78	57

The pH as well as the ionic strength, exerts a major effect on the resin surface. Since amino acids have both an acidic and basic site, and *XAD16* copolymers are aromatic and possess good electron-donor properties, it is expected that polar and electrostatic forces occur together with dispersion forces. The interaction between the non-ionisable amino acid R group and the *XAD16* surface is considered to be constant throughout the entire pH range (Doulia et al., 2001). Therefore, the variations in adsorption efficiency should be the result of the ionisation of COOH and/or NH_2 groups. Generally, adsorption is favoured by acidic conditions, where the COOH group exists as the undissociated form and the NH_2 is ionised. In basic solution, where the COOH group is ionised and the NH_2 group is unionised, and at intermediate pH, where both groups are ionised, adsorption is lower. Also, if any additional ionisable sites are present on the amino acids side chain, ionisation at these sites will also contribute to the overall charge on the species as a function of pH and to the extent of its adsorption (Myers, 2006). At acidic pH, when the electron acceptor COOH group is uncharged, amino acids can interact with resin taking advantage of the electron donor properties of its surface, apart the normal interaction by the R chain (Kroeff & Pietrzyk, 1976).

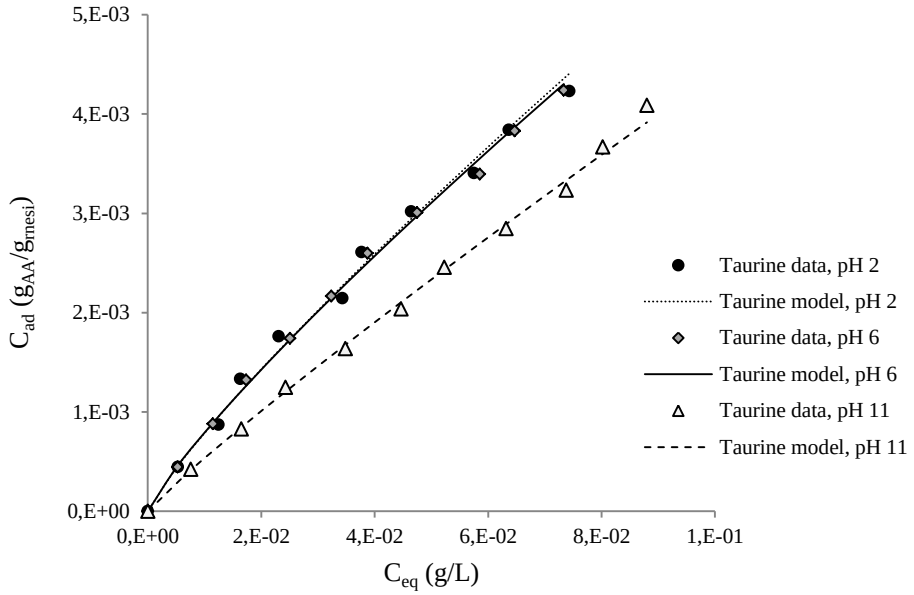


Figure 4.9 Adsorption isotherms of taurine at 20 ºC at pH 2, 6 and 11 (mean values ± standard deviation bars, which are not visible) with 10 g of resin for 100 ml of solution, and theoretical model data set

In general, the pH has no or little effect on adsorption of strong acids, so called either because are almost completely dissociated in aqueous solutions or due the higher stability of the respective conjugated bases (Pietrzyk & Chu, 1977). That has been apparently the case of taurine. Being a sulfonic acid, taurine is classified as a strong acid since naturally tends to loss a proton in aqueous solutions, in all the pH range (Hamborg et al., 2007).

4.3.6 Effects of initial concentration on amino acid recovery

The effect of initial concentration of amino acids has been tested in the range 0.05 g/L to 0.5 g/L, same used for adsorption isotherms study. Results show that adsorption decreases by increasing initial concentration for all the amino acid, however reduction is less significant for tryptophan (Figure 4.10). Recovery of taurine decreased from 90 down to 85 % by increasing concentration from 0.05 to 0.5 g/L; same reduction of recovery – from 86 to 81 % – has been observed for glycine, and even more for lysine –

from 76 to 63 %. A reduction of just 2% (from 100 to 98 %) has been observed in the case of tryptophan.

Results obtained are expected and in accord with the amino acids affinity for the resin. The initial concentration is important since a given mass of adsorbent can only adsorb a fixed amount of a specific compound. Therefore, the more concentrated the solution the smaller the recovery of compound (Meško et al., 1999). Results for taurine showed that recovery is constant up to an initial concentration of 0.1 g/L.

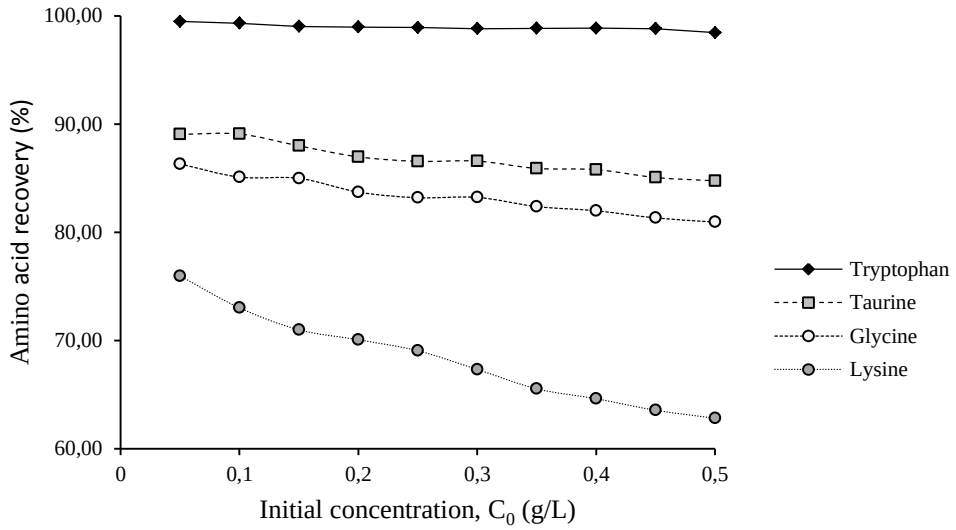


Figure 4.10 Effect of initial concentration on the recovery of tested amino acids, at 20 ºC, 10 g of resin for 100 ml of solution

Results for glycine and lysine showed that to obtain the highest recovery the lowest initial concentration 0.05 g/L must be achieved. Finally 100 % of tryptophan can be recovered from solution for an initial concentration up to 0.1 g/L.

4.3.7 Effects of *AmberliteXAD16* dose upon amino acids recovery

Results for the recovery of tested amino acids depending on adsorbent dose revealed that best value for adsorption is 10 g of dry *Amberlite XAD16* for 100 ml of solution. As represented in Figure 4.11 and Figure 4.12, a lower value of 5 g/100 ml results in a

significant reduction of adsorption, but a further increase above 10 g/100 ml does not show any significant effect in amino acids recovery.

From the results it is revealed that the percentage of amino acids adsorbed upon resin is determined by the sorption capacity of *XAD16* itself (Kumar et al., 2010), so as adsorption augmented by increasing the adsorbent dose. However, at high resin dosages the mobility of amino acids is solution is reduced (Argarwal et al., 2010), which results in any significant recovery increment, or worse, in a decrease in adsorption, as in the case of tryptophan and taurine.

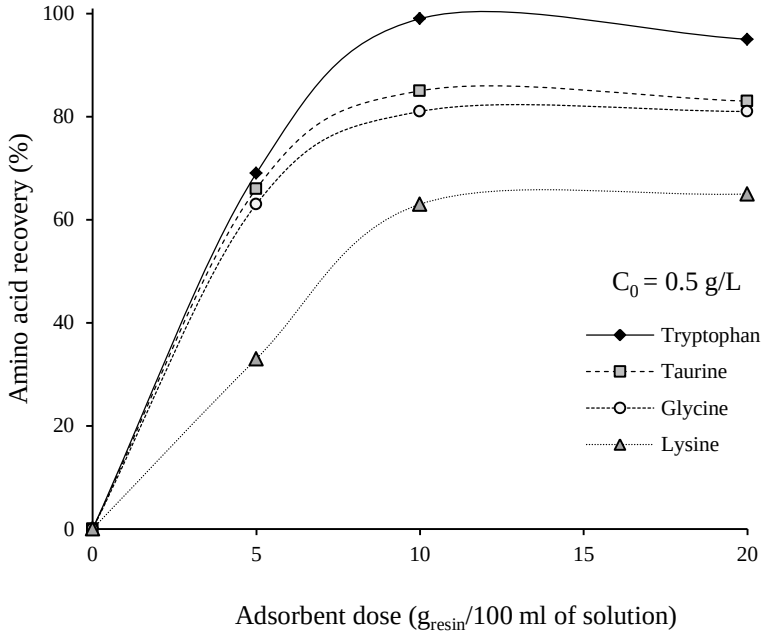


Figure 4.11 Effect of adsorbent dose on amino acid recovery from a solution at C_0=0.5 g/L and at 20 ºC

From Figure 4.11 and 4.12 it is evident the effect of the initial concentration upon amino acid recovery; however, 10 g is confirmed to be the optimal resin amount for the higher recovery of taurine, glycine and lysine even at C_0=0.05 g/L, while tryptophan achieves its plateau with a lower amount of adsorbent, 7 g instead of 10 g.

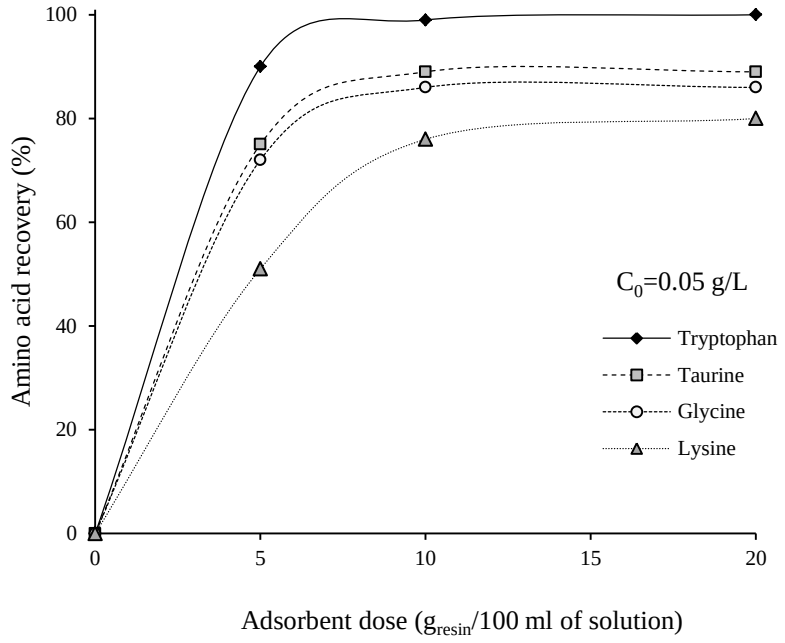


Figure 4.12 Effect of adsorbent dose on amino acid recovery from a solution at C_0=0.05 g/L and at 20ºC

4.3.8 Effect of parameters interaction upon adsorption

Analysing results, it can be observed a maximum recovery of amino acids at a specific value of each parameter investigated. In the following Table 4.10 is reported the parameter which allowed for the maximum yield, when one parameter at a time has been studied, at C_0=0.5 g/L and with a resin dose of 10 g of resin for 100 ml of solution.

Table 4.10 Maximum amino acids recovery for each parameter investigated

Parameter	Maximum Recovery (%)			
	Tryptophan	Taurine	Glycine	Lysine
10 ºC (*at*: 0 M NaCl, 0% EtOH, pH 6)	100	87	83	69
1.5 M NaCl (*at*: 20ºC, 0% EtOH, pH 6)	100	89	84	60
50% (v/v) EtOH (*at*: 20ºC, 0 M NaCl, pH 6)	100	88	85	64
pH 2 (*at*: 20ºC, 0 M NaCl, 0% EtOH)	100	85*	84	69

(*) Also at pH 6

The effect of parameters combination on the adsorption of taurine, the target amino acid, has been then investigated. Effects of interaction between pH and ionic strength upon adsorption of taurine have been then studied. For the temperature of 10 ºC results for recovery are reported in Table 4.11.

Table 4.11 Recovery of taurine by combining pH with ionic strength, at 10 ºC

Ionic strength (M)	pH		
	2	6	11
0	85	85	81
1.5	90	90	80
4.3	79	79	78

From the results showed in Table 4.11 it can be noticed that no differences in adsorption occurred by acidifying the taurine solution. An increase in ionic strength from 0 to 1.5 M resulted in the same increase in adsorption at pH 2 and pH 6, and this result could be apparently not specific for taurine. As reported by Kyriakopoulos *et al*. (2006), adsorption onto uncharged and not functionalised resins is generally highly influenced by a pH change at a low ionic strength (generally $\leq$ 1 M), while the pH factor is not significant at higher ionic strength values (generally > 1 M).

The effects of combination between ionic strength and ethanol have been not evaluated since there is dependence between the two parameters, i.e. the solubility of NaCl (and salt in general) is dependent on the amount of ethanol in solution (Pinho & Macedo, 1996).

A 3^2 factorial design has then been performed considering just two factors, temperature and ionic strength, at three levels: 10, 20 and 30 ºC for temperature, and 0, 1.5 and 4.3 M for ionic strength. Matrix of experiments and matrix of data for factorial design are reported in the Table 4.12a and 4.12b, respectively.

Table 4.12a Matrix of experiments for factorial design

Temperature (ºC) (Factor 1)	Ionic strength (Factor 2)		
	0 (Level 1)	1.5 (Level 2)	4.3 (Level 3)
10 (Level 1)	+	–	–
20 (Level 2)	+	+	+
30 (Level 3)	+	–	–

Table 4.12b Matrix of data for factorial design

Temperature (ºC) (Factor 1)	Ionic strength (Factor 2)		
	0 (Level 1)	1.5 (Level 2)	4.3 (Level 3)
10 (Level 1)	87	91	76
20 (Level 2)	85	89	75
30 (Level 3)	79	79	65

In Figure 4.13 and Figure 4.14 are reported the second-order fitted surface and the second-order contour plot of response (R% of taurine), respectively. Blue circles in both figures represent experimental results.

Factorial design analysis allowed identifying a maximum recovery of taurine of 90.10 % at the temperature of 13.5 ºC and at an ionic strength of 1.32 M NaCl. These coordinates represent the maximum point of the surface and falls inside the more reddish circular area of Figure 4.13. All other points represented inside that area represent experimental conditions allowing for recoveries between 90 and 90.10 %.

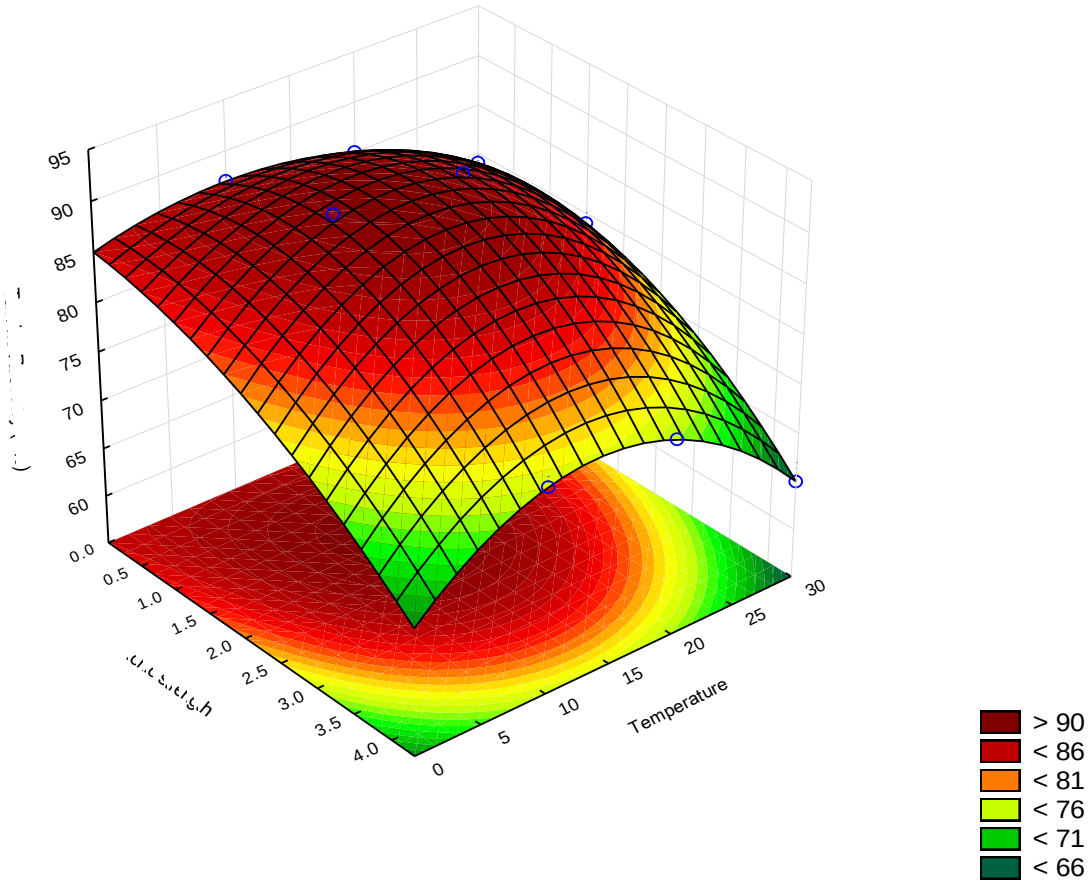


Figure 4.13 Second-order fitted surface of response (R% of taurine) for 3^2 factorial design

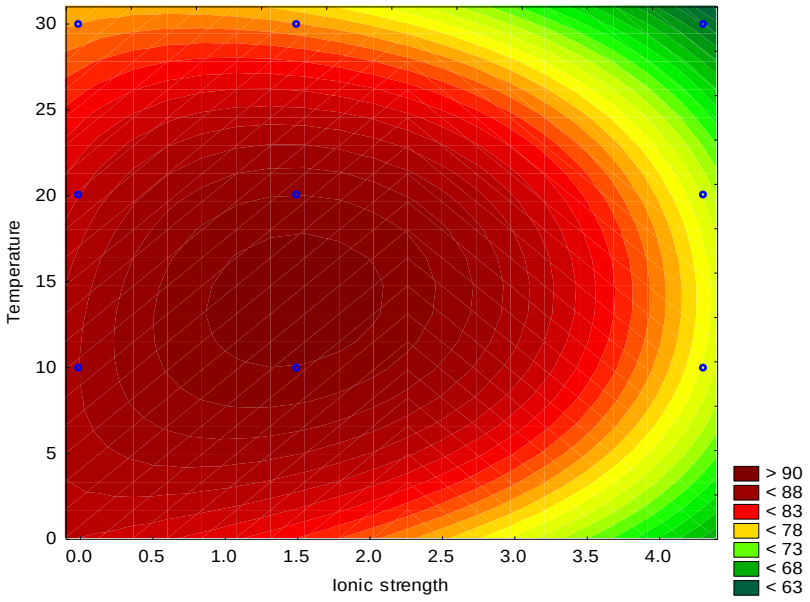


Figure 4.14 Second-order contour plot of response (R% of taurine) for 3^2 factorial design

Fitted function for the recovery of taurine is represented by the following equation:

(**Eq.** 4.11) $$R(\%) = 80.27 + 4.45 \cdot I_s - 1.52 \cdot I_s^2 + 1.03 \cdot T - 0.04 \cdot T^2 - 0.03 \cdot T \cdot I_s$$

where $R(\%)$ represents taurine recovery expressed in percent, I_s represents ionic strength expressed in molarities of NaCl, and T represent temperature expressed in Celsius degrees.

As it can be noticed from Equation 4.11 and in the Pareto graph (Figure 4.15), recovery of taurine is significantly dependent upon a linear and quadratic effect of each factor ($p>0.05$), however linear effect of ionic strength is the most significant.

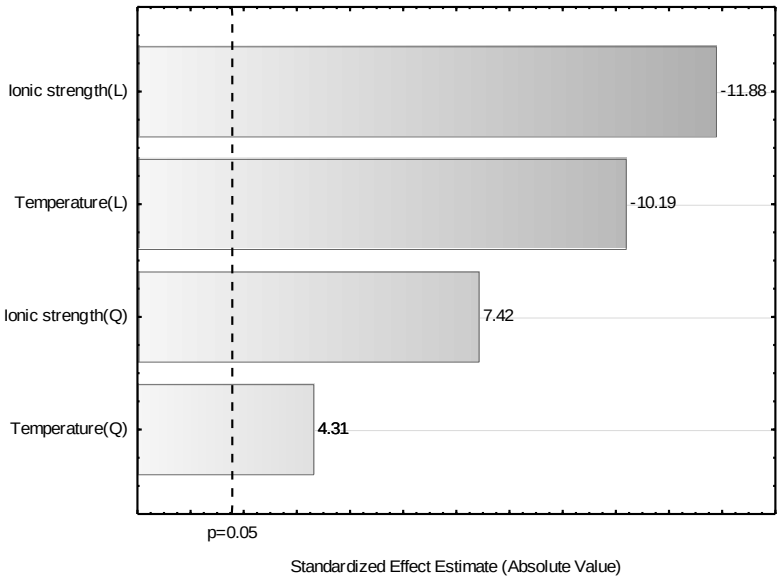


Figure 4.15 Pareto plot of the standardised effects of the two factors on the response (R% of taurine). "L" stands for "linear", "Q" stands for "quadratic". Vertical line defines significance limit expressed by the *p*-value at a confidence level of 95%

4.5 Conclusion

Adsorption of selected amino acids upon *Amberlite XAD16* resin has been demonstrated to be favourable and in accordance with the specific nature of each amino acid. The more the hydrophobicity of the amino acids the more the adsorption, so that adsorption decreased in the order tryptophan > taurine > glycine > lysine.

When one variable at a time has been investigated, an increase in adsorption for all the amino acids has been observed by decreasing temperature down to 10 ºC and by increasing ionic strength, to a limit of 1.5 M. Addition of ethanol resulted in an increase in adsorption for the hydrophobic amino acid tryptophan and for the neutral amino acids taurine and glycine, while not significant effect has been noticed for lysine. Adsorption of tryptophan, glycine and lysine has been found dependent upon pH when the ionic strength of solution is null. On the contrary, taurine adsorption was not dependent upon pH. An adsorbent dose of 10 g for 100 ml of solution showed to be the optimal amount for the highest recovery of all amino acids investigated, within a range of concentration of 0.05–0.5 g/L.

Factorial design analysis showed that, in order to maximize the recovery of taurine, adsorption should be carried out at the temperature of 13.5 ºC and at the ionic strength of 1.32 M NaCl. Factorial design also showed that ionic strength effect are more pronounced that temperature effects. These findings can be regarded as an important conclusion since salt is actually contained in the codfish salting wastewater (however, still at high concentration).

In the following chapter, the precipitation of salt from codfish salting processing wastewater by using ethanol will be discussed.

CHAPTER 5

ETHANOLIC PRECIPITATION OF SODIUM CHLORIDE FROM CODFISH SALTING PROCESSING WASTEWATER

5.1 Introduction

During dry salting, alternate layers of codfish and food-grade marine salt are stacked in a tank for 6 days. At the end of this period, approximately 200 L of heavy salted wastewater are drained away from each ton of codfish, carrying ca. 250 g/L of salt (sodium chloride – NaCl) and ca. 10 g/L of organic compounds, namely muscle proteins, peptides and free amino acids (Ferraro et al., 2011). Due to the high load of chloride, this wastewater is regarded as ecotoxic and can not be disposed to the sea, which could result, in the long run, in detrimental effects on the aquatic life as well as the quality of the seawater (El-Naas et al., 2010).

It has to be highlighted that the food processing industry has in general great interest in lowering or removing excessive inorganic salt content from the matrix to be processed in order to avoid corrosion problems generated by salt deposits inside the equipments, and also in unit operations such as extractive or azeotropic distillation, adsorption, crystallisation or liquid-liquid extraction, which are salt-sensitive (Farelo, 2004).

In this chapter is reported the extraction of NaCl dissolved in codfish salting processing wastewater, assuring at same time the required concentration in solution of proteins and free amino acids. According to results reported in previous chapter, best recovery of amino acids has been demonstrated to be reached at a NaCl concentration lower than the 4.3 M NaCl actually present in the wastewater.

Salt precipitation by ethanol, a food-grade solvent, has been chosen as an adequate strategy also encouraged by results for adsorption that demonstrated the positive effect of ethanol upon amino acids recovery. Organic solvents are frequently used to precipitate inorganic species from aqueous solutions either in method of analysis or in industrial applications (Bader, 1998; Pinto & Macedo, 2004). Pure aliphatic alcohols dissolve, in fact, inorganic salt very hardly and, for instance, just 0.18 % (w/w) of NaCl can be dissolved in pure ethanol at 20ºC (Jurkiewicz, 2007). Amino acids and proteins, although with lower solubility in alcohols than in water, are however much more soluble than sodium chloride under same operating conditions and ethanol concentration (Ji et al., 2009; van Oss et al., 1986).

In this study, the effects of wastewater-ethanol ratio, temperature, pH and time on salt precipitation by ethanol addition have been investigated. The effect of pH was also examined in absence of ethanol. The study has been carried out first by one-variable-at-

a-time approach and then by an experiment design performing a 3^2 fractional factorial design (2 variables at 3 levels). Compared to the one-variable-at-a-time approach, the fractional factorial design methodology allows to study at the same time the effects of each factor and the effects of interaction between factors on the response variable. In addition, the total number of runs in factorial design is much less as compared to the one-variable-at-a-time method (Jiju, 2003). Results obtained for wastewater have been compared with those of a blank solution composed of salt in water at same NaCl concentration of wastewater. The behavior and the role of proteins and amino acids in the wastewater-alcohol solution along salt precipitation have been also studied by determining concentration in wastewater before and after addition of ethanol.

5.2 Materials and Methods

5.2.1 Wastewater, blank solution and ethanol

Codfish salting process wastewater has been obtained as described in paragraph 3.2.1 of Chapter 3, at page 53. A blank solution at 254 g/L sodium chloride (resembling concentration of wastewater) has been prepared with food-grade marine salt, same used for codfish salting and supplied by *Pascoal & Filhos, S.A.* An amount of 319 g of salt has been added to 1 L deionized water and the mixture was placed into an ultrasound bath to accelerate salt dissolution. Absolute ethanol (99.9 % purity) of food-grade used for salt precipitation has been supplied by Aga (Porto, Portugal).

5.2.2 Sodium chloride precipitation procedure

The following procedure was carried out for both wastewater and blank solution. The effects of pH and of ethanol addition on salt precipitation were assessed. Effects of pH were studied in presence and in absence of ethanol. For the sample-ethanol mixtures, beside the effects of pH, the effects of settling time, temperature and ethanol in solution were also studied.

The effect of pH changes in both blank and wastewater were assessed by acidifying samples down to pH 2, using 1 M hydrochloric acid (Sigma-Aldrich; Sintra, Portugal),

and by alkalinizing up to pH 9 and pH 11, using 5 M sodium hydroxide (Sigma-Aldrich).

A volume of sample of 100 mL was transferred into a 250 mL cone shaped funnel. Then, acid or alkali was mixed with the sample in order to study the effect of pH. When the effect of ethanol was investigated (without changing the original sample pH), an aliquot of that solvent (different for each experiment, and in the range 20-140 ml of ethanol added to 100 ml of sample) was added to the funnel and the mixture was shaken for 1 min. Instantaneous extraction of sodium chloride following shaking was visible by the formation of a turbid and white fluid generated by the sample-ethanol mixture as a consequence of Na^+ and Cl^- ions association. After salt extraction, the funnel was transferred into a water-bath to allow the salt to settle. In order to study the effect of temperature on the amount of salt removed, precipitation runs were carried out at 0, 10, 15 and 27 ºC (this last as the actual ambient temperature). For the temperature of 0 ºC, the water was replaced by ice. Effects of temperature were assessed at a sample-ethanol ratio of 1:1. The effect of time on the amount of salt precipitated was also evaluated, where NaCl concentration in upper phase was determined at different times – 6.5, 13, 20, 25, 30, 60 and 120 min after precipitation started – at the temperature of 27 ºC and at a sample-ethanol ratio of 1:1. The effects of pH changes in the sample:ethanol 1:1 mixtures were finally investigated at the temperature of 27 ºC.

After the salt was settled, the supernatant was gently separated from the precipitate with a pipette. Both supernatant mixture and precipitate were centrifuged at 5000 rpm for 10 min. After that, the sample-ethanol mixture supernatant and the supernatant arising from centrifugation of the precipitated phase were mixed and kept at ambient temperature until analysis. Similarly, both heavy phases generated by centrifugation of sample-ethanol mixture and the precipitate were mixed and kept at ambient temperature until analysis.

Each combination of parameters was tested in duplicated: 12 mixtures (6 for wastewater and 6 for blank) for 3 different pH in absence of ethanol, 12 mixtures (6 for wastewater and 6 for blank) for 3 different pH value in the presence of ethanol, 28 mixtures (14 for wastewater and 14 for blank solution) for 7 different times, 28 mixtures (14 for wastewater and 14 for blank solution) for 7 different amounts of ethanol added to sample, 16 mixtures (8 for wastewater and 8 for blank solution) for 4 different

temperatures, and 8 mixtures for factorial design (for wastewater only) were prepared, giving a total of 104 experiments. Amount of salt precipitated was calculated as the percentage ratio of the difference between initial and final NaCl concentration in sample-ethanol mixture and the initial NaCl concentration in the sample-ethanol mixture.

5.2.3 HPLC analysis

5.2.3.1 Analysis of free amino acids and actin

Chromatographic analysis of free amino acids and actin have been performed as reported in paragraph 3.2.2 of Chapter 3, at pages 54 and 55.

5.2.3.2 HPLC-RID analysis of ethanol

Ethanol analysis has been performed using a Beckman & Coulter 168 series HPLC system interfaced with a Waters R-401 differential refractometer detector (Beckman&Coulter; Fullerton, CA, USA) and by an Aminex HPX-87H Ion Exclusion column (300 × 7.8 mm) (Bio-Rad; Lisbon, Portgual). Isocratic elution of ethanol has been performed using an 8 mM H_2SO_4 (Fluka; Lisbon, Portugal) mobile phase, at a flow rate of 0.8 ml/min and at 25ºC. Standards solutions for calibration have been in the range 5-0.5 %(w/w) of absolute ethanol (Fluka) in ultra-pure water; retention time has been 17.5 min. Samples to be analysed have been dissolved in ultra-pure water and filtered through a 0.45 µm cellulose membrane (Millipore-Interface; Amadora, Portugal). Mobile phase has been filtered and degassed in an ultrasound bath (Millipore-Interface) for 15 min prior to elution. All determinations have been carried out in triplicate.

5.2.4 Assessment of protein and electrophoretic analysis

The concentration of water–soluble proteins has been determined as reported in paragraph 3.2.4 of Chapter 3 at page 56. Electrophoretic analysis has been performed as reported in paragraph 3.2.5 of Chapter 3 at page 56.

5.2.5 Determination of salt and dry matter

Total salt content and dry matter have been determined as reported in paragraph 3.2.7 of Chapter 3, at page 57.

5.2.6 Design of experiments ans statistical analysis

Design of experiments was performed by a fractional factorial design. As known, fractional factorial design consists of a properly chosen subset of the experimental runs of the full factorial design. The subset is chosen in order to exploit the *sparsity-of-effects* principle, which states that a system is usually dominated by not more than two factors with low order interactions and not higher than the second order. Higher order interactions such as three factors interactions, are very rare (Juji, 2003).

Fractional factorial design has been performed with the software STATISTICA v.9.0 (*StatSoft* Co.). Two factors at three levels have been considered for a total of 9 (3^2) factorial experiments. Factors examined have been temperature and wastewater-ethanol ratio. Levels considered for temperature have been 0, 10 and 27 ºC. For the factor ratio, levels studied have been 20, 60 and 100 % (v/v) of ethanol added to sample. The factor time has been discarded since it not affects salt solubility but only salt precipitation after solubility has been decreased by the ethanol or temperature. The matrix of experiments for factorial design is reported in Table 5.1. According to factorial design symbology, symbol "+" indicates a parameters combination whose response (% of salt precipitated) is known from the one-variable-at-a-time approach, while symbol "–" indicates a parameters combination whose response must be tested (Jiju, 2003).

One-way analysis of variance (*ANOVA*) has been carried out with the software STATISTICA v.9.0 as well, setting a confidence level of 95% ($p \leq 0.05$).

Table 5.1 Matrix of experiments for factorial design

Temperature (ºC) (Factor 1)	% (v/v) of Ethanol added (Factor 2)		
	20 (Level 1)	60 (Level 2)	100 (Level 3)
0 (Level 1)	–	–	+
10 (Level 2)	–	–	+
27 (Level 3)	+	+	+

5.3 Results and Discussion

5.3.1 Effects of pH

Alteration in pH to acid or basic values did not produce salt precipitation either in wastewater or blank solution. These results confirmed the finding that the common ion effect only occurs for sparingly soluble salts. That is not the case of chloride salts which are very soluble in water expect those of Ag^{+}, Hg^{2+}, Pb^{2+}. For sparingly soluble salts, in fact, solubility is reduced when another species containing the same ion (called common ion) is added in solution, which results in salt precipitation (Khoshkbarchi et al., 1996).

5.3.2. Effects of ethanol

Different yields of precipitation of NaCl have been achieved depending on the operating conditions. Differences in amount of salt precipitated in wastewater and blank solution have been observed for each combination of parameters examined.

At the best operating condition, 15 ml of precipitated phase were collected starting from 100 ml of wastewater, i.e. 200 ml of wastewater-ethanol mixture. As showed in Table 5.2, precipitated phase contains ca. 70 % of dry matter, constituted almost totally by salt, and 30 % liquid matter, composed of ethanol and water, in the same proportion of ethanol-wastewater mixture. No precipitation of free amino acids occurred while 1.44% of proteins felt down along with salt. Ionic strength of wastewater has been reduced from 4.3 M to ca. 1.42 M in the wastewater-ethanol mixture.

Precipitation of salt is attributed to the decrease of polarity in both wastewater and blank solution which occurs after ethanol addition (Jurkiewicz, 2007). Water and organic solvents have different polarities and different strengths of interaction with sodium chloride, which results in different solubility of NaCl itself in water and organic solvents. Addition of ethanol – whose polarity at 25 ºC (24.3) is approximately one third that of water at 25 ºC (78.41) – to both blank solution and wastewater induces significant reduction on salt solubility (Morlyoshi et al., 1990). For each parameter investigated, amount of salt precipitated has been significantly higher ($p \leq 0.05$) in the blank-ethanol mixture than in wastewater-ethanol mixture (Table 5.3 and Figure 5.1, Table 5.4 and Figure 5.2, Table 5.5 and Figure 5.3), where the difference is attributable to the presence of amino acids in the wastewater. It is known that the presence of

amino acids in solution significantly increases the polarity of a solution above that of the pure solvent (Orella & Kirwan, 1991).

Table 5.2 Composition of wastewater, wastewater-ethanol mixture and precipitated phase at the best operating conditions 0ºC, 30 min, wastewater:ethanol ratio 1:1 (mean±standard deviation)

Entity	Sample		
	W [a]	W – EtOH [b]	Precipitated
Volume before salt precipitation	100 ml	–	–
Volume after salt precipitation	–	185 ml	15 ml
Salt	254.21±0.58 g/l	83.12±0.13 g/l	670±0.21 g/l
Dry matter	25.09±0.2 % w/w	8.93±0.21 % w/w	70.09±0.31 % w/w
Water	74.91±0.17 % w/w	44.35±0.21% w/w	14.01±0.14 % w/w
Ethanol	–	46.72±0.13 % w/w	15.9±0.11 % w/w
Protein	3.67±1.21 g/l	1.95±1.02 g/l	0.37±0.98 g/l
Free amino acids:			
Aspartic acid	0.765±0.05 g/l	0.412±0.03 g/l	0 g/l
Glutamic acid	1.171±0.02 g/l	0.631±0.04 g/l	0 g/l
Arginine	0.108±0.07 g/l	0.059±0.09 g/l	0 g/l
Creatine	2.687±0.01 g/l	1.45±0.05 g/l	0 g/l
Glycine	0.097±0.03 g/l	0.052±0.09 g/l	0 g/l
Lysine	0.486±0.07 g/l	0.263±0.06 g/l	0 g/l
Methionine	0.230±0.11 g/l	0.124±0.07 g/l	0 g/l
Phenylalanine	0.369±0.04 g/l	0.200±0.08 g/l	0 g/l
Taurine	0.228±0.04 g/l	0.123± 0.01g/l	0 g/l
Tryptophan	0.184±0.02 g/l	0.100±0.03 g/l	0 g/l

[a] Wastewater before ethanol addition
[b] Wastewater-ethanol mixture after salt precipitation

As such, the lower amount of salt precipitated in wastewater can be partially attributed to the higher polarity of the wastewater-ethanol mixture, which increased salt solubility above the blank-ethanol mixture. At same time, NaCl and ethanol contributed to the solubilisation of some amino acids in the wastewater-ethanol mixture. Aqueous solubility of a nonelectrolyte, like amino acids, is generally dependent on the concentration and type of salt present in solution, and the salt effect is described by the Setschenow equation (Ni & Yalkowsky, 2003). According to the latter, solubility of

polar amino acids increases by increasing NaCl concentration while the solubility of nonpolar analogues is decreased, and this happens because polar salts, like NaCl, produce a continuum that is more polar than pure water. An identical effect on the solubility of amino acids is generated by the addition of ethanol to water (Palecz & Nadolna, 2006). Codfish salting process wastewater contains 10 major free amino acids having different physicochemical behaviour, and the amount of hydrophilic amino acids − aspartic acid, glutamic acid, creatine and lysine − predominated (Table 5.2). HPLC-UV analysis of wastewater before ethanol addition and in wastewater-ethanol mixture after salt precipitation showed that no precipitation of amino acids occurred (Table 5.2), which account for the higher dielectric constant of wastewater-ethanol mixture in respect to the blank-ethanol one. The pH of the wastewater-ethanol mixture, pH 6, might also account for this result because the zwitterion form of the amino acids − which exist at intermediate pH − has a large effect on increasing their solubility since they are strong dipoles that easily bond to water and ethanol (Orella & Kirwan, 1991).

Table 5.3 NaCl precipitated along 120 min at 27 ºC (ambient temperature) and at a sample:ethanol ratio of 1:1 (v/v). Values are expressed in % (mean ± standard deviation)

Sample	Precipitation of NaCl depending on time						
	6.5 min	13 min	20 min	25 min	30 min	60 min	120 min
Blank solution	$34.05\pm0.48^{a, A}$	$32.25\pm0.67^{b, A}$	$31.21\pm0.46^{c, A}$	$30.78\pm0.48^{c, A}$	$29.53\pm0.48^{c, A}$	$29.67\pm0.51^{d, A}$	$29.11\pm0.61^{d, A}$
Wastewater	$14.94\pm0.55^{a, B}$	$18.61\pm0.67^{b, B}$	$25.10\pm0.34^{c, B}$	$25.53\pm0.22^{c, B}$	$26.17\pm0.35^{d, B}$	$26.21\pm0.44^{d, B}$	$26.20\pm0.61^{d, B}$

Values in the same line that are not followed by the same lowercase superscript letter are statistically different ($p\leq0.05$)
Values in the same columns that have not the same capital superscript letter are statistically different ($p\leq0.05$)

Table 5.4 NaCl precipitated depending on ethanol added to sample (v/v) at 27 ºC (ambient temperature) and after 30 min. Values are expressed in % (mean ± standard deviation)

Sample	Precipitation of NaCl depending on percentage of ethanol added						
	20 %(v/v)	40 %(v/v)	60 %(v/v)	80 %(v/v)	100 %(v/v)	120 %(v/v)	140 %(v/v)
Blank solution	$7.14\pm0.11^{a, A}$	$12.69\pm0.14^{b. A}$	$19.12\pm0.34^{c, A}$	$24.39\pm0.45^{d, A}$	$30.24\pm0.55^{e, A}$	$35.12\pm0.51^{f, A}$	$40.32\pm0.49^{g, A}$
Wastewater	$1.71\pm0.07^{a, A}$	$5.42\pm0.26^{b, B}$	$11.05\pm0.13^{c, B}$	$19.22\pm0.53^{d, B}$	$26.12\pm0.48^{e, B}$	$26.15\pm0.29^{e, B}$	$26.21\pm0.56^{e, B}$

Values in the same line that are not followed by the same lowercase superscript letter are statistically different ($p\leq0.05$)
Values in the same columns that have not the same capital superscript letter are statistically different ($p\leq0.05$)

Table 5.5 NaCl precipitated depending on temperature at a sample:ethanol ratio of 1:1 (v/v) and after 30 min. Values are expressed in % (mean ± standard deviation)

Sample	Temperature			
	0 ºC	10 ºC	15 ºC	27 ºC
Blank solution	$37.15\pm0.36^{ab, A}$	$33.56\pm0.78^{bc, A}$	$32.35\pm0.58^{cd, A}$	$30.23\pm0.63^{d, A}$
Wastewater	$32.71\pm0.52^{a, B}$	$29.34\pm0.47^{b, B}$	$28.81\pm0.55^{b, B}$	$26.15\pm0.58^{c, B}$

Values in the same line that are not followed by the same lowercase superscript letter are statistically different ($p\leq0.05$). Values in the same columns that have not the same capital superscript letter are statistically different ($p\leq0.05$).

Regarding proteins, 1.44 %(w/w) has precipitated with salt at the best extracting conditions, and precipitated phase shows the same protein composition of wastewater (recall paraghraph 3.3 of Chapter 3) and wastewater-ethanol mixture supernatant (Figure 5.1). In Figure 5.1, myosin heavy chains (205 kDa) and other bands originated from myosin heavy chain fragmentation between 205 and 97.4 kDa, which account for heavy meromyosin (and its subunits) and light meromyosin, are clearly visible. The band appearing at 78 kDa may be either tropomyosin or a remaining of light meromyosin, as reported elsewhere (Thorarinsdottir et al., 2202). Actin appeared visible with a band at ca. 45 kDa. The peptide of ca. 8.2 kDa, can be a product of myosin degradation.

Myofibrillar proteins have been found in wastewater as a consequence of the "salting-out" effect throughout the codfish salting process (Ferraro et al., 2011). Protein solubility is a function of the dielectric constant, ionic strength, pH and temperature of a solution (Zayas, 1997). A salting-in/salting-out effect phenomenon is common to most proteins: increasing salt concentration initially improves solubility (salting-in), but after a maximum, solubility is depressed (salting-out) (Gagnon et al., 1997).

Protein solubility is a function of the dielectric constant, ionic strength, pH and temperature of a solution (Zayas, 1997). A salting-in/salting-out effect phenomenon is common to most proteins: increasing salt concentration initially improves solubility (salting-in), but after a maximum, solubility is depressed (salting-out) (Gagnon et al., 1997). As such, proteins of white muscles, like cod proteins, are completely soluble in diluted salt solution, i.e. in solution of physiological ionic strength (9 g/L NaCl) or less, at a neutral pH (Hultin et al.,2007).

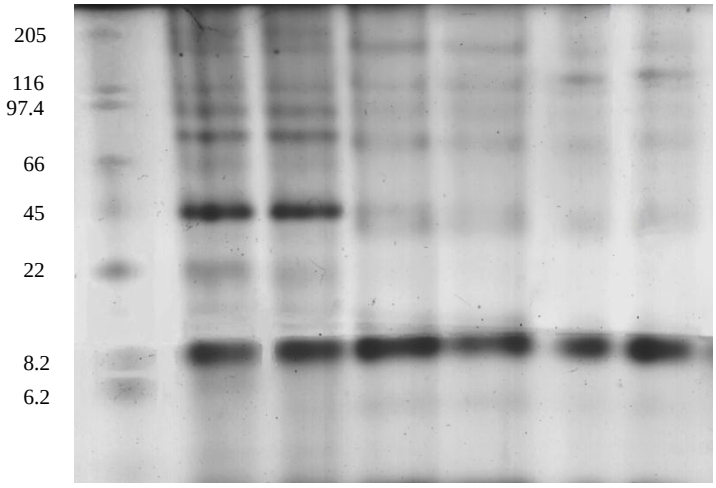


Figure 5.1 SDS-Page electropherogram of samples. First lane represents standards mixture with weight expressed in kDa. Second and third lanes refer to wastewater; fourth and fifth lanes refer to wastewater-ethanol mixture supernatant; sixth and seventh lanes represent the precipitated phase

Myosin, the major myofibrillar protein, is an expecption since being soluble at higher ionic strength (> 18 g/L NaCl) because of the hydrophobicity of its surface (Gagnon et al., 1997). Weakly polar solvents, like ethanol, methanol or acetone, cause denaturation of secondary and tertiary structure of proteins. As such, their solubility decreases markedly due to an increase in strength and number of intra and intermolecular electrostatic attraction, which result in proteins aggregation and precipitation (Hultin et al.,2007). As reported by Simpson (2004), most proteins larger than 20 kDa precipitate from aqueous solutions when concentration of organic solvent reaches ca. 50 %(w/w). Typically, larger proteins and more hydrophilic proteins (like myosin rod) precipitate at a lower organic solvents concentration, while higher solvent concentrations are required for smaller and hydrophobic proteins (like myosin head) (Smith, 2010). However, performing salt precipitation at 0 ºC should limit protein precipitation since protein solubility increases by decreasing temperature, due to increasing of the dielectric constant (Zayas, 1997). Also, denaturation induced by ethanol can be reduced by the low temperature of salt precipitation since codfish proteins in physiological solution dos not undergone denaturation at temperatures in the range - 4ºC < T < 30ºC (Arai et al., 1976).

5.3.2.1 One-variable-at-time-approach methodology

5.3.2.1.1 Amount of NaCl precipitated depending on time

When the effect of time on salt settling has been investigated results showed that blank- and wastewater-ethanol mixtures needed the same period of time to reach the equilibrium, ca. 30 min. However, precipitation trends of salt in blank- and wastewater-ethanol mixtures have been different (Figure 5.2). In the wastewater-ethanol mixture, the amount of salt precipitated increased with time (from ca. 15 to ca. 26.%) ($p \leq 0.05$) up to 30 min, and remained constant between 30 and 120 min ($p > 0.05$) (Table 5.3). On the contrary, in the blank-ethanol mixture, salt has easily precipitated during the first 6.5 min (34 % NaCl precipitated), but it has been dissolved again until reaching an equilibrium value at 30 min (ca. 30 % NaCl precipitated), after which has remained constant up to 120 min ($p > 0.05$) (Table 5.3).

At the equilibrium time of 30 min, the amount of salt precipitated has been higher in blank than in wastewater-ethanol mixture and a difference of ca. 3% was observed.

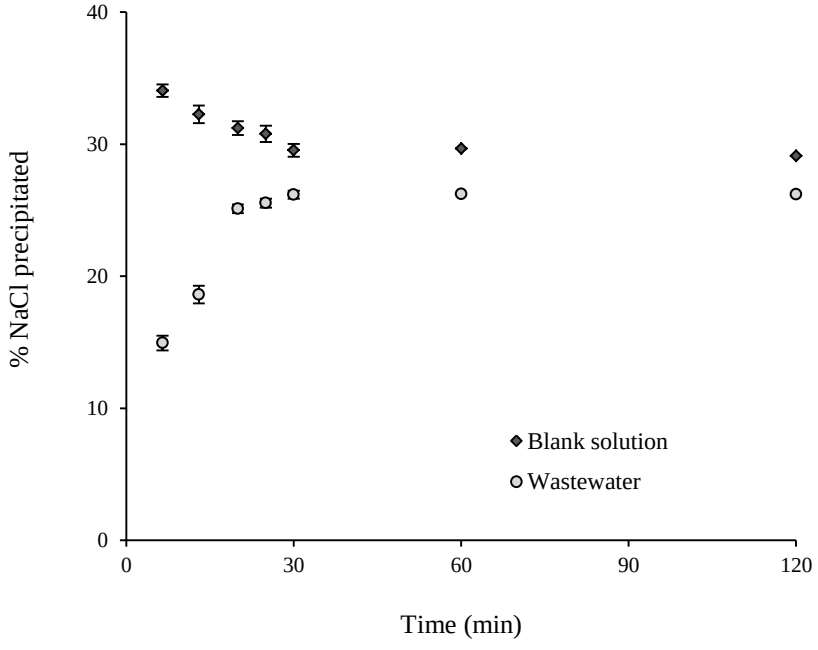


Figure 5.2 Salt precipitation trends in blank solution and wastewater after 100 ml of ethanol added to 100 of sample, along 120 min (mean values ± standard deviation bars)

Although in the blank-ethanol solution salt precipitated more quickly, NaCl particles that have been rapidly pulled down by gravity tended to migrate to region of lower concentration according to the Fick's law and until establishing a dynamic equilibrium with NaCl in the supernatant phase (Sivasankar, 2008); that condition was achieved at 30% of NaCl precipitated (Figure 5.2). The slower precipitation rate and the lower amount of NaCl precipitated in the wastewater-ethanol solution could be attributed to the presence of proteins and hydrophobic amino acids, and two phenomena can be hypothesised. First, the large surface of proteins in solution made the precipitation of NaCl particles difficult, and as such an amount of the salt settled onto proteins and floated along with them. On the other hand, proteins and hydrophobic amino acids were kept in solution due to NaCl bridges with water – the salting-in phenomenon (Hultin, 2007) – so that an amount of NaCl continued bonded to protein and amino acids even after ethanol addition, limiting salt precipitation.

5.3.2.1.2 Amount of NaCl precipitated depending on ethanol added to samples

The effects of variable volumes of ethanol added to the samples on salt precipitation have been studied at 27 ºC and at a defined time period of 30 min. As the volume of ethanol increased, the amount of salt has precipitated significantly and has linearly increased ($p \leq 0.05$) in blank-ethanol mixture (from ca. 7 to ca. 40 %) in all the range studied (20-140 % ethanol added). In the wastewater-ethanol mixture the amount of salt precipitated has significantly increased only up to 100 % ethanol added (from ca. 1.7 to ca. 26 %); no significant differences ($p > 0.05$) in amounts of sodium chloride precipitated in wastewater have been observed by increasing the proportion to ethanol over 1:1 (v/v) (Table 5.4 and Figure 5.3). As such, it is possible to conclude that ethanol has had a positive effect on salt settling in the wastewater-ethanol mixture, however only up to a ratio 1:1. At that proportion, yield of precipitation has been 4% higher in blank- in respect to wastewater-ethanol mixture.

At constant temperature, solubility of hydrophilic amino acids increases by increasing ethanol concentration, while solubility of hydrophobic amino acids decreases, however only up to 45% (w/w) of ethanol in water, since no significant change in polarity occurs over this concentration (Orella & Kirwan, 1991; Ji et al., 2009). This phenomenon can

largely explains why salt precipitated in the wastewater-ethanol mixture has not increased by increasing ethanol addition up 100% v/v (Figure 5.3).

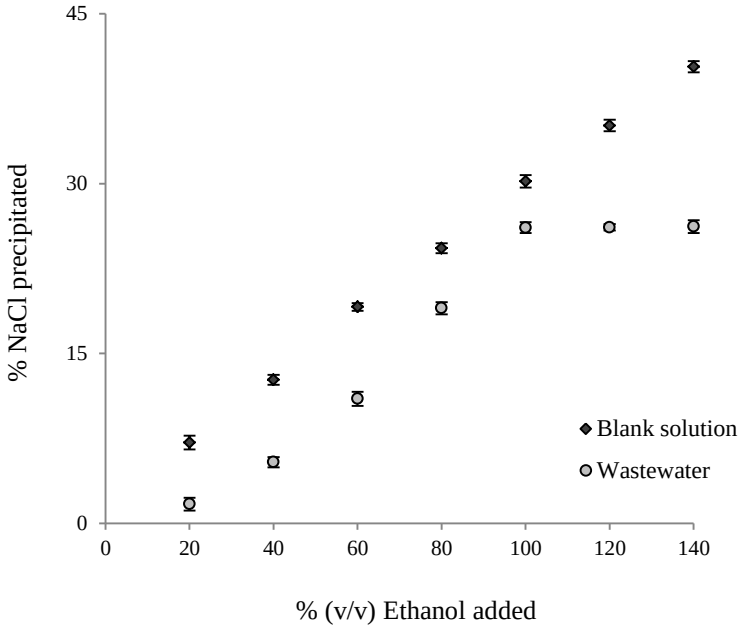


Figure 5.3 Salt precipitation trends in blank solution and wastewater along ethanol addition, after 30 min and at 27 ºC (mean values ± standard deviation bars)

5.3.2.1.3 Amount of NaCl precipitated depending on temperature

The effects of temperature have been analysed at a sample:ethanol ratio 1:1 (v/v) and at the plateau time of 30 min. Both samples have shown the same tendency: linear and significant increasing ($p \leq 0.05$) of percentage of salt precipitated have been observed in blank-ethanol mixture (from ca. 30 to ca. 37%) and wastewater-ethanol mixture (from ca. 26 to ca. 33 %) by decreasing temperature from 27 ºC to 0 ºC (Table 5.5 and Figure 5.4).

For each value of temperature tested, yield of precipitation of sodium chloride has been ca. 4% higher in blank- than in wastewater-ethanol mixture; however, the increase in amount of salt precipitated by decreasing temperature from 27 to 0 ºC has been the same in both samples, ca. 7% (Table 5.5). Sodium chloride solubility in water is slightly

affected by temperature: Pinho and Machedo (2005) reported a decrease of ca. 0.051 % for a decrease of 1 ºC, in the range 0-30 ºC. On the contrary, in aqueous-ethanol solutions, Farelo (2004) reported a decrease of solubility of ca. 0.26 % for a decrease of 1 ºC in the same range of temperature, 0-30 ºC, and for a ratio ethanol to water of 1:1 (v/v).

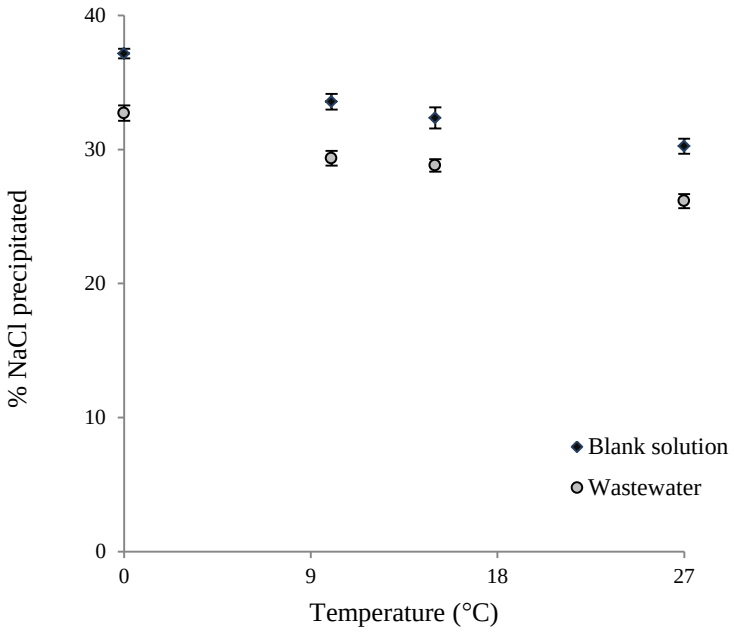


Figure 5.4 Salt precipitation trends in blank solution and wastewater along temperature increasing, after 30 min and for 100 ml of ethanol added to 100 ml of sample (mean values ± standard deviation bars)

5.3.2.1.4 Amount of NaCl precipitated depending on pH

The effects of pH change were examined at 27 ºC and at a sample:ethanol ratio of 1:1. Results revealed that no increase in salt precipitation occurred either by acidifying or alkalinizing the samples, which can be attributed to the absence of the common ion effect as discussed in section 3.1.

5.3.2.2 Experimental design methodology

Fractional factorial design has been established only for codfish salting wastewater, and by the assumption that not significant differences in salt precipitated from the wastewater-ethanol mixture are noticed by increasing ethanol up 100 % (v/v) (Figure 5.3). The second-order fitted surface of response (Figure 5.5) generated by factorial design data (Table 5.6) shows no maximum points but a trend, which corroborates the results found by the one-variable-at-a-time approach: ca. 33 % salt precipitated at the optimum operating conditions of 0 ºC, 100 % (v/v) ethanol added and 30 min duration. As can be seen in the Pareto graph of Figure 5.6, the response (% salt precipitated) is significantly dependent upon a linear effect of each factor ($p>0.05$), and ethanol showed the greatest effect. As expected, temperature dependence of response is not as pronounced as ethanol (Figure 5.6).

Table 5.6 Matrix of data (mean values) for factorial design

Temperature (ºC)	% (v/v) of Ethanol added		
	20	60	100
0	9.06	25.35	32.71
10	7.31	23.23	29.34
27	1.71	11.05	26.15

Quadratic effects of variables, which can be expected for factor levels greater than 2 (Jiju, 2003), have been not significant as well as has not been the interaction between variables ($p\leq0.05$) (Figure 5.6 and Figure 5.7).

Results of experimental design have confirmed the one-variable-at-a-time approach outcomes. however, only 9 experiments were needed for the fractional factorial design versus the 18 runs performed in the one-variable-at-a-time methodology

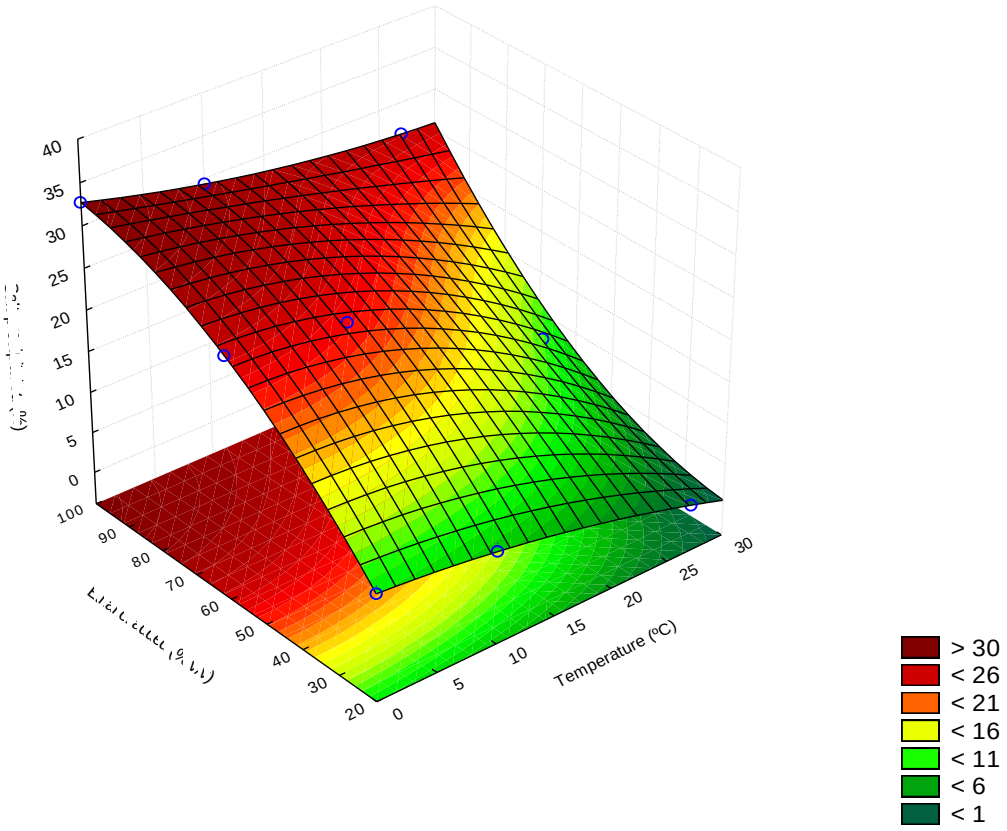


Figure 5.5 Second-order fitted surface of response (% salt precipitated)

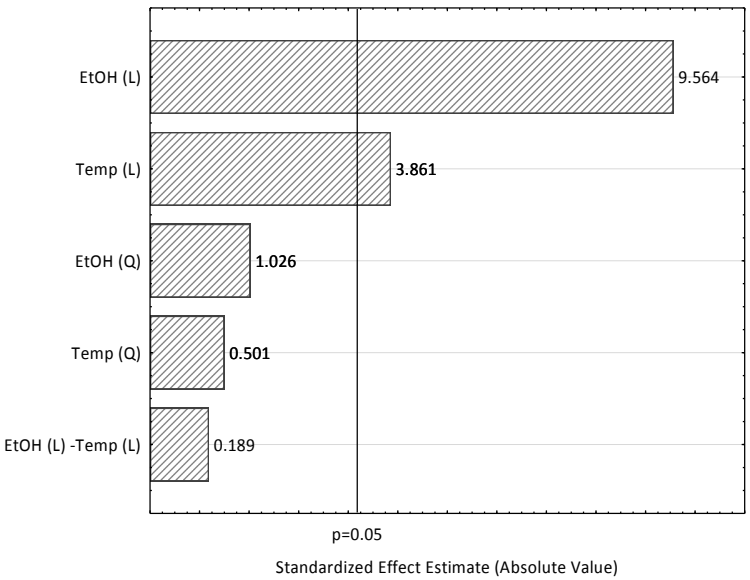


Figure 5.6 Pareto plot of the standardised effects of the two factors ("EtOH"- ethanol; "Temp"- temperature) on the response (salt precipitated). "L" stands for "linear", "Q" stands for "quadratic". Vertical line defines significance limit expressed by the *p*-value at a confidence level of 95%

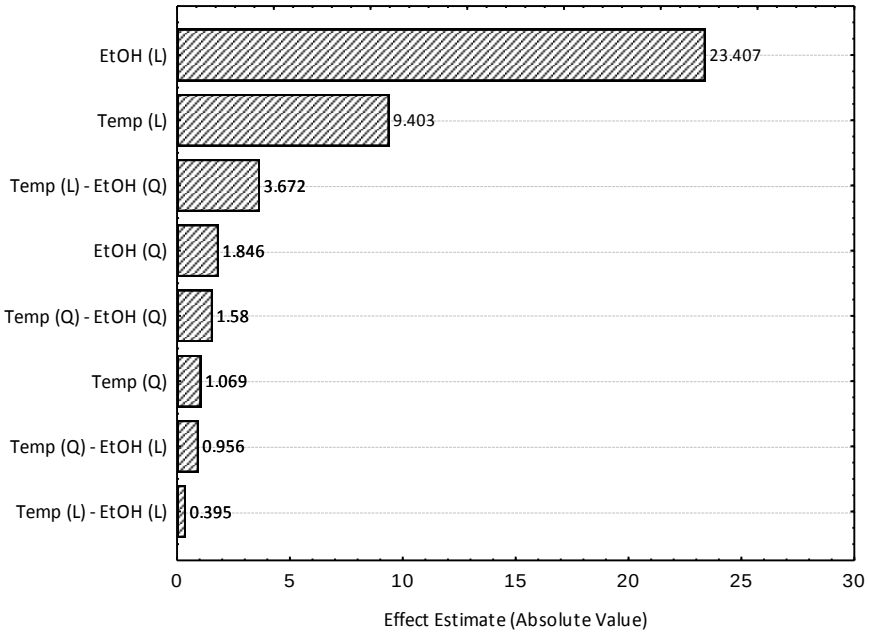


Figure 5.7 Pareto plot of the effects of the two factors ("EtOH" – ethanol; "Temp" - temperature) on the response (salt precipitated). "L" stands for "linear", "Q" stands for "quadratic"

5.4 Conclusion

Extraction of salt from codfish salting wastewater by ethanol has been investigated and successfully carried out. Ca. 33 % of salt has been precipitated at the optimal operating conditions of 0 ºC, 100 % (v/v) ethanol added to the wastewater and at a settling time of 30 min. Ionic strenght has been reduced from 4.3 M to ca. 1.4 M in the wastewater-ethanol mixture; no precipitation of amino acids occured, while 1.44 % of proteins felt down with salt in the precipitated phase.

Proteins and free amino acids significantly limited salt precipitation in wastewater; in a blank solution (salt in water at 254 g/L) a higher amount of salt, ca. 37%, has been in fact precipitated in the same conditions. Solvent and temperature have a linear effect on salt precipitation for both wastewater and blank solution. However, ethanol has showed to be the driving factor in salt precipitation while the low temperature helped avoiding proteins precipitation and denaturation. Changes in pH did not exert any effect on salt precipitation.

Amount of salt precipitated could be increased by several extraction stages, each followed by ethanol distillation. Salt contained in the supernatant allows avoiding the

azeotrope formed by water and ethanol, making the recovery of pure ethanol feasible which could be recycled in the next precipitation stage.

Salt precipitation from codfish wastewater by ethanol addition has been demonstrated to be a good strategy in the view of the recovery of amino acids and proteins by adsorption, as well as for the recovery of salt itself.

CHAPTER 6

KINETICS OF ADSORPTION AND DESORPTION OF AMINO ACIDS AND PROTEINS ON *AMBERLITE XAD16* RESIN

6.1 Introduction

Adsorption equilibrium data are very important for understanding the affinity of a compound towards a specific adsorbent, for determining the adsorbent uptaking capacity and the for estimating the effects of environmental conditions on the adsorption yield (as discussed in Chapter 4). At same time, since sorption is a time-dependent process, it is also crucial to know the kinetics and the mechanism of adsorption and desorption of adsorbate from the resin (Rajic et al., 2010). Overall kinetic model describing the amounts of compound adsorbed on the resin along time is not enough, *per se*, to elucidate neither the adsorption mechanisms nor the specific rate-controlling step (Ruthen, 1984). So that, some specific mathematical models must be tested in order to clarify the mechanisms of adsorption. Rate limiting step elucidation is crucial for the design of sorption systems; it is strongly dependent on the porosity of the adsorbent and, in a lower amount, on the operating conditions at which sorption is carried out (Valiullin & Dvoyashkin, 2007).

Regarding desorption, elution can be accomplished in a number of ways depending on the nature of compounds to be desorbed. Four basic methods are in common use although only two are suitable for desorption of liquid: the thermal swing and the displacement desorption that may also be combined with advantage in some situations (Ruthven, 1984). In thermal swing, the adsorbent is regenerated by heating, at a constant pressure, usually with a stream of hot gases or liquids, at a temperature suitable for the removing of adsorbed species. In displacement desorption, pressure and temperature are maintained constant (at the normal ambient values) and the adsorbed species are displaced by a stream containing a competitively adsorbed species. With respect to *Amberlite XAD16*, methanol, ethanol, acetone and isopropanol have been proved to be useful solvents for amino acids displacement. Concentrated bases (2-4% NaOH) have been demonstrated to be efficient for proteins and peptides desorption, where a dilute oxidising agent (< 0.5%) can be also added to enhance the removal of protein fouling (Rohm&Haas, 2010). Since physical desorption is an endothermic process (McCabe et al., 1993), the combination of thermal swing and displacement desorption could improve compounds recovery.

In this chapter are reported kinetics and mechanism of adsorption and desorption of free amino acids and proteins from the ethanol-wastewater mixture obtained after salt precipitation from the codfish salting processing wastewater as described in Chapter 5.

6.2 Theoretical backgound of kinetics and mechanism of adsorption and desorption

Kinetics of physical adsorption, based on the overall uptaking rate by adsorbent, has been found generally described by a pseudo-first-order rate expression also called "*Lagergren firs-order rate model*" (Tan & Hameed, 2010), as follow:

(**Eq** 6.1) $$r_{ad} = \frac{dC_{ad}}{dt} = k_{ad} \cdot (C_{ad\infty} - C_{ad})$$

where C_{ad} (g/g) represents the amount adsorbed on the resin at time t (h), k_{ad} (h^{-1}) is the time dependent adsorption constant, and $C_{ad\infty}$ (g/g) is the amount adsorbed on the resin when equilibrium is reached. Integration of Equation 6.1 by separating variables leads to the following relation:

(**Eq.** 6.2) $$C_{ad} = C_{ad\infty} \cdot (1 - e^{-k_1 \cdot t})$$

Kinetics expression for the overall adsorption rate describes the evolution of compounds uptaking through time but do not supply any information about the mechanism of adsorption. According to the well established theory proposed by Boyd *et al.* (1947) adsorption of organic or inorganic compounds over porous adsorbents occurs in three sequential steps:

1. film diffusion, where adsorbate molecules travel towards the external surface of the adsorbent;
2. particle diffusion, where adsorbate molecules travel within the pores of the adsorbent;

3. adsorption of the compounds on the interior surface of adsorbent (the small amount of the adsorption that occurs on the exterior surface of the adsorbent is generally considered not significant).

Each one of these steps is characterised by a specific resistance whose relative value determines whether the step is limiting the overall adsorption process (McKay et al., 1987). If the external transport resistance is higher than the internal transport resistance, rate is controlled by film diffusion; if the internal transport resistance is higher than the external transport resistance, rate is controlled by particle diffusion. If the external and internal resistances are similar, the transport of a compound through the boundary adsorbent layer may not be possible at a significant rate, and formation of a liquid film surrounding the adsorbent particles takes place through the proper concentration gradient (Tan & Hameed, 2010). Adsorption itself (third step) is much faster than both diffusion steps so that it cannot be treated as rate limiting step (Valiullin & Dvoyashkin, 2007). Consequently, a two resistances model based on external film mass transfer and internal pore diffusion is generally applied over a wide range of physical adsorpiton phenomena (McKay et al., 1987).

Specifically, intraparticle diffusion includes two phenomenon: (a) diffusion within the pore volume (known as pore diffusion), and (b) diffusion along the surface of pore (known as surface diffusion) (Meško et al., 1999). Internal diffusion rate increases with the dimension of the adsorbent pores (which can be micropores, mesopores or macropores or even etherogeneous pores). *Amberlite XAD16* is a mesoporous resin (2-50 nm pore diameter) with average pore dimension of ca. 10 nm, and for which the surface diffusion has been demonstrated to be the dominant intraparticle diffusion mechanism (O'Connor et al., 2006; Valiullin & Dvoyashkin, 2007).

Kinetic data obtained by the batch adsorption method can be elucidated by the mathematical models proposed by Reichenberg (1952) and by Weber and Morris (1962), both based on the observations of Boyd *et al.* (1947). These mathematical models allow identifying rate-limiting steps: film diffusion or intraparticle diffusion, or eventually both.

The model proposed by *Reinchenberg* is based on the following expression:

(**Eq.** 6.3) $$F = \frac{C_{ad}(t)}{C_{ad}(\infty)} = 1 - \frac{6}{\pi}\sum_{n=1}^{\infty}\frac{e^{-n^2 \cdot Bt}}{n^2}$$

where F represents the amount adsorbed on the resin as a fraction of the amount adsorbed after infinite time; $C_{ad}(t)$ and $C_{ad}(\infty)$ (both expressed in g/g) represent the compound concentration on the resin at each time and at infinite time, respectively; B_t is the Boyd dimensionless time-dependent parameter. Depending on the F value, relation between F and B_t can be expressed in two different ways, as follow:

(**Eq.** 6.4) $$B_t = 6.2832 - 3.2899 \cdot F - 6.2832 \cdot (1 - 1.0470 \cdot F)^{1/2} \quad \text{for} \quad 0 \le F \le 0.85$$

(**Eq.** 6.5) $$B_t = -\ln(1 - F) - 0.4977 \quad \text{for} \quad 0.86 \le F \le 1$$

The plot obtained by representing Boyd parameter B_t versus time allows identifying the adsorption controlling step. If the plot is a straight line and passes through the axis origin, the adsorption process is governed by particle diffusion. If the plot is not a straight line and does not pass through the axis origin, the adsorption process is governed by film diffusion. If the plot is not a straight line but passes through the origin, the adsorption process is governed by film diffusion up to a certain time, after which pore diffusion becomes the controlling mechanism.

The model proposed by *Weber* and *Morris* is based on the following mass-transfer model:

(**Eq.** 6.6) $$C_{ad}(t) = k_d \cdot t^{1/2} + I$$

where $C_{ad}(t)$ represents the amount of compounds adsorbed on the resin at time t, and is expressed in g/g; t is time expressed in min; k_d is the diffusion rate constant and is expressed in $g \cdot g^{-1} \cdot min^{-1/2}$; I is the intercept and give an idea of the boundary layer thickness, i.e. the larger the intercept the grater the boundary layer effect. If the plot of $C_{ad}(t)$ versus $t^{1/2}$ is a straight line over the all processing time and passes through the origin, then intraparticle diffusion is the sole adsorption limiting step. Otherwise some

other mechanism along with intraparticle diffusion is also involved. In this study, both theories have been employed to elucidate the mechanism of adsorption.

The adsorbed chemicals will remain on the resin surface nearly indefinitely, provided that no forces are applied on the resin surface (heat, pressure, solvents, etc.). As resin environment is modified desorption occurs, and the overall rate of the process can be expressed by the follow equation (Ruthven, 1984):

(**Eq.** 6.7) $$r_{des} = -\frac{dC_{ad}}{dt} = k_{des} \cdot C_{ad}{}^{n}$$

where k_{des} is the time dependent desorption constant, C_{ad} (g/g) is the concentration of the compounds on the resin at each time, and n is the order of the desorption rate. Physical desorption generally occurs according to a first-order kinetic so that n=1 (Paserba & Gellman, 2000; Ruthven, 1984). Integration of equation 6.7 by separating variables leads to the expression:

(**Eq.** 6.8) $$C_{ad} = C_{ad}(0) \cdot e^{-k_{des} \cdot t}$$

where $C_{ad}(0)$ (g/g) is the amount adsorbed on the resin at the zero desorption time, which corresponds to the amount adsorbed on the resin after the adsorption step. However, for a practical point of view, it is easier to use the kinetic expression correlating the concentration of desorbed compound in the eluting mixture with time, because of it is determined directly by sample analysis. So that equation 6.8 will be substituted by the following pseudo-first-order expression:

(**Eq.** 6.9) $$C_{des} = C_{des\infty}(1 - e^{-k_{des} \cdot t})$$

where C_{des} (g/L) is the concentration of the desorbed compound in the eluting mixture and $C_{des\infty}$ (g/L) is the concentration of the desorbed compound in the eluting mixture at equilibrium.

The mechanism of desorption is the result of at least two possible contributions. The first is competition of the eluting solvent with the adsorbed solute for the adsorbent

surface. The second one is the effect of the eluting solvent on the activity of the solute in the liquid phase, based on the preferential partitioning of the solute between resin and solvent (Grzegorczyk & Carta, 1996).

6.3 Materials and Methods

6.3.1 Solution of free amino acids and proteins

Solution containing free amino acids and proteins to be recovered by sorption is that described in the second column of Table 5.2, as obtained after partial salt precipitation by ethanol addition to the codfish salting processing wastewater. That mixture contained 50% (v/v) ethanol and 1.42 M NaCl (83 g/L NaCl).

6.3.2 Batch adsorption and desorption of free amino acids and proteins

Experimental adsorption and desoprtion data have been obtained with the following procedures. The highest recovery has been achieved carrying out two runs of adsorption and two runs of desorption.

6.3.2.1 Adsorption

Adsorption has been executed at the conditions optimised for the recovery of taurine, as reported in Chapter 4. Temperature allowing for the highest recovery has been determined by Equation 4.11 and taking in account the ionic strength of the actual solution. When equation 4.11 is solved for the maximum recovery and at 1.42 M ionic strength, a temperature of 12 ºC is obtained.

A volume of 100 ml of solution of free amino acids and proteins has been added to a 250 ml capacity Erlenmeyer flask, placed onto a 12 ºC thermostated stirring plate, where mixing has been achieved by a magnetic bar. When temperature of solution has reached the desired value of 12ºC, an amount of 10 g of dry *Amberlite XAD16* resin pre-treated as reported in paragraph 4.4.3.1 at page 86, has been added to the flask and adsorption started. Once steady-state has been reached – as determined by sample analysis along time – the process has been stopped, the resin has been separated from

the solution by filtration with a 230 Mesh (0.053 mm) stainless steel strainer and desorption has been carried out thereafter. Mixture remained in Erlenmeyer flask, and partially depleted of amino acids and proteins, has been keep away until undergoing a second adsorption step.

The effect of stirring regime on adsorption kinetic and mechanism has been evaluated by studying three different agitation extents: 150, 300 and 500 rpm.

Percent adsorption yield, Y_{ad}(%), has been determined as follow:

(**Eq.** 6.5) $$Y_{ad}(\%) = \frac{C_{ad} \cdot W_d}{(C_0 / 1000) \cdot V} \cdot 100$$

where C_{ad} (g/g) represents the concentration of compound on the resin and has been calculated by the following equation:

(**Eq.** 6.6) $$C_{ad} = \frac{C_0 \cdot V_0 - C \cdot (V_0 + \Delta V)}{1000 \cdot W_d}$$

where C_0 (g/L) is the initial concentration of compund in the wastewater-ethanol mixture, C (g/L) is the compound concentration after adsorption, V_0 is the initial volume of amino acid solution (ml), ΔV is the dilution brought into amino acid solution by addition of conditioned and wet resin, and expressed as $\Delta V = (W_w - W_d)/\rho_{H2O}$, where W_w is the wet resin weight (g), W_d is the dry resin weight (g) and ρ_{H2O} is the density of water (g/ml) at the experimental temperature.

6.3.2.2 Desorption

Resin retained in the strainer has been added to a 250 ml capacity Erlenmeyer containing the eluting solution. Free amino acids and proteins have been desorbed selectively, where free amino acids have been desorbed first.

For free amino acids elution two different food-grade solvents have been tested, namely acetone and ethanol (Sigma-Aldrich). The effects of two different temperatures – 20 and 30 ºC, and the effects of three different volumes of eluting solvent – 20, 40 and 60 ml –

on amino acids desorption yield have been also studied. Desorption has been carried out at 300 rpm.

After free amino acids have been desorbed, the resin has been separated from the eluted solution and has been transferred to a 250 ml capacity Erlenmeyer flask for proteins elution. Proteins have then been desorbed by adding to the *Amberlite XAD16* a strong solution of sodium hydroxide (NaOH) (Sigma-Aldrich), at a rate of 4% (w/v) (4 g of NaOH in 100 ml of solution) in distilled water. The effects of two different temperatures and three different volumes of eluting solvent on proteins desorption yield have been studied: 20 and 30ºC, and 20, 40, 60 ml, respectively. Desorption has been carried out at 300 rpm.

Percent desorption yield, Y_{des}(%), has been determined as follow:

(**Eq.** 6.7) $$Y_{des}(\%) = \frac{(C_{des}/1000)\cdot V_{el}}{C_{ad}\cdot W_r}\cdot 100$$

where C_{des} (g/L) is the compound concentration in the eluting solvent volume V_{el} (ml), C_{ad} is the amount of compound (g/g) retained on the resin and W_d is the dry resin weight (g).

After the first desorption cycle has been concluded, the resin retained in the strained has been washed with distilled water to displace the NaOH solution and a second adsorption step has been accomplished as described in previous paragraph.

Total process yield in percent, Y_{total}(%), has been calculated as follow:

(**Eq.** 6.8) $$Y_{total}(\%) = \frac{C_{des}\cdot V_{el}}{C_0\cdot V_0}\cdot 100 \qquad \text{or} \qquad Y_{total}(\%) = \frac{Y_{ad}\cdot Y_{des}}{100}$$

6.3.3 HPLC-UV/Vis analysis

Chromatographic analysis of free amino acids have been performed as reported in paragraph 3.2.2 of Chapter 3, at page 54. In the case of HPLC analysis of eluted amino acids solution, 5 ml of have been dryied with nitrogen and dry matter has been redissolved in dislilled water prior to derivatisation.

6.3.4 Assessment of protein and electrophoretic analysis

Proteins have been determined as reported in paragraph 3.2.4 of Chapter 3, page 56. Electrophoretic analysis has been performed as reported in paragraph 3.2.5 of Chapter 3, at page 56. An amount of 5 ml of sample have been dryied with nitrogen and dry matter has been redissolved in dislilled water prior to the determination.

6.3.5 Determination of salt

Total salt content has been determined as reported in paragraph 3.2.7 of Chapter 3, at page 57.

6.3.6 Modelling of experimental data

Modelling of experimental data and analysis of variance (one-way ANOVA) have been performed with the software *STATISTICA v.9.0*.
Adequacy of kinetic models proposed for adsorption and desorption of free amino acids and proteins has been evaluated by two goodness-of-fit criteria, namely coefficient of determination (R^2) and standard error of regression (SER) (Shumaker 1992) assuming a confidence level of 95% ($p \leq 0.05$).

6.3.7 Scanning Electron Microscopy of *Amberlite XAD16*

Amberlite XAD16 resin has been scanned by electron microscopy before and after pretreatment, after adsorption, after desorption of amino acids and after desorption of peptides and proteins. The scanning electron microscope Jeol JSM-5600 (Jeol-IZASA, Carnaxide, Portugal) has been used for the purpose with the following scanning conditions: accelerating voltage 15 kV, vacuum 10 Pa, magnification 50X.

6.4 Results and Discussion

Codfish wastewater-ethanol mixture to be adsorbed carried a salt load of ca. 83 g/L and an organic load of 5.36 g/L, composed of 3.41 g/L of free amino acids and 1.95 g/L of proteins. So that 100 ml of initial mixture contained ca. 0.54 g of organic load.
Proteins have been totally removed from the wastewater-ethanol mixture while a rate of 76 % has been achieved for total free amino acids. Of these, hydrophobic free amino acids and taurine have been removed at a rate of 100%. All the organic matter collected on the resin through adsorption has been totally removed by desorption.
The overall sorption process – constituted of two adsorption cycles and two desorption cycles – has needed a time of 380 min (or 4 h and 20 min) with a total recovery of 83 % of organic matter. In Figure 6.1 are reported some pictures of *Amberlite XAD16* along the overall sorption process.

6.4.1 Adsorption

Adsorption of free amino acids and proteins has been accomplished by two adsorption cycles carried out at 12º C and 300 rpm. For each adsorption cycle, maximum yield has been achieved after 70 min. Second adsorption cycle has been carried out after the first desorption stage which is discussed in next session.
By the end of the first adsorption cycle 42 % of the organic matter present in the mixture has been collected on the resin: an amount of 0.23 g has been retained on *Amberlite XAD16* of which 55 % (0.13 g) have been proteins and remaining 45 % (0.10 g) have been amino acids, where the higher hydrophobicity nature of myofibrillar proteins accounted for the higher adsorption yield with respect to amino acids. After the first adsorption cycle, 65 % of proteins and 29 % of amino acids present in the wastewater-ethanol mixture have been collected with the resin. An amount of 0.6 g of NaCl has been retained by the resin, corresponding to 7.3 % of salt contained in the wastewater-ethanol mixture.
Second adsorption cycle allowed the recovery of 83 % of organic matter remained in the mixture after the first adsorption cycle, equivalent to 41 % of organic load contained in the initial wastewater-ethanol mixture. An amount of 0.07 g of proteins and 0.14 g of free amino acids has been retained on the resin, corresponding to 35 and 65 % of

organic matter in respectively. Same amount of salt as first adsorption cycle, 0.6 g, has been retained on *Amberlite XAD16*.

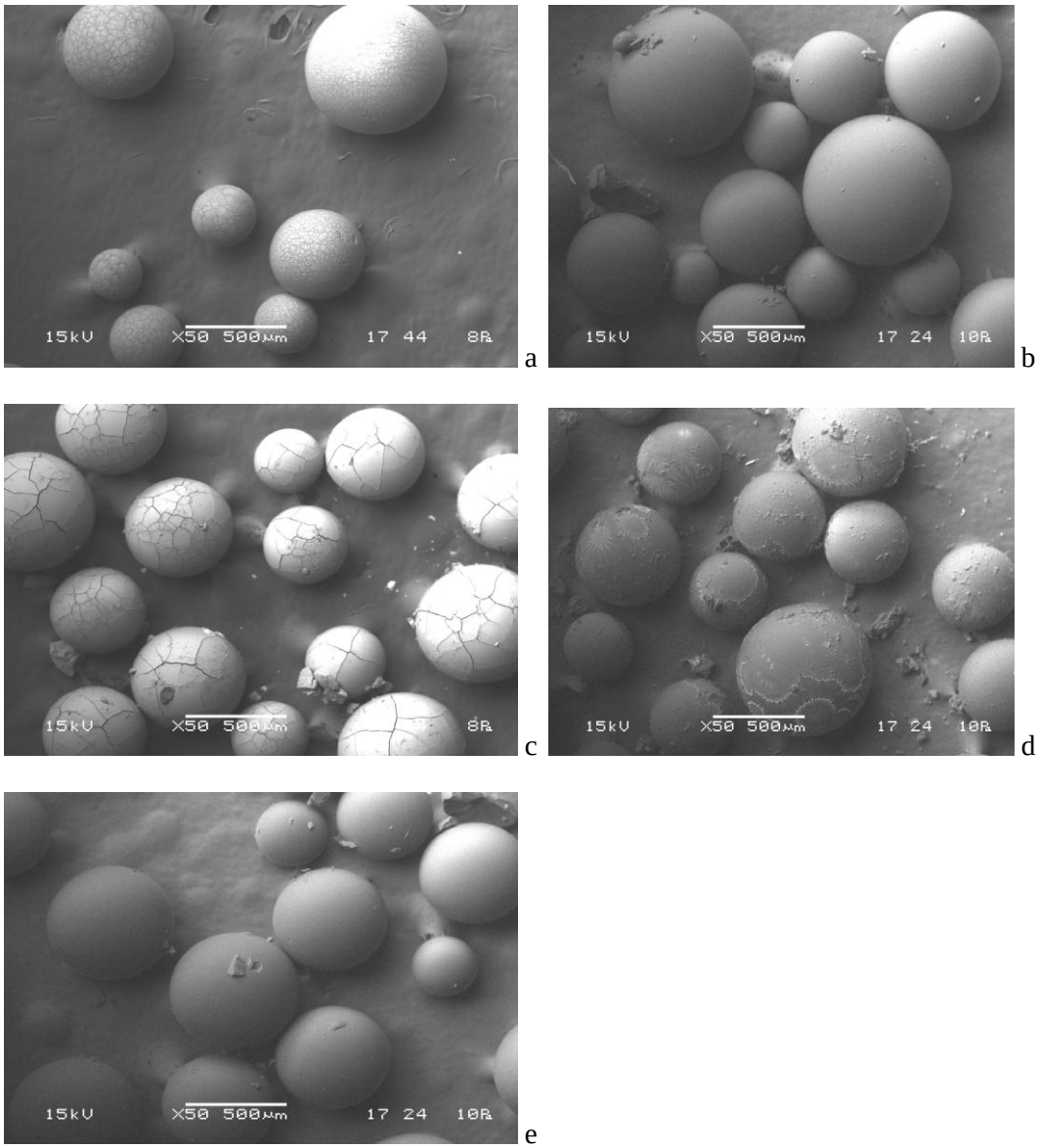


Figure 6.1 Scanning electron microscopy images of *Amberlite XAD16* along the sorption process: (a) *Amberlite XAD16* before washing; (b) washed *Amberlite XAD16*; (c) *Amberlite XAD16* after adsorption; (d) *Amberlite XAD16* after free amino acids desorption; (e) *Amberlite XAD16* after proteins desorption

Chromatographic and spectroscopic analysis of composition of mixtures undergoing adsorption showed that adsorption yields have been higher for hydrophobic species – proteins, phenylalanine, tryptophan and methionine – and for taurine, than for glycine and hydrophilic amino acids (Table 6.1). This result is in accordance with *Amberlite XAD16* specificity toward hydrophobic compounds and with the results for equilibrium study reported in Chapter 4. In Figure 6.2 are reported the yields of adsorption cycle for proteins and free amino acids along time.

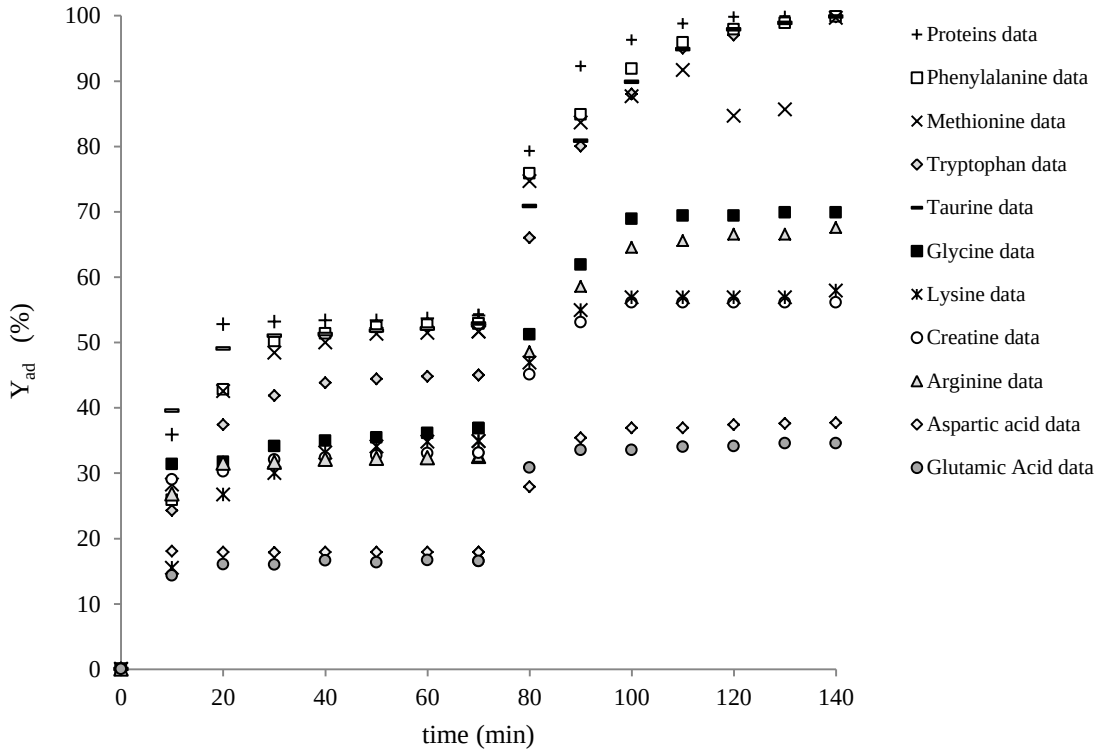


Figure 6.2 Yields of adsorption for proteins and free amino acids along time (mean values ± standard deviation) at 12ºC and 300 rpm. Data from 0 to 70 min represent yields along the first adsorption cycle; data from 70 to 140 min represents yields along the second adsorption cycle.

Table 6.1 Kinetic parameters ($C_{ad\infty}$ and k_{ad}, in g/g and min^{-1} respectively), statistic parameters (R^2 and SER) and yield after first and second adsorption cycles (in %) for proteins and free amino acids, at 12ºC and 300 rpm

Entity	Parameter							
	$C_{ad\infty} \cdot 10^4$ (I cycle)	k_{ad} (I cycle)	$C_{ad\infty} \cdot 10^4$ (II cycle)	k_{ad} (II cycle)	R^2	SER·10^4	Y_{Ad} (I cycle)	Y_{Ad} (II cycle)
Proteins	102	0.115	82	0.120	0.98	0.104	55	100
Aspartic acid	6.1	0.430	6.7	0.390	0.98	0.011	18	38
Glutamic acid	8.3	0.177	9.3	0.155	0.99	0.023	16	35
Arginine	1.8	0.166	1.9	0.178	0.97	0.005	33	68
Creatine	44	0.217	39	0.204	0.98	0.010	32	60
Glycine	1.8	0.206	1.8	0.187	0.98	0.011	35	70
Lysine	3.4	0.056	3.4	0.064	0.99	0.009	35	55
Methionine	6.2	0.077	5.7	0.067	0.98	0.004	52	100
Phenylalanine	10.5	0.070	9	0.067	0.97	0.022	54	100
Tryptophan	4.3	0.082	5.3	0.090	0.99	0.013	45	100
Taurine	6.3	0.130	5.6	0.144	0.99	0.009	53	100

Total recovery of proteins has been achieved by the two adsorption cycles while for free amino acids maximum adsorption yields have been obtained for phenylalanine, tryptophan, taurine and methionine (100 % recovery); lowest adsorption yields have been obtained for aspartic and glutamic acids (38 and 36 % respectively) (Table 6.1).
Free amino acids and proteins have been adsorbed according to a pseudo-first order kinetic (Eq. 6.1) as resulted from experimental data modelling. In Figure 6.3 are reported the amount of compounds adsorbed on the resin along time during the first adsorption cycle as an example. Kinetic and statistic parameters relative to the first and second theoretical adsorption model are summarised in Table 6.1.

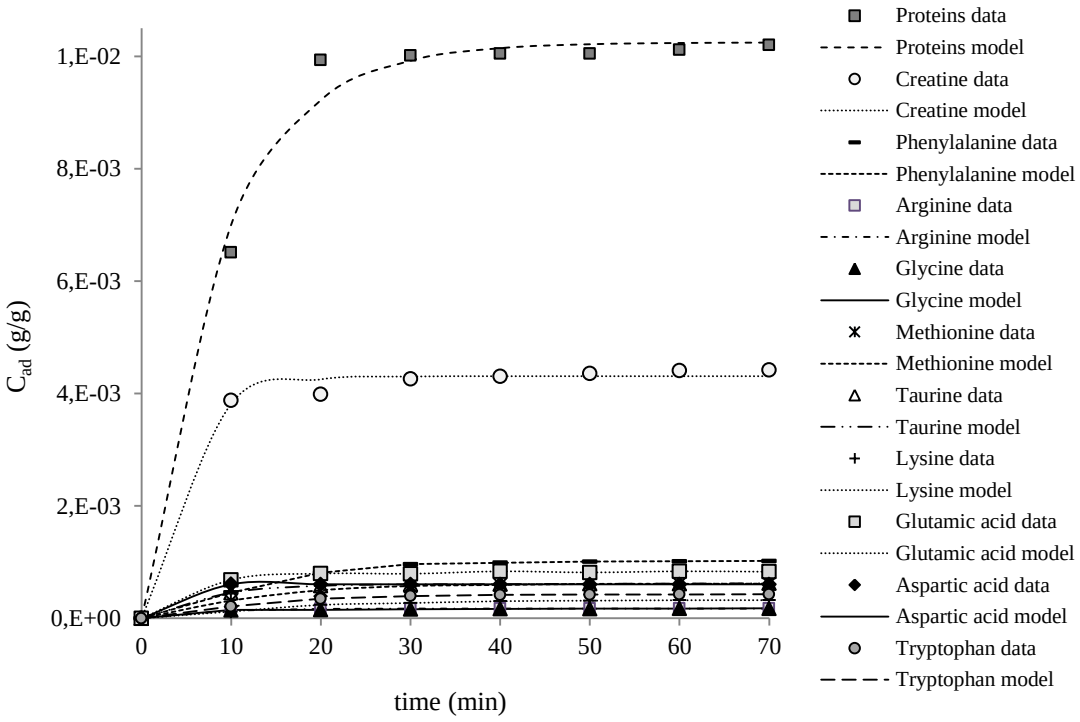


Figure 6.3 Amount of proteins and free amino acids adsorbed on the resin along time (mean values ± standard deviation) during the first adsorption cycle, at 12ºC and 300 rpm

6.4.1.1. Elucidation of mechanism of adsorption

In order to elucidate the mechanism of adsorption, the model of *Reinchenberg* (Eq. 6.3) and the model of *Weber* and *Morris* (Eq. 6.4) have been used.

When the *Reichenberg* model has been tested, the plot of B_t versus time has been a curve passing through the axis origin for all the amino acids and for proteins (Figure 6.4). Hence, it could be concluded that adsorption process has been controlled by two mechanisms, i.e. film and particle diffusion.

Results obtained by testing the *Weber* and *Morris* model lead to the same conclusion. As it is possible to see in Figure 6.5, the plots of C_{ad} versus time$^{1/2}$ are not linear, meaning that adsorption has been controlled by film and particle diffusion. Each plot consisted of two linear segments. The first segment, with a shaper slope, can be attributed to the diffusion through the external surface of the adsorbent, i.e. the boundary layer, or film diffusion. Second segment reflects a gradual adsorption stage which is characterised by the intra-particle diffusion into *Amberlite XAD16* channels and vacancies (Rajic et al., 2010). The slope of each linear portion indicates the rate of the corresponding adsorption, where a lower slope describing a slower adsorption process. Therefore, from Figure 6.4, it follows that the film diffusion (at the beginning stages) proceeded faster than the intraparticle diffusion (at later stages).

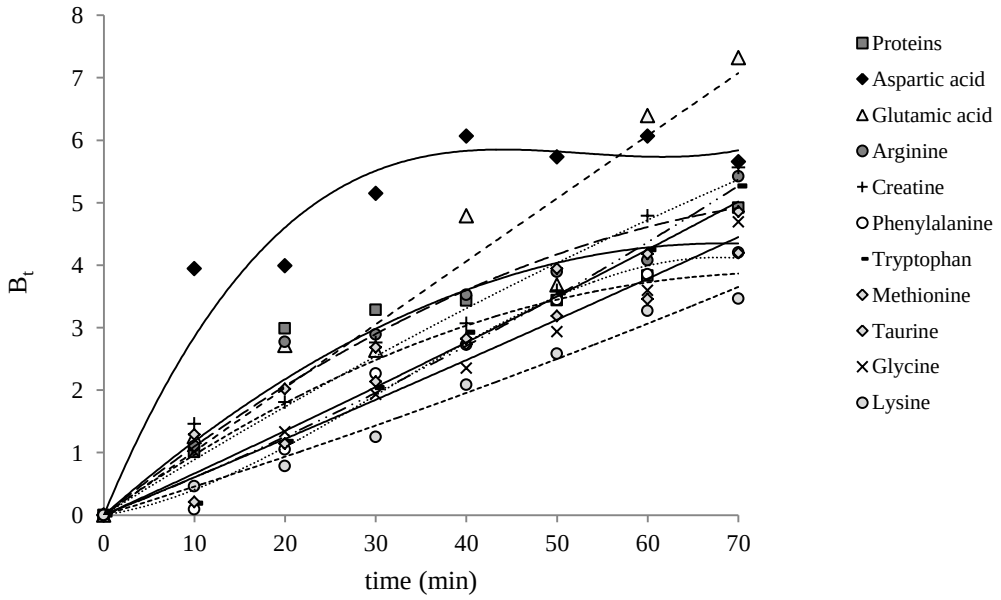


Figure 6.4 Plot of B_t versus time (*Reichenberg* model)

Boundary layer effect has been more important for proteins and for creatine than for remaining amino acids, as can be observed for the value of the intercept *I* (recall equation 6.6) of the second segment (Figure 6.5). The deviation from the origin of this segment, which reflects a near saturation, might be due to the difference in the mass transfer rate in the initial and final stage of adsorption (Mohanty et al., 2005).

The presence of ethanol in solution may have allowed for the reduction of intraparticle diffusion resistance. As reported by Kong and co-workers (2005), the solvent reduce the infiltration pressure of a solution into the pore of hydrophobic material, as in the case of *Amberlite XAD16*.

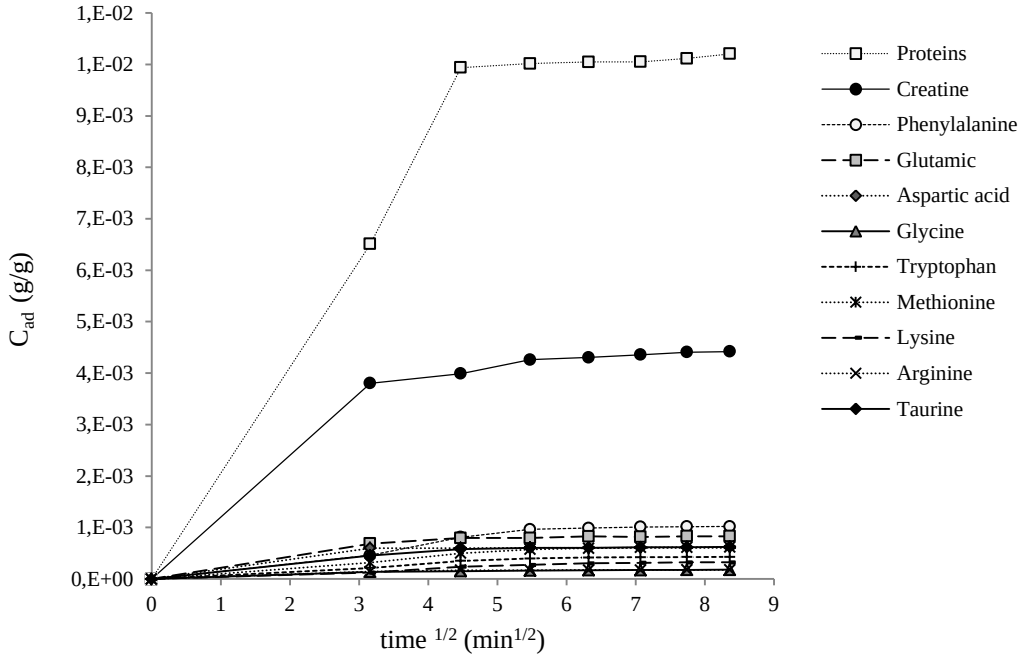


Figure 6.5 Plot of C_{ad} (g/g) versus time$^{1/2}$ (*Weber & Morris* model)

6.4.1.2 Effect of the agitation speed on adsorption

Results of effect of agitation speed onto adsorption yield showed that the optimum value is 300 rpm. The lower speed of 150 rpm and the higher speed of 500 rpm led to the same yields; however more time has been needed. When agitation speed is

decreased, mobility of both resin and compounds in solution is reduced, so that it can be hypnotised that interaction between them diminished, and the effect of the boundary layer has been more evident. On the contrary, when agitation speed has been increased, relative speed of resin toward solution has been high so as contact time between them could be not sufficient for the uptaking (Mckay et al., 1987). In Figure 6.6 are reported adsorption curves for taurine depending on agitation speed, for the suck of comparison.
Values of external mass transfer coefficient have been estimated by using the expression for the initial mass transfer rate, when the mass transfer resistance is restricted to the external boundary layer and the surface concentration is assumed negligible (Gogoi et al., 2010):

(**Eq.** 6.9) $r = k_f \cdot S_r \cdot (C_0 - C_s)$ where $C_s=0$.

Combination of equation 6.9 with equation 6.1, expressed as a function of compound in solution instead of amount of compound adsorbed on the resin, leads to the follow relation:

(**Eq.** 6.10) $k_f = -\frac{1}{S_r} \cdot \left[\frac{d(C_0 - C_t)/C_0}{dt} \right]_{t=0}$ where $S_r = \frac{6 \cdot W_d}{d_p \cdot \rho_p \cdot (1-\varepsilon_p)}$

S_r is the external surface of the resin (mm^2), C_0 and C_t (g/mm^3) are compound concentration in solution at $t=0$ and at a time t respectively, W_d is the resin weight (g), d_p is the average particles diameter (mm), ρ_p is the specific gravity (g/mm^3) and ε_p is the porosity of resin particles (mm^3/mm^3).
Best agitation speed has been found to be 300 rpm. At this regime, mass transfer coefficient for taurine is $k_f=1.94 \cdot 10^{-4}$ $mm^{-2}s^{-1}$, while smaller values have been found for 150 rpm and for 500 rpm, with $k_f = 1.51 \cdot 10^{-4}$ $mm^{-2}s^{-1}$ and $1.25 \cdot 10^{-4}$ $mm^{-2}s^{-1}$ respectively.

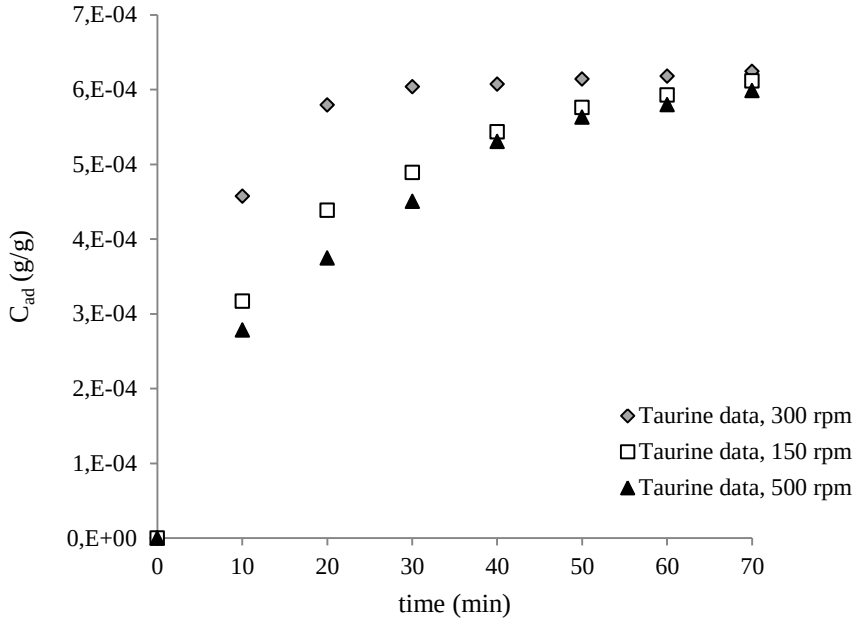


Figure 6.6 Amount of taurine adsorbed on *Amberlite XAD16* (mean values ± standard deviation) along time depending on agitation speed

6.4.2 Desorption

Free amino acids and proteins have been desorbed selectively by using acetone and a strong alkaline solution of NaOH (4% w/v) respectively. Maximum yields have been achieved at the optimal operating conditions consisting of a temperature of 30ºC and a volume of eluting solvent of 40 ml. Each desorption cycle of both free amino acids and proteins required a time of 120 min.

Selective desorption of free amino acids and proteins can be attributed to the stronger hydrophobicity character of proteins. Even though proteins are soluble in acetone, like free amino acids, their hydrophobic interactions with *Amberlite XAD16* could not be broken just by acetone so that a stronger medium, as the alkaline NaOH solution, has been needed. In Figure 6.7 is reported an electopherogram of wastewater-ethanol mixture, of free amino acids mixture eluted with acetone and of proteins mixture eluted with NaOH solution, all after second desorption cycle.

Desorption occurred according to a pseudo-first order kinetic (Eq. 6.9) as resulted from experimental data modelling. In Figure 6.8 are reported desorption yields along time while in Figure 6.9 are reported concentration of free amino acids and proteins in the

respective eluting solvents during the first desorption cycle as an example of desorption trend. Kinetic and statistic parameters of the process, as well as yields of desorption and yields of the overall sorption process are summarised in Table 6.2.

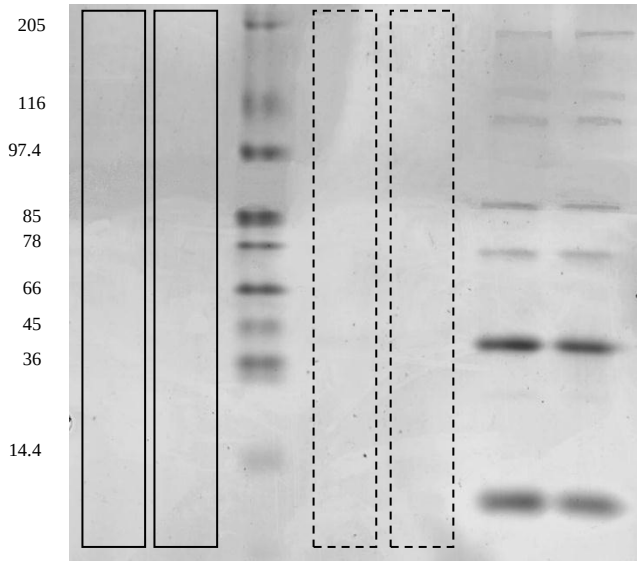


Figure 6.7 SDS-Page electopherogram of samples. From left to right, first and second lanes (enclosed by the first and the second solid line rectangles respectively) represent mixture of free amino acids eluted by acetone; third lane represents standard mixture with molecular weight expressed in kDa; fourth and fifth lanes (enclosed by the third and the fourth dotted line rectangles respectively) represent the wastewater-ethanol mixture after second adsorption cycle; sixth and seventh lanes represent the proteins mixture eluted by the NaOH solution.

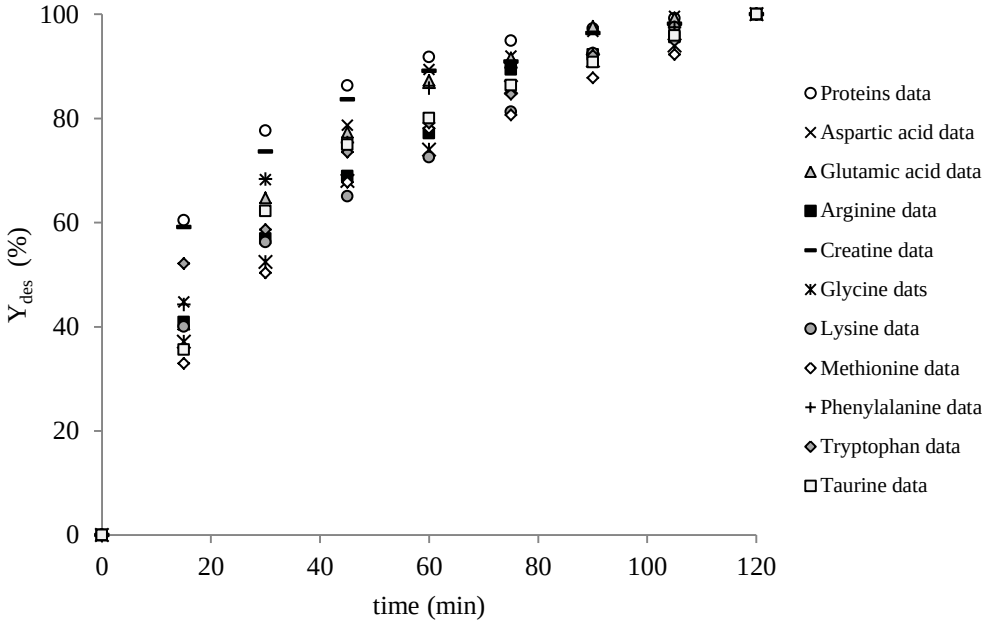


Figure 6.8 Yields of desorption cycle for proteins and free amino acids along time. Experimental data (mean values ± standard deviation) at 30ºC, 40 ml of eluting solvents and 300 rpm

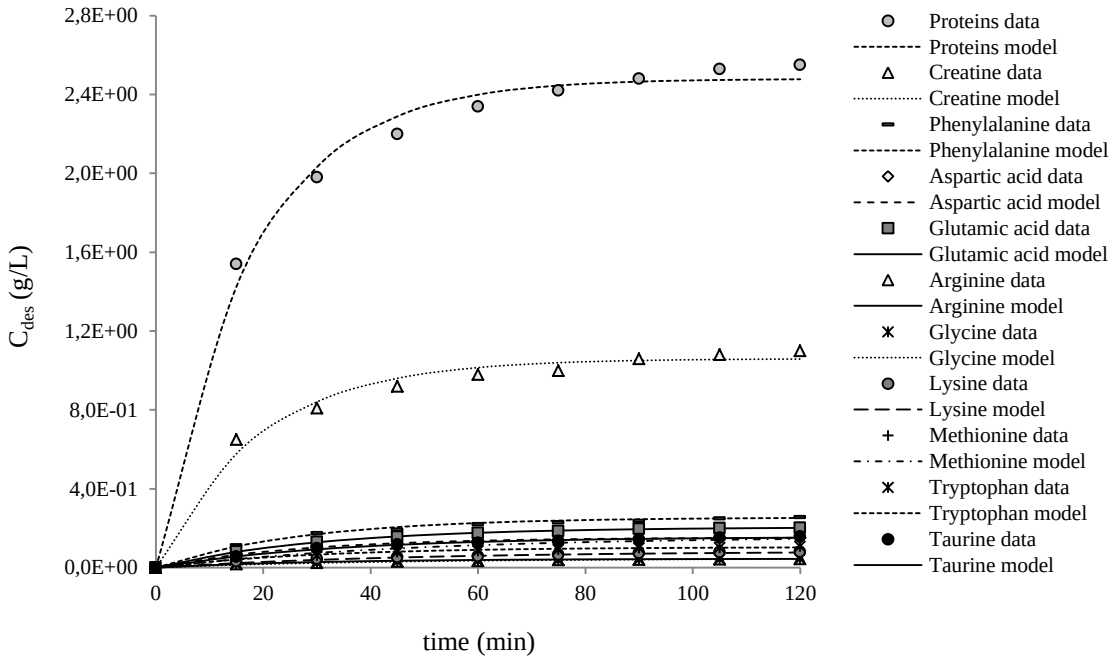


Figure 6.9 Concentration of proteins and free amino acids (mean values ± standard deviation), in 40 ml 4% NaOH solution and 40 ml of acetone respectively, along time and during the first desorption cycle, at 30ºC and 300 rpm

Table 6.2 Kinetic parameters ($C_{des\infty}$ and k_{des}, in g/L and min^{-1} respectively), statistic parameters (R^2 and SER), yields of desorption cycles and total sorption yields (%) for free amino acids and proteins, at the best operating condition

Entity	Parameter								
	$C_{des\infty}$ (I cycle)	k_{des} (I cycle)	$C_{des\infty}$ (II cycle)	k_{des} (II cycle)	R^2	SER·10^4	Y_{Des} (I cycle)	Y_{Des} (II cycle)	Y_{total}
Proteins	2.53	0.057	2.16	0.063	0.99	0.321	100	100	100
Aspartic acid	0.20	0.037	0.22	0.045	0.98	0.011	100	100	38
Glutamic acid	0.29	0.033	0.35	0.034	0.99	0.023	100	100	34
Arginine	0.05	0.026	0.05	0.035	0.97	0.005	100	100	68
Creatine	1.5	0.053	1.3	0.054	0.98	0.010	100	100	60
Glycine	0.05	0.023	0.05	0.025	0.98	0.011	100	100	70
Lysine	0.23	0.021	0.13	0.023	0.99	0.009	100	100	55
Methionine	0.18	0.023	0.16	0.025	0.98	0.004	100	100	100
Phenylalanine	0.30	0.036	0.26	0.035	0.97	0.022	100	100	100
Tryptophan	0.12	0.033	0.14	0.031	0.99	0.013	100	100	100
Taurine	0.17	0.030	0.15	0.029	0.99	0.009	100	100	100

Mixture of free amino acids eluted by acetone contained NaCl at a concentration of 15 g/L and amount released in the eluent corresponded to the total amount of NaCl retained on *Amberlite XAD16*. Sodium chloride is scarcely soluble in pure acetone (ca. 0.3 % w/w) and other organic solvents like ethanol (Pinho & Machedo, 2005); however, two factors contributed to solubilisation. First, the amount of water adsorbed on the resin has been solubilised in acetone along with free amino acids and has dissolved NaCl. Second, the presence of free amino acids in solution significantly increased salt solubility in acetone. As reported in previous chapter, it is known that the presence of amino acids in solution significantly increases the polarity of the solution above that of the pure solvent, resulting in an increased solubility of NaCl (Orella & Kirwan, 1991). A less amount of salt has been found in the proteins eluted solution with a concentration of 0.26 g/L.

6.4.2.1 Effect of temperature on desorption yields

Physical desorption is an exothermic process and is favoured by lower temperature than adsorption (McCabe et al., 1993). Optimal temperature for maximum desorption of free amino acids and proteins has resulted to be 30ºC. In Figure 6.10 are reported desorption yields at 30 and 20ºC for proteins and for taurine, as an example for free amino acids.

6.4.2.2 Effects of different volume of eluting solvents on desorption yield

Amount of solute dissolved in the eluent is a function of eluent volume. For both free amino acids and proteins minimum amount of eluent allowing for 100% desorption has been 40 ml. In Figure 6.11 are reported desorption yields for proteins and for taurine, as an example for free amino acids.

6.4.2.3 Effects of eluent nature on desorption yield of free amino acids

Acetone resulted more effective than ethanol in free amino acids removing. Although the solvents have similar polarity, expressed by the dielectric constant (ε) – acetone ε=21, ethanol ε=24 – acetone is an aprotic liquid and for this reason has higher affinity for the aromatic structure of *Amberlite XAD16* than ethanol, which has a protic nature

(Manin et al., 2003). In Figure 6.12 are reported desorption yields for taurine depending on eluent used – ethanol and acetone.

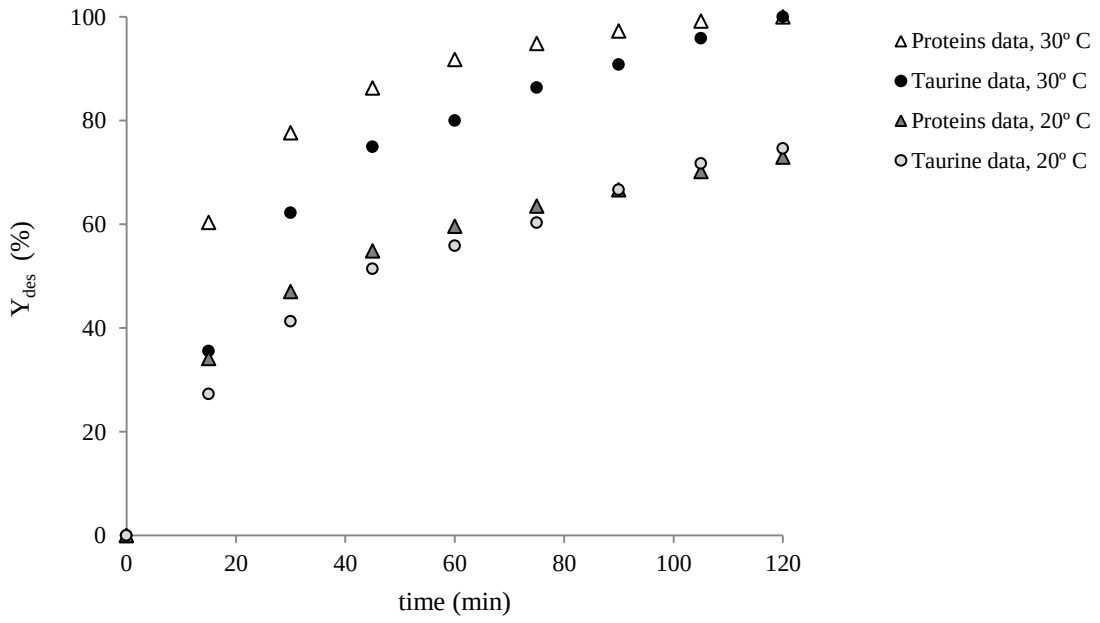


Figure 6.10 Effect of temperature upon proteins and taurine desorption, using 40 ml of acetone for elution

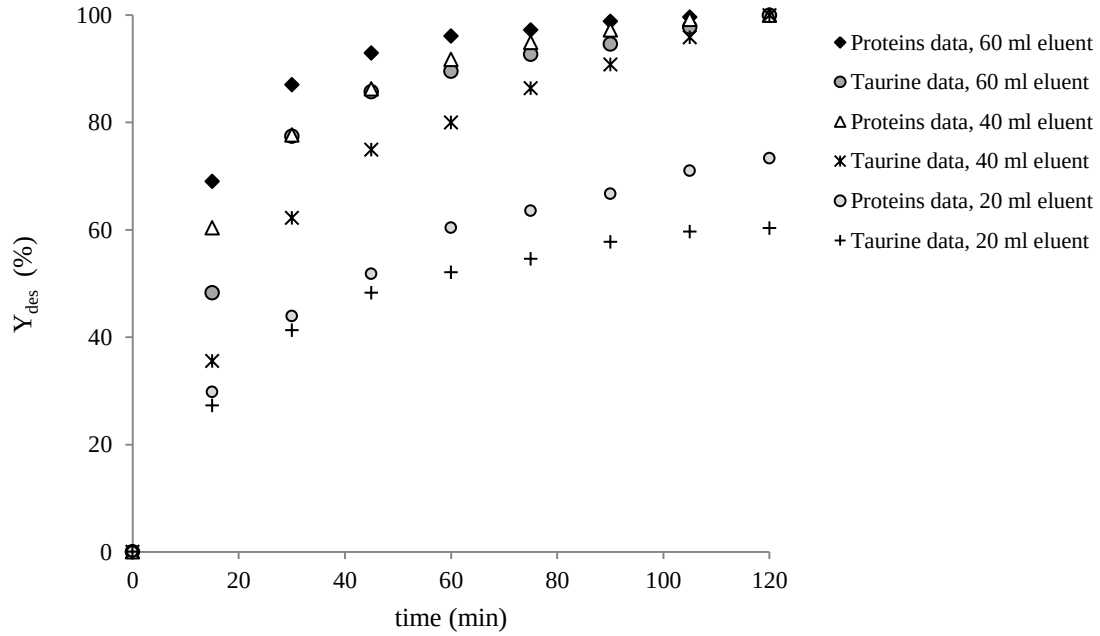


Figure 6.11 Yield of desorption of proteins and taurine depending on volume of eluting solvents

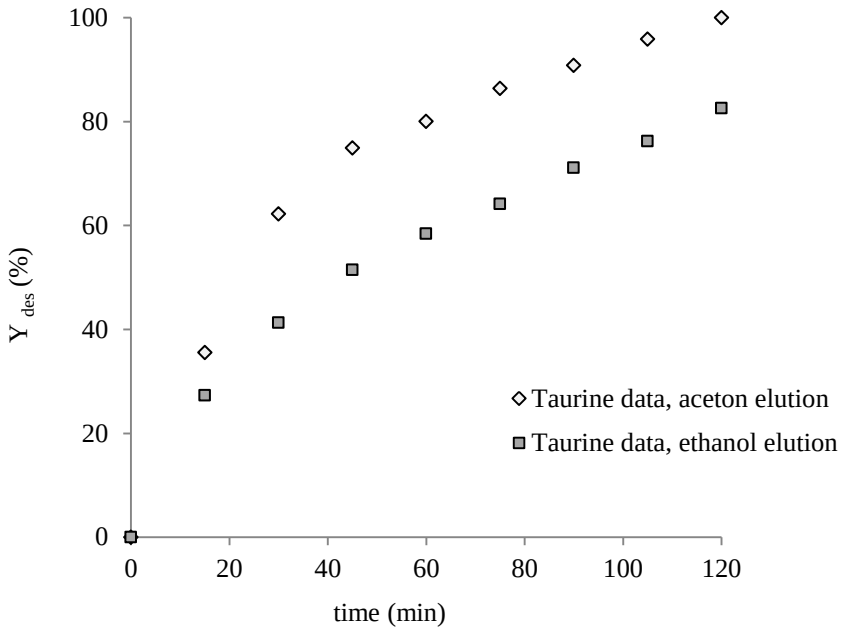


Figure 6.12 Effect of eluent nature upon taurine desorption yield, at 30ºC, 40 ml of eluting solvent (acetone and ethanol)

6.5 Purification of free amino acids and proteins and summary of the overall purification process

Solutions of free amino acids eluted in the first and second desorption cycles have been coupled and the resulting mixture has been evaporated in order to remove acetone. Same operations have been accomplished for proteins solutions. Composition of the respective mixtures on wet (in the respective eluent) and on dry base is reported in Table 6.3.

The overall process for the recovery of free amino acids and proteins from codfish salting processing wastewater is described in the flow diagram reported in Figure 6.13. Purification has been achieved by 12 stages, as follow: storage of codfish salting processing wastewater, centrifugation of wastewater, precipitation of salt from the supernatant obtained by centrifugation, first *Amberlite XAD16* pretreatment, first adsorption cycle of free amino acids and proteins, desorption of free amino acids by acetone, desorption of proteins by an aqueous solution of NaOH at 4% (w/v), second *Amberlite XAD16* pretreatment, second adsorption cycle of free amino acids and proteins remained in wastewater-ethanol mixture after the first adsorption cycle, second desorption cycle of proteins, evaporation of eluents from the desorbed mixtures.

Table 6.3 Concentration of free amino acids and proteins in the eluents and on dry base after evaporation

Mixture	Composition	
Acetone eluted	Wet base (g/L)	Dry base (%)
Aspartic acid	0.21	1.3
Glutamic acid	0.32	1.8
Arginine	0.05	0.3
Creatine	1.41	7.8
Glycine	0.05	0.3
Lysine	0.25	1.4
Methionine	0.17	1
Phenylalanine	0.28	1.6
Taurine	0.16	0.9
Tryptophan	0.13	0.8
NaCl	15	82
NaOH solution eluted	Solution (g/L)	Dry base (%)
Proteins	2.35	90
NaCl	0.26	10

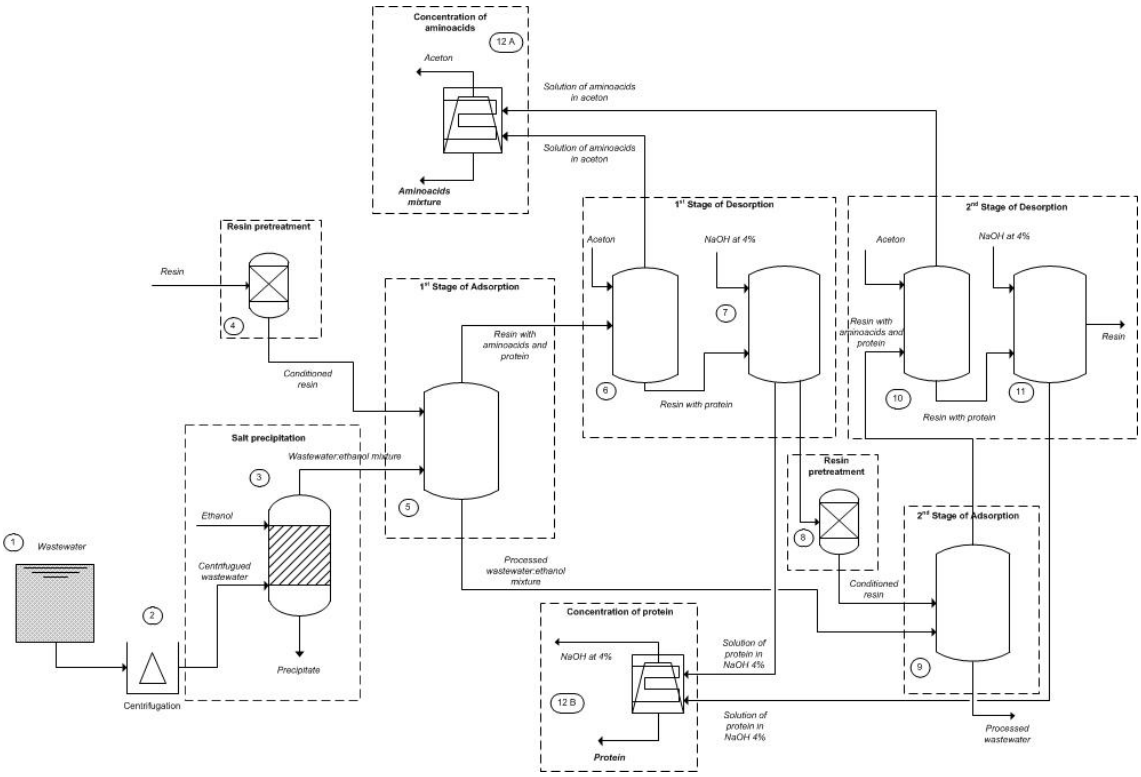


Figure 6.13 Process flow diagram for the extraction of free amino acids and proteins from codfish salting processing wastewater

6.6 Conclusion

The sorption process implemented allowed for the selective recovery of proteins and free amino acids from codfish salting processing wastewater. Total amount of proteins has been extracted; for the free amino acids fraction, a total recovery of 76 % has been obtained where 100 % recovery has been achieved for hydrophobic amino acids. As expected, hydrophobic amino acids – phenylalanine, tryptophan and methionine – and taurine have been totally removed from the mixture, while a lowest yield of recovery has been observed for aspartic and glutamic acid. Acetone resulted to be the best solvent for free amino acids elution when compared with another food-grade solvent such as ethanol.

PART IV

CHARACTERISATION OF FREE AMINO ACIDS EXTRACT

Abstract

Free amino acids extracted from codfish salting processing wastewater can be regarded as a valuable mixture to be introduced into food, nutraceutical or pharmaceutical formulations because it contains mainly essential (methionine, lysine, phenylalanine and tryptophan) and conditional-essential (arginine, creatine and taurine) amino acids, while only three are not-essential (aspartic and glutamic acids, and glycine). Hence, some important aspects have been analysed, namely antioxidant capacity and bioavailability.

Antioxidant capacity of free amino acids mixture has been assessed by ABTS, TEAC and ORAC tests and by the DNA oxidative damage inhibition assay. Results for antioxidant capacity showed that free amino acids mixture does not exhibit pro-oxidant activity upon DNA; also, the mixture can prevent DNA from oxidation if the salinity – 15 g/L – is reduced down to an isotonic level of 9 g/L NaCl. The effect of salinity may be attributed to the generation of reactive oxygen species promoted by the hypertonic levels of NaCl, an effect that has been also visible on the results for ABTS, TEAC and ORAC, where antioxidant capacity was higher for the isotonic mixture of free amino acids than for the hypertonic ones.

In vitro permeability through intestinal epithelium monolayer (obtained by culturing Caco-2 cell line) has been studied in order to predict the *in vivo* bioavailability of amino acids in human, where the term bioavailability means the degree at which a compound is taken up by a specific tissue or organ after administration. Results for bioavailability showed that all the amino acids extracted from codfish salting processing wastewater were able to cross the intestinal epithelium but, in order to avoid cytotoxic effect on intestinal cells, the salinity must be adjusted, by dilution, to an isotonic level. Values for apparent permeability coefficient of isotonic mixture of amino acids, $P_{app} > 10^{-5}$ cm/s, showed that all are very likely to be well absorbed by human intestine. Amino acids have been transported through the epithelium depending on their initial concentration and transport rate has been at least 95%, all expect creatine whose transport has been of 6%.

CHAPTER 7

EVALUATION OF ANTIOXIDANT ACTIVITY OF FREE AMINO ACIDS EXTRACT

7.1 Introduction

The increasing evidence that oxygen reactive species and oxidative damage are involved in several inflammatory and degenerative diseases has stimulated much interest and concern, and has encouraged to more and more in-depth study and research on the antioxidants subject. The word "antioxidant" can be defined in various ways. According to a broader definition, an antioxidant is "any substance that when present at low concentration compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate" (Halliwell, 1990). The term "oxidizable substrate" includes almost everything found in living cells, such as proteins, lipids, carbohydrates and DNA. In a normal cell there is an appropriate pro-oxidant/antioxidant balance, where a pro-oxidant is any toxic oxygen species. Oxidising substances are normally generated by all aerobic organisms, as unwanted by-products of their regular oxygen metabolism, and those organisms accordingly generate antioxidant molecules, aimed at inhibiting damages brought by oxidants in their living tissues (Gião et al., 2007). When production of oxygen species is increased (e.g. following the ingestion of some chemicals or drug) or when levels of antioxidants are diminished, the balance can shifts towards the pro-oxidants, generating an "oxidative stress" state responsible for the alteration of DNA and proteins, and peroxidation of lipids, which can lead, in the long term, to the appearance of some illnesses such as cancer, arteriosclerosis and coronary heart diseases (Ohshima et al, 2006; Barzilay & Yamamoto, 2004; Sohal, 2002).

When an antioxidant is going to be characterised, various questions must be addressed, such as what kind of molecules it is supposed to protect, what the concentration needed, what the mechanism of protection, and also whether the antioxidant cause damages in biological system different from those in which it exerts protection or whether the antioxidant-derived compounds cause biological damage (Halliwell, 1990). In testing putative antioxidant activity it is important to use biologically-relevant reactive oxygen species which are known to be formed *in vivo*, such as O_2 and reactive oxygen species (ROS) such as superoxide, $O_2^{\bullet-}$, that convert into H_2O_2 and the highly reactive species hydroxyl radical, $OH^{\bullet}$, peroxyl radical, $RO_2\bullet$, nitric oxide radical, NO•, thiyl radical, RS•, etc.(Somogyi et al., 2007). Free radicals are capable of independent existence and are highly unstable molecules, having electrons available to react with various organic substrates. Mechanism by which antioxidants act includes decrease or depletion of O_2,

removal of ROS and $O_2^{\bullet}$, scavenging of radicals $RO_2\bullet$ and $OH^{\bullet}$, disruption of chain reactions that have been already initiated, quenching of singlet oxygen, repair of oxidative damage brought by radicals, and enhancing the elimination of damaged molecules (Schlesier et al. 2008).

Apart endogenous (internally synthesised) antioxidants, there are several consumed compounds that have been proposed to act as antioxidant *in vivo*, playing an important role in antioxidant defence. They include β-carotene, albumin, retinol, flavonoids and other phenolic compounds, coenzyme Q, uric acid, bilirubin, phytic acid, mucus, carnosine and related compounds, retinol, polyamines, superoxide dismutase, vitamin E, glutathione peroxidase, and ascorbic acid, a compound that has been reported to have both antioxidant and pro-oxidant properties (Thaipong et al., 2006). Some sulphur-containing compounds have been also recognised as valuable antioxidants, represented by substances such as garlic oil, diallylsulphides, glutathione, lipoic acid, mercapto-propionyl-glycine, and the sulphur containing amino acids — cysteine, methione, taurine and taurine derivatives (Atmaca, 2004). Sulphur-containing antioxidants may be beneficial in a number of oxidative stress states, such as ischemia injury, diabetes, cataracts formation, neurodegeneration and radiation injury. The amino acid methionine is an efficient scavenger of almost all oxidising substances under physiological conditions, such as H_2O_2, OH•, peroxynitrite, chloramines and hypochlorous acids; moreover, absence of methionine has been demonstrated to be directly correlated with endogenous antioxidants deficiencies in the heart (Seneviratne et al., 1999). Regarding taurine, there is evidence that the amino acid blocks toxicity caused by oxidative stress, but the mechanism underlying the antioxidant activity remains unclear. Some studies (Obrosova, et al., 2001; Hwang et al., 2000) reveal that taurine is not able to directly scavenge ROS, but it is an effective inhibitor of ROS generation. In a recent research, Shaffer et al., (2009), reported a novel antioxidant activity of taurine related to respiratory chain components, and deficiency of taurine is associated to depletion of oxygen toward the formation of superoxide anions. Among the not sulphur-containing amino acids having direct antioxidant activity, arginine and creatine have reported to be the most important, essentially due to their chemical structure. It has been noticed, in fact, that the well-marked cationic properties of the guanidine group of arginine and

creatine allow for regulation of peroxidation processes in cell membrane (Lawler et al., 2002; Milyutina et al., 1991).

It is very appealing to researchers to have a convenient method for the quick quantification of antioxidant effectiveness in preventing diseases but, ironically, the main problem is the lack of a validated assay that can reliably measure the antioxidant capacity of foods and biological samples. Several methods have been proposed and the opinion on these varies considerably; there seems to be no consensus opinions, most probably due to the fact that the area of antioxidant is such a complex topic. It is extremely difficult to compare results from different assays therefore it is recommended to evaluate the antioxidant activity by various analytical methods (Frenkel and Meyer, 2000). In a general manner, antioxidant capacity assays can roughly be characterised into two types, depending upon reaction involved: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET) reactions (Re et al., 1999). Both methods can be applied for lipophilic and hydrophilic antioxidant. HAT-based methods are carried out by using a synthetic free radical, an oxidizable molecular probe and an antioxidant. A competitive reaction takes place, in which the antioxidant and the probe compete for thermally generated peroxyl radicals, through the decomposition of azo compounds, inhibiting or retarding probe oxidation. ORAC (Oxygen radical absorption capacity) is one of these assays. ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced, and the extent of colour change is correlated with antioxidant concentration. These methods involve two components in the reacting mixture: antioxidant and an oxidising probe. The oxidant abstracts an electron from the antioxidant, causing colour changes of the probe. The reaction endpoint is reached when colour change stops. ABTS (2,2'-azinobis-3(-ethyl-benzothiazoline-6-sulfonic-acid)) and TEAC (Trolox equivalent antioxidant capacity) (Cationic radical of 2,2'-azinobis-3(-ethyl-benzothiazoline-6-sulfonic-acid)), are examples of ET-based assays (Huang et al., 2005; Schlesier et al. 2008). Both assays use $ABTS^{+\bullet}$ (ABTS radical cation) as oxidant but the antioxidant capacity of a compound is standardised against ascorbic acid in ABTS and against Trolox in TEAC.

Some authors (Muñiz et al., 2001; Rivero et al., 2005) have also shown the usefulness of using DNA scission as an effective method to assess the antioxidant activity against

oxygen species *in vivo*, where DNA strands breaks induced by an oxidant can be adequately visualised by electrophoresis.

In this chapter is evaluated the antioxidant capacity of free amino acids mixture extracted from codfish salting processing wastewater by the HAT- and ET-based methods ORAC, ABTS and TEAC. Prevention of DNA from oxidative degradation, by analysing both anti-oxidant and pro-oxidant capacity of the free amino acids extract, is also assessed and discussed.

7.2 Materials and Methods

7.2.1 Samples

In-vitro antioxidant activity has been assessed for fourth samples, as described below.

The dry mixture of free amino acids extracted from codfish salting processing wastewater (obtained as described in Chapter 6) has been dissolved in ultrapure water until reaching the composition reported in Table 6.3, at a rate of salt of 15 g/L. Solution obtained has been called *sample I*. That mixture has been diluted 1.67 fold in order to obtain an isotonic solution of amino acids, at a rate of salt of 9 g/L, named *sample II*. Each of these mixtures has been compared with an identical solution made of synthetics free amino acids and salt (Sigma-Aldrich, Sintra, Portugal), named *sample III* and *sample IV*, respectively. Composition of samples is reported in Table 7.1.

Table 7.1 Composition of samples

Composition (g/L)	Samples	
	I (extracted) and III (synthetic)	II (extracted) and IV (synthetic)
	Hypertonic free amino acids mixtures	Isotonic free amino acids mixtures
Aspartic acid	0.21	0.13
Glutamic acid	0.32	0.18
Arginine	0.05	0.03
Creatine	1.41	0.84
Glycine	0.05	0.03
Lysine	0.25	0.11
Methionine	0.17	0.10
Phenylalanine	0.28	0.17
Taurine	0.16	0.10
Tryptophan	0.13	0.08
NaCl	15	9

7.2.2 ABTS radical cation decolourisation assays

ABTS radical cation decolourisation assay has been performed by using the method reported by Guimarães et al. (2011) and by Thaipong et al. (2006). These method are based on decolourisation of $ABTS^{\bullet+}$, measured as percent reduction of absorbance at 734 nm.

ABTS radical cation, $ABTS^{\bullet+}$ (a peroxyl radical, RO•), has been produced by mixing 7 mM ABTS (Sigma-Aldrich) solution with 2.54 mM potassium persulfate (Sigma-Aldrich) in the proportion 1:1 (v/v), and the mixture has been then allowed to react during 16 h in the dark, at room temperature. After this time, $ABTS^{\bullet+}$ solution has been diluted in ultrapure water so as to obtain an absorbance of 0.700±0.02 at 734 nm, using a UV-1203 mini spectrophotometer (Shymazu; Kyoto, Japan). Each sample – the standard antioxidant ascorbic acid, and an aliquot of free amino acids mixtures – has been added to 1 mL of $ABTS^{\bullet+}$ solution, using a volume such that the percent reduction in absorbance, by 6 min, would lie in the range 20% to 80%.

Standard solutions of ascorbic acid have been obtained as follows: an amount of 0.03 g of ascorbic acid (Sigma-Aldrich) has been dissolved in 100 mL of ultrapure water, and then 5 standard mixtures have been obtained by diluting 1, 1.25, 1.67, 2.5 and 5 times the stock solution with ultrapure water. 100 μL of each standard has been added to 1 mL of $ABTS^{\bullet+}$ solution and then absorbance has been read at 734 nm.

Standard solutions of Trolox have been obtained as follows: an amount of 0.01 g of Trolox (Merck; Hamburg, Germany) has been added to 100 mL of ultrapure water, and then 5 standard mixtures have been obtained by diluting 1.25, 1.43, 2, 4 and 5 times the stock solution with ultrapure water. 100 μL of each standard has been added to 1 mL of $ABTS^{\bullet+}$ solution then absorbance has been read at 734 nm. In the case of samples, an amount of 100 μL of each free amino acids mixture has been similarly added to 1 mL of $ABTS^{\bullet+}$ solution then absorbance has been read at 734 nm, after 6 min of inhibition.

All determinations have been done in triplicate. Results are expressed as μM of ascorbic acid equivalents, and μM of Trolox Equivalents, for mL of sample (μM AAE/mL and μM TE/mL, respectively).

7.2.3 ORAC assay

ORAC (oxygen radical absorbance capacity) has been performed by the method described by Ou et al. (2001) and Huang et al. (2002), and carried out on the 96-wells plate fluorescent reader equipment Fluorostat Optima (BMG-LABTECH, Biotech Cientifica; Madrid, Spain).

The oxidant, a peroxyl radical, has been generated by the compound AAPH (2,2´-azo-bis-2-methylpropionamide-dihydrochloride) (Sigma-Aldrich). The probe, used for monitoring reaction process, has been fluorescein disodium (Sigma-Aldrich), a fluorescent protein. Peroxyl radical damages the fluorescent molecule resulting in the loss of fluorescence, whose recording along time allows monitoring the oxidisation process and extent.

The assay is accomplished by using the reagents described below. The control sample has been obtained by adding 80 μL of 75 mM/L sodium phosphate buffer (pH 7.4) to 120 μL of 1.17 mmol/L fluorescein disodium. Sodium phosphate buffer at pH 7.4 has been prepared by adding 810 mL of 13.06 g/L K_2HPO_4 to 190 mL of 2.60 g/250 mL $NaH_2PO_4 \cdot H_2O$. Fluorescein solution has been obtained by adding 0.01097 g of fluorescein disodium to sodium phosphate buffer up to 25 mL. The blank sample has been obtained by mixing 20 μL sodium phosphate buffer, 120 μL of 1.17 mmol/L fluorescein disodium and 60 μL of AAPH.

Standard mixtures of antioxidant have been obtained by adding 20 μL of Trolox (at different concentration) to 120 μL of 1.17 mmol/L fluorescein disodium and 60 μL AAPH. Stock solution of Trolox has been prepared by adding 0.0125 g of Trolox to 1 mL of methanol (Merk) and to sodium phosphate buffer up to 50 mL; then 1 mL of this solution has been added to 9 mL of sodium phosphate buffer. Starting from this stock solution, 8 different standards have been prepared by diluting with sodium phosphate buffer and in the range 80-10 μM Trolox.

Sample mixtures to be tested have been obtained by adding 20 μL of each sample (at different concentrations) to 120 μL of 1.17 mmol/L fluorescein disodium and 60 μL AAPH. Different concentrations have been obtained by diluting 10, 25, 30, 40 and 50 times, 10 ml of samples with sodium phosphate buffer. The fluorescein intensity – 485 nm in excitation and 520 nm in emission – has been measured at 40ΌI C, at each min and

over 1 h and 40 min. The relative Trolox equivalent ORAC value was expressed in μM Trolox equivalents/mL sample (μM TE/mL)

7.2.4 DNA oxidative damage inhibition assay

DNA oxidative damage inhibition assay has been performed by using the method reported by Gião et al. (2007). This method includes assays for both controls and samples, and assesses both anti-oxidant and pro-oxidant capacities of the latter. The oxidant, hydroxyl radical OH•, is generated by a mixture of ascorbic acid and copper.

Positive control has been obtained by mixing 800 μL of 100 mmol/L sodium phosphate buffer (pH 7.4) and 200 μL of 0.25 mg/mL calf thymus DNA (Sigma-Aldrich). Sodium phosphate buffer at pH 7.4 has been prepared by adding 810 mL of 13.06 g/L K_2HPO_4 to 190 mL of 2.60 g/250 mL $NaH_2PO_4 \cdot H_2O$. DNA solution has been obtained by adding 0.0125 g of DNA to 50 ml sterile distilled water, and then allowing dissolving overnight in the dark and at room temperature. Negative control has been obtained by adding 690 μL of 100 mmol/L sodium phosphate buffer (pH 7.4) to 200 μL of 0.25 mg/mL DNA, 100 μL of 100 mmol/L ascorbic acid (Sigma-Aldrich) and 10 μL of 10 mmol/L $CuSO_4$ (Sigma-Aldrich). Antioxidant effect of samples has been obtained by a mixture of 290 of 100 mM/L sodium phosphate buffer (pH 7.4), 200 μL of 0.25 mg/mL DNA, 400 μL of sample, 100 μL of 100 mmol/L ascorbic acid and 10 μL of 10 mM/L $CuSO_4$. Pro-oxidant effect of samples has been obtained by a mixture of 400 μL of 100 mmol/L sodium phosphate buffer (pH 7.4), 200 μL of 0.25 mg/mL of DNA and 200 μL of sample. Final volume has been 1 mL in all the cases, and all the mixtures have been incubated at 37º C for 1 h.

Following incubation, an aliquot of 10 μL of each mixture has been mixed with 1 μL of bromophenol blue buffer (20 % (w/w) glycerol and 0.1 % (w/w) bromophenol blue (Sigma-Aldrich) in distilled water) and placed on top of wells of 0.2 % (p/v) agarose gel. This gel has been prepared by adding 0.6 g of agarose type I (Sigma-Aldrich) to 30 ml TBE (Tris/Borate/EDTA) buffer (Sigma-Aldrich). After running, the gel has been submerged in a 10 mg/L solution of ethidium bromide (Amresco; Solon, OH, USA). Electrophoresis has been run using a Bio-Rad (Richmond, CA, USA) well and power supply model 1000/500, at 150 V and 400 mA. DNA bands have been finally digitalised using the Gel Doc XR System (Bio-Rad).

7.2.5 Statistical analysis

Analysis of variance (one-way *ANOVA*) has been performed with the software STATISTICA v.9.0 at a confidence level of 95%. Analysis of variance has been used to test differences in the antioxidant capacity resulting for three different methods – ABTS, TEAC and ORAC.

7.3 Results and Discussion

7.3.1 ORAC and ABTS assays

Results for antioxidant capacity are reported in Table 7.2. ABTS based assays showed that isotonic mixtures allowed for higher percent inhibition of peroxyl radicals than hypertonic mixtures – 65 % against 46 %. Not significant differences ($p>0.05$) have been found between extracted and synthetic mixtures of amino acids, either isotonic or hypertonic.

Table 7.2 Antioxidant capacity of samples

Sample	ABTS (μM AAE/mL)	TEAC (μM TE/mL)	ORAC (μM TE/mL)
I	1164±56 $^{A, a}$	1638±86 $^{A, b}$	1665±71 $^{A, b}$
II	1749±78 $^{B, a}$	2676±113 $^{B, b}$	2968±102 $^{B, c}$
III	1171±64 $^{A, a}$	1702±78 $^{A, b}$	1715±84 $^{A, b}$
IV	1701±92 $^{B, a}$	2387±76 $^{B, b}$	2895± 85 $^{B, c}$

Values in the same line that are not followed by the same lowercase superscript letter are significantly different ($p\leq0.05$). Values in the same columns that have not the same capital superscript letter are significantly different ($p\leq0.05$)

Values for ABTS, TEAC and ORAC have been found significantly lower ($p\leq0.05$) for hypertonic solutions. For these mixtures values for ORAC and TEAC have been found not significantly different ($p>0.05$); conversely, for the isotonic solutions, values for ORAC have been significantly higher ($p\leq0.05$) than TEAC, where the result can be attributed to the effects of NaCl on the antioxidant capacity, and to the different nature of the assays. Values for ABTS have been found significantly lower ($p\leq0.05$) than

ORAC and TEAC for both extracted and synthetic mixtures, either hypertonic or isotonic.

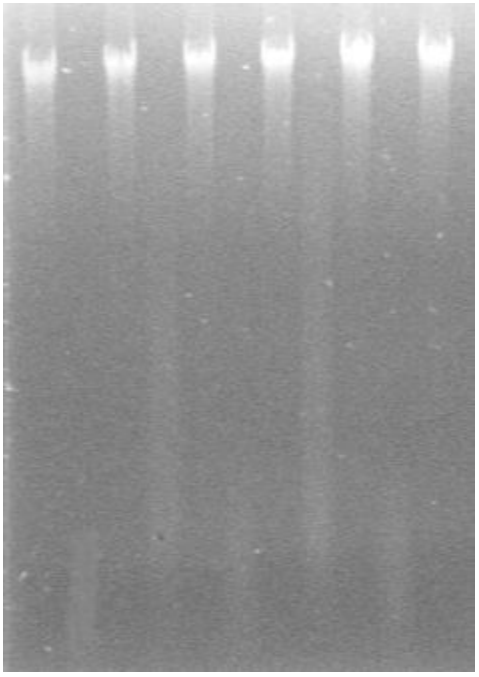
Even though hypertonic mixtures have higher concentration (1.67 times) of amino acids than the isotonic solutions, the lower antioxidant capacity must be attributed to the partial oxidation of free amino acids themselves induced by the high level of NaCl. As reported elsewhere (Ketonen & Mervaala, 2008; Mishra et al., 2009), level of NaCl higher than the normal concentration (9 g/L) promotes the generation of ROS, particularly sodium hypochlorite, NaClO.

The found that for the isotonic solutions ORAC values have been higher than both ABTS and TEAC assessment can be attributed to the different nature of the assays. ORAC test is based on the transfer of hydrogen atoms from the antioxidant to the oxidant, which is neutralised so as to preserve the substrate (the probe) from oxidative damage. Therefore, hydrogen donor compounds are very likely to show antioxidant activity by using the ORAC assay. Regarding free amino acids mixture, it must be noticed that the lateral chain of arginine, creatine, lysine and tryptophan easily loose a hydrogen atom in all the pH range, while the acidic group of aspartic and glutamic acid loose the hydrogen atom at pH > pK_a, as in the case of the free amino acids isotonic mixtures, whose pH is 5.3 (Zulueta et al., 2009).

The lower values for ABTS with respect to TEAC are in accordance with the assays mechanism and with the differences between the standard antioxidants, where ascorbic acid is a stronger antioxidant than Trolox. In the case of isotonic mixtures, 1.82 g/L total amino acids allowed for the same inhibition of $ABTS^{+\bullet}$ (65 %) promoted by 0.03 g/L ascorbic acid, or by 0.067 g/L Trolox.

7.3.2 DNA oxidative damage inhibition assay

Results for inhibition of oxidative damage of DNA are depicted in Figure 7.1. The assay revealed that all samples tested do not have pro-oxidant effect on DNA. This result can be noticed by observing that DNA bands of lanes (c), (e), (g) and (i) (Figure 7.1), relative to mixtures DNA+sample, are equivalent to DNA positive control band of lane (a), demonstrating that sample are not able to oxidise DNA.



a b c d e f g h i l m

Figure 7.1 Effects of samples on DNA oxidative damage. Lane (a) represents DNA alone (positive control); lane (b) represents DNA+Cu(II)-ascorbic acid (negative control); lane (c) represents DNA+sample III; lane (d) represents DNA+Cu(II)-ascorbic acid+sample III; lane (e) represents DNA+sample IV; lane (f) represents DNA+Cu(II)-ascorbic acid+sample IV; lane (g) represents DNA+sample II; lane (h) represents DNA+Cu(II)-ascorbic acid+sample II; lane (i) represents DNA+sample I; lane (l) represents DNA+Cu(II)-ascorbic acid+sample I; lane (m) represents again DNA alone

Regarding antioxidant capacity, hypertonic mixtures of free amino acids showed less antioxidant capacity than the isotonic solutions. Degradation of DNA is in fact more evident in lanes (d) and (h) representing hypertonic mixtures – than in lanes (f) and (l) – representing isotonic solutions. Evidence that saline hypertonicity generates genotoxic effects has evolved over many years (Dmitrieva et el, 2005). Hypertonic NaCl causes DNA damage in cell, yet the cell survives and functions in both cultured and *in vivo* conditions. Cells are also able to rapidly repair DNA breaks if NaCl concentration is lowered to iso- or ipotonic concentrations (Dmitrieva & Burg, 2007). As aforementioned, high levels of NaCl promote ROS formation, that result in oxidation of proteins and lipids. Extracted and synthetic free amino acids mixtures, both hypertonic and isotonic, showed an equivalent effect.

7.4 Conclusion

Free amino acids mixture extracted from codfish salting processing wastewater showed significant antioxidant activity in competitive and not-competitive antioxidation mechanisms. Not pro-oxidant activity has been noticed. Hypertonic mixtures showed, however, lower antioxidant activity than isotonic solution. The differences could be attributed to the partial oxidation of amino acids themselves and due to oxygen reactive species, like NaClO, promoted by the high NaCl level.

CHAPTER 8

IN-VITRO BIOAVAILABILITY OF FREE AMINO ACIDS EXTRACT

8.1 Introduction

The relevance of amino acids lies in their biological importance; all cellular tissue and fluid in living organisms contain a reservoir of free amino acids, named "*amino acids pool*", which take part in metabolic reactions such as biosyntheses of peptides and proteins and synthesis of nucleotides (Wang et al., 2009). In the human body, some amino acids are ready available in tissues and organs because they are synthesised from α-ketoacids and glutamate by the aminotransferase enzyme, and for this reason are called "*non-essential*". However, human beings are not able to synthesise all the amino acids they need for the normal function of the body and is of relevant importance the intake of such "*essential*" amino acids from diet.

Dietary amino acids become available to the cells of specific tissues and organs – brain, kidney, eye, muscle – after having passed into the blood through the intestinal walls. Introduction into the blood circulation is the last stage of digestion, called absorption, and regards amino acids as well as other small compounds derived from digestion of macromolecules such as glucose, glycerol, vitamins and mineral salts (Chakrabarti, 1994). Dietary amino acids develop important functions yet in the intestine; they are a major fuel for the small intestinal mucosa (epithelium) being obligatory precursors for intestinal syntheses of glutathione, nitric oxide, polyamines, purine and pyrimidine nucleotides and some other important compounds. Also, amino acids are essential for maintaining intestinal mucosal mass and integrity (Wu, 1998). Recent studies support potential therapeutic role for specific amino acids (including arginine, glycine, lysine and sulphur containing amino acids) in gut-related diseases. Results of the new lines of work indicate trophic and cytoprotective effects of amino acids on gut integrity, growth and health in animals and humans (Wang et al., 2009).

Due to the importance of dietary amino acids, it is crucial to evaluate their bioavailability, more accurately termed oral bioavailability (Chakrabarti, 1994). Bioloavailabily is the degree of which a substance is taken up by a specific tissue or organ after administration. For a pharmacokinetics point of view, definition is more specific, meaning the extent and rate at which a substance becomes available in the general circulation after a number of routes inside intestine and liver (Ungell & Artursson, 2009). In the case of amino acids, before reaching the target tissues and

organs, they have to pass the small intestine epithelial cells, a continuous monolayer of which a primary function is to act as a barrier Chakrabarti, 1994).

In-vivo bioavailability, or absorption, is currently predicted by using *in-vitro* measurements of permeability through specific cell culture systems, whose selection is of a paramount importance in order to successful mimic a biological barrier such as that of small intestine (Grés et al., 1998). Caco-2, a continuous line of heterogeneous human epithelial colorectal adenocarcinome cells, has frequently been used to study the characteristic and regulation of nutrient and drug absorption in the small intestine at a cellular level (Artursson et al., 1991). It has been possible to establishing a good relationship between *in-vitro* permeability and human absorption *in-vivo* by the Caco-2 cell culture (Artursson, 1990); for this reason Caco-2 cells monolayers currently represents a reference *in-vitro* model in physico-chemical and theoretical prediction of drug and nutrients absorption, and also allowing for the study of drug and nutrient absorption mechanism under controlled conditions and the screening of approaches for improving absorption itself (Artursson et al., 2001; Moyes et al., 2010).

Caco-2 cells are commonly used to not as individual cell but as a confluent monolayer on a cell culture insert filter (Bailey et al, 1996). Although derived from a colon (large intestine) carcinoma, when cultured under specific conditions, the cells become differentiated and polarised such that their phenotype morphologically and functionally resembles the enterocytes (intestinal absorptive cells) which lines the small intestine, providing a physical and biochemical barrier to passage of ions and small molecules, such as amino acids and monosaccharides. Caco-2 cells monolayer express tight junctions – microvillus – and a number of enzymes and transporters that are characteristics of such enterocytes: peptidases, esterases, P-glycoprotein, uptake transporters of amino acids, bile acids, carboxylic acids, etc. (Satsu et al., 1997).

Compared to in vivo animal model, the use of Caco-2 cell line has the advantage of being performed on human cells. In addition it also minimise the use of time-consuming, expensive, and some time controversial animal studies (Grés et al., 1998).

Strictly related with *in-vitro* bioavailability study is the integrity of intestinal epithelium, expressed by a parameter named trans-epithelial electrical resistance (TEER). TEER is a measure of mucosal integrity and epithelium continuity, and changes in its value are indicative of alterations in epithelial barrier functions or in the

transcellular permeability of ions (Pinton et al., 2009). For this reason TEER is used a very sensitive parameter for evaluating epithelial monolayer perturbation as induced from a substance before and during permeation though the intestinal wall (Velarde et al., 1999).

In this chapter is discussed the permeability of free amino acids extracted from codfish salting processing wastewater thought the small intestine epithelium expressed by Caco-2 cell monolayer, as well as the integrity of epithelial monolayer – measured by TEER – as affected by the free amino acids mixture. A comparison of results obtained with an identical mixture of synthetic free amino acids and the effect of salt on monolayer integrity are also debated.

8.2 Materials and Methods

8.2.1 Samples

In-vitro bioavailability and TEER measurements have been assessed for seventh different samples, as described below.

The dry mixture of free amino acids extracted from codfish salting processing wastewater (obtained as described in Chapter 6) has been dissolved in ultrapure water until reaching the composition reported in Table 6.3, at a rate of salt of 15 g/L. Solution obtained has been called *sample I*. That mixture has been diluted 1.67 fold in order to obtain an isotonic solution of amino acids, at a rate of salt of 9 g/L, named *sample II*. Each of these mixtures has been compared with an identical solution made of synthetics free amino acids and salt (Sigma-Aldrich; Sintra, Portugal), named *sample III* and *sample IV* respectively. A mixture of synthetic free amino acids without salt, called *sample V*, has been also studied in order to assess the effect of NaCl on amino acids transport across epithelium.

In order to assess the effects of salt on the monolayer integrity, TEER has been also measured for Caco-2 cells monolayers exposed to a blank saline solution at a rate of 15 g/L, named *sample V*, resembling salt rate of *sample I* and *sample III*, and a blank saline solution at a rate of 9 g/L NaCl, named *sample VI*, resembling salt rate of *sample II* and

sample IV. All determinations been carried out in triplicate. Composition of all samples is reported in Table 8.1.

Table 8.1 Composition of samples

Composition (g/L)	Sample						
	I	II	III	IV	V	VI	VII
	Natural FAA* mixture	Natural FAA mixture	Synthetic FAA mixture	Synthetic FAA mixture	Synthetic FAA mixture	Blank solution	Blank solution
Aspartic acid	0.21	0.13	0.21	0.13	0.13	-	-
Glutamic acid	0.32	0.18	0.32	0.18	0.18	-	-
Arginine	0.05	0.03	0.05	0.03	0.03	-	-
Creatine	1.41	0.84	1.41	0.84	0.84	-	-
Glycine	0.05	0.03	0.05	0.03	0.03	-	-
Lysine	0.25	0.11	0.25	0.11	0.11	-	-
Methionine	0.17	0.10	0.17	0.10	0.10	-	-
Phenylalanine	0.28	0.17	0.28	0.17	0.17	-	-
Taurine	0.16	0.10	0.16	0.10	0.10	-	-
Tryptophan	0.13	0.08	0.13	0.08	0.08	-	-
NaCl	15	9	15	9	0	15	9

*FAA – Free amino acids

8.2.2 Cell culture

Caco-2 cells (HTB-37, line 29-38) have been obtained from the American Type Culture Collection (ATCC) (Rockville; MD, USA).

Cell cultures have been seeded as described elsewhere (Fonte et al, 2011). Briefly, caco-2 cells have been seeded at a density of 3×10^6 cells/cm^2 and cultured in a polyester Transwell insert (Sigma-Aldrich; Sintra, Portugal), containing 6 wells with a dish area of 4.52 cm^2 and a dish porosity of 3μm. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) GlutaMax (Sigma-Aldrich) with the addition of penicillin/streptomycin 1%, non-essential amino acids 1%, and heat inactivated foetal bovine serum 10% (Sigma-Aldrich). Upper (donor) and lower (receiver) wells have been filled with an amount of 1.5 ml and 2.5 of culture medium respectively. The cells have been cultured to confluence over 21 days by incubation at 37º C. During the incubation period culture medium has been renewed on alternate days in upper and lower wells. After 21 days, medium has been retired and the monolayer the cells have

formed on the wells dish has been washed using 4 ml of Hank's balanced salt solution (HBBS) (Sigma-Aldrich).

8.2.3 TEER determination

Before cell seeding, trans-epithelial electrical resistance of Transwell insert with culture medium ($TEER_{insert}$) has been measured using a Millicell-ERS instrument (Millipore; Amadora, Portugal). On day 21 of culture, the TEER of the epithelium on the insert ($TEER_{control}$) has been measured to confirm the confluence of the cell layer. Only wells with $TEER_{control}$ on day 21 of at least 200 $\Omega \cdot cm^2$ higher than $TEER_{insert}$ must be considered confluent (Fonte et al., 2011).
The TEER of the epithelium alone has been expressed in $\Omega \cdot cm^2$ and determined as $[(TEER_{total} \times 4.52) - (TEER_{control} - TEER_{insert})]$, equivalent to $[(TEER_{total} \times 4.52) - 200]$, where $TEER_{total}$ represents the value of transepithelial electrical resistance of each insert containing monolayer and sample, and the term 4.52 represent cell monolayer area. TEER measurements have been done after 0, 15, 30, 60, 120 and 180 min of epithelium monolayer exposure to sample. Increased or constant TEER along time indicates monolayer tightening and integrity while decreased TEER along time indicates loss of monolayer tightening and integrity (Moyes et al., 2010).

8.2.4 Permeability and transport determination

Permeability measurements of natural and synthetic free amino acids mixtures have been performed as described below. After 21 days of culture and monolayer washing, lower (receiver) wells of the Transwell insert have been filled with 2.5 ml of HBSS solution while an amount of 1.5 ml of sample has been loaded on the upper (donor) wells containing the Caco-2 cells monolayer. After 15, 30, 60, 120 and 180 min, 0.3 ml of solution has been retired from the lower wells and free amino acids have been determined by HPLC-UV as reported in Chapter 3, paragraph 3.2.2, at pages 54 and 55. The volume retired has been replaced with 0.3 ml of HBSS loaded in the lower wells.
Permeability has been determined according the following equation:

(**Eq.** 8.1) $$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot C_o}$$

where P_{app} is the apparent permeability coefficient expressed in cm/s, Q (expressed in mg) is the amount of amino acid transported in the receiver well, t (expressed in seconds) is time, A is the monolayer area (4.52 cm^2) and C_0 (expressed in mg/cm^3) is the concentration of amino acid in the donor well. Compounds with high apparent permeability coefficient in Caco-2 cells monolayers, $P_{app} > 10^{-5}$ cm/s, are likely to be well absorbed in human, while compound with low apparent permeability coefficient, $P_{app} < 10^{-5}$ cm/s, are less likely to be absorbed (Ungell & Artursson, 2009).
Transport of amino acids from the donor to the receiver wells has been expressed as follow:

(**Eq.** 8.2) $$T(\%) = \frac{Q}{C_0 \cdot V} \cdot 100$$

where Q (g) is the amount of amino acid transported in the receiver well, C_0 (g/L) is the concentration of amino acid in the donor well and V is the volume of samples loaded in the donor well ($0.3 \cdot 10^{-3}$ L). All determinations have been done in triplicate.

8.2.5 Statistical analysis

Statistical analysis have been carried out on permeability and on TEER, in order to access differences along time for each samples, and differeces between samples at each time. One-way analysis of variance (*ANOVA*) has been carried out with the software STATISTICA v.9.0 setting a confidence level of 95% ($p \leq 0.05$).

8.3 Results and Discussion

Results from TEER measurements showed that all samples with isotonic concentration of salt (II, IV and VII) exhibited higher transepithelial electrical resistance than samples with hypertonic salt concentration (I, III and VI) ($p \leq 0.5$). For each sample, values of TEER decreased from 0 to 30 min ($p \leq 0.05$) and remained constant up to 180 min ($p > 0.05$). For each time, no significant differences in TEER ($p > 0.05$) have been noticed between extracted and synthetic amino acids mixtures and blank solution at 9 g/L NaCl (II, IV and V), as well as between extracted and synthetic amino acids mixture and

blank solution at 15 g/L (I, III, VI); a higher value of TEER ($p>0.05$) has been found for mixture of amino acids without salt (V). In Table 8.2 are reported TEER values at each time while in Figure 8.1 are reported TEER trend along time, for each sample.

Table 8.2 TEER values (mean values ± standard deviation) along time for each sample. TEER is expressed in $\Omega \cdot cm^2$, time is expressed in min

Sample	TEER values depending on time					
	0 min	15 min	30 min	60 min	120 min	180 min
I	207 ± 39.4^{aA}	205 ± 21.37^{aA}	157 ± 27.49^{bA}	157 ± 21.36^{bA}	139 ± 19.7^{bA}	135 ± 16.3^{bA}
II	365 ± 39.14^{aB}	332 ± 39.64^{aB}	317 ± 35.95^{abB}	300 ± 44.59^{bB}	297 ± 33.57^{bB}	301 ± 42.33^{bB}
III	207 ± 49.79^{aA}	193 ± 35.88^{aC}	161 ± 13.05^{aA}	151 ± 9.41^{aA}	136 ± 6.90^{abA}	127 ± 6.90^{bA}
IV	379 ± 22.6^{aB}	330 ± 42^{abB}	311 ± 43.12^{bB}	306 ± 45.87^{bB}	293 ± 36.16^{bB}	243 ± 31.64^{bC}
V	406 ± 23.4^{aC}	387 ± 32.3^{aD}	369 ± 19.3^{abC}	348 ± 23.6^{bC}	316 ± 25.4^{bB}	306 ± 24.2^{bB}
VI	204 ± 18.27^{aA}	201 ± 14.53^{aC}	169 ± 34.52^{abC}	153 ± 19.70^{abA}	144 ± 4.52^{bE}	127 ± 5.22^{bA}
VII	374 ± 23.92^{aB}	339 ± 23.92^{abB}	321 ± 33.93^{bB}	314 ± 58.12^{bB}	299 ± 42.72^{bB}	254 ± 24.89^{bC}

Values in the same line that are not followed by the same lowercase superscript letter are significantly different ($p\leq0.05$). Values in the same columns that have not the same capital superscript letter are significantly different ($p\leq0.05$)

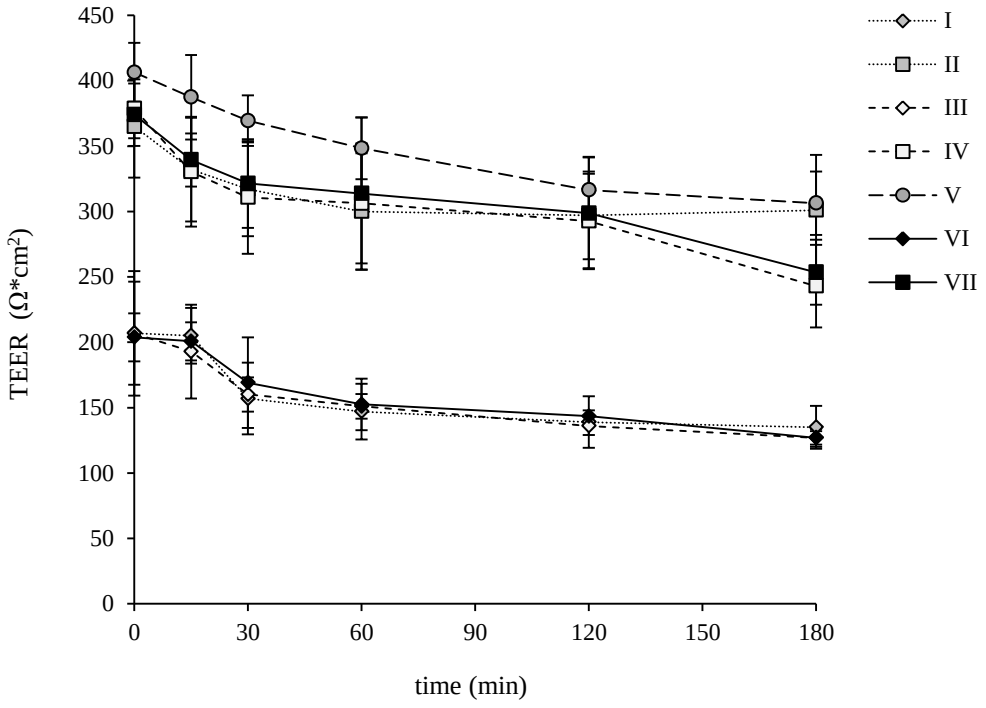


Figure 8.1 TEER along time for each sample (mean values ± standar deviation bars)

Cell monolayer integrity has been lost between 15 and 30 min for all samples at 15 g/L (when TEER has gone below to 200 $\Omega \cdot cm^2$, Table 8.2), while samples at 9 g/L and sample saltless allowed for monolayer integrity up to the end of the determination (180 min). The TEER decrease caused by the hypertonic salt solutions of amino acids (I, III and VI) is associated with an increase in transepithelial permeability of the cell monolayer as well as subtle change in cytoskeletal structure; this suggests that membrane perturbation induced by hypertonic content of NaCl has resulted in disruption of the tight junctions of the monolayer and a subsequent increase in paracellular permeability (Narai et al., 1998). In the light or results, it has been concluded that amino acids mixture at 15 g/L is not viable either for pharmaceutical or for food or feed applications.

Results from transport of amino acids in the mixtures at 9 g/L NaCl showed that all, except creatine, have passed from the apical (upper) to the basolateral (lower) well by permeating the cell monolayer, at a rate $\geq$ 95 % by the end of 180 min. Value for transport and apparent permeability coefficient for all amino acids mixtures at 9 g/L NaCl, and for amino acids mixture without salt, are reported in Table 8.3. For each time, no significant differences in transport ($p>0.05$) have been found between extracted and synthetic amino acids mixtures at 9 g/L NaCl (samples II and IV respectively), while a significant lower transport value ($p\leq0.05$) has been obtained for amino acids mixture without salt. Total transport (100 %) has been observed for amino acids arginine, glycine, lysine, methionine and tryptophan, followed by aspartic acid and taurine (ca. 98 %). Lower transport has been observed for creatine (6 %). In Figure 8.2 is reported transport of amino acids of extracted mixture at 9 g/L NaCl depending on time.

Table 8.3 Values for apparent permeability coefficient (cm/s) and Transport (%) for free amino acids mixture extracted from codfish wastewater (sample I) and for synthetic free amino acids mixture with salt at 9 g/L NaCl and withou salt (sample IV and sample V respectively)

Amino acid	Parameter	P_{app} and T for samples II, IV and V depending on time														
		15 min			30 min			60 min			120 min			180 min		
		II	IV	V	II	IV	V	II	IV	V	II	IV	V	II	IV	V
Aspartic acid	P_{app}	8.7E-05^{a}	8.2E-05^{a}	7.1E-05^{b}	2.3E-04^{c}	2.5E-04^{c}	1.9E-04^{d}	3.1E-04^{e}	3.3E-04^{e}	2.8E-04^{f}	3.3E-04 ^{g}	3.2E-04 ^{g}	2.9E-04^{h}	3.6E-04 ^{i}	3.5E-04 ^{i}	3E-04^{l}
	Trasport	23 ^{A}	23 ^{A}	20 ^{B}	63 ^{C}	64 ^{C}	60 ^{D}	83 ^{E}	83 ^{E}	80 ^{F}	85 ^{G}	86 ^{G}	81 ^{H}	99 ^{I}	98 ^{I}	94 ^{L}
Glutamic acid	P_{app}	9.8E-05^{a}	9.6E-05^{a}	8.5E-05^{b}	1.9E-04^{c}	1.8E-04^{c}	1.5E-04^{d}	2.3E-04^{e}	2.5E-04 ^{e}	1.8E-04^{f}	2.9E-04 ^{g}	2.8E-04 ^{g}	2.5E-04^{h}	3.6E-04 ^{i}	3.7E-04 ^{i}	3E-04^{l}
	Trasport	27 ^{A}	26 ^{A}	23 ^{B}	52 ^{C}	52 ^{C}	47 ^{D}	63 ^{E}	62 ^{E}	58 ^{F}	78 ^{G}	79 ^{G}	75 ^{H}	96 ^{I}	97 ^{I}	92 ^{L}
Arginine	P_{app}	2.1E-04^{a}	2.2E-04^{a}	1.7E-04^{b}	3.1E-04^{c}	3E-04 ^{c}	2.8E-04^{d}	3.3E-04^{c}	3.2E-04^{b}	2.9E-04^{c}	3.5E-04 ^{d}	3.5E-04 ^{d}	3.1E-04^{f}	3.7E-04 ^{g}	3.7E-04 ^{g}	3.2E-04^{h}
	Trasport	56 ^{A}	55 ^{A}	50 ^{B}	84 ^{C}	85 ^{C}	81 ^{D}	88 ^{E}	87 ^{E}	83 ^{F}	95 ^{G}	96 ^{G}	92 ^{H}	100 ^{I}	100 ^{I}	95 ^{L}
Creatine	P_{app}	3.1E-06^{a}	3E-06 ^{a}	2.5E-06^{b}	9E-06 ^{c}	8.9E-06^{c}	8.3E-06^{d}	1.9E-05^{e}	1.8E-05 ^{e}	1.5E-05^{f}	2.1E-05 ^{g}	2.2E-05 ^{g}	1.8E-05^{h}	2.1E-05 ^{i}	2E-05 ^{i}	1.7E-05^{l}
	Trasport	0.8 ^{A}	0.9 ^{A}	0.5 ^{B}	2.4 ^{C}	2.6 ^{C}	2.1 ^{D}	5 ^{E}	5.1 ^{E}	4.3 ^{F}	6 ^{G}	5.9 ^{G}	5 ^{H}	6 ^{I}	6.1 ^{I}	5.1 ^{L}
Glycine	P_{app}	1.7E-04^{a}	1.6E-04^{a}	1.3E-04^{b}	2.8E-04^{c}	2.9E-04^{c}	2.5E-05^{d}	3.2E-04^{e}	3.1E-04^{e}	2.9E-04^{f}	3.6E-04 ^{g}	3.5E-04 ^{g}	3.1E-04^{h}	3.7E-04 ^{i}	3.6E-04 ^{i}	3.2E-04^{l}
	Trasport	47 ^{A}	48 ^{A}	45 ^{B}	74 ^{C}	75 ^{C}	71 ^{D}	88 ^{E}	87 ^{E}	83 ^{F}	97 ^{G}	97 ^{G}	93 ^{H}	100 ^{I}	100 ^{I}	96 ^{L}
Lysine	P_{app}	2.6E-04^{a}	2.7E-04^{a}	2.3E-04^{b}	2.7E-04^{a}	2.6E-04^{a}	2.4E-04	3.5E-04^{b}	3.5E-04^{b}	3.1E-04	3.5E-04 ^{c}	3.6E-04 ^{d}	3.2E-04	3.7E-04 ^{d}	3.7E-04 ^{d}	3.3E-04
	Trasport	69 ^{A}	68 ^{A}	64 ^{B}	75 ^{C}	76 ^{C}	71 ^{D}	95 ^{E}	96 ^{E}	91 ^{F}	95 ^{G}	95 ^{G}	92 ^{H}	99 ^{I}	100 ^{I}	95 ^{L}
Methionine	P_{app}	2.2E-04 ^{a}	2.1E-04^{a}	1.7E-04^{b}	3E-04 ^{c}	3.1E-04^{c}	2.7E-04^{d}	3.4E-04^{e}	3.3E-04 ^{e}	2.9E-04^{f}	3.6E-04 ^{g}	3.5E-04 ^{g}	3.1E-04^{h}	3.6E-04 ^{i}	3.7E-04 ^{i}	3.2E-04^{l}
	Trasport	59 ^{A}	58 ^{A}	55 ^{B}	82 ^{C}	83 ^{C}	78 ^{D}	90 ^{E}	89 ^{E}	85 ^{F}	97 ^{G}	96 ^{G}	92 ^{H}	99 ^{I}	100 ^{I}	95 ^{L}
Phenylalanine	P_{app}	2.3E-04^{a}	2.2E-04^{a}	1.6E-04^{b}	2.7E-04^{b}	2.7E-04^{c}	2.2E-04^{d}	3.3E-04^{e}	3.2E-04 ^{e}	2.8E-04^{f}	3.4E-04 ^{g}	3.5E-04 ^{g}	3E-04^{h}	3.5E-04 ^{i}	3.6E-04 ^{i}	3.1E-04^{h}
	Trasport	63 ^{A}	64 ^{A}	61 ^{B}	73 ^{C}	74 ^{C}	70 ^{D}	90 ^{E}	91 ^{E}	86 ^{F}	92 ^{G}	91 ^{G}	86 ^{H}	95 ^{I}	96 ^{I}	91 ^{L}
Taurine	P_{app}	2.2E-04^{a}	2.3E-04^{a}	1.7E-04^{b}	2.8E-04^{c}	2.9E-04^{c}	2.4E-04^{d}	3.4E-04^{e}	3.4E-04 ^{e}	3E-04 ^{f}	3.6E-04 ^{g}	3.7E-04 ^{g}	3.1E-04^{h}	3.7E-04 ^{i}	3.6E-04 ^{i}	3.2E-04^{L}
	Trasport	63 ^{A}	64 ^{A}	59 ^{B}	73 ^{C}	72 ^{C}	69 ^{D}	91 ^{E}	90 ^{E}	86 ^{F}	92 ^{G}	93 ^{G}	87 ^{H}	97 ^{I}	98 ^{I}	93 ^{L}
Tryptophan	P_{app}	2.6E-05^{a}	2.6E-05^{a}	23-05^{b}	9.3E-05^{c}	9.4E-05^{c}	8.9E-05^{d}	3E-04 ^{e}	3.1E-04 ^{e}	2.7E-04^{f}	3.7E-04 ^{g}	3.6E-04 ^{g}	3.1E-04^{h}	3.7E-04 ^{i}	3.8E-04 ^{i}	3.2E-04^{l}
	Trasport	7 ^{A}	7 ^{A}	5 ^{B}	25 ^{C}	24 ^{C}	20 ^{D}	80 ^{E}	79 ^{E}	74 ^{F}	98 ^{G}	97 ^{G}	95 ^{H}	100 ^{I}	100 ^{I}	95 ^{L}

For P_{app}, values in the same line that are not followed by the same lowercase superscript letter are statistical different ($p \leq 0.05$)

For T, values in the same line that are not followed by the same lowercase superscript letter are statistical different ($p \leq 0.05$

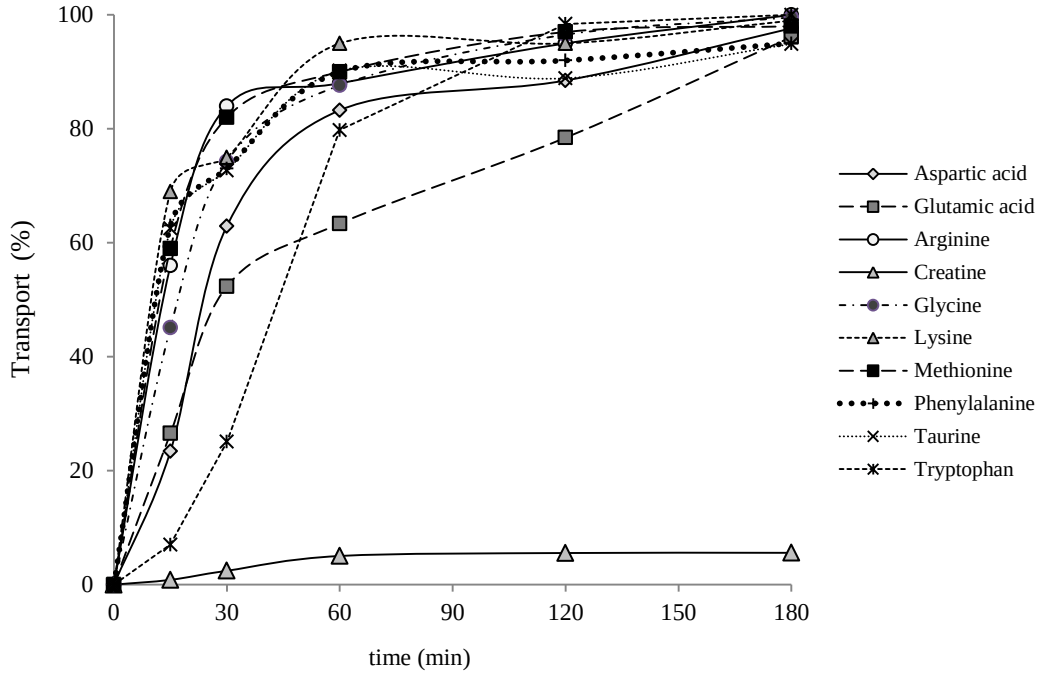


Figure 8.2 Transport of free amino acids extracted from codfish salting processing wastewater (sample II) through the Caco-2 cells monolayer (mean values±standard deviation bars)

Amount of amino acids permeated through the cell monolayer, and relative to 1.5 ml of sample, has been: 0.32 mg for aspartic acid, 0.46 mg for glutamic acid, 0.08 mg for arginine, 0.13 for mg creatine, 0.08 for glycine, 0.38 mg for lysine, 0.25 mg for methionine, 0.42 mg for phenylalanine, 0.20 mg for tryptophan and 0.19 mg for taurine. According to the research work reported by some authors (Dash et al., 2001; Satsu et al., 1997), absorption of amino acids is governed by a dose-limiting mechanism, a saturation process mathematically obeying to the *Michaelis-Menten* kinetics. This find can be confirmed by results obtained and noticing that creatine transport rate was significantly lower than other amino acids transport rate (recall Table 8.1), and attributable to the higher dose of creatine in the apical well.

For all the amino acids permeability has been high, $P_{app}>10^{-5}$ cm/s (Table 8.3), suggesting that they are likely to be well absorbed by the human intestine (Ungell & Artursson, 2009). For each time, no significant differences have been observed between extracted and synthetic amino acids ($p>0.05$), while a significantly lower value ($p\leq0.05$) has been found for amino acids mixture without salt. Creatine showed the lowest

permeability in all mixtures. In Figure 8.3 is reported the apparent permeability coefficient along time for each amino acid contained in extracted mixture at 9 g/L NaCl. It is well known amino acids are absorbed via active transport and co-transport, both carriers-mediated, whereas taurine has been reported to be transported by a system which mainly accepts β-amino acids (β amino acids) (Satsu et al. 1997). Active transport occurs by a concentration gradient, for α-amino acids and neutral amino acid with the amino group, like glycine, where they accumulate on the cell mucosa and then diffuse by mean of a carrier. Neutral amino acids analogue without the amino group, such as taurine, does not undergone active transport and an electrochemical gradient is required for absorption (Thwaites & Stevens, 1999). Also all the negatively charged amino acids, like glutamic acids, lysine and arginine, do not permeate by active transport.

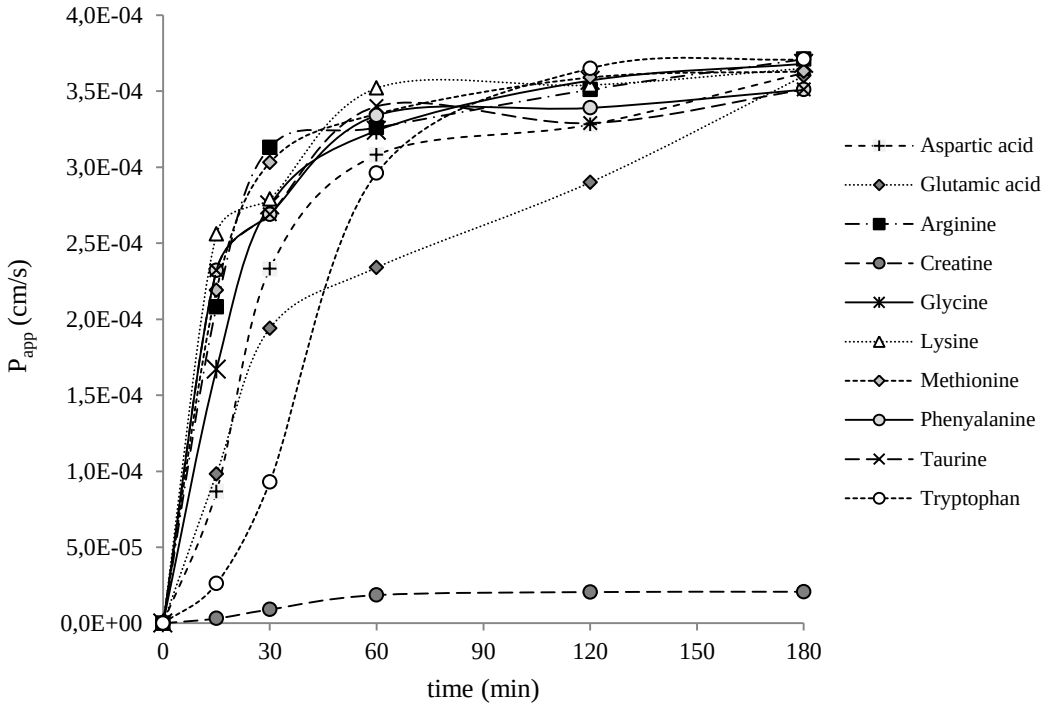


Figure 8.3 Apparent permeability of free amino acids extracted from codfish salting processing wastewater (sample II) through the Caco-2 cells monolayer (mean values ±standard deviation bars)

Amino acids transport through the epithelial monolayer, and various other tissues, has been found strongly dependent on the Na^+ concentration in both active and co-transport (Ferruzza et al., 1995). The Na^+ electrochemical gradient is recognized as the primary driving force for nutrient and solutes transport across the plasma membrane of mammalian cells (Martín-Venegas et al., 2008) while, in contrast, many plant, yeast, fungi and bacteria utilize the H^+ electrochemical gradient (Thwaites & Stevens, 1999). This found can explain the higher transport and permeability values for amino acids mixture at a 9 g/L NaCl with respect to mixture without salt.

As reported by Chakrabarti (1994), absorption of an amino acid when alone in solution may be different that absorption when other amino acids are present, either in equimolar proportion or proportion simulating the composition of a protein, because some competition processes can occur. Methionine is the most rapidly absorbed amino acids, both alone or in mixture, and it acts as a carrier for some other amino acids (leucine, iso-leucine and valine). Trasport of lysine is inhibited from arginine, and this two dicarboxilic amino acids are absorbed slowest of all; arginine is absorbed more rapidly in solution than alone, probably because the trasport is mediated by other amino acids; on the contraty, glycine is more rapidly absorbed when alone; basic amino acids, like glutamic acid, dos not inhibit trasport of other amino acids (Frenhani & Burini, 1999).

Absorption of an amino acid with time depends on its concentration, character and side chain. Amino acids with short side-chain, such as glycine and taurine, are slowly transported when in low concentration (assuming as a reference the normal concentration in the intestinal lumen); amino acids with lipophilic side chain, such as methionine, phenylalanine and tryptophan, are rapidly transported when in low concentration; acidic amino acids, such as aspartic acid and glutamic acid, are rapidly transported when in high concentration; dibasic amino acids, such lysine and arginine, are always slowly transported (Matthews 1972; Martín-Venegas et al., 2008). In the light on this find and by observing the transport trend of amino acids reported in Figure 8.2, it can be concluded that most rapidly transported amino acids have been creatine, glycine, lysine, methionine, phenylalanine and taurine, while glutamic acid and tryptophan have been the most slowly transported.

8.4 Conclusion

In-vitro permeability study by using Caco-2 cell line demonstrated the bioavailability of free amino acids mixture extracted from codfish salting processing wastewater, however only for isotonic level of NaCl, 9 g/L. The high value of the apparent permeability coefficient, $P_{app}>10^{-5}$ cm/s, allowed to conclude that all the amino acids in the isotonic solution are likely to be well absorbed by the human intestine. No significant differences have been found between the synthetic and the natural amino acids mixture. Hypertonic saline mixture of amino acids, at 15 g/L NaCl, can not be proposed for pharmaceutical, food and feed application because they negatively affect the intestinal monolayer structure inducing a loss of the integrity, and just after 15-30 min of exposure.

Absorption of amino acids has been demonstrated to be salt-dependent as resulted from the higher transport and permeability values for the isotonic saline amino acids mixture with respect to the mixture without salt.

PART V

SYNTHESIS AND FOLLOW UP

Abstract

In this Part, a general conclusion to the research accomplished for the valorisation of codfish salting processing wastewater in presented.

Further research works following the investigation described in this thesis are also debated.

CHAPTER 9

GENERAL CONCLUSION AND FUTURE WORK

9.1 Conclusion

The research work reported in this PhD thesis allowed concluding that valorisation of codfish salting processing wastewater, through the extraction of high added value compounds in it contained, can be successfully carried out.

Among all liquid waste generated during codfish salting, drying and re-hydration processes, the water drained away from codfish tissues through salting has been shown to have the highest potential for upgrading, carrying important compounds – free amino acids and muscle proteins – that can be valorised for pharmaceutical, cosmetic and food applications. By the end of the salting process, 1000 g of free amino acids and ca. 570 g of muscle proteins are released in 155 L of wastewater produced starting from one ton on codfish after the typical 6 days process.

The extraction process implemented allowed for the recovery of the organic matter as well as for the partial recovery of sodium chloride used in salting process. Amino acids and proteins have been selectively recovered by batch sorption, using the commercial *Amberlite XAD16* resin. Process parameters – temperature, pH, ionic strength, addition of organic solvents, stirring rate and adsorbent dose – were optimized by the factorial design methodology and in order to maximize the recovery of amino acid taurine. Equilibrium study conducted showed that the ionic strength of codfish salting processing wastewater (260 g/L NaCl) had to be reduced in order to allow amino acids interaction with resin. Therefore, a salt extraction process has been studied allowing for the reduction of NaCl from 4.3 M to 1.4 M by food-grade ethanol at the temperature of 0º C. No precipitation of amino acids occurred, while 1.44 % of proteins felt down with salt in the precipitated phase. Ethanol has shown to be the driving factor in salt precipitation while the low temperature helped reducing proteins precipitation and denaturation.

Along the sorption process, free amino acids and proteins have been adsorbed at the same time, while desorption has been accomplished selectively. Proteins have been totally extracted, while for the total free amino acids fraction, a recovery of 76 % has been obtained. Among all amino acids the hydrophobic ones – phenylalanine, tryptophan and methionine – and taurine have been totally removed from the mixture, while a lower yield of recovery has been observed for aspartic and glutamic acid, as expected. Acetone resulted to be the best solvent for free amino acids elution when

compared with another food-grade solvent such as ethanol. A strong alkaline solution of 4% sodium hydroxide in water has been used for proteins desorption.

Free amino acids extracted from codfish salting processing wastewater can be regarded as a valuable mixture to be introduced into food, nutraceutical or pharmaceutical formulations because it contains mainly essential (methionine, lysine, phenylalanine and tryptophan) and conditional-essential (arginine, creatine and taurine) amino acids, while just three are not-essential (aspartic and glutamic acids, and glycine).

Hence, some important aspects have been elucidates, namely antioxidant capacity and bioavailability. Antioxidant capacity of free amino acids mixture has been assessed by ABTS, TEAC and ORAC tests and by the DNA oxidative damage inhibition assay.

In vitro permeability though intestinal epithelium monolayer (obtained by culturing Caco-2 cell line) has been studied in order to assess the *in vivo* bioavailability of amino acids in human, where the term bioavailability means the degree at which a compound is taken up by a specific tissue or organ after administration. Results for antioxidant capacity evaluation showed that free amino acids mixture does not show pro-oxidant activity of DNA; also, it can prevent DNA from oxidisation if salinity is reduced down to an isotonic level of 9 g/L NaCl. The effect of salinity has to be attributed to the generation of reactive oxygen species promoted by the hypertonic levels of NaCl, an effect that has been also visible on the results for ABTS, TEAC and ORAC where antioxidant capacity values have been higher for the isotonic mixture of free amino acids than for the hypertonic ones. Results for bioavailability showed that all the amino acids extracted from codfish salting processing wastewater have been able to cross the intestinal epithelium but, in order to avoid cytotoxic effect on intestinal cells, the salinity must be adjusted, by dilution, to an isotonic level. Values for apparent permeability coefficient of isotonic mixture of amino acids, $P_{app} > 10^{-5}$ cm/s, showed that all are very likely to be well absorbed by human. Amino acids have been transported through the epithelium depending on their initial concentration where transport rate has been at least 95%, for except for creatine whose transport has been 6 %. Hypertonic saline mixtures of amino acids, at 15 g/L NaCl, cannot be proposed for pharmaceutical, food and feed application because negatively affect intestinal monolayer structure by inducing a loss of the integrity and just after 15-30 min of exposure.

Absorption of amino acids has been demonstrated to be salt-dependent as resulted from the higher transport and permeability values obtained for the isotonic saline amino acids mixtures with respect to the analogue synthetic mixtures without salt.
On the bases of the results obtained, it can be concluded that the process implemented is very likely to be successful, due to the simplicity of methodology and equipments, and to the useful applications of the extracts. It has to be highlighted that the process has been accomplished by using food-grade solvents only, without any toxic residue, which doubtless contributes to the high added value of the compound extracted.

9.2 Future work

Results obtained can be considered very promising however further research works are needed.
In vitro studies of free amino acids extract, regarding antioxidant activity, protection against oxidation of DNA and bioavailability, have been assessed, however some other tests *in vivo* are needed in order to establish effects on human tissues, and particularly therapeutics developments.
Muscle proteins extract must be characterised, where the effects of salt on the rheology and gel forming ability must be assessed. Eventually, salt must be retired by dialysis or ultrafiltration and recycled in the salting procedure.
Salt extract, obtained by precipitation with ethanol before the sorption process, must be purified from the mixture ethanol-water trapped in it. Extractive distillation may allow for the purpose and also for the recovery of pure ethanol, which can be recycled in the precipitation procedure.

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