CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

HYALURONIC ACID AND COLLAGEN EXTRACTION FROM CHICKEN COMBS

by

Gonçalo Costa Soares

June 2017

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biomedical Engineering

by

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Resumo

A avicultura industrial é uma atividade pecuária em ascensão, requerendo novas alternativas para a valorização dos subprodutos gerados. O ácido hialurónico (HA) é um biopolímero importante, que é encontrado em elevadas concentrações nas cristas de galinha. O HA desempenha importantes funções no corpo humano, ao nível da matriz extracelular, pele, cartilagem, fluído sinovial, fertilização, entre outras. Pode ser utilizado na indústria médica e cosmética, com aplicações para engenharia de tecidos, tratamento de feridas, tratamentos estéticos, redução de adesões e cicatrizes, transporte de moléculas, controlo de células estaminais e oftalmologia. O colagénio é uma das proteínas mais importantes, sendo utilizado em diversas aplicações médicas enquanto biomaterial, tais como, engenharia de tecidos, sistemas de libertação controlada de fármacos, oftalmologia, tratamento de feridas e outras.

Esta tese teve como objetivo investigar a possibilidade de extrair conjuntamente HA e colagénio a partir de cristas de galinha e otimizar esse processo. A otimização da extração foi baseada nas análises feitas por *Fast Protein Liquid Chromatography*, que também serviram para confirmar a integridade do colagénio extraído. Na metodologia de extração otimizada, as cristas de galinha foram sujeitas a um processo de delipidação com acetona, seguido de três extrações consecutivas com ácido acético 0.5 M durante 24 horas, a 4 ºC, resultando numa extração conjunta de HA e colagénio com rendimentos de 0.06 % e 2.71 %, respetivamente, e em termos de peso seco. As técnicas de *Fourier Transform Infrared Spectroscopy* e *Differential Scanning Calorimetry* confirmaram a presença de HA e colagénio tipo I nos liofilizados.

Confirmou-se que o HA e o colagénio podem ser extraídos por um processo viável a partir de cristas de galinha, podendo ser utilizados em inúmeras aplicações na área da biomedicina.

Palavras-Chave: Cristas de galinha, Ácido hialurónico, Colagénio, Extração.

Abstract

Poultry industry is a rising agricultural activity, demanding new alternatives for the valorisation of the generated by-products. Hyaluronic acid (HA) is an important biopolymer which is present in high concentrations in chicken combs. It plays many important roles in the human body, such as in the extracellular matrix, in the skin, in cartilage, in synovial fluid, in fertilization, among others. HA can be used in medical and cosmetic industries with applications for tissue engineering, wound healing, aesthetic treatments, adhesions and scar reduction, molecule delivery, control of stem cell behaviour and ophthalmology. Collagen is one of the most important proteins, having many medical applications as a biomaterial, such as in tissue engineering, in drug delivery systems, in ophthalmology, in wound healing, and others.

This thesis aimed to investigate the possibility and optimization of a conjoint extraction of HA and collagen from chicken combs. Extraction optimization was based on Fast Protein Liquid Chromatography analyses, which was also used to confirm the integrity of extracted collagen. In the optimized extraction methodology, chicken combs were subjected to a delipidation process with acetone, followed by three consecutive extractions with 0.5 M acetic acid for 24 hours, at 4 ºC, which allowed the conjoint extraction of HA and collagen with extractions yields of 0.06 % and 2.71 % in terms of dry weight, respectively. Fourier Transform Infrared Spectroscopy and Differential Scanning Calorimetry confirmed the presence of both HA and collagen type I in lyophilized powders.

HA and collagen can thus be viably extracted from chicken combs, with many possible applications in the biomedical field.

Keywords: Chicken combs, Hyaluronic acid, Collagen; Extraction.

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List of Abbreviations

3D – Three Dimension

4-Hyp – 4-hydroxiproline

AA – Acetic Acid

Ala – Alanine

Asp – Aspartate

BDNF – Brain-derived Neurotrophic Factor

BMP-2 – Bone Morphogenetic Protein-2

Ch – Chondroitin

ChS – Chondroitin Sulphate

CPC – Cetylpyridinium Chloride

DSC – Differential Scanning Calorimetry

ECM – Extracellular Matrix

EU – European Union

FDA – Food and Drug Administration

Fe^{n+} – Iron cation

FGF-β – Fibroblast growth factor beta

FPLC – Fast Protein Liquid Chromatography

FTIR – Fourier Transform Infrared Spectroscopy

GAGs – Glycosaminoglycans

Gly – Glycine

HA – Hyaluronic Acid

HABPs – Hyaladherins

HARE – Hyaluronan receptor for endocytosis

HAS – Hyaluronan Synthases

HCl – Hydrogen Chloride

HPLC – High Performance Liquid Chromatography

HYALs – Hyaluronoglucosaminidases

Hyp – Hydroxyproline

Hz – Hertz

IαI – Inter-α-inhibitor

ICAM-1 – Intracellular adhesion molecule-1

IL-1β – Interleukin 1 beta

kDa – kilo Dalton

LFA-1 – Leukocyte Integrins Lymphocyte Function Associated-1

LIVE-1 – Lymphatic Vessel Endothelial Hyaluronan receptor 1

Lys – Lysine

M – Molar

MMPs – Matrix Metalloproteinases

MW – Molecular Weight

Na^+ – Sodium cation

NaCl – Sodium Chloride

OECD – Organisation for Economic Co-operation and Development

OVD – Ophthalmic Viscosurgical Devices

PEG – Poly(ethylene glycol)

PEGDGE – Poly(ethylene glycol) diglycidyl ether

pH – Potential of Hydrogen

PMMA – Poly(methyl methacrylate)

ppm – Parts per million

Pro – Proline

RHAMM – Receptor for Hyaluronan-Mediated Motility

SPAM – Sperm Adhesion Molecule

TE – Tissue Engineering

TEMED – Tetramethylethylenediamine

TLRs – Toll-like Receptors

TNF-α – Tumour Necrosis Factor-α

Tris-HCl – Tris(hydroxymethyl)aminomethane hydrochloride

TSG-6 – Tumour Necrosis Factor-stimulated gene-6

UV – Ultraviolet

V – Volume

VICs – Valvular Interstitial Cells

vol. – Volumes

w – Weight

Chapter 1: Introduction

Poultry industry has one of the highest levels of development worldwide, considering agricultural activities. This occurs, mostly, due to the use of animal origin proteins in Human nutrition [1]. Poultry meat consumption per capita in the Organisation for Economic Co-operation and Development (OECD) countries and European Union (EU) has suffered a growth from 2000 to 2015 with some oscillations throughout the years.

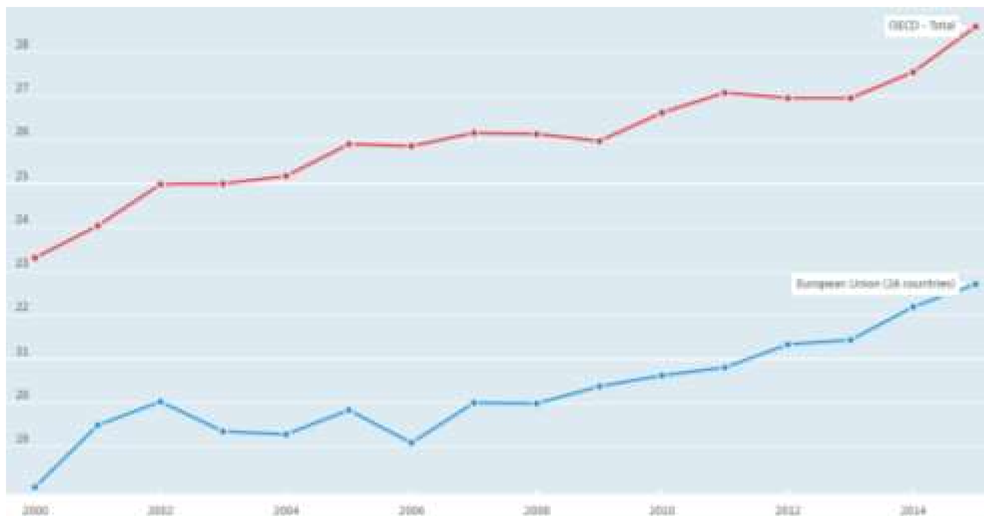

Figure 1.1 - Poultry meat consumption per capita in OECD and European Union (28 countries) from 2000 to 2015 (x-axis: kg/per capita; y-axis: Years). (Obtained from [2])

Slaughtering generates two by-products types: solid and liquid. Solid by-products include feathers, viscera, heads, feet, skin, fat, bones, carcasses unfit for consumption, among other items. On the other hand, liquid by-products mainly include blood and other effluents. These by-products can be transformed in raw materials for animal feed such as poultry flours and fats. According to information provided by the holding *Soja de Portugal* (Ovar, Portugal), each kilogram of solid by-products (only to consider the global mixture of indiscriminate chicken parts) generates 180 g of poultry flour and 120 g of poultry fat, which represents a yield of 18 and 12 %, respectively. Poultry fat can be used to produce soaps, bath soaps and chemical products, while poultry flour is specifically used in the production of animal feeding (together with poultry fat), since its use in Human nutrition is considered unsafe due to the risk of avian disease contamination.

Commercial price for sodium hyaluronate from rooster combs is ca. 555 €/g (Sigma-Aldrich, Inc., St. Louis, MO, USA). This high price will certainly justify the investment in

extraction of hyaluronic acid (HA) from rooster combs. As case study, this thesis will have the holding company *Soja de Portugal*, which administrates *Savinor, S.A.* (Trofa, Portugal) and *Avicasal, S.A.* (São Pedro do Sul, Portugal) in the field of poultry industry and valorisation of by-products, respectively. These two companies together have an annual production capacity close to 9 tons of chicken combs, considering that in 2015 they processed 22 million chickens and the medium weight of a chicken comb is 0.4 g. Also, considering that it is possible to extract 1 g of HA from 1 kg of chicken combs, according to the yields of HA extraction from chicken combs reported in the literature [1], [3], the income obtained by selling the extracted HA would be close to 5 million euro. This fact would turn the industrial exploitation of chicken combs for noble and higher added-value productive uses extremely profitable, fully compensating the displacement of this by-product from the production of poultry flour and fat.

On the other hand, besides the economic aspects, it's important to consider the important impact on scientific progress, with evident positive impacts in people's well-being, since this allows the adoption of procedures to solve pressing problems such as osteoarthritis. At this point, it should be noticed that HA and collagen can be used together in biomedical applications related to cartilage, skin and other tissues. Thus, the possible benefits that can be obtained by the clinical use of these two macromolecules are undeniable.

At last there is a strong innovative character when combining HA and collagen from chicken combs, which will certainly enable their exploitation by an industrial and productive point of view. To date only HA is extracted from chicken combs and the protein content (mainly collagen) is wasted. The joint exploitation of HA and collagen would allow the production of innovative and cutting-edge biomaterials at a relative lower cost.

Considering the solid by-products, this thesis will focus in the valorisation of chicken combs. Chicken combs are known for their richness in HA and collagen [4]–[6]. Nevertheless, this by-product is neglected, together with the head, and directed towards the transformation into poultry flour and fat. However, it is important to mention that both HA and collagen possess countless physicochemical properties that make them extremely relevant for purposeful applications in the medical, pharmaceutical and cosmetics fields.

1. Hyaluronic Acid

Hyaluronic acid (HA or Hyaluronan – the “modern name”) is a natural biopolymer that belongs to the glycosaminoglycans (GAGs) family. This family is composed of aminosugar-containing polysaccharides, such as chondroitin (Ch) and chondroitin sulphate (ChS) [7] and their major biochemical features are: linear non-branched polysaccharides, repeated disaccharide units (heteropolysaccharides) and acetylated amino group of the amino sugars (which leads to the disappearance of the positive charge) [8].

The first isolation of HA goes back as 1918, when Leven and Lopez-Suarez isolated a new polysaccharide from the vitreous body and cord blood, and called it “mucoitin-sulfuric acid”. It consisted of glucosamine, glucuronic acid and sulphate ions, but today is clear that isolated compound was in fact HA extracted with a mixture of sulphated glycosaminoglycans. According to literature, the discovery of HA belongs to Karl Meyer and John Palmer, in 1934, who mentioned about an “unusual polysaccharide with an extremely high molecular weight isolated from the vitreous of bovine eyes”. They attributed the name of hyaluronic acid to the new polysaccharide, a designation derived from “hyaloid” (glassy glass-like in appearance) and “uronic acid” (a class of sugar acids, present in HA)[8].

The precise chemical structure of HA was published only after 20 years from its discovery (in 1934), in 1954 by Linker and Meyer. The chemical structure of the molecule of HA (Figure 1.2) was described as 5 000 – 30 000 disaccharide units of β-1→4-linked D-glucuronic acid and (β-1→3) *N*-acetyl-D-glucosamine.

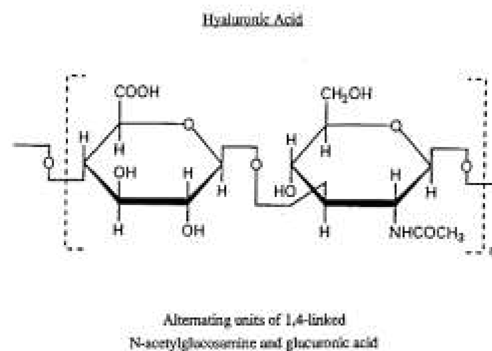

Figure 1.2 - Structure of the disaccharide unit of HA. Obtained from the web.

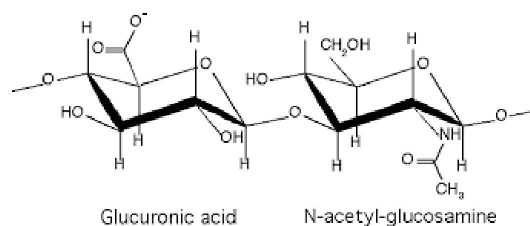

Figure 1.3 - Three-dimensional structure of the disaccharide unit of HA. Obtained from the web.

From a chemical point of view, each disaccharide unit of HA has four different types of functional groups: acetamide, carboxylic acid, hydroxyl and terminal aldehyde. According to Figure 1.3, it is possible to observe that the hydrogen atoms are in an axial position, which is sterically less favourable, and explains why part of the molecule is hydrophobic. On the other hand, carboxyl, hydroxyl and acetamide groups provide the hydrophilic properties to the molecule. The stereochemistry of HA disaccharides justifies why HA is energetically stable [8].

HA can be considered a glycosaminoglycan based on the anionic charge in each glucuronic acid unit at physiological pH and the presence of negative charges associated with the carboxylate group, which are balanced by mobile cations such as Na^+ [9]. Although being a glycosaminoglycan, HA has some distinguish biochemical features: most simple GAG, the only one not sulphated, not covalently associated with a core protein (can be found in a free state), not synthesized in Golgi apparatus, not chemically modified after synthesis and the one with the highest molar mass (up to $8x10^6$ Da) [8], [10]. Two of the most important properties of glycosaminoglycans are their solubility in water (a differentiation from other polysaccharides) and the ability to bind significant amounts of water. When HA is dissolved at pH around 7.0, its carboxylic groups are dissociated, with a low pK value, and the polymer molecules have high-density negative charges, causing an attraction of sodium, potassium, magnesium, calcium and other osmotically active cations. This allows HA molecules to bind up to a thousand times more water than the weight of the macromolecules themselves. Because of these properties, HA molecules can adopt an enlarged conformation, occupy an extremely large volume and form gels at very low concentrations [8].

1.1. HA synthesis and degradation

Enzymes called hyaluronan synthases (HAS) are responsible for the synthesis of HA. These enzymes are membrane proteins integrated into the inner surface of the citoplasmatic cell membrane, who enlarge the HA molecule by adding glucuronic acid and *N*-acetylaminoglucose, repeatedly, to the initial polysaccharide. After that, the polymeric chain is transferred through pore-like structures of the membrane directly on the outer cell surface, into extracellular matrix (ECM) (HA can also be transported into the nucleus of the cell, from the ECM by endocytosis). There are three forms of HAS (HAS_1, HAS_2 and HAS_3) present in the human and vertebrate bodies. They perform synthesis of different molecular weight HA: HAS_1, which performs slow synthesis of high molecular mass HA; HAS_2 which is more active than HAS_1 and responsible for the synthesis of HA with molecular weights up to 2000 kDa; finally

HAS_3 which is the most active enzyme, responsible for the synthesis of short chains of HA (from 200 – 300 kDa) [8].

These enzymes contain cytoplasmic domains with sites that are targets for phosphorylation by protein kinases A and C. Both these protein kinases stimulation leads to activation of the synthesis of HA.

Some factors affect the synthesis of HA. For example, the fibroblast growth factor beta (FGF-β) can stimulate it, in cell culture, by activating the genes of HAS or by activating HAS by phosphorylation through protein kinase C activation [8].

On the other hand, HA is a very sensitive molecule degradable by enzymatic, mechanical, thermal and chemical reactions, due to its long straight linear structures up to 2.4 mm. This could result in a decrease of molecular weight or degree of polymerization. The main factors that can degrade HA are radiation, ultrasound, high temperature, extreme pH, oxidative reagents, free radicals and dynamic motion. Nevertheless, enzymatic degradation is the primary factor of HA's degradation, which initially takes place in the animal tissues, by the action of hyaluronidases, beta-glucuronidase and beta-N-acetylhexozaminidase (exoglycolase). The most relevant class of enzymes that decompose HA's chains are the hyaluronidases (hyaluronoglucosaminidases; HYALs) [8], [11]. HYALs actions lead to the hydrolysis of the hexosaminidic β(1→4) linkages between *N*-acetyl-D-glucosamine and D-glucuronic acid residues of HA, while other enzymes affect the chain terminus, decomposing the molecule at the end of the polymer. Although the name suggests to be reactive specifically with HA, hyaluronidases can also hydrolyse β(1→4) glycosidic linkages between N-acetylgalactosamine sulphate and glucuronic acid in chondroitin, chondroitin-4-sulphate, chondroitin-6-sulphate and dermatan-sulphate, albeit at slower rate. A plausible explanation for the broader specificity of HYALs can be that chondroitins preceded HA in evolution. The reaction rate of these enzymes is directly proportional to the length of the polymer chain – longer chains suffer higher decomposition [8], [11]–[13].

The systematization of HYALs was first proposed by Karl Meyer and is still relevant nowadays. According to Meyer's classifications, hyaluronidases could be divided into three groups [8], [14], as described in Table 1.1.

Table 1.1 - Systematization of hyaluronidases adapted from Meyer [9].

Type	Designation	Characteristics
Type 1	HYALs of the testicular type (hyaluronate-endo-β-*N*-acetyl-hexosaminidases).	Degrade HA through a non-processive endo-lytic process, with the final products of the reaction being predominantly tetrasaccharides. It's said in literature that vertebrate HYALs (testicular and lysosomal HYALs) also have trans-glycosylase activities, with the ability to cross-link chains of HA and chains of HA with Ch or ChS, although the mechanism of the reaction is not known and there is no evidence of the cross-linked chains.
Type 1a	Testicular HYAL, contained in the animal seminal glands and sperm.	
Type 1b	Lysosomal HYAL, present in the lysosomes of the different cells, blood serum, synovial liquid.	
Type 1c	Submandibular HYAL, present in the animal saliva and saliva glands.	
Type 2	HYALs from leech saliva and certain crustaceans (endo- β-glucuronidases).	This type utilizes the hydrolysis mechanism that cleaves the β(1→3) glycosidic bond. They're more similar to the first one than the third, and the final products are also tetrasaccharides, which possess amino sugar at their reducing terminus.
Type 3	Microbial HYALs (hyaluronate-endo-β-*N*-acetyl-hexosaminidases).	Microbial HYALs include the eliminases (also referred as lyases or HA lyases), which hydrolyse endo-β-1,4-glycoside bonds via the β-elimination reaction, with introducing of an unsaturated bond and dehydrate the residue of uronic acid at the non-reducing terminus of the molecule. The result is a 4,5-unsaturated disaccharide.

There are also HYALs activities associated with some species of fungi, although there is no sequence data, neither characterization of these enzymes [13].

In the human body, there are six genes coding for five HYALs. The HYALs are the following ones: HYALs 1–4, Sperm adhesion molecule (SPAM1, former PH-20). The sixth gene – HYALP1 – is a pseudogene that is transcribed, but not translated. Studies based on the 3D models of these enzymes indicated a big similarity, with differences in their C-terminal domains. Active sites and catalytic clefs are highly conserved regions [8], [13].

HYAL-1 is found in the major parenchymal organs (e.g. liver, kidneys, spleen and heart), serum and urine. It can degrade high molecular weight HA to small oligomers and is active at acidic pH.

HYAL-2 has a broader pH optimum, being best when it's less than 4. It can only hydrolyse high molecular weight HA to intermediate size HA fragments of approximately 20 kDa (~50 disaccharide units).

HYAL-3 is also an acid-active enzyme whose degradation mechanism isn't clear yet.

HYAL-4 is also a chondroitinase and its properties are also not studied.

SPAM1 is a testicular enzyme located on the human sperm surface and inner acrosomal membrane. It was thought that this enzyme was tissue-specific, but it was found in the epididymis, breast in the female reproductive tract and associated with a number of malignancies [13]. This glycosylphosphatidylinositol-anchored enzyme hydrolyses HA through the formation of the saturated oligosaccharides. It's active within the pH range of 4.0-7.0 and has high thermal stability (up to 50 ºC). SPAM1 is very important for the fertilization in most mammalians. Upon contact with the oocyte, the sperm cell releases this hyaluronidase by the acrosome, which hydrolyses the HA-rich cumulus layer surrounding the oocyte and the HA in the *zona pellucida*, thus facilitating the conception [8], [11], [13], as seen in Figure 1.4.

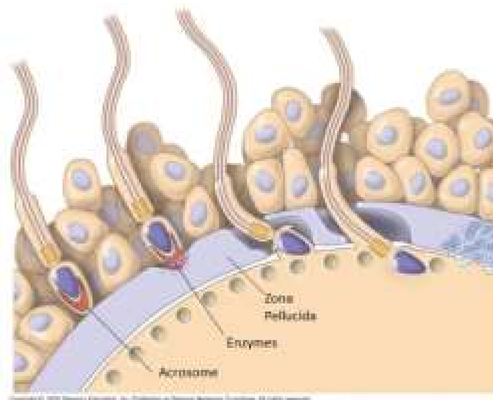

Figure 1.4 - Schematic figure representing the fertilization in mammals. Obtained from the web.

HA is also sensitive to acid-alkaline hydrolysis, which can decrease, irreversibly, its viscosity (e.g. viscosity reduced in 2.5 times by acidification with acetic acid solution.). It can also be depolymerized by oxidation-reduction. For example, papain SH-groups and Fe^{2+} and Fe^{3+}, in the presence of reducing agents, could depolymerize HA, thus reducing its viscosity [8].

The rate of synthesis and decomposition of HA molecules differs according to the tissue where it's present. In skin (epidermis and dermis), HA's half-life is 24-48 h, which means that, during this time, 50% of HA content decomposes and the same amount is synthesized. In the joints the half-life is 1-30 weeks and in the bloodstream just 2-5 minutes. During one day, approximately 5-7 g of HA are synthesized and cleaved in the body of a 70 kg's adult man (~1/3 of the whole amount of HA in the human body of an average adult person – 15 g) [8]. This actions are performed, predominantly, by the reticuloendothelial system [15].

1.2. Where is HA present?

HA is present in almost all human body, in all mammals and in other invertebrates (although in relative small amounts), with the highest amounts being found in the ECM of tissues, such as synovial joint fluid (3-4 mg/mL), vitreous humour of the human eye (0.1-0.4 mg/g wet weight), vitreous body, cartilage, umbilical cords, heart valves, skeletal tissues and lungs (~10 % of the proteoglycan content). The skin (both epidermis and dermis) contains almost half of the human's body HA, being most of it located in the intracellular space (~2.5 g/L). Also, it is present in the matrix produced by the cumulus cells around the oocyte prior to ovulation (~0.5 mg/mL) and in the capsules of some bacteria such as strains of *Streptococcus*. The highest amount of HA is found in rooster combs, mainly localized in the mucous fibres of the subcutaneous layer [8]–[11]. Table 1.2 summarizes the occurrence of HA in different animal tissues.

Table 1.2 - Occurrence of HA in different animal tissues and its content (adapted from [4]).

Tissue or Body fluid	Concentration (µg/mL)	Remarks
Rooster Comb	7500	The animal tissue with by far the highest HA content.
Human Umbilical Cord	4100	Contains primarily HA with a relatively high molar mass.
Human Joint (synovial) Fluid	1400 – 3600	The volume of the synovial fluid increases under inflammatory conditions. This leads to a decreased HA concentration.
Bovine Nasal Cartilage	1200	Often used as a cartilage model in experimental studies.
Human Vitreous Body	140 – 340	HA concentration increases upon the maturation of this tissue.
Human Dermis	200 – 500	Suggested as a "rejuvenating" agent in cosmetic dermatology.
Human Epidermis	100	HA concentration is much higher around the cells that synthesize HA.
Rabbit Brain	65	HA is supposed to reduce the probability of occurrence of brain tumours.
Rabbit Heart	27	HA is a major constituent in the pathological matrix that occludes the artery in coronary restenosis.
Human Thoracic Lymph	0.2 – 50	The low molar mass of this HA is explained by the preferential uptake of the larger molecules by the liver endothelial cells.
Human Urine	0.1 – 0.3	Urine is also an important source of hyaluronidase.
Human Serum	0.01 – 0.1	HA concentrations increase in serum from elderly people as well as in patients with rheumatoid arthritis and liver cirrhosis.

1.2.1. Hyaluronic Acid in Skin

Skin is a stratified and highly organized structure that serves as a barrier between the external environment and the inside of the body. This organ, which many authors refer as "the body's largest organ" (corroborated by Sontheimer) [16], consists of two layers of different tissue: epidermis (the superficial epithelium layer) and dermis.

Epidermis is composed by multiple layers of epithelial cells with approximately 75-150 µm thickness, in total. It consists mainly of outward moving cells called keratinocytes (flat squamous cells) [17]. HA was found to be present around these cells [18].

The stratification of epidermis goes as follows, from top to bottom, starting in stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basal (or germinativum) [17], [18]. The dermis is a denser connective tissue consisting of collagen, elastin, reticulin and a ground substance (composed of glycosaminoglycans, including HA). The cells present in this tissue are fibroblasts, mast cells and dendritic cells. This skin's layer contains blood vessels, lymphatic vessels, nerves, such as parasympathetic and sympathetic nerves, hair follicles, small hair muscles, sebaceous glands and sweat glands [17].

HA is mostly located between collagen fibres and elastin, in the ECM of the skin. It fills the ECM by forming a polymeric web with hyaladherins (proteins that have specific binding sites with polysaccharides) [8]. Its ability to bind water allows the formation of gel structures that maintain a high level of skin elasticity and regulates the diffusion rate of the compounds according to their molecular weight, hydrodynamic volume and charge. It also plays a role in the protection of skin, preventing penetration of microorganisms through the wounded surface and by acting as a free-radical scavenger of reactive oxygen species [8].

In the epidermis, HA can be synthesized by keratinocytes, whose growth factor activates HAS_1 and HAS_3. In the opposite way, HA is involved in keratinocyte proliferation, migration and differentiation. As an example, retinoic acid, which is an inhibitor of epidermal terminal differentiation, also stimulates the synthesis of HA, doubling its content in the epidermis of human skin organ culture [19]. When retinoic acid is present, HA fills the intercellular space, contributing for a reduced number of desmosomes and preventing the tight adhesion of keratinocytes. This results in an inhibition of proper terminal differentiation, a demonstration of HA action on keratinocytes' differentiation. Another factor that supports the role of HA in epidermal differentiation is the action of hydrocortisone (cortisol) over the regulation of HA. Hydrocortisone inhibits the catabolism of HA at all doses, but when used at pharmacological doses, it also inhibits HA synthesis, thus reducing the content of HA in epidermis. This results

in the enhancement of normal differentiation of keratinocytes. It can be concluded that high content of HA inhibits differentiation whereas low content enhances it, so HA may have a role in the regulation of this process. HA can also delay the differentiation of keratinocytes by interacting with its cell surface receptors, sending a survival signal. It was shown that in transgenic mice with inhibited expression of the receptor for HA-mediated motility (RHAMM) in epidermal keratinocytes, the wound healing was delayed. It was also shown, by a complementary experience in mice, that CD44 receptor, when inhibited, affects the wound healing response in the same way. Another function of HA is associated with its rapid turnover in skin (24-48 h), that may help to remove and clear noxious compounds from the epidermis. It may also play an important role in immune responses, because its ability to create extracellular space facilitates the movement of cells from the immune systems, such as Langerhans cells and lymphocytes [18].

1.3. HA Functions

HA is implicated in many biological processes, where it plays important roles. According to Camenisch and McDonald [15], "HA performs three functions: expands the extracellular space by binding salt and water, interacts with a variety of extracellular molecules to form a composite ECM and is recognized by several cell surface receptors that activate intracellular signalling pathways in response to HA or function in HA internalization.".

The ECM (intercellular or pericellular matrix) is a spontaneously organized structure composed mainly by fibrous protein structures of collagen and elastin, which are plunged into a hydrated polysaccharide gel of HA and other glycosaminoglycans. It produces highly specialized structures such as tendon cartilage, basal membrane, and others [8]. HA plays an important role in maintaining the pH and osmotic pressure of the ECM within the physiological norms. It's also involved in the structure and organization of the ECM, maintenance of extracellular space, by binding water and salt, in the active exchange of metabolites, ions and gases between blood and tissues and performs the role of "structural mediator" during cell interaction, creating channels for their migration [7], [8], [10].

In cartilage, HA forms an aggregation centre for aggrecan (a large chondroitin sulphate proteoglycan that retains its macromolecular assembly in the matrix due to specific HA-protein interactions), which is immobilized in the collagen network. This structure is responsible for the structural and mechanical characteristics of cartilage [10], [20].

HA is also present in high concentrations of high molecular mass in synovial fluid, where it provides the necessary lubrication for the joint and resiliency under static conditions. Thus, it is a shock absorber, reducing friction and diminishing wear, caused by the movement of the bones. This is possible due to the ability of HA to bind large amounts of water, which creates a swelling pressure (turgor) that resists compression forces. The explanation of the deteriorated joint movement and pain associated to arthritic diseases, such as osteoarthritis and rheumatoid arthritis, is based on HA's degradation by reactive oxygen species, which results in a reduction of viscosity [8], [10], [11].

HA also plays an important role in fertilization, since it is present in the *zona pellucida* and in the *corona radiata* of the egg, with other glycosaminoglycans, preventing the adhesion to the wall of fallopian tube and reducing the risk of ectopic pregnancy [8].

Also, there is an involvement in cell division, migration and differentiation. It's capable of accelerating (or slowing) the growth of cells and their proliferation (e.g. stimulates the proliferation of human fibroblasts through the collagen of the ECM). A fact that sustains these roles is the coincidence of the accumulation of HA with periods of the division and migration of cells in tissues. It can also participate in the regulation of the transcription of genes and plays an important role in selecting and determining the status of the cell and its adaptation properties [8].

When looking at it from a medical and biomedical point of view, HA promotes epithelial regeneration, prevents the formation of granulation tissue, adhesions and scars, reduces swelling and itching, normalizes blood circulation, promotes scarring of venous ulcers and protects internal eye tissue. In the form of a gel, it provides protection from penetration of high molecular weight toxins and microbial invasion, due to its enlarged conformation [8].

1.3.1. Dependence on the size

HA plays many important functions, but these are dependent on the size of HA molecules, as summarized in Figure 1.5.

High molecular weight HA (>500 kDa) is space-filling, anti-angiogenic, immunosuppressive [12], and supresses cell proliferation and migration. According to Selyanin *et al.* [8], it carries out the functions of intercellular communications between the cells separated by ECM.

When this polymer is on intermediate size, 10-50 disaccharides (50 – 100 kDa), it plays opposite roles from the above mentioned, being inflammatory, immuno-stimulating and

angiogenic [12]. Perng and co-workers [21] demonstrated that HA of 6.5 kDa scaffolds, with collagen, revascularize faster than those of 200 kDa. A similar HA fragment, of 6.9 kDa, also promoted tumour cell motility in a CD44-dependent manner [22]. Ohkawara and co-workers [23] showed that HA of about 200 kDa improves survival of peripheral blood eosinophils *in vivo*, and HA of 3000 to 6000 kDa has much less effect. The inflammatory role was also proved by Jiang and co-workers [11], who found that HA of less than 500 kDa induce inflammatory responses in inflammatory, but not resident, macrophages. West and Kumar [24] showed that HA of 3-16 disaccharides stimulates angiogenesis *in vivo* and endothelial cell proliferation *in vitro*, while native and high molecular weight HA has no effect.

The smaller oligosaccharides are antiapoptotic and induce heat shock proteins, appearing to function as endogenous danger signals, in conditions of thermal shock and slow cell death. Termeer and co-workers [25] demonstrated that HA oligomers of 4-6 disaccharides induced immunophenotypic maturation of human monocyte-derived dendritic cells.

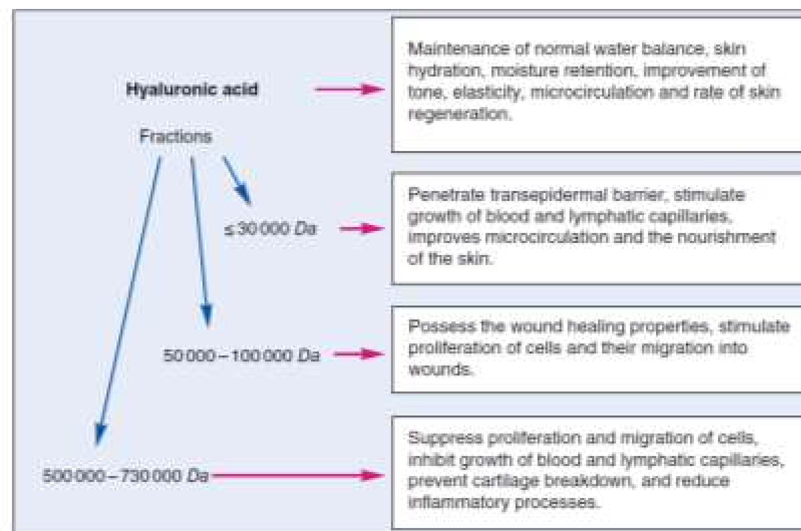

Figure 1.5 - Functions of HA fragments according to their molecular weight (Adapted from [2]).

1.3.2. HA in Wound Healing Process

When an injury occurs, there are a series of sequential events in order to repair the damaged tissue. This response is called wound healing process and occurs as follows: inflammation, granulation tissue formation, reepithelization and remodelling. HA is likely to play important roles in these events, as summarized in Table 1.3.

Table 1.3 - Wound Healing biological processes involving HA [14].

Stage	Process	Mechanism
Inflammatory phase	Inflammation activation	Enhancement of cell infiltration; increase of proinflammatory cytokines TNF-α, IL-1β and IL-8 via a CD44-mediated mechanism; facilitates primary adhesion of cytokine-activated lymphocytes to endothelium.
	Inflammation moderation	Free radical scavenging and antioxidant properties; TSG-6 and IαI mediated inhibition of inflammatory proteinases.
Granulation phase	Cell Proliferation	HA synthesis facilitates cell detachment and mitosis.
	Cell migration	Increased HA synthesis; HA-rich granulation tissue provides open, hydrated matrix that facilitates cell migration; receptor mediated cell migration, e.g., CD44, RHAMM.
	Angiogenesis	Angiogenic properties of low molecular weight HA oligosaccharides.
Reepithelization	Keratinocyte functions	HA-rich matrix is associated with proliferating basal keratinocytes migration via a CD44-mediated mechanism.
Remodelling	Scarring	HA-rich matrix may reduce collagen deposition, leading to reduced scarring as seen in fetal wound healing.

Inflammation is the first response to tissue injury. In the early phase of this process, the injured tissue is very rich in HA. This can act as a promoter of early inflammation, and its synthesis is also improved in response to inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), and bacterial lipopolysaccharides. This phenomenon only happens in microvascular endothelial cells, where it facilitates primary adhesion of cytokine-activated lymphocytes expressing the HA-binding variants of CD44, working in a positive feedback loop. There is an enhancement of cellular infiltration due to HA, which can facilitate the movement of lymphocytes to the injured tissue [26]. HA can also moderate the inflammatory response, which is contradictory but may help in the stabilization of granulation tissue matrix [20].

The granulation tissue has a rich matrix in HA that provides an enhancement of cell migration and proliferation and organization of the granulation matrix itself. This HA-rich matrix facilitates cell migration through specific cell interaction via cell surface receptors for HA, directed migration and control of the cell locomotion mechanisms (e.g. RHAMM forms links with protein kinases associated with cell locomotion). HA plays an important role in cell proliferation in the way that an increase in HA's content is essential for fibroblast detachment

from the matrix and mitosis [27]. It also facilitates cell mitosis in response to mitogenic factors, which can be understood as a justification for HA's role in cell proliferation. In order to the normal tissue repair proceed, the inflammation needs to be moderated, so the granulation tissue is stabilized. As it was said previously, HA plays this function. In fact, it acts as a free-radical scavenger and may also moderate the inflammation through its specific biological interactions with the tumour necrosis factor-stimulated gene-6 (TSG-6)/inter-α-inhibitor (IαI) complex, inhibiting plasmin activity, thus functioning as a negative feedback. Another step of the granulation phase is the angiogenesis (process of new blood-vessels formation from pre-existing ones). Although high molecular weight HA inhibits angiogenesis, it has been shown that low molecular weight HA oligosaccharides promote angiogenesis and enhance the production of collagen by endothelial cells. These low molecular weight HA oligosaccharides may be formed by the action of free-radicals and enzymes onto native HA [20]. In addition, HA is implicated in the control of keratinocyte proliferation, thus playing an important role during reepithelization of wounded tissue repair. In these cases, HA is expressed in the wound margin, in the connective tissue matrix, being associated with CD44 and providing migration of keratinocytes [20]. The last step of wound healing mechanism is the remodulation, when collagen is laid up onto the wound site, which makes the tissue fibrous and results in a scar. The study of fetal wound healing suggest that HA may reduce the deposition of collagen, reducing the scar. In this type of wound healing process, there is no fibrous scarring, which may be correlated with the high content of HA. The application of HA in wounded tympanic membranes resulted in scarless healing, supporting the proposed role of HA in remodelling, as shown by Laurent and co-workers [28].

1.3.3. HA-binding proteins

HA can covalently bind to some protein receptors that specifically bind biopolymers on the citoplasmatic membrane surface or in the extracellular space. These proteins are referred as hyaladherins (HABPs) and are divided in three types: soluble proteins; proteins that bind HA with other polymers of ECM; and other proteins. They include: **CD44**, **RHAMM**, lymphatic vessel endothelial HA receptor-1 (**LYVE-1**), intracellular adhesion molecule-1 (**ICAM-1**), HA receptor for endocytosis (**HARE**), **TSG-6**, **IαI**, toll-like recptor-2 (**TLR_2**), **TLR_4**, and probably more to be discovered. The general feature of them is the binding of HA with other cells or matrix components. One feature of particular interest is the fact that hyaladherins can form complexes with proteinase inhibitors.

The major cell-surface receptor for HA is CD44. This protein is expressed by many cells, such as fibroblasts, epithelial cells, smooth muscle cells, neutrophils, macrophages and lymphocytes, mostly in the standard isoform – 85 kDa protein that undergoes posttranslational modification. The interaction of HA with CD44 may play important roles in what concerns to development of cells, inflammation, apoptosis, T-cell recruitment and activation, tumour growth, metastasis, cell-cell and cell-substrate adhesion, cell migration, proliferation and activation, uptake of HA and regulation of HA content. It is also important in the regulation of keratinocytes proliferation and in the maintenance of HA homeostasis, in the skin. The binding of CD44 with HA stimulates signalling mediated via Rac and Ras. CD44 is also responsible for mediating the binding of HA with chondrocytes. As can be seen in Figure 1.6, the binding of HA with CD44 can cause a cascade of signals that affect the activation of gene transcription, resulting in the switching off of the "differentiation" group of genes and turning on the "proliferation" group, thus changing the cell cytoskeleton, which leads to the alteration of the migrational activity of cells [8], [9], [11], [15], [20].

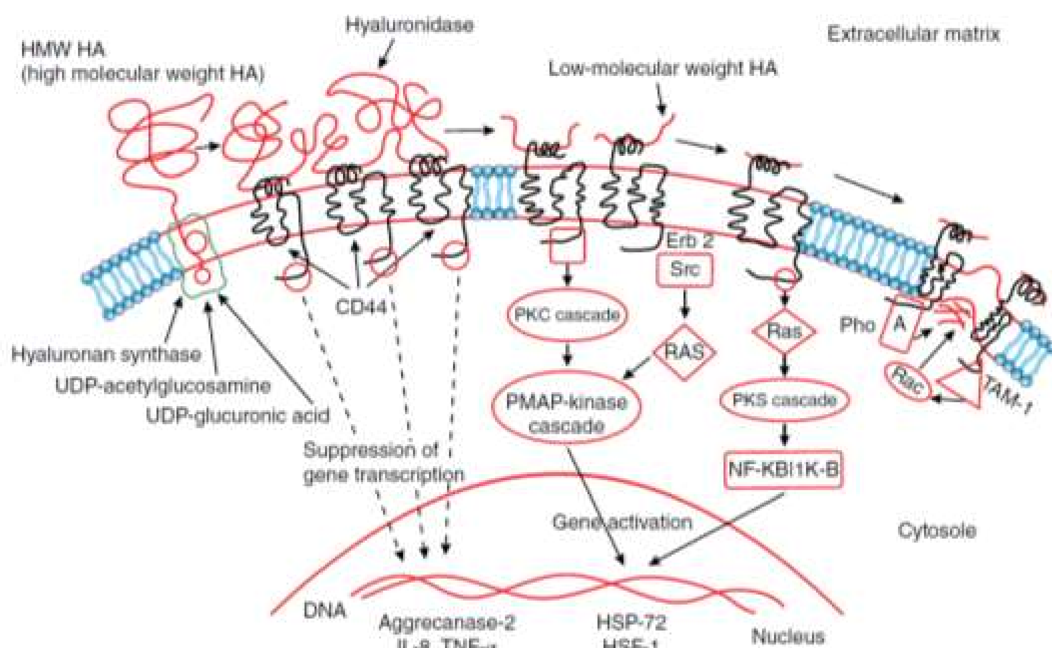

Figure 1.6 - "Signalling scheme of transformation from hyaluronan through receptor CD44 and into the cell, nucleus and genetic system" (obtained from [2]).

RHAMM is also an important HABP that is expressed on cells membrane's surface, as well in the cytosol and in the nuclei of different cells, and associated with cell locomotion, having been identified in many mobile cells, such as migrating fibroblasts and highly metastatic tumour cells. Because of that, RHAMM plays a role in cell migration of fibroblasts and smooth

muscle cells. It was shown by Hardwick and co-workers [29] that RHAMM binds to biotinylated HA (biotinylation is the process of attaching biotin (vitamin-D) to proteins and other macromolecules, allowing the resulting conjugate to discretely bind to avidin, in a complex mixture.). There are experiments that suggest that RHAMM plays a role in Ras-dependent oncogenesis. When linked to HA it plays an important role in tissue injury and repair. It can also participate in HA endocytosis and in the regulation of cell response to growth factors stimulation [11], [20].

LYVE-1 is a type I integral membrane glycoprotein that contains a putative link module and binds soluble and immobilized HA. It is present on the lymph vessel wall and in normal hepatic blood sinusoidal endothelial cells. It is important the transportation of HA from tissue to lymph by uptaking HA via lymphatic endothelial cells [11].

ICAM-1 is a cell adhesion molecule present on endothelial cells, macrophages and others. Its binding with HA may affect the binding of HA with other receptors such as leukocyte integrins lymphocyte function associated-1 (LFA-1) and Mac-1. It is possible that ICAM-1 binding to HA may contribute to the control of the inflammatory activation mediated by ICAM-1 [20].

HARE (also called stabilin-2) is present in sinusoidal endothelial cells of liver, lymph node and spleen. Its active site for linking HA can be blocked by an antibody [11].

TSG-6 is an important HABP closely related to CD44. Its expression occurs in neutrophils and is induced by TNF-α. It can form a stable complex with IαI (a serine proteinase inhibitor in serum) while it binds to HA. This complex, which can be additionally organized by matrix HA, is from extremely importance to the wound healing process, since it may form a negative feedback mechanism in the control of inflammation and stabilization of the ECM during the latter part of the inflammation process. There are also some evidences that IαI can form a stable covalent linkage with HA and this complex may have a role in the formation of pericellular matrix [20].

Toll-like receptors (TLRs) are part of the innate immune system. The recognition of microbial components by them initiates signal transduction pathways that result in expression of genes, whose products control innate immune responses and further instruct development of antigen-specific acquired immunity. There is evidence suggesting that degradation products of HA are generated from inflammation, induce signals that are transduced by TLR_2 and TLR_4 to macrophages and dendrite cells [8], [12], [30].

1.4. Extraction of HA

As it was mentioned before, HA is present in almost every vertebrate animal and some species of bacteria. It can thus be extracted from many sources, both animal and bacterial.

Nowadays, the most viable way of obtaining HA is from microorganisms, such as certain strains of *Streptococcus*, which can have an extraction yield of 1-6 g of HA per litre of cultural liquid. As an example, there is a promising new strain of genetically-modified bacteria – *Bacillus subtilis* – who can encode the enzyme HA synthase and is able to produce HA in the 1000 kDa range of molecular weight, with the advantage of not producing hyaluronidases, neither exotoxins or endotoxins [10]. Although being so economically viable, HA produced through this via is only approved for treatment of superficial wounds and in cosmetic industry, due to the risk of mutations of bacterial strains, co-production of various toxins, pyrogens, immunogens and others.

Hence, when injection of HA is required for specific treatments, the HA extracted from rooster combs is still preferred. This source of HA was one of the firsts to be approved by the Food and Drug Administration (FDA, responsible for protecting the public health, and others.) [10]. Rooster combs are the best animal source of HA, having the largest content of this biopolymer (Table 1.2), which can be extracted with the highest molecular weight, in the range of 2500 kDa.

The extraction process for obtaining HA, specifically from rooster combs, comprises three main stages: preparation, extraction and purification [8]. The preparation step consists in removing the blood and other impurities that come from the avian industries. After this, the blood-free samples could be stored for up to 24 months in 95 % ethanol at 4-22 ºC. To continue the process, the tissues should be grounded in a homogenizer, disintegrator or ball mill. Then, the combs can be placed under acetone, 95 % ethanol or a mixture of ethanol and chloroform, many times as necessary till transparent solution, for dehydration and delipidation. It's also possible to wash the combs directly in acetone (or in the other solutions stated) and then grind them [31]. The extraction step is intended to separate the HA molecules from the combs tissues. In order to achieve this, one can use many methods such as described in Table 1.4. It is also possible to use others, like sodium acetate 5 % solution [32], [33], papain [34], [35] and even crude proteolytic enzymes from chicken intestine and pancreas [3]. Finally, the purification, since, after extraction, the solution containing HA may also contain proteins that can be in the natural form, complexed with HA or between them, and in the form of small peptides (usually when the extraction is carried out with proteolytic enzymes), nucleic acids, lipids,

mucopolysaccharides and low molecular weight precursors. Table 1.5 states some methods for the separation of HA from proteins and the breakdown of the mucopolysaccharides complexes. Examples of the overall methodology usually applied can be seen in Table 1.4. After this step, the protein content must be 0.5 % or less in a commercial product, so it can be considered safe.

Table 1.4 - Methods for HA extraction and purification [2].

#	Source	Extraction	Purification
1	Rooster combs	Water 100 °C, 6 times.	Papain; ultrafiltration in 40 % water-ethanol mixture.
2	Rooster combs	Water.	Extract heating at 90-100 °C; lipid removal; filtration; treatment with activated carbon.
3	Rooster and chicken combs	Water acidified to pH = 3-4, 90-100 °C, 40-50 min.	Treatment with activated carbon, then cellulose; filtration.
4	Rooster and chicken combs	Water, 2 extractions.	Treatment with chloroform; precipitation with ethanol.
5	Chicken combs	Aqueous solution of n-propyl or *tert*-butyl alcohol twice (5-25 %) liquid module 1:(10-15).	Sodium chloride addition (two-phase system); precipitation with ethanol.
6	Rooster combs	Physiological solution, 80-90 °C, 2 extractions.	Filtration; precipitation with saponified acetic acid with sodium hydroxide to pH = 7.0-7.3; heating to 80-90 °C; repeatable filtration.
7	Rooster combs	Water extraction.	Multiple treatments with a mixture chloroform and sodium chloride 4-5 °C for 3-5 h; treatment with pronaze; precipitation with ethanol.
8	Rooster combs	Water, 3 extractions. Tissue: water 1:(4-6), 2-4 h.	Precipitation with trichloroacetic acid (1-2 %) from the extract volume at 20-22 °C for 1-2 h; lipid and water removal with acetone and ether three times.
9	Rooster combs	1-15 % solution of sodium chloride at 60 °C, 18 h. Yield 1.92 % from the starting material.	Centrifugation; lyophilisation.
10	Rooster and chicken combs	Wash of the grounded raw material with ethanol with 1% chloroform. Extraction with 3-3.5 volumes of water, acidified to pH = 3-4 at 90-100 °C during 40-60 min. Yield 0.09 %.	Extracts filtration and proteins removal at 60-80 °C, 1-2 h with charcoal, then diethylaminoethyl-cellulose (1-1.5 % from the extract volume); filtration at 30-40 °C through polyvinyl-chloride membranes.
11	Rooster combs	Before grinding, the tissue is treated with ethanol in a ration 1:2, then grind, treat with ultrasound (16-20 kHz) 20-25 min. Extraction with	Vacuum filtration of the extracts; HA 95 % purity precipitation with ethanol at the ration 1:3, drying.

		water at 45-50 ºC 20-25 min. 55 % of HA could be extracted.	
12	Rooster combs or umbilical cord	Grinded raw material is frozen to (-20-70 ºC), 2 parts of water by weight added and the mixture is heated for 15-25 min at 95-100 ºC. Method increases the yield of HA in 3-4 times.	HA precipitation with acetic acid; ultrafiltration; lyophilisation.
13	Rooster combs	The tissue is treated with ethanol in ratio 1:2, extracted with water with collagenase 0.03-0.04 % to the tissue weight for 45-50 min, at 45-50 ºC, pH = 6.8-7.0. Increased yield and better quality of HA.	Precipitation with ethanol at the ration 1:3; vacuum filtration, vacuum drying or sublimation.
14	Rooster combs	Frozen tissue treated with water at 55 ºC, grinded and adjust pH to 7.5. Proteinase added and proteolysis carried out for 3.5 h at 37 ºC. After filtration, 5.6 g of the final product is obtained from 1 kg of the tissue.	Precipitation with cetylpyridinium chloride; the precipitated powder dissolved in 30 % of ethanol with sodium chloride and re-precipitated with ethanol.
15	Rooster combs or umbilical cord	The combs are boiled in water for 45 min, grinded and heated for 4 h at 50 ºC and pH = 7.5 with pronaze. Yield 6.7 g from 1 kg of the tissue.	Filtration; precipitation with cetylpyridinium chloride (CPC); the precipitant is dissolved in 30 % ethanol with sodium chloride and treated with ammonium chloride in order to precipitate the final product.

Table 1.5 - Methods for separating HA from proteins and mucopolysaccharides complexes [23].

Denaturation and Separation of Proteins	**Removal and Breakdown of Complexes with Mucopolysaccharides**
Extraction by chloroform with amyl alcohol	Hydrolysis with 18 % hydrochloric acid
Extraction with 90 % phenol	Treatment with cetylpyridinium
Extraction with sodium acetate	Addition of heavy metal salts
Enzymatic hydrolysis with papain Precipitation with cetylpyridinium Ultrafiltration	Enzymatic hydrolysis with: Pepsin; Pronase; Trypsin.
Adsorption on activated carbon	Ion-exchange chromatography
Electrodialysis	Electrophoresis

In biomedical applications, HA solutions must be sterilized, which is usually achieved by autoclaving at 120-130 ºC or by ionizing gamma radiation [8]. These processes, along with extraction and purification methodologies, cause a fragmentation of HA molecules, which can lead to a change in the therapeutic activity that was initially desired for the biomedical product [8]. For that reason, it is of major importance to analyse the molecular weight of the biopolymer through the entire process of obtaining HA. Another important measure is the viscosity, because it is directly correlated with the molecular weight of HA and its reduction is proportional to the thermal degradation. Nevertheless, there are some solutions for this problem, such as the addition of amino acids, boric acid and glycerol, hydroproline sulphate, uric acid, phenolic compounds (e.g. pyrogallol) and 8-hydroxyquinoline (last one prevents HA viscosity reduction) [8].

1.5. HA applications and uses

As mentioned above, HA has important physicochemical properties and biological functions. Such characteristics make this molecule highly biocompatible and biodegradable, proving that HA is a very suitable biomaterial for many biomedical applications [9]. If not, there are chemical modifications that can alter some HA's properties, which target three functional groups of the molecule – glucuronic acid's carboxylic acid, both primary and secondary hydroxyl groups and *N*-acetyl group (following deamidation) [36], [37]. The resulting derivatives can be considered to be "monolithic" or "living", meaning that they do not form new chemical bonds or that they form new covalent bonds in the presence of cells or molecules, respectively [38].

There are already many medical applications using commercially available HA, while others are being developed and investigated. The next paragraphs will review some of these applications according to the field of use.

1.5.1. Tissue Engineering

The purpose of tissue engineering (TE) is to aid in the regeneration of a damaged tissue, and for that it is possible to use many forms of biomaterials such as scaffolds, hydrogels, injectable materials and others.

HA is considered a very interesting candidate for TE strategies as it plays a major role in tissue organization, especially in skin, where it is involved in keratinocytes proliferation,

migration and differentiation. Some other studies show that HA is implicated in morphogenesis, being a major constituent of the ECM surrounding migrating and proliferating cells during embryonic development [39], [40].

In order to create an adequate scaffold to induce cell regeneration in an architecturally complex tissue, a dual-crosslinked HA hydrogel scaffold was engineered with photopatterned anisotropic swelling [41]. Another approach to obtain a more complex tissue regeneration could be the production of a HA–tetrabutylammonium salt based sponge-like scaffold with a porous structure [42]. A similar work was made by Ko and co-workers [43].

It was found possible, by Bhattacharyya *et al.* [44], to incorporate single-walled carbon nanotubes in an HA hydrogel, enhancing the mechanical properties and maintaining the characteristic swelling behaviour of normal HA hydrogels.

1.5.1.1. Cartilage Tissue Engineering

Since the 1980's, HA has been used to treat many patients suffering from osteoarthritis – a degenerative joint disease –, as a viscosupplementation agent. There are studies that demonstrated the ability of HA to inhibit cartilage degradation [45], protect the surface of articular cartilage [46], [47], normalize the synovial fluid properties [48] (through HA's anti-inflammatory action [49]) and to reduce the pain [50]–[53]. In fact, this is one of the major applications of HA, with many products on the market like, for example, Synvisc® (Produced by Sanofi, Paris, France), Hyalgan® (Produced by Fidia, Abano Terme PD, Italy) and Durolane (Produced by Q-Med AB, Uppsala, Sweden). Although this is not a TE application, one will consider this as the base step into cartilage tissue engineering.

For cartilage repair and regeneration, the products must promote cellular growth.

Back in 2001, a modified HA-based scaffold was used to growth human chondrocytes. These cells maintained a proper phenotype, producing type II collagen and aggrecan, which is useful to repair articular cartilage defects [54].

Another approach could be the encapsulation of cells, such as auricular chondrocytes, in photopolymerized HA-based scaffolds [55]–[57] or freeze dried HA/Chitosan scaffolds [58]. Cultivation of human meniscus cells was achieved on a freeze dried HA/Polyglycolic acid scaffold by Freymann *et al.* [59].

A co-crosslinked synthetic ECM, prepared by combining polyethylene glycol diacrylate (PEGDA) with thiolated HA and thiolated gelatin, has been used to deliver mesenchymal stem

cells for osteochondral defect repair in the patellar groove of rabbit femoral articular cartilage, with promising results [60].

1.5.1.2. Cardiovascular Tissue Engineering

Hydrogels composed of HA, alginate and carboxymethylcellulose presented suitable properties as cardiovascular biomaterials [61].

HA-based hydrogels have been showing benefits when used in heart regeneration after a myocardial infarction, in preclinical models [62]–[64]. A HA-based scaffold (HYAFF®11) has been used to deliver mesenchymal stem cells to the infarcted myocardial area [65], [66].

A recent review explains the benefits of this therapy and refers to more studies on this subject [67].

It is of great importance to find a biological substitute for heart valves, since the natural ones suffer from diseases or damage inflicted during the hole life of a person and the synthetics can be rejected more easily by the organism.

Once HA is present within the structure of the native heart valve, studies were also made to culture valvular interstitial cells (VICs) on photopolymerized HA-based hydrogels. It was found that these cells adhered and proliferated on this matrix [68] and the low molecular weight HA gels and their degradation products significantly increased the cellular proliferation [69]. The encapsulation of VICs in crosslinked hydrogels of HA and poly(ethylene glycol) (PEG) was shown to be viable and allowed some control over the produced matrix [70].

1.5.1.3. Central Neural Tissue Engineering

It is known that transplantation of stem/progenitor cells after a stroke improves recovery, on rodent models [71]. However, these cells die after transplantation, especially if they are transplanted to the stroke cavity, which is the best area since transplantation may damage the normal brain tissue.

To solve this problem, a hydrogel composed of cross-linked HA and heparin sulphate was created to support stem cells when transplanted into the stroke cavity. This support matrix significantly promoted the survival of 2 different neural progenitor cell lines both *in vitro* and *in vivo* conditions, when compared to stem cell injection without any support matrix. [71]

A biphasic biomaterial that consisted of electrospun poly(L-lactic acid) with an HA hydrogel core was used to seed human mesenchymal stem cells, obtaining good results in terms

of histology, biochemistry, immunohistochemistry and gene expression. This showed to be a potential biological substitute for degenerated intervertebral discs. [72]

A freeze dried methacrylate functionalised HA scaffold was used as a brain-mimetic model ECM to investigate the mechanobiological regulation of brain tumours progression [73].

An HA-based hydrogel containing peptides and growth factors was used to culture *in vitro* human mesenchymal stem cells, which enhanced neural differentiation of the cells. This hydrogel was also used on spinal cord injured rat models, showing a positive influence on the regeneration of motor function [74].

1.5.1.4. Other tissues

HA/gelatin scaffolds have been also produced with tuneable porous structures for other soft tissue applications [75].

A copolymer of HA and poly(2-hydroxyethylmethacrylate) was analysed for supporting alveolar cell adhesion and growth, proving to be suitable for many cellular types [76]. This could be used as a scaffold for lung tissue engineering.

Espandar and co-workers [77] evaluated the ability of human adipose-derived stem cells to survive and express human cornea-specific proteins on HA-based scaffolds. The positive results obtained with this *in vivo* experience opens up the possibility of using these scaffolds as a source of keratocytes to regenerate the cornea stroma.

Renal regeneration was found to be improved by *in vivo* delivery of embryonic endothelial progenitor cells encapsulated in HA hydrogels [78].

A solid free-form fabricated scaffold of poly(lactic-co-glycolic acid) grafted HA encapsulating intact bone morphogenetic protein-2 (BMP-2)/PEG complex has been successfully used to delivery this protein with a controllable release for up to a month, which contribute to an enhancement in bone regeneration [79].

Synthesized high molecular weight HA scaffold was used to deliver brain-derived neurotrophic factor (BDNF), as a substitute for the typical bovine collagen-derived atelocollagen. It proved to be a proper scaffold for periodontal tissue regeneration. [80]

1.5.2. Wound Healing

Wound healing is a dynamic process that involves the replacement of missing or devitalized cellular structures and tissues.

HA preparations are useful for the enhancement of wound healing process and have been widely used ever since the first records.

Probably the first use of HA in this application came in the 1960's, when the product Hyalgan® (Produced by Fidia, Abano Terme PD, Italy) has been applied topically for the treatment of burns and skin ulcers [10].

The treatment of deep cutaneous acute lesions, such as burns, ulcers or traumas, is a very important application of HA, since autologous skin is not always readily available. There are some products on the market that have been widely used for these treatments, such as Hyalomatrix® (Produced by Anika Therapeutics Inc., Bedford, MA, U.S.), mostly used for treating burns [81]–[83]. Most of these products are made of HA derivatives, being HYAFF® ("HA derivative polymer obtained by the esterification of the free carboxylic group of glucuronic acid with benzylic alcohol" [83]) the most used.

There are some studies about the use of HA to treat burns that showed promising results [84]–[86].

Another important application of HA derivatives is the management of chronic wounds, such as diabetic foot ulcers [87]–[89], neuropathic leg ulcers [90] and venous and vascular leg ulcers [91], [92]. Traumatic injuries can also be similar to the chronic foot ulcers, thus it is possible to treat them in a similar way with HA. Cervelli *et al.* used HA and platelet rich plasma – an autologous plasma with a platelet concentration of 10^6 platelets/µL and a high concentration of native growth factors [93] – to heal wounds with exposed tendons [94].

Interstitial cystitis – a chronic disease characterized by pain in the bladder and pollakiuria [95] – has been treated with intravesical injection of HA [96]. These treatments accelerated epithelial healing of the vesical mucosa, inhibited vesical fibrosis [97] and reduced the pain and urinary frequency [98].

Sodium hyaluronate (sodium salt of HA) accelerates the wound healing of the corneal epithelium by binding to CD44 [99], which promotes migration of corneal epithelium cells to the denuded cornea [100].

Glottal insufficiency – a condition that keeps the vocal folds from closing completely – is usually treated by surgery or by vocal fold injection with augmentative substances, like bovine collagen. Hylaform® (Hylan B gel; Produced by Genzyme Corporation, Cambridge, MA, USA) is a good and safe alternative to the usually used augmentative substance, proving to be effective, even after 2 years of treatment [101], [102].

Two commercial biomaterial products, composed of HYAFF® – EpiFilm® and EpiDisc® Otologic Laminae (Produced by Medtronic, Minneapolis, MN, USA) –, have been used in a

surgical procedure called *Hyaluronic Acid Fat Graft Myringoplasty*, to treat tympanic membrane perforations [103].

In this context, TE poses as a more suitable medical treatment by delivering cells and tissue constructs to the body.

It was found possible to mimic epidermis by culturing keratinocytes on an HA-based scaffold, forming a sheet-like structure [104]. Another HA-based scaffold has been used to culture fibroblasts in a three-dimensional structure that simulates dermis [105]. These two studies point to the possibility of forming a biological skin equivalent.

1.5.3. Aesthetic Treatments

The most common nonsurgical aesthetic treatments are rejuvenation procedures such as botulinum toxin and dermal filler injections. According to *American Society of Plastic Surgeons*, in 2015 there were performed 2.4 million treatments with soft tissue fillers, being 1.95 million of this made with HA-derived products [106]. The majority of this treatments are applied to correct age-related temporal volume loss, wrinkles, folds, HIV-associated facial lipoatrophy and acne scars or to augment facial structures and improve nasal function [107].

For these purposes there are several products on the market, e.g.: Restylane® (Produced by Galderma Laboratories, L.P., TX, USA), who was the first FDA approved product and proved to have advantages when compared to collagen products [108]; Emervel® (Produced by Galderma Laboratories, L.P., TX, USA); Macrolane® (Produced by Galderma Laboratories, L.P., TX, USA); Perlane® (Produced by Galderma Laboratories, L.P., TX, USA); Prevelle™ Silk (Produced by Genzyme Corporation, Cambridge, MA, USA); Hylaform®; Captique® (Produced by Genzyme Biosurgery, Ridgefield, NJ, USA); Juvéderm® (Produced by Allergan, Irvine, CA, USA); Elevess™ (Produced by Anika Therapeutics Inc., Bedford, MA, U.S.), among others.

1.5.4. Adhesions and Scars Reduction

Postoperative adhesions are a complication of invasive surgical procedures and can lead to many problems, such as infertility, bowel obstruction, morbidity and even mortality [109].

One way to prevent adhesion is using a barrier that will keep tissue planes separated until normal wound healing has taken place. HA-based hydrogels have been used as a barrier for adhesion reduction and prevention [110]–[113]. GYNECARE INTERGEL® Adhesion

Prevention Solution (Produced by Lifecore Biomedical, Chaska, MN, USA) is a FDA approved product used as a barrier during gynaecological surgery to prevent adhesions. Seprafilm® (Produced by Genzyme Corporation, Cambridge, MA, USA) is used in abdominal or pelvic laparotomy to reduce adhesions. MeroGel® (Produced by Medtronic, Minneapolis, MN, USA) is a gel stent used after functional endoscopic sinus surgery or nasal trauma to reduce adhesion.

In a similar way, HA prevents scar formation in skin incision wounds [114], after brain damage [115] and after spinal cord injury [116]. It is also capable of preventing epidural scarring [117], as well as keloid scarring [118].

Some positive results have been obtained when using HA after flexor tendon repair to prevent peritendinous adhesions [119]. The formation of de-novo intrauterine adhesions after hysteroscopy was significantly reduced with an auto-cross-linked HA gel [120].

1.5.5. Molecule Delivery

Since HA can be easily chemically modified, it's possible to link it to many molecules – such as drugs or drug carriers – and cells, which makes it a nice "vehicle" for molecule delivery.

A work from Pouyani and Prestwich [121] demonstrated how HA can be functionalized in order to covalently attach to steroidal and nonsteroidal anti-inflammatory drugs.

Apart from that, in what concerns to drug delivery, it has been suggested that HA improves the half-life of a drug in the blood plasma [122]. Moreover, HA is particularly suitable for anticancer therapy since CD44 and RHAMM are overexpressed in tumour cells, which enhances binding and internalization of HA, thus improving drug selectivity versus target cells [123], [124].

As an antitumor conjugate, HA has been tested with Paclitaxel (Taxol® – Produced by Bristol-Myers Squibb, New York, NY, USA), showing selective toxicity toward human cancer cell lines [125], which requires receptor-mediated cellular uptake of the bioconjugate [126]. A previous work reported antitumor activity and anti-metastatic effects with a HA-Mitomycin C complex, being selectively toxic to a lung carcinoma xenograft [127]. For the purpose of making an antitumor conjugate, HA has been combined with some other drugs: sodium butyrate [128], camptothecin [129], doxorubicin [130], [131], cisplatin [132], [133] and quercetin [134].

Another research work has used HA modified chitosan nanoparticles loaded with solution of dorzolamide hydrochloride and timolol maleate to evaluate the potential of this drug delivery system in the treatment of glaucoma [135].

In summary, HA can be used to deliver growth factors [74], [136], [137]; small molecules, such as steroidal anti-inflammatory drugs stated above [121] and others [138]; and large molecules, such as proteins [139], [140] or DNA [141]. More insights regarding this subject are provided in the reviews of Prestwich and Dosio *et al.* [142], [143].

1.5.6. Control of Stem Cell Behaviour

HA has been used to control the differentiation of entrapped cells, as described in a study previously mentioned, where human chondrocytes seeded on a HA scaffold produced collagen type II and aggrecan, while reducing the production of collagen type I [54]. But it would be by far more interesting if HA could be used to control the differentiation of stem cells.

A more recent study permitted chondrogenesis of mesenchymal stem cells when cultured in a 3D HA hydrogel with production of cartilage specific matrix proteins [144].

Another work reported a long-term self-renewal of human embryonic stem cells when encapsulated in a 3D HA hydrogel, with the possibility of inducing differentiation by altering soluble factors. Introduction of angiogenic factors induced vascular differentiation [145].

1.5.7. Ophthalmology

Being a major component of the vitreous of the eye, HA is widely used in ophthalmology for its viscoelastic properties. HA's products are mainly used as ophthalmic viscosurgical devices (OVD) to protect delicate tissues of the eye, like the endothelial layer of the cornea, and to provide space during surgeries [10].

The first product of HA for ophthalmology came on the market in 1980 – it's called Healon® (sodium hyaluronate 1%) and was developed by Endre Balazs; now it's manufactured by Abbott Laboratories, Inc. (Abbott Park, IL, USA), in at least five different formulations – with the purpose of protecting the corneal endothelium during intraocular surgery [146].

Healon® has been used to position capsular flaps in posterior chamber lens insertion procedure [147], to maintain the anterior chamber space during intraocular lens implantation [148], helping to reduce the loss of endothelial cells during this procedures [149] and as a tear substitute [150], [151].

It is a common procedure to use OVD's during cataract surgery, being sodium hyaluronate's products the most widely used. For this purpose there are two types of HA: high

molecular weight HA (1900-3900 kDa) is used to maintain operative space and lower molecular weight HA (600-1200 kDa) is used to protect the endothelial layer [152, Ch. 21].

Viscoat® (Produced by Alcon, Fort Worth, TX, USA), a product composed of sodium hyaluronate 3 % and chondroitin sulphate 4 %, is widely used for cataract surgery. There are reports of different formulations using sodium hyaluronate that better fulfil the requirements for an OVD – Maltese *et al.* [153] created an OVD composed of HA 2.3 % and hydroxypropylmethylcellulose 0.8 % that showed good results when compared to Viscoat®[153]; Kretz and co-workers [154] reported that Healon EndoCoat® (3 % sodium hyaluronate) showed great adherence to the endothelial surface than the standardly used Healon GV® (1.4 % sodium hyaluronate).

It is important to mention that the use of HA in cataract surgery and intraocular lens implantation has some contraindications, like the postoperative rise of intraocular pressure, which can be managed using hyaluronidases to aid in the removal of HA [155]. It has also been reported that introducing exogenous HA during cataract surgery may contribute to posterior capsular opacification formation *in vivo* [156]. Wang and co-workers modified poly(methyl methacrylate) (PMMA) intraocular lenses with HA-lysozyme composite coating that can be used to prevent posterior capsular opacification and endophthalmitis [157].

On the other hand, it has been suggested by Knepper *et al.* that a depletion of HA and the accumulation of chondroitin sulphate may increase intraocular pressure, causing a primary open-angle glaucoma [158]. Recent studies confirmed that a depletion of HA results in a reduction in activities of matrix metalloproteinases (MMPs), which can lead to the pathogenesis of primary open-angle glaucoma [159]. Back in 1998, McCarty suggested that adequate supplementation of glucosamines could prevent primary open-angle glaucoma, as well as the normal age-related rise in intraocular pressure [160]. A recent study [161] concluded that nonpenetrating very deep sclerectomy with the use of HA implant is an effective surgical option for patients with medically uncontrolled glaucoma when compared to trabeculectomy – the treatment for all types of glaucoma introduced by Cairns in 1968 [162].

HA is also used to treat dry eye syndrome. The product Vismed® (Produced by TRB Chemedica Int. SA, Genève, Switzerland) – hypotonic 0.18 % sodium hyaluronate – proved to be well tolerated and resulted in low incidence of adverse events when compared to saline HA drops and 0.3 % carbomer drops [163]. Another study concluded that 0.3 % sodium hyaluronate would be preferable to treat dry eye syndrome over 0.1 % sodium hyaluronate, carboxymethylcellulose or hydroxypropyl methylcellulose [164]. There are some products on the market to treat dry eyes, like Hylo® (Produced by Ursapharm GmbH, Saarbrücken,

Germany), Vislube® (Produced by TRB Chemedica Int. SA, Genève, Switzerland) or Hylovis® (Produced by TRB Chemedica Int. SA, Genève, Switzerland).

Sodium hyaluronate has also been reported to be useful in the removal of silicon oil in retinal detachment surgery [165], as well as to reduce the risk of opacifying vitreous haemorrhage after vitrectomy in phakic eyes [166].

2. Collagen

Collagens are the most abundant proteins in the mammal body, constituting about 30 % of total protein mass. The collagen "superfamily" comprises 28 distinct genetic types numbered with Roman numerals (I – XXVIII) in vertebrates that can be further classified as fibrillar or non-fibrillar types. Although there is no reference to the first discovery of collagen, it is known that researchers started studying its molecular structure since the 1920's. Collagen type II has been discovered in 1969, by Miller and Matukas [167], and since then, the discovery of other 26 types has been accelerated by molecular biology and gene cloning. Including, in 2007, a novel epidermal collagen has been called collagen XXIX [168], but it was further concluded to be a novel collagen VI chain [169]. This is an example of molecular diversity of the collagen family caused by the existence of several molecular isoforms for the same collagen type. There are also hybrid collagen isoforms, formed by α chains belonging to two different collagen types, as happens in the so-called collagen type V/XI [170], [171].

A good definition for collagen is a protein that contains a triple-helix domain – ranging from 96 % in collagen type I to less than 10 % in collagen type XII – and plays a structural role in the ECM, although there are not well-defined criteria to name a protein collagen [172].

The most common collagen is type I, accounting for 90 % of the collagen present in the body and is mainly located in bones (is the main constituent of the organic part), skin, teeth, tendons, ligaments, vascular ligatures and organs. Type II is the main constituent of cartilage and appears in the eyes. Collagen type III is the main constituent of reticular fibers and also appears on skin, muscle and blood vessels, but this collagen is dependent on age, which means that its quantity reduces with age [173], [174].

2.1. Molecular Structure

The common structural feature of collagens is the formation of a triple-helix, which was proposed in the 1950's based on fibre X-ray diffraction studies and amino acid composition/sequence data [175], [176]. The triple-helix of collagen is rod-shaped, right-handed superhelical twisting with approximately 3000 Å long and 15 Å thick [177]. Three α chains of fibril-forming collagens compose this helix, which are left-handed and have three amino acid residues per turn, varying in size from 662 [human α1(X)] up to 3152 amino acids [human α3(VI)] [178].

This three polyproline II-like polypeptide chains can be either identical – forming homotrimers (e.g. collagen II) –, or different – forming heterotrimers (e.g. collagen IX). They are supercoiled about a common axis and require glycine (Gly) as every third residue in order to be close packed near the central axis [179]. The characteristic pattern of an α chain is thus $(Gly\text{-}X\text{-}Y)_n$, where X and Y are frequently proline (Pro) and 4-hydroxiproline (4-Hyp), respectively. This triplet (Pro-[4-Hyp]-Gly) is the most stabilizer of the triple-helix, and is required $(Pro\text{-}[4\text{-}Hyp]\text{-}Gly)_6$ as the minimum length for triple-helix formation [180]. Although fibril forming homotrimers collagens have identical amino acid sequences, the three chains within the triple-helix are spread out by one residue, creating a non-equivalent environment between amino acids at the same axial level, which leads to the possibility of recognition of different faces of a triple-helix. On the other hand, all non-fibrillar collagens have interruptions on the typical repeating pattern (up to 21-26 interruptions in the collagen IV chains [181]) [182]. This causes local distortions on the triple-helix that are associated with regions of considerable plasticity and flexibility and may serve as molecular recognition sites within non-fibrillar collagens [183].

Residues in the X position are more prone to interaction, since they are more exposed when compared to those in Y position. Charged residues (~15-20 % content) and hydrophobic residues (~6 % content), characteristic of fibrillar collagens, are important for interactions with other collagen molecules, receptors and matrix components [184], [185].

Stability of the triple-helix is due to the presence of Gly as every third residue, a high content of Pro and hydroxyproline (Hyp), interchain hydrogen bonds, salt bridges, electrostatic interactions involving lysine (Lys) and aspartate (Asp) and the presence of alanine (Ala) and imino acids [185]–[187]. Notwithstanding, collagen triple-helix is modulated depending on Pro and Hyp content, varying from a tighter to looser superhelix twist along the sequence [172].

2.2. Extraction of Collagen

Collagen extraction process consists in a two-step procedure. First, a mild chemical treatment – called pre-treatment – is necessary to partial hydrolyse the collagen and to remove non-collagenous substances. This process maintains the collagen chains intact but cleaves the cross-linkers [188]. It can be performed by an acidic process, e.g. mineral acid – which is more suitable for fragile raw materials [189]–, or by an alkaline process, usually applying sodium hydroxide – better for thicker raw materials [190]. The second step is the extraction procedure, which can be performed either by chemical hydrolysis, enzymatic hydrolysis or using ultrasounds.

Chemical hydrolysis is the most adopted procedure by industry. It can be performed with neutral saline solutions – e.g. sodium chloride, phosphates, citrates or tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) [191] – when collagen is salt soluble. The most common process is acid hydrolysis, which uses organic acids – e.g. acetic acid (AA), lactic acid or citric acid [192] – or inorganic acids, such as hydrochloric acid [193]. On the other hand, enzymatic hydrolysis allows a better extraction yield, generates less waste and can reduce the extraction time, but it is more expensive [173]. The most used enzyme for this purpose is pepsin, which can come from different sources such as bovine or even marine [194]. In many cases, enzymatic hydrolysis is performed after an acidic extraction [195]. The use of ultrasound in collagen extraction has proved to increase the yield of extraction and to reduce the extraction time [196]–[198].

Some examples of the diversity of raw materials used for collagen extraction, as well as the corresponding extraction procedure, are highlighted in Table 1.6.

Table 1.6 - Reported examples of raw materials and extraction procedures used to obtain collagen.

Raw material	Extraction procedure	Reference
Silky fowl feet	Extraction with 10 volumes (vol.) (V/w) AA 0.5 M and 0.1 % pepsin at 4 ºC for 24 h.	[192]
Chicken skin	Extraction with AA 0.5 M below 10 ºC for 3 days with stirring.	[199]
Chicken feet	Extraction with AA 0.5 M containing 0.1 % papain or pepsin at 4 ºC for 24 h.	[200]
Chicken feet	Extraction in a salt solution (0.45 M NaCl in 0.05 M Tris-HCl, pH = 7.5) at a ratio of 1:80 (w/V) for 48 h. Further extraction in AA 0.5 M 1:80 (w/V) for 48 h. Further extraction in AA 0.5	[201]

	M 1:80 (w/V) (pH = 2) containing 0.1 % (w/V) pepsin for 48 h.	
Chick sternal cartilage	Extraction using 1.0 M NaCl in 0.05M Tris–HCl (pH = 7.5) at 4 ºC for 24 h. Further 0.5 % pepsin added for 32 h at 20 ºC.	[202]
Bovine pericardium	Extraction using pepsin-to-bovine pericardium ratio of 1:20 solubilized in 10 mM HCl (pH = 2) for 12 h at 4 ºC.	[203]
Bovine dermis and cornea	Extraction with AA 0.5 M (pH = 2.5) containing pepsin (1:100) at 4 ºC for 72 h, with stirring.	[204]
Limed bovine split wastes	Extraction with 30 vol. AA 0.5 M containing 2 % pepsin at 4 ºC for 48 h.	[205]
Bovine Achilles tendon	Extraction in HCl 0.5 M (pH = 2) with pepsin 20:1 (w/w) at 4 ºC for 24 h with stirring.	[206]
Porcine Achilles tendon	Extraction in HCl 0.5 M (pH = 2) with pepsin 20:1 (w/w) at 4 ºC for 24 h with stirring.	[207]
Buffalo skin	Extraction with AA 0.5 M 1:30 (w/V) at 4 ºC for 24 h with stirring.	[208]
Rat tail	Extraction in HCl 0.5 M (pH = 2) with pepsin 20:1 (w/w) at 4 ºC for 24 h with stirring.	[206]
Rat tail tendon	Extraction with urea 9 M at 25 ºC for 20 h.	[209]
Alligator bones	Two consecutive extractions with 10 vol. of AA 0.5 M for 3 days at room temperature to obtain acid-soluble collagen. Two consecutive extractions with 10 vol. of AA 0.5 M containing 0.1 % (w/V) pepsin for 3 days at room temperature.	[210]
Bullfrog skin	Extraction with AA 0.5 M and 0.1 g pepsin at 4 ºC for 8 h with stirring.	[211]
Bullfrog (*Rana catesbeiana*) fallopian tube	Extraction with AA 0.5 M containing 10 % pepsin at 4 ºC for 2 days.	[212]
Sipunculida coelomic wall	Extraction with AA 0.5 M at 4 ºC for 72 h.	[213]
Chilean Mussels (*Mytilus Chilensis*) Byssus	Extraction in distilled water (2:3 w/V) with pH adjusted to 4.0, with HCl, at 80 ºC for 24 h.	[214]
Jellyfish	Acid-soluble collagen extraction with AA 0.5 M for 3 days with stirring. Pepsin-solubilised collagen extraction with 10 vol. of AA 0.5 M containing 0.1 % (w/V) pepsin for 3 days.	[215]
Eel bone	Extraction with AA 0.5 M containing 2.5 % (1:45 w/V) pepsin at 37 ºC for 28 h with stirring.	[216]
Squid (*Doryteuthis singhalensis*) skin	Acid-soluble collagen isolated with AA 0.5 M 1:10 (w/V) at 4 ºC for 3 days with stirring. Pepsin-soluble collagen extracted in	[217]

	5 vol. of AA 0.5 M containing 10 % (w/V) pepsin at 4 ºC for 48 h with stirring.	
Sea Cucumber (*Holothuria parva*) skin	Extraction with AA 0.5 M containing 1 % (w/w) porcine pepsin at 4 ºC for 48 h with stirring.	[218]
Cuttlefish (*Sepiella inermis*) skin	Acid-soluble collagen extracted with AA 0.5 M at 4 ºC for 3 days. Pepsin-soluble collagen extracted in AA 0.5 M with 10 % (w/V) pepsin at 4 ºC for 48 h.	[219]
Starfish (*Asterias amurensis*)	Extraction in AA 0.5 M with 10 % (w/V) pepsin at 4 ºC for 2 days.	[220]
Silvertip Shark (*Carcharhinus albimarginatus*) skeletal and head bone	Extraction with AA 0.5 M containing 1 % pepsin (1:6 w/V) at 4 ºC for 4 days, with stirring.	[221]
Brownbanded Bamboo Shark (*Chiloscyllium punctatum*) cartilage	Acid-soluble collagen extracted with AA 0.5 M (1:15 w/V) at 4 ºC for 48 h with stirring. Pepsin-soluble collagen extracted in AA 0.5 M (1:15 w/V) containing porcine pepsin at 4 ºC for 48 h with stirring.	[222]
Balloon fish (*Diodon holocanthus*) skin	Acid-soluble collagen extracted with AA 0.5 M (1:10 w/V) at 4 ºC for 1 day and then for 12 h (in same conditions), with stirring. Pepsin-soluble collagen extracted in 2 vol. of AA 0.5 M containing 1.5 % (w/w) pepsin at 4 ºC for 30 h with stirring.	[223]
Malaysian Cultured Catfish (Hybrid *Clarias* sp.)	Extraction in AA containing pepsin 1:40 (w/w) at 4 ºC for 20 h.	[224]
Sea Bass (*Lateolabrax japonicas*) skins	Extraction in AA 0.1 M with ultrasonic treatment with a frequency of 20 kHz and 80 % amplitude at 4 ºC for 3 h.	[197]
Bester Sturgeon (*Huso huso x Acipenser ruthenus*)	Extraction in HCl solution (1:10 w/V) (pH=2) containing 0.1 % (w/V) porcine pepsin at 10 ºC for 48 h with stirring.	[193]
Yellowfin Tuna (*Thunnus albacares*) swim bladder	Acid-soluble collagen extracted in AA 0.5 M (1:10 w/V) at 4 ºC for 48 h with stirring. Pepsin-soluble collagen extracted in AA 0.5 M (1:10 w/V) containing crude stomach extract at 4 ºC for 48 h.	[225]
Spotted golden Goatfish (*Parupeneus heptacanthus*) scales	Acid-soluble collagen extracted in AA 0.5 M at 4 ºC for 48 h. Pepsin-soluble collagen extracted in AA 0.5 M containing 1 % pepsin (w/w) at a ratio of 1:10 (w/V) at 4 ºC for 48 h.	[226]

Pacific Cod (*Gadus macrocephalus*) bone	Extraction with AA 0.5 M (1:15 w/V) at 4 ºC for 3 days with stirring. Residue re-extracted in 10 vol. of AA 0.5 M in the same conditions.	[227]
Arabesque Greenling (*Pleurogrammus azonus*) skin	Acid-soluble collagen extracted with AA 0.5 M (1:30 w/V) at 4 ºC for 24 h with stirring. Precipitate re-extracted by the same method. Pepsin-soluble collagen extracted in 3 vol. of AA 0.5 M containing albacore tuna pepsin at 4 ºC for 24 h with stirring.	[228]
Bighead Carp (*Hypophthalmichthys nobilis*)	Extraction with AA 0.5 M (1:10 w/V) containing 0.1 % (w/V) pepsin at 4 ºC for 3 days.	[229]
Unicorn Leatherjacket (*Aluterus monocerous*) skin	Extraction with AA 0.5 M (1:15 w/V) containing acidified tuna stomach extracts or acidified porcine pepsin at 4 ºC for 48 h with stirring.	[230]
Bigeye Snapper skin	Extraction in AA 0.5 M (1:15 w/V) containing tongol tuna stomach extract containing pepsin or porcine pepsin at 4 ºC for 48 h with stirring.	[194]
Sailfish (*Istiophorus platypterus*) skin	Acid-soluble collagen extracted with AA 0.5 M at 4 ºC for 72 h with stirring. Precipitate re-extracted in the same conditions. Pepsin-soluble collagen extracted in AA 0.5 M containing 0.1 % (w/V) pepsin at 4 ºC for 72 h with stirring. Precipitate re-extracted in the same conditions.	[231]
Spanish Mackerel (*Scomberomorous niphonius*) skin and bone	Skin – Acid-soluble collagen extracted with AA 0.5 M (1:15 w/V) at 4 ºC for 24 h with stirring. Residue re-extracted in same conditions. Pepsin-soluble collagen extracted in AA 0.5 M (1:15 w/V) containing pepsin from porcine stomach mucosa at 4 ºC for 2 days with stirring. Bone – Acid-soluble collagen extracted with AA 0.5 M (1:15 w/V) at 4ºC for 3 days with stirring. Re-extraction in same conditions. Pepsin-soluble collagen extracted in 10 vol. of AA 0.5 M containing porcine pepsin at 4 ºC for 2 days with stirring.	[232]
Cobia (*Rachycentron canadum*) skin	Acid-soluble collagen extracted in 50 vol. of AA 0.5 M for 3 days with stirring. Residue re-extracted in 30 vol. of AA 0.5 M for 2 days with stirring, Pepsin-soluble collagen extracted in 20 vol. of AA 0.5 M containing 0.1 % (w/V) pepsin for 24 h with stirring. Residue re-extracted in 10 vol. of AA 0.5 M containing 0.1 % (w/V) pepsin for 24 h with stirring.	[233]
Silver-line Grunt skin	Extraction with AA 0.5 M (1:10 w/V) containing 1 mg/mL pepsin at 10 ºC for 6 h in a shaking water bath,	[234]
Ornate Threadfin Bream (*Nemipterus hexodon*) skin	Extraction with AA 0.5 M (1:100 w/V) at 4 ºC for 48 h with stirring.	[235]

2.3. Characteristics and biomedical applications

Collagen has been widely used as a biomaterial due to its unique properties. It plays an important role in tissues and organs formation, but also contributes to their molecular architecture and mechanical properties [236]. This protein bears good mechanical properties such as high tensile strength due to fibers formation and stability through self-aggregation and cross-linking, which is exhibited by its presence in skin, ligaments, tendons, bones, cartilage and others. It is involved in cell biological functions, such as cell survival, proliferation and differentiation and helps healing bones and blood vessels [237]. Collagen is biocompatible and exhibits weak antigenicity and biodegradability [238].

The main application for collagen has been drug delivery systems, including shields for ophthalmology, sponges for burns and chronic wounds, mini-pellets or tablets for protein delivery, nanoparticles for gene delivery, gel formulations for drug delivery, antibiotic dressings and as controlling material for transdermal delivery [172], [238]. It has also been used in TE [239], as artificial blood vessels and valves [240] and as surgical sutures [241], to state a few examples.

3. Hyaluronic acid-Collagen Biomedical Applications

In the recent years, many have been using HA and collagen together for diverse biomedical applications, as shown in Table 1.7.

Table 1.7 – Examples of existent scaffolds composed of HA and Collagen.

Tissue	Formulation	Cross-linking Reagent	Application	Reference
Adipose tissue	Collagen I/ HA (HA: 7.5 % and 15 %)	1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride	Mammary stromal tissue development	[242]
Brain tissue	Collagen I/ HA 1:2	1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride/ N-hydroxysuccinimide 5:2	Neural stem cells growth and differentiation	[243]
Cartilage	Collagen II/ HA 1000 µL:350 µL	4SPEG (100 µL)	Chondrocyte delivery	[244]
Cartilage	Collagen I/HA(1)/icariin 6 (mg/mL):10 (mg/mL):$5x10^{-4}$ (mol/L)	2-methyl-1-[4(hydroxyethoxy)phenyl]-2-methyl-1-propanone (I2959)/ UV light	Cartilage formation	[245]

Cartilage	PEGDM with 1% collagen I/ 1% HA	Irgacure 2959/ UV light	ECM production	[246]
Cartilage	Collagen I/ HA(1)/ Chondroitin sulphate(1) 5.0:0.3:2.2 (mg/mL)	Tetramethylethylenediamine (TEMED)	3D Cell Culture/ TE	[247]
Cartilage	Collagen I/ HA(1)/ Chondroitin sulphate(1)	Genipin (0.75 mM)	TE	[248]
Cartilage	Poly(propylene fumarate) scaffold with 5 % collagen I/ HA (0.23 mg/mL)	-	Encapsulation /Cartilage repair	[249]
Intervertebral Disc	Collagen II/ HA Weight ratio: 9:1	1-ethyl-3(3-dimethyl aminopropyl) carbodiimide/ N-hydroxysuccinimide	Mesenchymal Stem Cells Carrier	[250]
Skin	Collagen I+III/ HA/ sericin 40 %:20 %:40 %	1-ethyl-3(3-dimethyl aminopropyl) carbodiimide / N-hydroxysuccinimide	TE	[251]
Skin	Collagen I/ HA Weight ratio: 3:7; 5:5; 7:3	Poly(ethylene glycol) diglycidyl ether (PEGDGE)	Wound Dressing	[252]
Skin	Collagen/ HA 2:1	1-ethyl-3(3-dimethylaminopropyl)-carbodiimide	Chronic wounds and diabetic foot ulcers	[253]
Soft tissues	Human-like Collagen/ HA 2:10	1,4-butanediol diglycidyl ether	Filling and Repair	[254]
Spinal Disc	Collagen scaffold/ HA(1)	Irgacure 2959 (1000 ppm)/ UV light	Limit disc degeneration	[255]
Tissues in general	Collagen/ HA(2) 0.19 (mg/mL): 2 % (w/V)	PEGDGE	Cell growth and differentiation	[256]
Tissues in general	Collagen I/HA(1) 3:30 (mg/mL)	Irgacure 2959/ UV light	Cell Culture	[257]
Tissues in general	Collagen I/ HA/ Chitosan 9:1:1	1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride	TE	[258]
Vascular tissue	Poly(ε-caprolactone)/ Collagen/ HA	Hepracil™	Growth factor incorporation/ TE	[259]
-	Collagen II/ HA Weight ratio: 8:1; 1:1	1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride	-	[260]
-	Collagen I/ HA (MW = 6.5 kDa; 8 %)	1-ethyl-3(3-dimethyl aminopropyl) carbodiimide	Angiogenesis	[21]

(1)Pre-treated
(2)Or HA-biotin

4. Aim of this thesis

Nowadays several reports are available in the literature referring HA extraction from chicken combs and collagen extraction from different sources, especially from marine origin. Significant advances are being made in this area, encouraging the search for new sources. Collagen has never been extracted from chicken combs, neither both collagen and HA have been valorised together as a unique extracted compound.

Given the added value of these two compounds for different applications, and the availability of considerable amounts of chicken combs as a by-product, the overall aim of this thesis was the optimization of an extraction process for both HA and collagen from chicken combs and its further characterization.

The specific objectives are:

- HA and collagen extraction from chicken combs,
- Optimization of the extraction methodology,
- Characterization of the extracts through Fast Protein Liquid Chromatography analysis,
- Characterization of lyophilized powders through total collagen determination, total HA determination, Fourier Transform Infrared Spectroscopy analysis and Differential Scanning Calorimetry analysis.

Chapter 2: Materials and Methods

1. Materials

Standards of sodium hyaluronate (97.3 %) and native collagen solution (509 µg/mL hydroxyproline content) were purchased from *Acofarma*, Spain. Acetone (*Sigma-Aldrich*, St. Louis, MO, EUA) and acetic acid 96 % (AA) (*Merck*, Darmstadt, Germany) were used for the extraction methods. For the determination of total collagen content, the following chemicals were used: sulfuric acid 96.3 % (Panreac, Barcelona, Spain), sodium acetate anhydrous (*EMD Millipore*, Billerica, MA, EUA), citric acid (*Sigma-Aldrich*), chloramine T trihydrate 98 % (*Sigma-Aldrich*) and Ehrlich's solution (*Sigma-Aldrich*). Determination of HA content required the following chemicals: di-sodium tetraborate decahydrate extra pure (*Merck*), carbazole >95 % (*Sigma-Aldrich*) and ethanol absolute (*Panreac*).

Chicken combs were provided by *SAVINOR – Sociedade Avícola do Norte, S.A.* – on two periods, October 2015 and February 2016. They were collected from animals with ages between 32-52 weeks, in the processing line, after animals slaughtering. The collected combs were stored at -18 ºC.

2. Chemical composition of the chicken combs

2.1. Dry Weight

The dry weight determination was based on the loss of water and volatile substances at 105 ºC, for at least 24 h, until constant mass.

2.2. Total protein content

This analysis was performed using Kjeldahl method with a conversion factor of 6.25.

2.3. Total lipid content

This analysis was performed using the Soxhlet technique.

3. Extraction of hyaluronic acid and collagen from chicken combs

Three experimental extractions were performed. The first extraction of HA and collagen from chicken combs was performed in an attempt to separate HA from collagen as described in Figure 2.1.

Figure 2.1 - First extraction methodology applied. AA - Acetic Acid

Extraction was performed at 4 ºC to avoid collagen degradation. Between each delipidation the supernatant was always discarded. After the first extraction with AA, the supernatant was discarded. Grinding of the combs was performed using *Moulinex A320* chopper. Every centrifugation was performed at 4000 rpm for 30 min (at 4 ºC), in a super-speed rotor. The second extraction is described in Figure 2.2. This extraction followed all the steps of the first extraction with the exception that chopped combs were put into the previous supernatant for more 24 h.

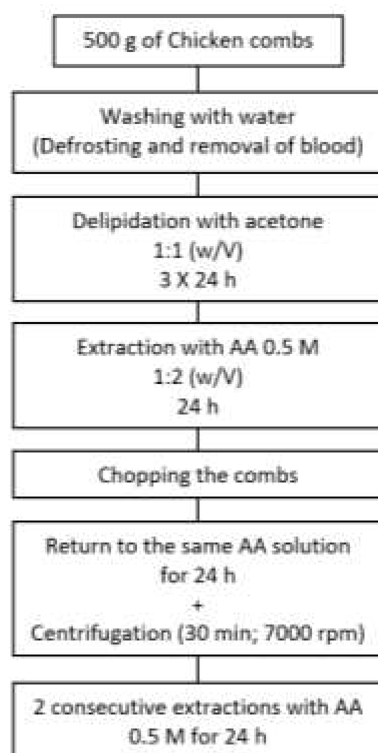

Figure 2.2 - Second extraction methodology applied. AA - Acetic Acid

In addition, this methodology was performed another time but the extraction times with AA 0.5 M were changed, where the second extraction was performed during 48 h, while the third extraction was performed for 72 h.

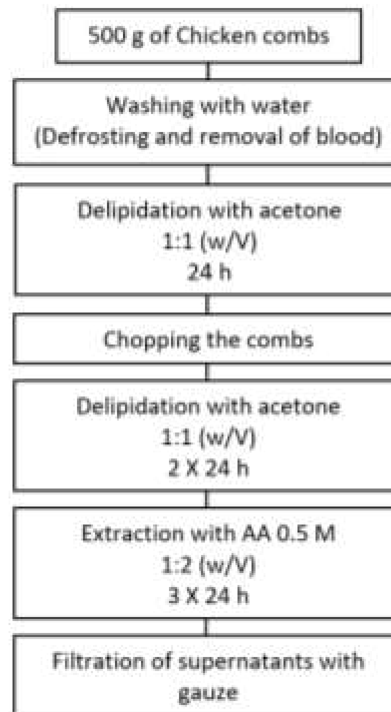

Figure 2.3 - Third extraction methodology applied. AA - Acetic Acid

A third and optimized extraction was performed (Figure 2.3). On this methodology, the chopping step was carried out using a hand blender and after the first 24 h delipidation, which allowed for a better removal of lipids. Between each extraction with AA 0.5 M, the supernatants were filtered with gauze, by squeezing. This proved to be as effective as centrifugation and less time consuming. After extraction, every supernatant (rich in HA and collagen) was conserved at -18 ºC or at -80 ºC and further lyophilised. Lyophilisation was performed in a *Christ Alpha 1-4* equipment (*B. Braun*, Melsungen, Germany), at -53 ºC with a pressure of 0.04 bar for at least 72 h, depending on the quantity of material that was being lyophilised.

4. Liquid extracts characterization

For each extraction procedure, the extracts (supernatants) were characterized through fast protein liquid chromatography (FPLC) and high performance liquid chromatography (HPLC) analysis.

4.1. Fast Protein Liquid Chromatography (FPLC)

FPLC analyses were carried out on *Äkta Pure 25 L* equipment (from *GE Healthcare Life Sciences*, Little Chalfont, UK) with a stationary phase consisting on a *Superdex™ 200 Increase 10/300 GL* column or on a *Superdex™ Peptides 10/300 GL* and a *Superdex™ 200 Increase 10/300 GL* columns in a row. The mobile phase used on every analysis was a phosphate buffer solution with pH = 7 prepared in the laboratory. The volume of sample injected was 100 µL. Samples were previously filtered through a 0.45 µm porosity syringe filter and some were even diluted with ultra-pure water. Elution time was 60 min for the one column analysis and 200 min for the two columns in a row analysis, with a flow of 0.5 mL/min. UV lamp was set to 280 nm. Data was acquired using *Unicorn™* software (from *GE Healthcare Life Sciences*).

4.2. High Performance Liquid Chromatography (HPLC)

HPLC analysis were performed on an equipment composed by an HPLC Pump K-1001, an autosampler and a UV detector k-2501 (all from *Knauer*, Berlin, Germany). Parameters used were adapted from a methodology developed for the determination of sodium hyaluronate in pharmaceutical formulations [261]. Stationary phase used was *Ultrahydrogel™ 250* 7.8 x 300 mm column (from *Waters Corp.*, MA, USA). Mobile phase consisted of a 0.05 M potassium dihydrogen phosphate, with pH adjusted to 7.0. Constant flow rate of 0.6 mL/min and 30 min of elution time were employed throughout the analysis. The wavelength of the UV detector was set to 205 nm and the volume of sample injected was 10 µL. Data was obtained and analysed using *Clarity™* software.

A calibration curve was made using standard solutions consisting of sodium hyaluronate 97.3 % pure dissolved in ultra-pure water. Standard solutions were prepared in triplicate with the following concentrations: 1.0 mg/mL, 0.75 mg/mL, 0.50 mg/mL and 0 mg/mL.

Samples were all previously filtered through a 0.45 µm porosity syringe filter.

5. Lyophilized powders characterization

5.1. Yield of lyophilisation

The yield of lyophilisation was determined by weighing the supernatants obtained by the third extraction methodology and the resulting lyophilized powders, in an analytical balance.

5.2. Total collagen determination: Hydroxyproline quantification

For the determination of total collagen content, lyophilized samples were analysed for their hydroxyproline content, as proposed by *Neuman and Logan* [262].

In brief, first a hydrolysis treatment of samples was performed. 0.1 g (named P_a) of the dried powders were weighted and homogenized with 30 mL of sulphuric acid (3.5 M), and then covered with watch glasses and heated at 105 ºC for the occurrence of hydrolysis. After 16 h, the solutions were transferred into bigger volumetric flasks and the volume was completed with distilled water until 500 mL. After cooling, part of these solutions was filtered with paper filters. An aliquot (named υ) of these filtered solutions were diluted with distilled water in 100 mL volumetric flaks. For the quantification of hydroxyproline, 2 mL of every final dilution solution were pipetted for a test tube. Control was made with 2 mL distilled water. 1 mL of oxidizing solution (10 mL of chloramine T solution diluted in 40 mL citrate buffer [30 g of citric acid, 15 g of sodium hydroxide and 90 g of sodium acetate dissolved in 500 mL distilled water. 290 mL of isopropanol were added to this solution and pH adjusted to 6.0. This solution was diluted with distilled water in a 1 L volumetric flask]) was added to each test tube and then were shaken and let in rest for exactly 20 min. 1 mL of Ehrlich solution was added to each tube and then shaken and put inside a 60 ºC water bath for exactly 15 min. After this, test tubes were cooled in running water for 3 min. Absorbance was read at 558 nm, using the control as the blank. Standard curve was obtained by reading the absorbance of standard hydroxyproline (trans-4-Hydroxi-L-Proline from *Sigma-Aldrich*) solutions with 0.6, 1.2, 1.8, 2.4, 3.0, 3.6, 4.2 and 4.8 µg/mL. Previous steps for hydroxyproline quantification were applied to each solution. The standard curve was obtained by plotting µg of hydroxyproline *vs* absorbance and applying a linear regression to these data. For the calculation of total collagen content, the following equation was applied.

Total collagen content (g of collagen/ 100 g of sample): $C = \frac{2.5 \times h}{v \times P_a} \times f_c$ (2.5.1.1),

where h is the value of µg of hydroxyproline that each sample contains (obtained from the standard curve equation) and f_c is the conversion factor from hydroxyproline to collagen (the value used was 8.0).

5.3. Total hyaluronic acid determination: Carbazole method

HA content was determined by measuring the concentration of uronic acids, using a modified carbazole reaction [263]. Thus, 5 mL of sulphuric acid (0.025 M di-sodium tetraborate decahydrate in sulfuric acid) was placed in test tubes and cooled to 4 ºC. Approximately 10 mg of sample where layered onto the acid. Test tubes were closed with Teflon stoppers and shaken with constant cooling, with ice. Then, test tubes were heated in a vigorously boiling water bath for 10 min. 0.2 mL of carbazole reagent (0.125 % of carbazole in absolute ethanol) were added to each test tube and they were shaken again. The tubes were then heated in the boiling bath for 15 min and cooled to room temperature. Optical density was read at 530 nm in a 1 cm cell. The blank was made against sulphuric acid. For the standard curve, readings of absorbance were performed with standard sodium hyaluronate solutions with 0.125, 0.250, 0.500, 0.750 and 1.000 mg/mL. The procedure was followed equally for the standard solutions, except that was layered 1 mL of each sample onto the acid instead of 10 mg. The standard curve was obtained by plotting mg of HA *vs* absorbance and applying a linear regression to these data. For the calculation of total HA content, the concentration value was divided per weight of the sample.

5.4. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were obtained using the *Spectrum 100 FT-IR Spectrometer* (*PerkinElmer*, USA) in the range of wavenumber from 4000 to 450 cm^{-1} during 8 scans, with 4 cm^{-1} resolution. The FTIR spectra were normalized and smoothed to minimize the interferences, using the *Spectrum* software. Commercial sodium hyaluronate and native collagen solution were also analysed by FTIR to compare with lyophilised powders.

5.5. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry *DSC-60* equipment (from *Shimadzu Corp.*, Kyoto, Japan) was used for the thermal analysis. Sample mass used in all cases was about 7 mg and samples were closed inside aluminium pans. The heating rate was 10 ºC/min from 30-350 ºC. Nitrogen was used to keep an inert atmosphere at a flow rate of 40 mL/min. Data was collected at a sampling interval of 1 second using the *TA-60WS* (from *Shimadzu*) software.

Chapter 3: Results and Discussion

1. Chemical composition of the chicken combs

Chemical composition analyses performed to chicken combs include dry weight assessments and determination of total protein content and total lipid content, which are presented in Table 3.1. According to literature [4], defatted combs have a dry weight of about 10 %. Since no hydration was performed after delipidation, it is not possible to directly correlate this information with the obtained values, but it is possible to observe that this value is similar to the dry weight of the combs after treatment with AA.

Table 3.1 – Chemical composition of the chicken combs.

Sample	Dry Weight (%)	Total protein content (g/ 100 g)	Total lipid content (g/ 100 g)
Washed combs	30.82	15.7	15.2
Combs in AA 0.5 M 48 h	16.00	-	-

AA – Acetic acid.

2. Optimization of Extraction methodology

From the first extraction methodology, it was possible to make some considerations about the presence of collagen in the supernatants, based on FPLC analysis. Since the supernatant obtained after the first extraction with AA 0.5 M for 24 h was discarded, due to an excessive amount of fat, the extraction with AA after grinding the combs was considered as the first extraction. Based on FPLC analysis was possible to verify that collagen was solubilized into the supernatant (Figure 3.1). Nevertheless, it was also present in the sediment, as confirmed by analysing the supernatant obtained after the extraction with AA on the 1st half of the sediment (Figure 3.2).

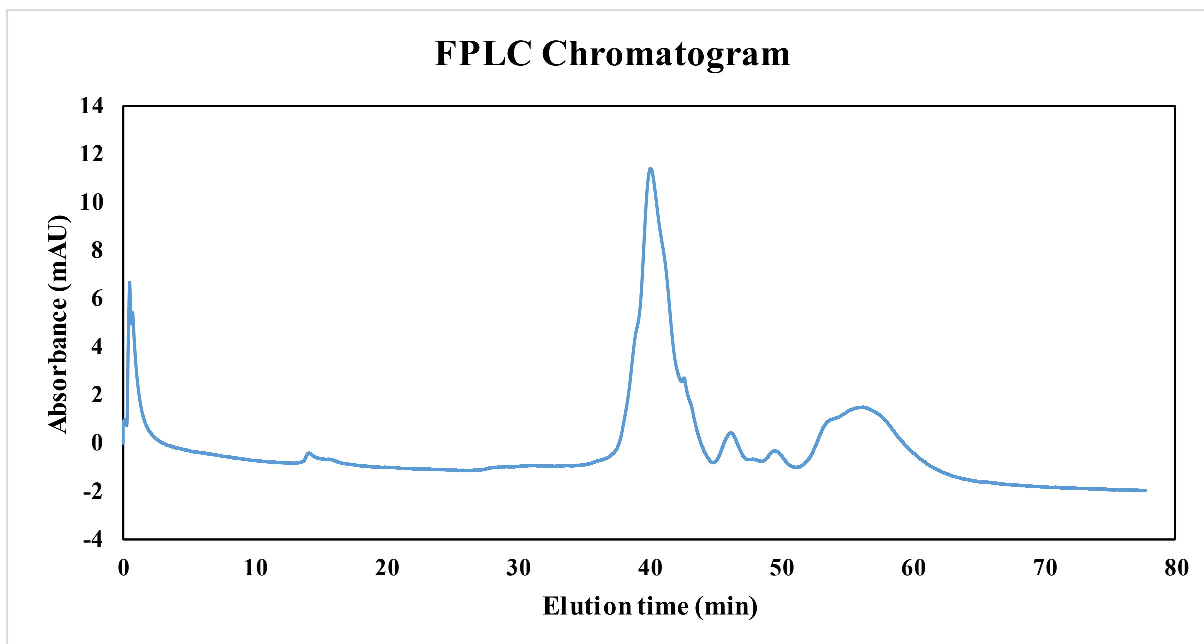

Figure 3.1 - FPLC chromatogram of the supernatant obtained after first extraction.

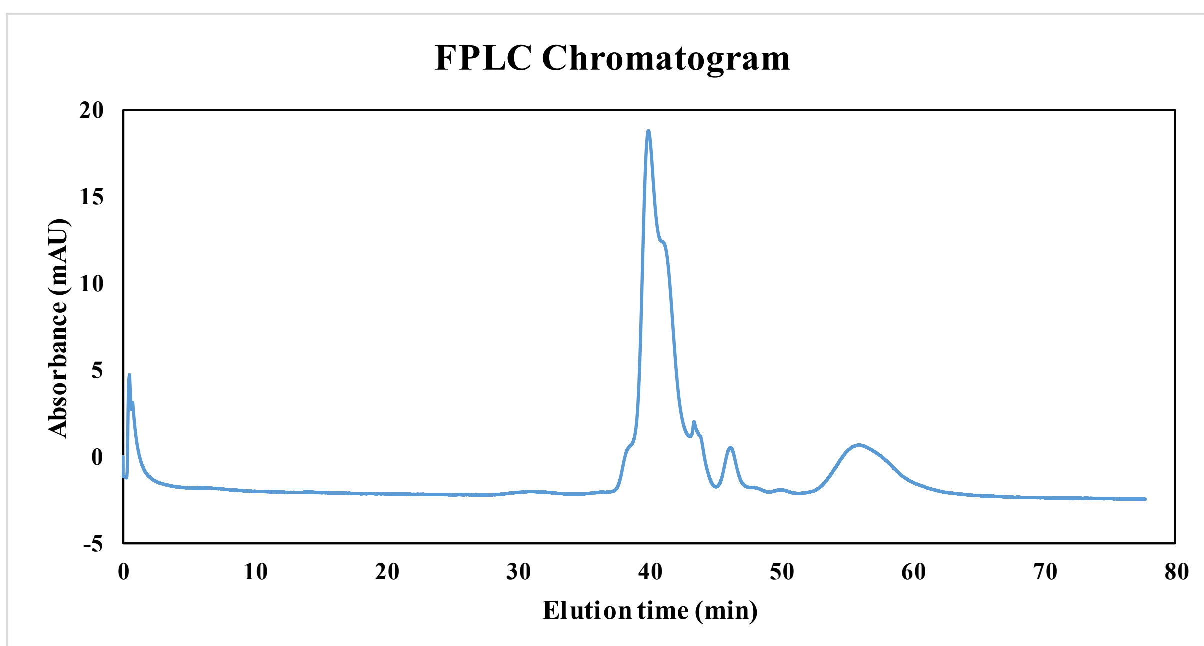

Figure 3.2 - FPLC chromatogram of the supernatant obtained after extraction on the first half of the sediment.

The addition of a NaCl solution to the 2nd half of the sediment solubilized part of the collagen, but most of it remained in the sediment and was solubilized after extraction with AA 0.5 M. Proved by FPLC analysis to the supernatant obtained after centrifugation of the solution with NaCl (Figure 3.3) and to the supernatant obtained after centrifugation of the extraction solution (Figure 3.4).

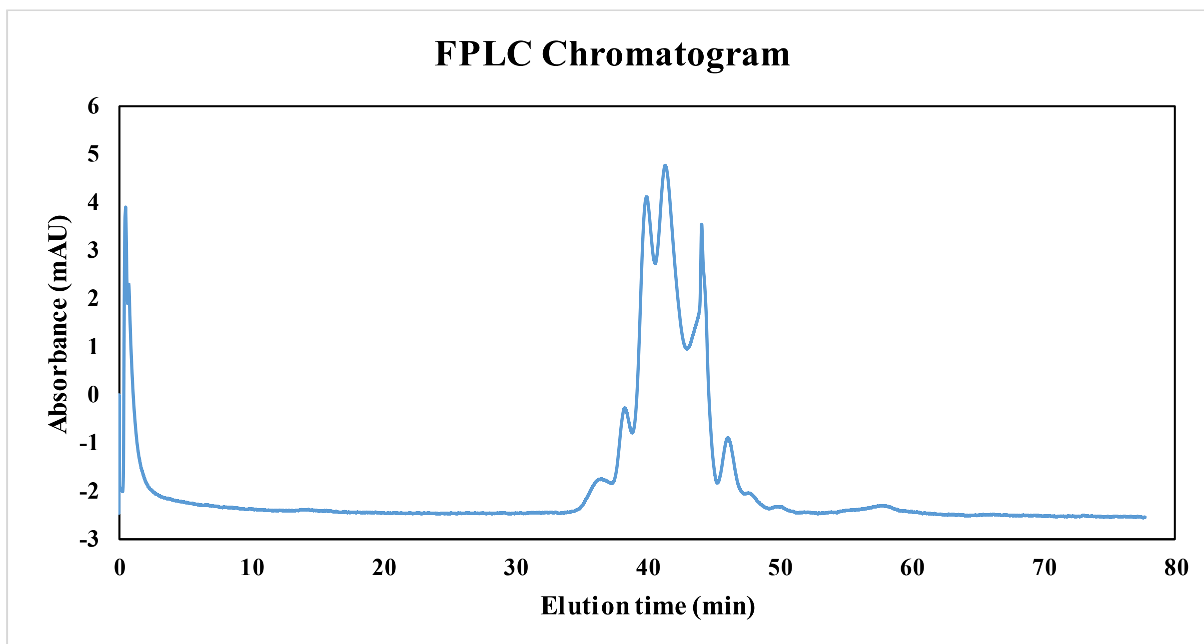

Figure 3.3 - FPLC chromatogram of the supernatant obtained after NaCl addition.

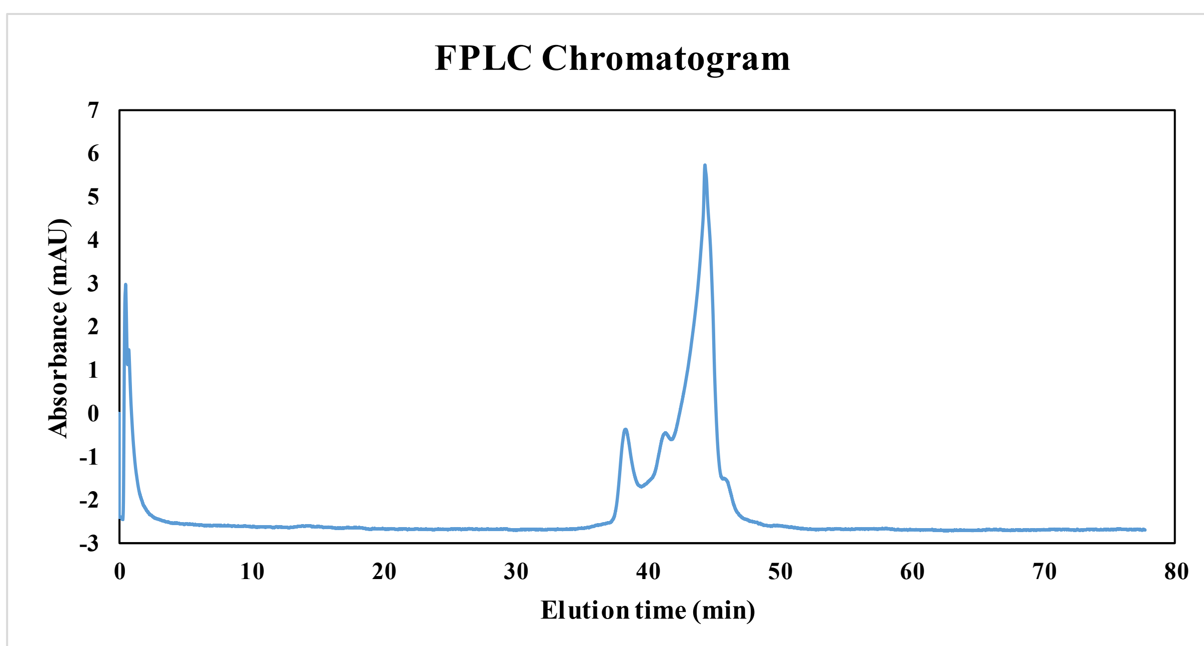

Figure 3.4 - FPLC chromatogram of the supernatant obtained by diluting the sediment treated with NaCl on AA 0.5 M.

Addition of sodium acetate to the supernatant resulting from the first extraction precipitated part of the collagen (Figure 3.5). Addition of ethanol to this last precipitate solubilized the collagen (Figure 3.6). The above mentioned is proved by the presence of a peak with an elution time around 40 min in both figures.

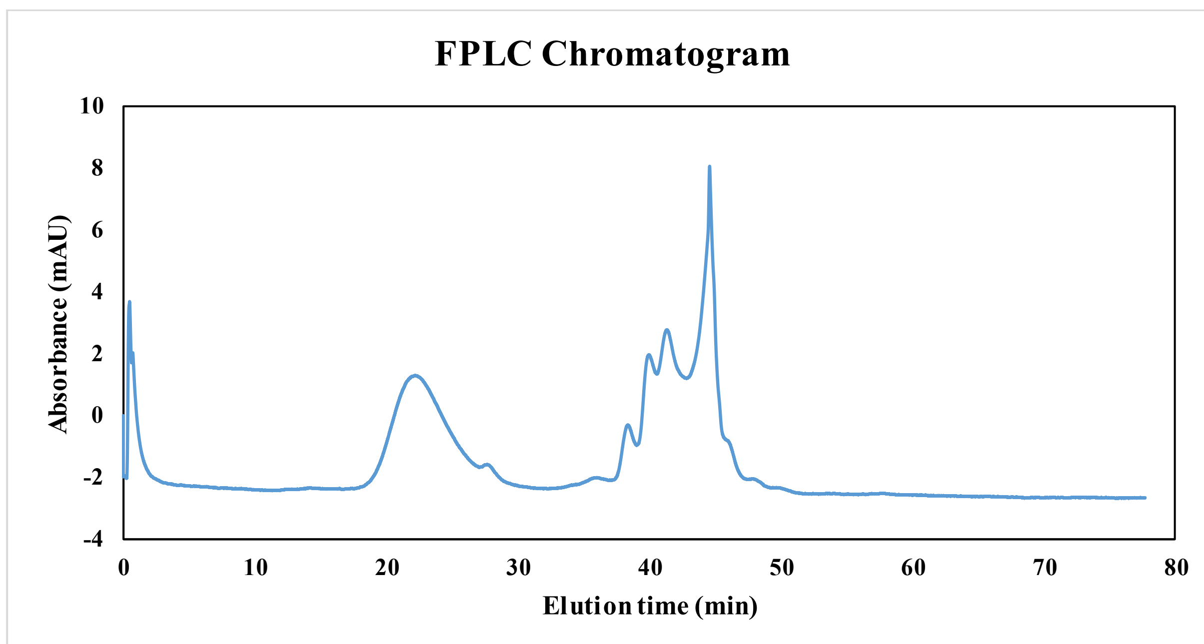

Figure 3.5 - FPLC chromatogram of the supernatant obtained after addition of sodium acetate.

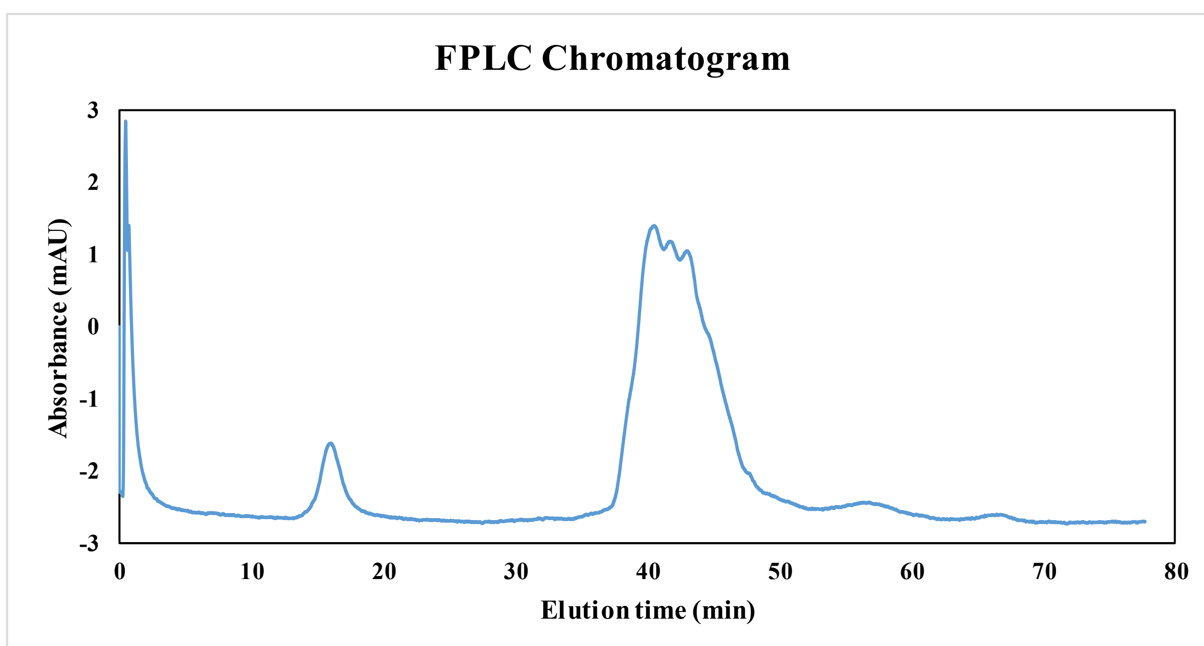

Figure 3.6 - FPLC chromatogram of the supernatant obtained after addition of ethanol.

In summary, it was not possible to separate the collagen from the first supernatant obtained, by any of the tested procedures. The steps including the addition of sodium acetate and dissolution with NaCl solution were thus eliminated. In addition, HPLC analyses weren't able to detect the presence of HA.

Based on these findings, the second methodology (Figure 2.2) was applied.

FPLC analyses of the supernatants resulting from extractions with AA 0.5 M showed a high concentration of collagen in every extract (Figures 3.7 – 3.9). The area of the peaks with a retention time at 40 min is directly proportional with the concentration of collagen on the supernatant, which means that the concentration of collagen in the third extract (Figure 3.9) was much lower than the concentration in the second extract (Figure 3.8). Based on these results it was established a limit of three consecutive extractions with AA to decrease the time of extraction defined in the methodology.

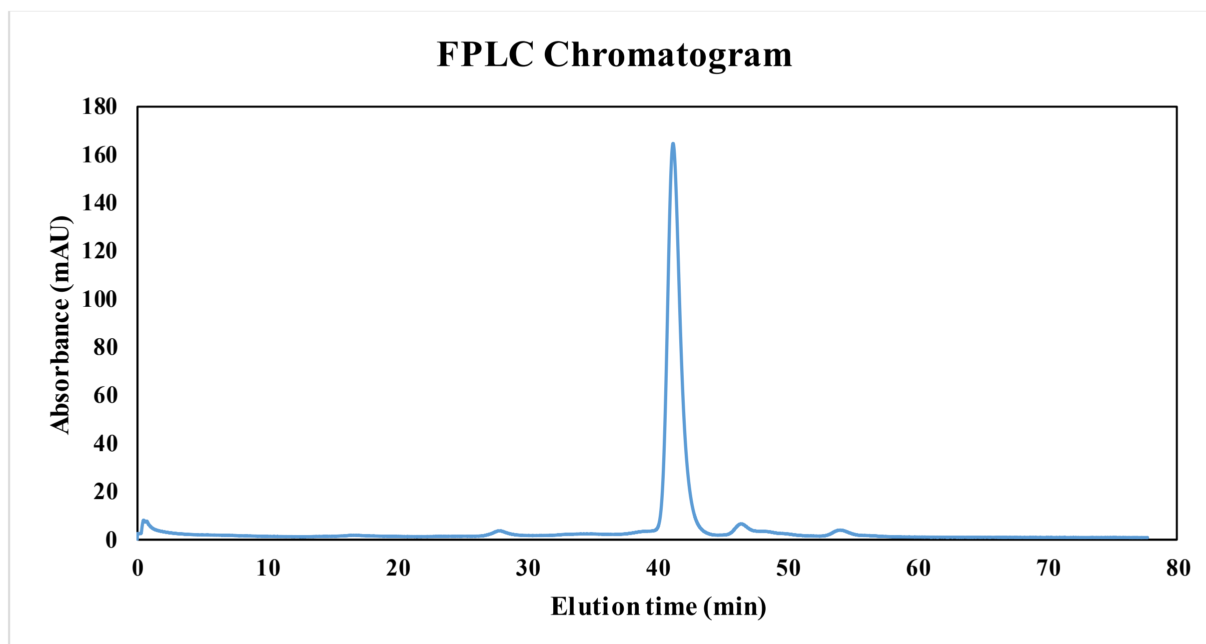

Figure 3.7 - FPLC chromatogram of the supernatant obtained after first extraction (2nd methodology) [Dilution Factor = 2].

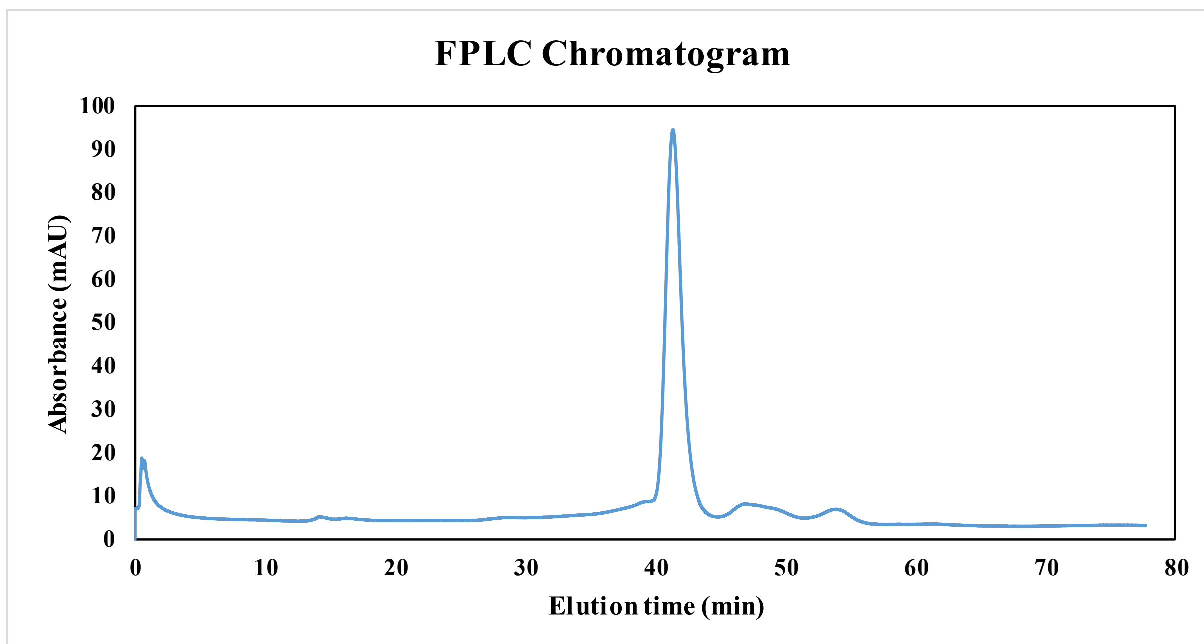

Figure 3.8 - FPLC chromatogram of the supernatant obtained after second extraction (2nd methodology) [Dilution Factor = 2].

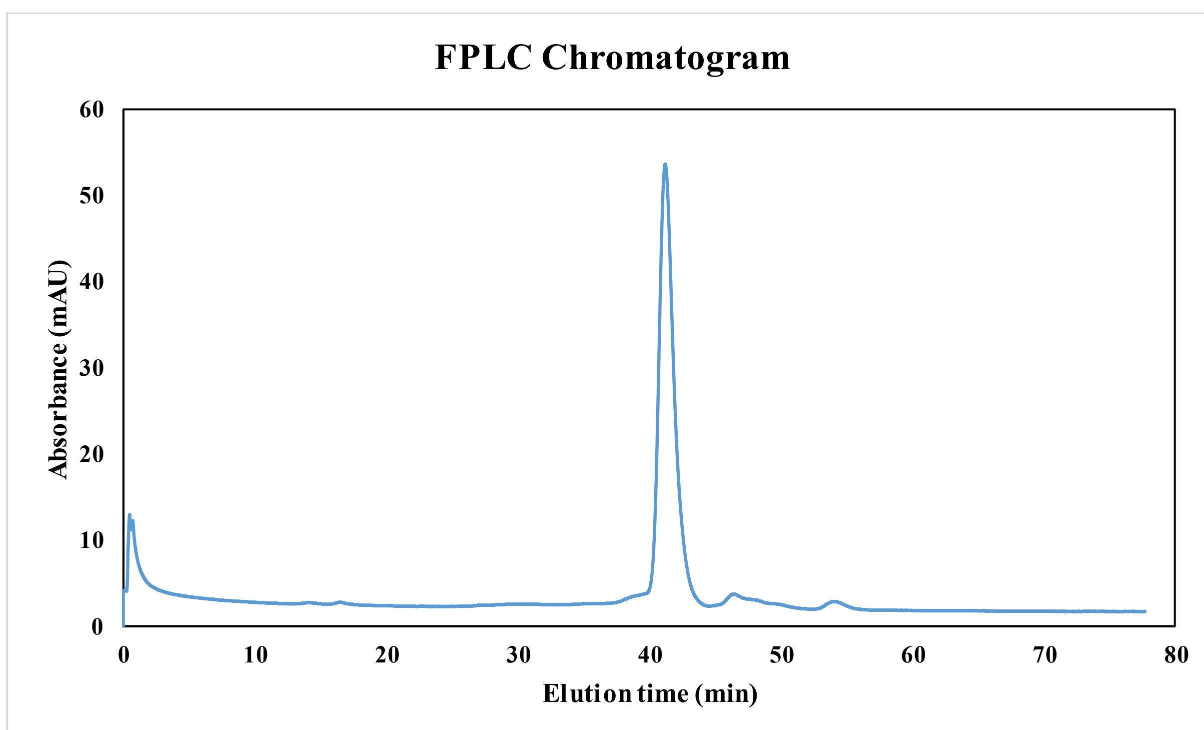

Figure 3.9 - FPLC chromatogram of the supernatant obtained after third extraction (2nd methodology) [Dilution Factor = 2].

This second methodology was then applied another time but with the previously mentioned alterations on the time of extractions, to evaluate if it was possible to extract more

collagen by increasing the time of extraction and if degradation of collagen occurred with the increase of time.

As previously mentioned, the first extraction with AA 0.5 M was performed for 48 h in total, but the supernatant was tested on FPLC after 24 h of extraction (Figure 3.10) and after 48 h (Figure 3.11). The results suggested a slight increase on the peaks' area with a retention time of 41 min – from 301.36 min×mAU to 352.6 min×mAU (considering non-diluted solutions) –, which is directly correlated to a better yield for collagen extraction. Nevertheless, it was expected the double of the collagen concentration after 48 h extraction when compared to 24 h extraction, so these results indicate that grinding the combs did not affect the yield of extraction (at least on the first extraction) and that it is preferable to perform 2 extractions of 24 h each rather than a single 48 h extraction. It is important to mention that no evidence of collagen degradation was observed after 48 h of extraction when compared to 24 h.

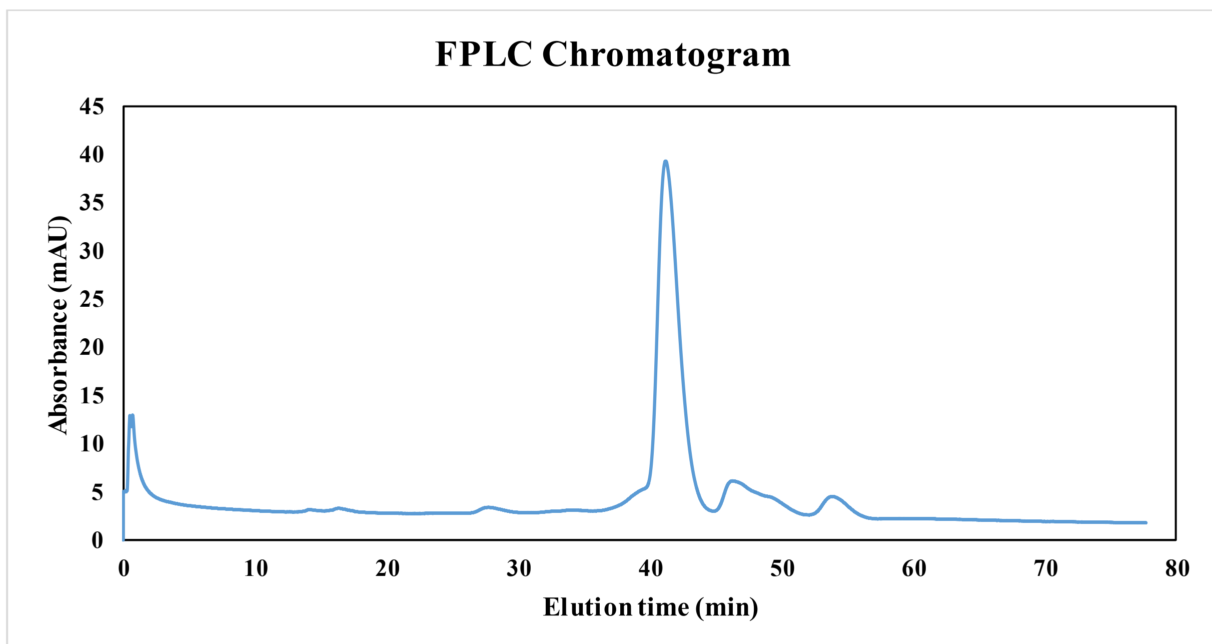

Figure 3.10 - FPLC chromatogram of the supernatant obtained after 24 h extraction (2nd methodology altered) [Dilution Factor = 4].

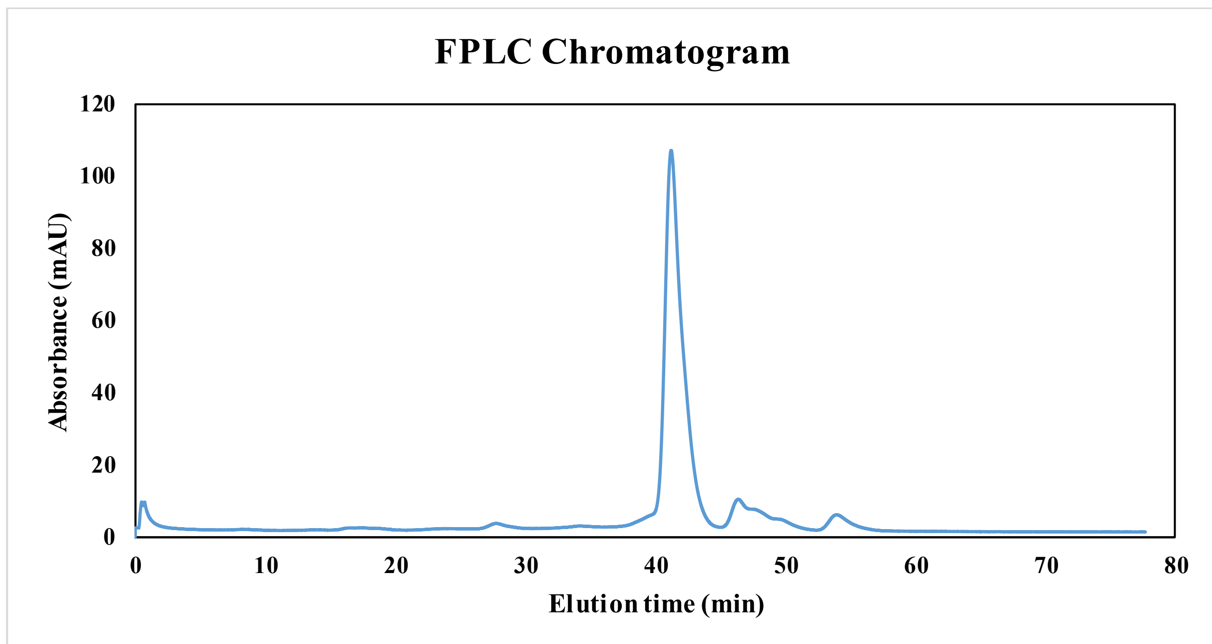

Figure 3.11 - FPLC chromatogram of the supernatant obtained after 48 h extraction (2nd methodology altered) [Dilution Factor = 2].

FPLC analysis performed to the second extraction in AA (Figure 3.12) shows a 50 % reduction on the area of the peak with a retention time of 41 min when compared with previous analysis. This is equivalent to what was observed previously from the second to third extraction when the extraction time was 24 h. Moreover, the area of the peaks with a retention time of 46 min and 53 min (Figure 3.12) reduced to 70 % of the previous analysis (Figure 3.11), suggesting the occurrence of degradation of collagen.

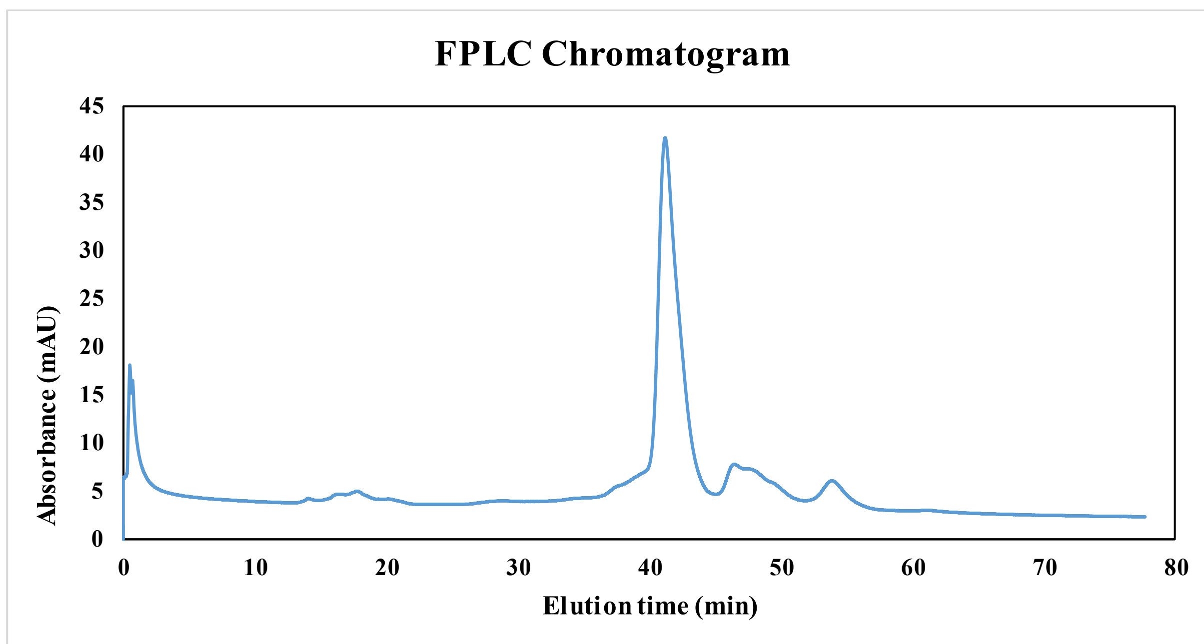

Figure 3.12 - FPLC chromatogram of the supernatant obtained after second extraction (2nd methodology altered) [Dilution Factor = 2].

After the third extraction in AA, FPLC analysis shows a greater reduction on collagen concentration – to half of the previous supernatant concentration – and a lot of degradation, characterized by multiple peaks (Figure 3.13).

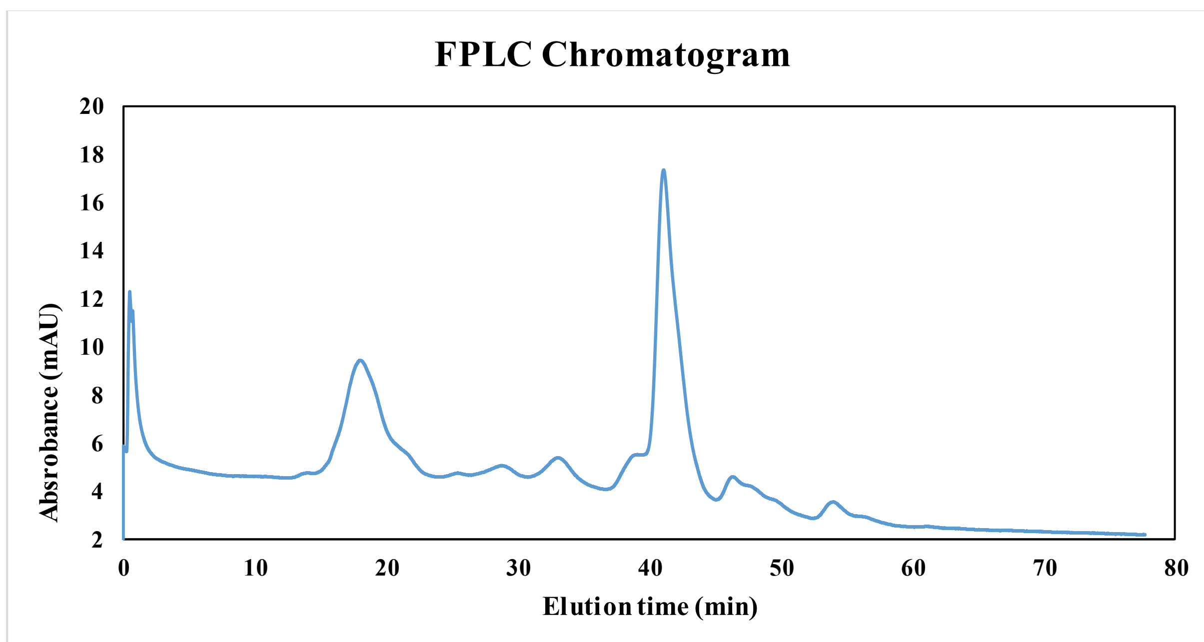

Figure 3.13 - FPLC chromatogram of the supernatant obtained after third extraction (2nd methodology altered) [Dilution Factor = 2].

Based on these results, it was concluded that three extractions in AA 0.5 M for 24 h would be the best alternative in terms of collagen integrity and time of the process. Besides this, another problem has occurred – the supernatant obtained after the first extraction in AA had high amounts of lipids due to an ineffectiveness on the delipidation process.

The third methodology applied (Figure 2.3) was based on these conclusions. Here, the combs were chopped after first delipidation with acetone. This step allowed a much better delipidation and the supernatant obtained after the first extraction with AA had much less content of lipids, as can be compared in Figures 3.14 and 3.15.

Figure 3.14 - Lyophilized powder from the supernatant obtained after first extraction with AA (2nd methodology).

Figure 3.15 - Lyophilized powder from the supernatant obtained after first extraction with AA (3rd methodology).

The results from FPLC analyses performed to the supernatants showed much well-defined peaks with minor signs of degradation. The first supernatant obtained had a high intensity peak with an area of 1806 min×mAU (Figure 3.16), which is much higher than the previous analyses but could be due to the FPLC methodology applied to these samples – 2 columns in a row and an elution time of 200 min, contrarily to the previous analysis where only one column was used with an elution time of 60 min.

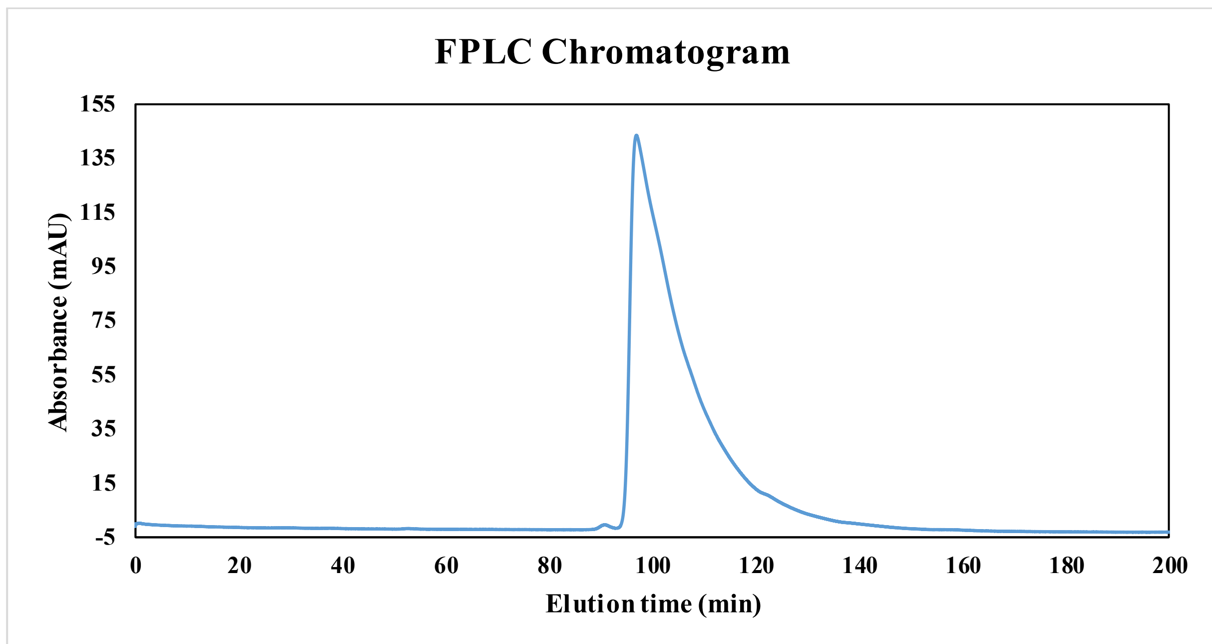

Figure 3.16 - FPLC chromatogram of the supernatant obtained after first extraction (3rd methodology).

FPLC chromatogram from the second extraction with AA (Figure 3.17) has also a well-defined peak for collagen and a minor peak for collagen degradation, but the area of the peak correspondent to collagen is much smaller – 455 min×mAU – and the retention time was 78 min instead of 96 min. This result could suggest that a better delipidation allows a better first extraction yield, which could explain why the second extraction supernatant has a much smaller collagen concentration. The alteration on the retention time can be explained by the accumulation of detritus on the column, leading to a delay in the retention time – column was cleaner when the second supernatant was analysed. When comparing the chromatograms using the elution volume instead of elution time on the x-axis (Section 3.1, Chapter 3), the peaks are coincident, which sustains the explanation given before for the alteration of the retention time.

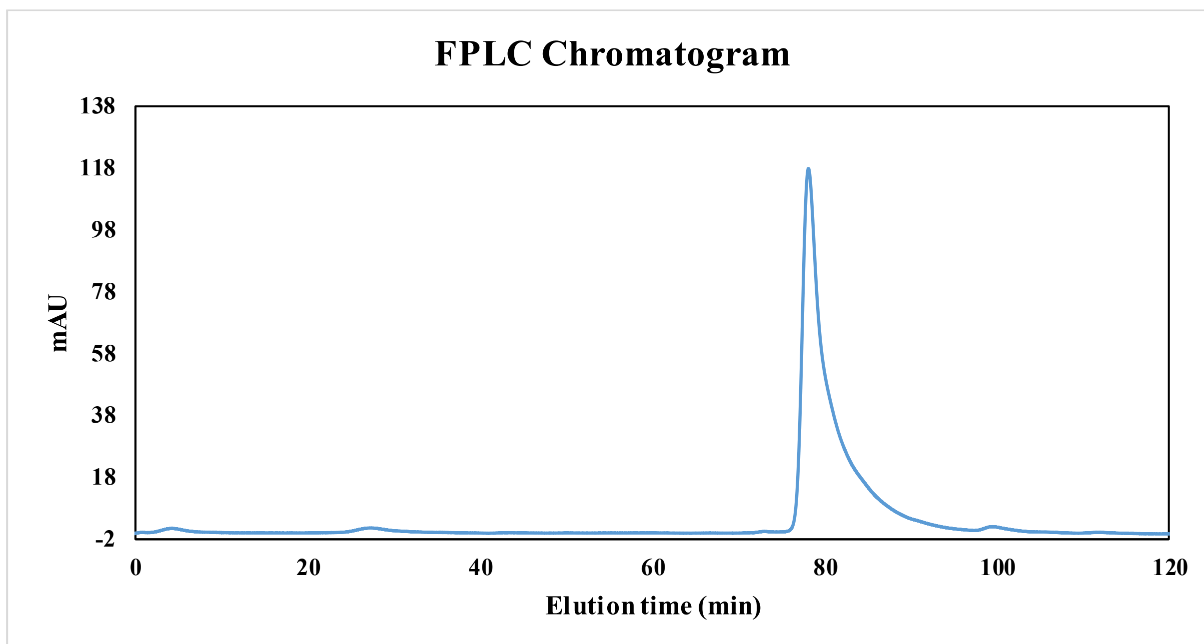

Figure 3.17 - FPLC chromatogram of the supernatant obtained after second extraction (3rd methodology).

The results from the FPLC analysis to the third supernatant (Figure 3.18) show a much smaller yield of extraction – 37 % of the second extraction and 9 % of the first extraction. Nevertheless, the peak corresponding to collagen appears at the same elution time as on the first supernatant analysis (first and third supernatants where analysed on the same day) and there are no major evidences of collagen degradation.

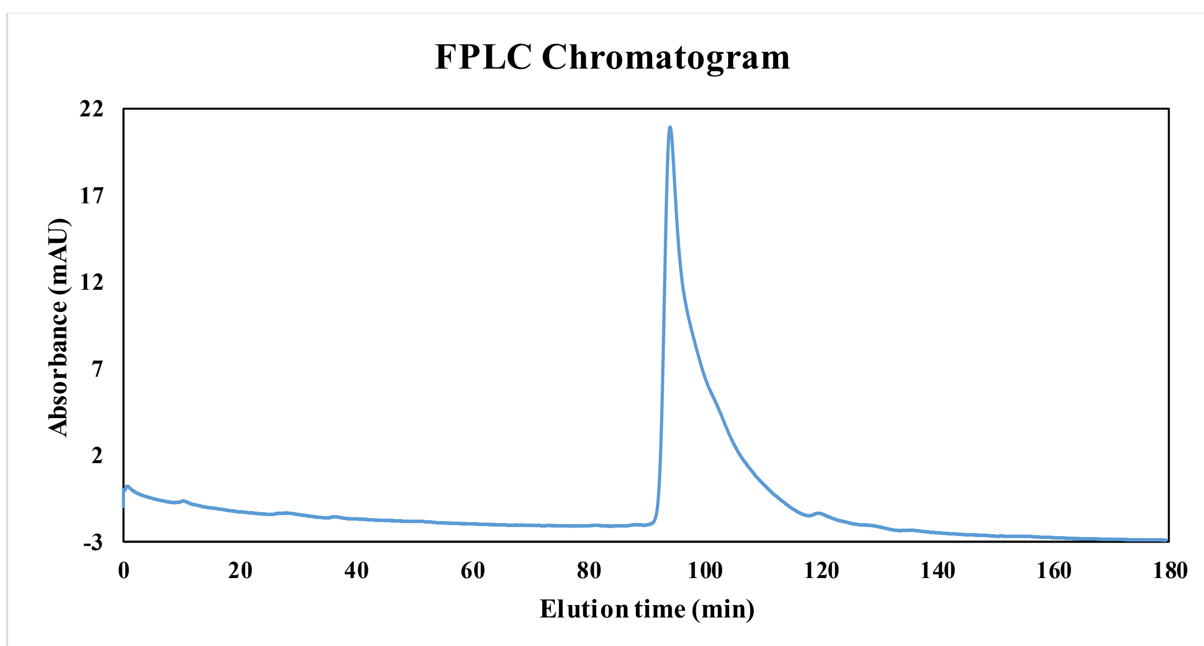

Figure 3.18 - FPLC chromatogram of the supernatant obtained after third extraction (3rd methodology).

Summing up, it was possible to optimize the extraction methodology based on FPLC results, which allowed to achieve a more efficient and less time consuming methodology. Also, the quality of the supernatants obtained was much higher when comparing with those obtained with the first procedures, since degradation peaks were almost inexistent and collagen peaks were perfectly coincident.

3. Extracts characterization

3.1. Fast Protein Liquid Chromatography (FPLC)

FPLC analyses were carried out using size exclusion chromatography columns, which means that molecular weight varies in the inverse ratio of elution time or volume.

Chromatograms from the first extraction methodology (Figures 3.1 – 3.6) will not be subject of discussion here, since degradation ratio was high for these samples. As mentioned above, these analyses had the only purpose of tracking the presence of collagen between each step of the extraction methodology, which was confirmed by the peaks with a retention time of about 40 min.

In this section, chromatograms will be presented as function of the elution volume instead of elution time, to eliminate the influence of contamination in the columns.

When comparing the three FPLC chromatograms obtained from the supernatants of the second extraction methodology (Figure 3.19), it is possible to conclude that no major degradation is occurring through this methodology since all the three peaks referring to collagen are perfectly aligned with the same elution volume.

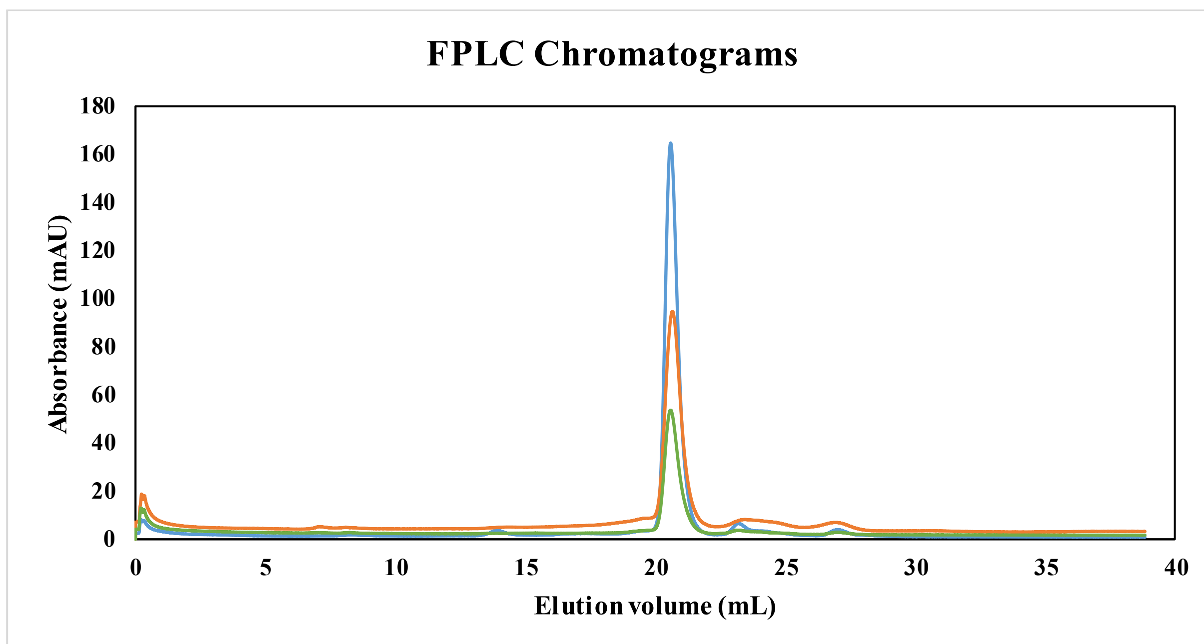

Figure 3.19 - Comparison of the chromatograms obtained by the 2nd extraction methodology. Blue - 1st supernatant; Orange - 2nd supernatant; Green - 3rd supernatant. [Dilution Factor = 2].

Also, when comparing these chromatograms with the chromatogram of the commercial native collagen (solution containing roughly 4 mg/mL collagen content) (Figure 3.20), one can confirm that extracted collagen has a higher molecular weight than the commercial one and that the first and second supernatants have a much higher collagen concentration than the commercial – in terms of comparison, second supernatant chromatogram would have a peak area of 281.6 min×mAU, if not diluted, while commercial collagen chromatogram has a peak area of just 156.4 min×mAU. It is also possible to observe that commercial collagen chromatogram has a narrow peak while the peaks of the supernatants' chromatograms are more broad, meaning that extracted collagen is still less pure than the commercial one. Since any purification procedure was applied after extraction, this is a plausible assumption.

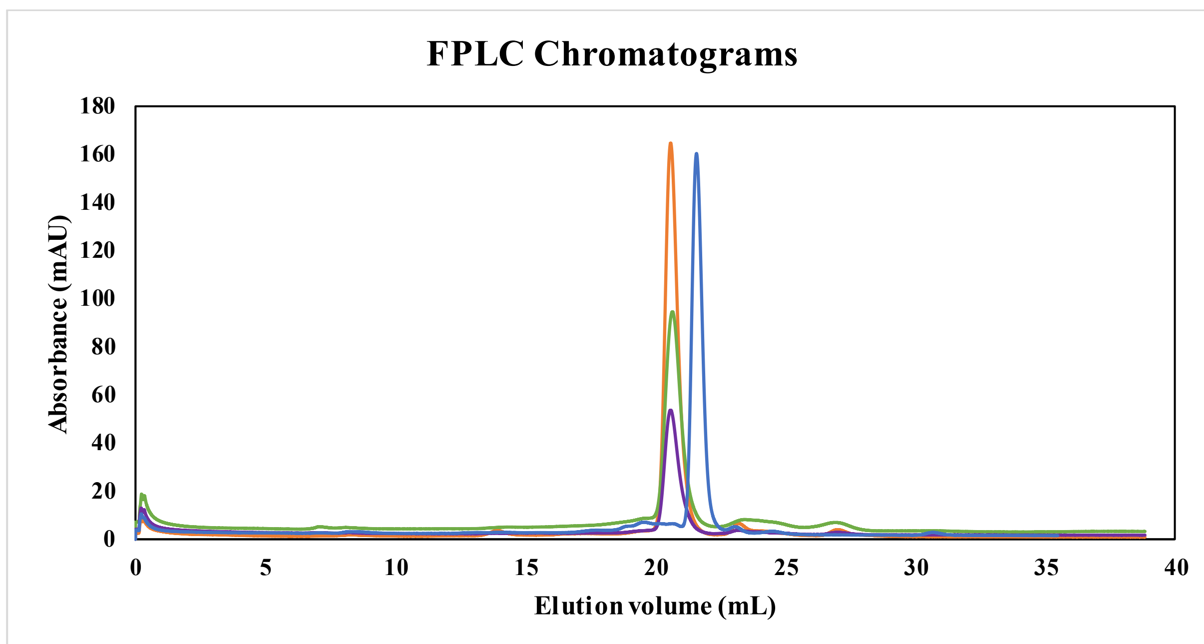

Figure 3.20 - Comparison of the chromatograms obtained by the 2nd extraction methodology with commercial collagen. Blue - Commercial collagen (not diluted); Orange - 1st supernatant; Green - 2nd supernatant; Purple - 3rd supernatant. Dilution Factor = 2.

In the same way, chromatograms from the modified second extraction methodology (Figure 3.21) have the same elution volume for the collagen peak. But, on these it is possible to notice considerable minor peaks that eluted later, meaning that more degradation occurred.

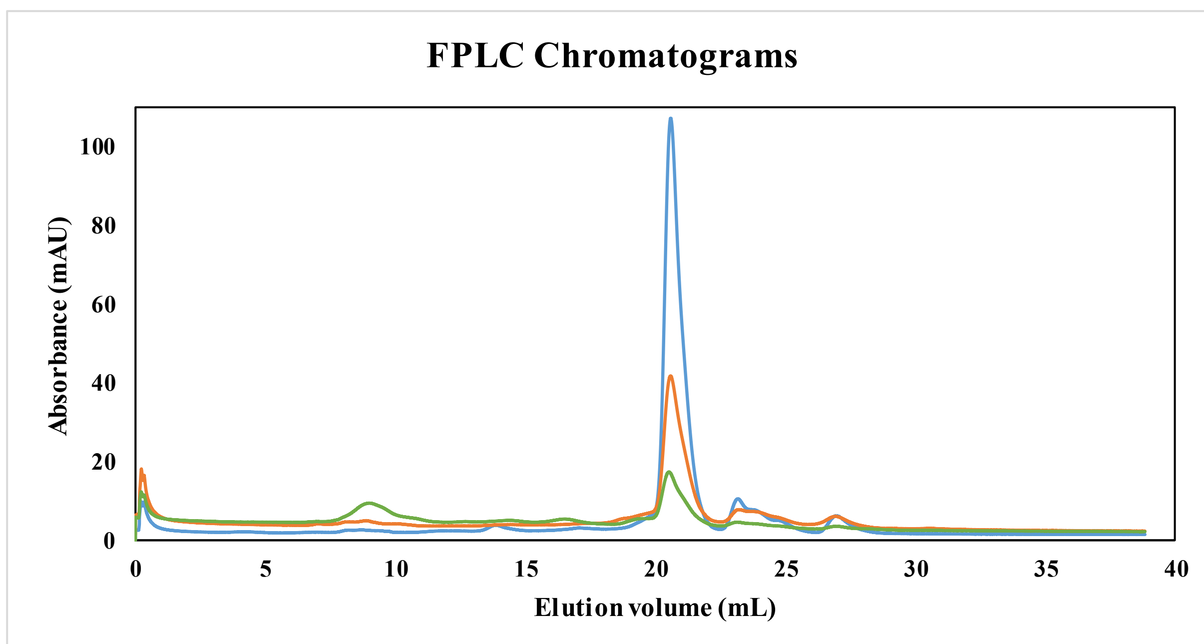

Figure 3.21 - Comparison of the chromatograms obtained by the modified 2nd extraction methodology. Blue - 1st supernatant; Orange - 2nd supernatant; Green - 3rd supernatant. [Dilution Factor = 2].

Moreover, when comparing the chromatograms from the second extraction with the chromatograms from the modified second extraction methodology (Figure 3.22), collagen peaks are all perfectly aligned at the same elution volume. When looking only to the coincident elution volume, it is possible to state that AA extraction is reproducible, since it assures that the extracted collagen has the same molecular weight.

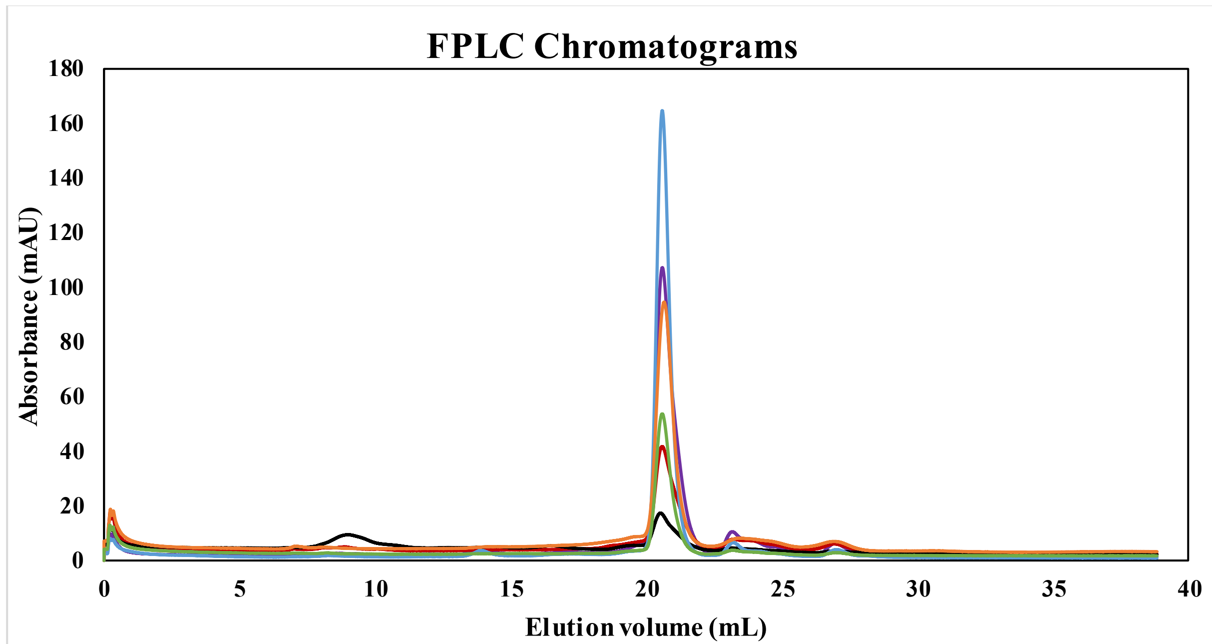

Figure 3.22 - Comparison of the chromatograms obtained by the 2nd extraction and by the modified 2nd extraction methodologies. Blue - 1st supernatant; Orange - 2nd supernatant; Green - 3rd supernatant; Purple - 1st supernatant (modified); Red – 2nd supernatant (modified); Black – 3rd supernatant (modified). [Dilution Factor = 2].

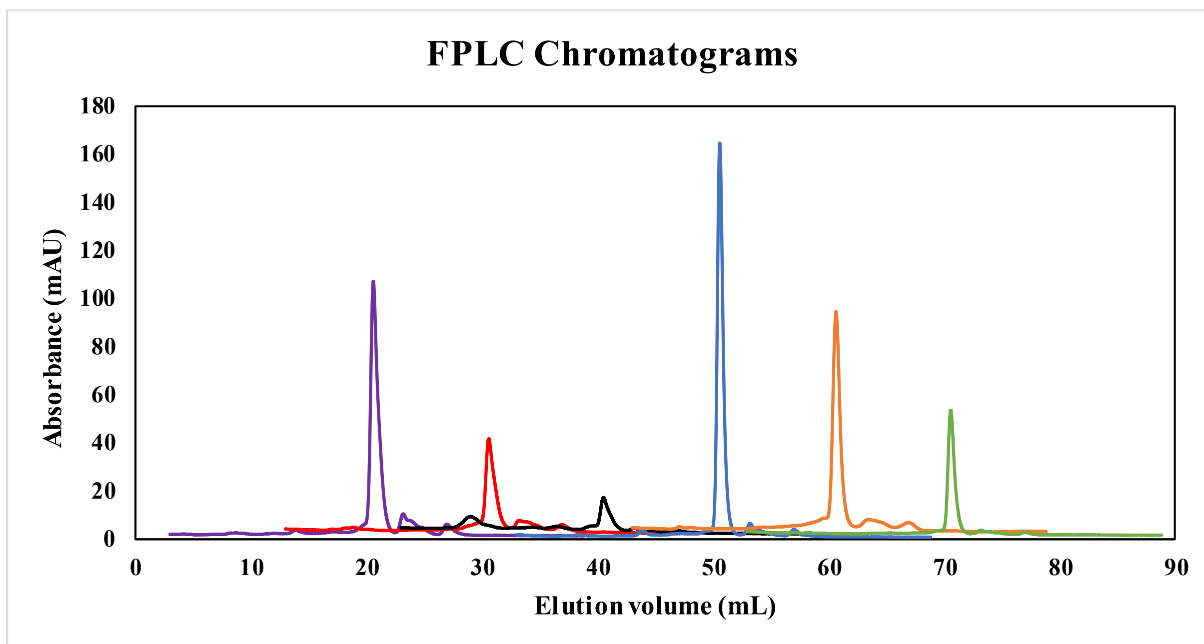

Figure 3.23 - Comparison of the chromatograms obtained by the 2nd extraction and by the modified 2nd extraction methodologies. Blue - 1st supernatant; Orange - 2nd supernatant; Green - 3rd supernatant; Purple - 1st supernatant (modified); Red – 2nd supernatant (mod modified); Black – 3rd supernatant (modified). Dilution Factor = 2. Peaks shifted by increments of 10.

As observed in the previous section, the yield of collagen extraction using the modified second extraction methodology was lower when comparing the chromatograms from both second extraction methodologies (normal and modified) – Figure 3.23.

When looking to the chromatograms from the third extraction methodology (Figure 3.24), collagen peaks are all perfectly aligned and well defined, being also more broad than previous extractions, which can be due to a better extraction yield. These supernatants weren't previously diluted before.

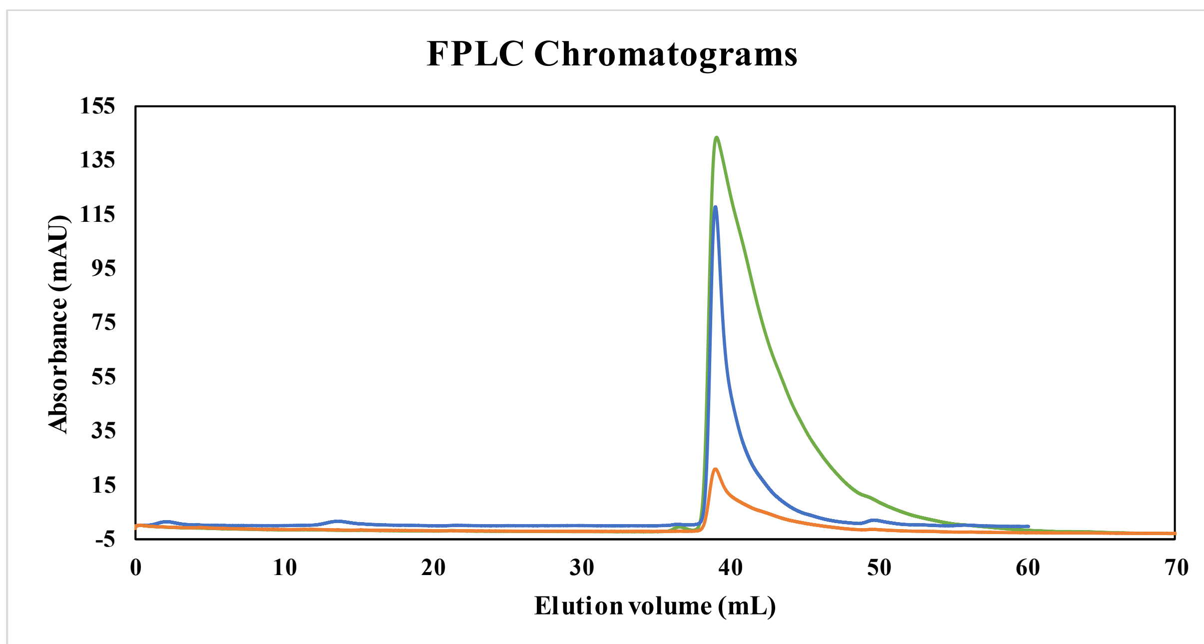

Figure 3.24 - Comparison of the chromatograms obtained by the 3rd extraction methodology. Green - 1st supernatant; Blue - 2nd supernatant; Orange - 3rd supernatant.

Furthermore, when comparing these chromatograms with commercial native collagen (Figure 3.25), it is possible to observe the same tendency observed before – collagen from the supernatants has a closer but slightly higher molecular weight than the commercial.

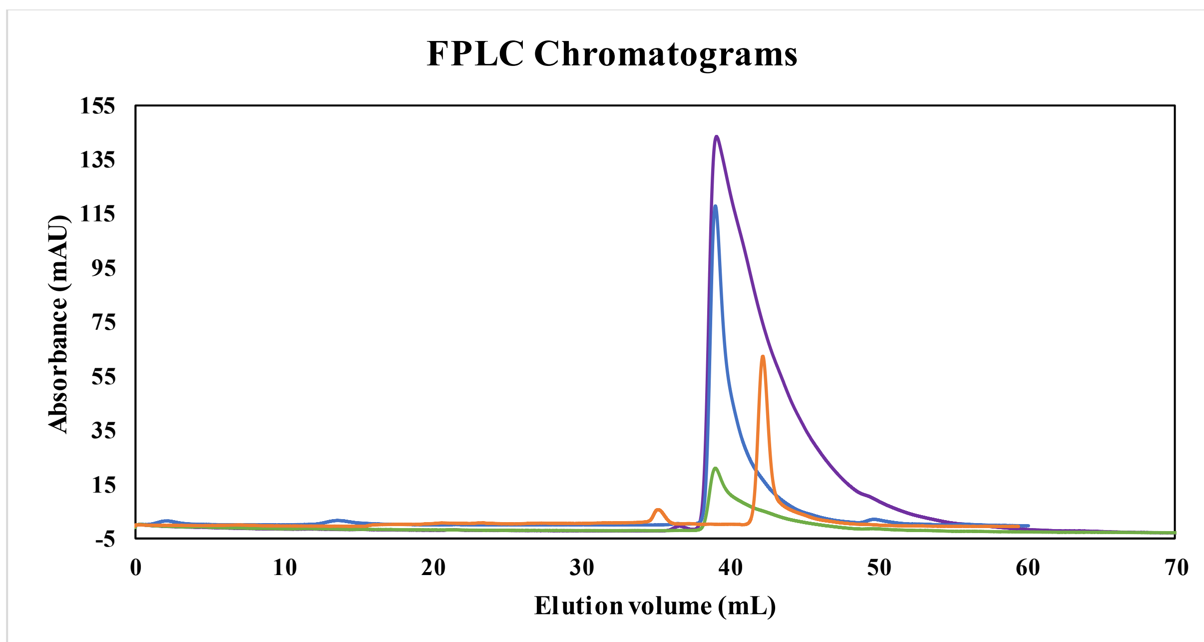

Figure 3.25 - Comparison of the chromatograms obtained by the 3rd extraction methodology with commercial collagen. Orange - Commercial collagen; Purple - 1st supernatant; Blue- 2nd supernatant; Green - 3rd supernatant.

3.2. High Performance Liquid Chromatography (HPLC)

As mentioned before (section 4.2, Chapter 2), HPLC methodology applied was adapted from a previous work in the literature [261], to determine sodium hyaluronate, meaning that there was no guarantee that this would provide a determination of HA in supernatants. The first approach was to mimic the results obtained by Ruckmani *et al.* [261] with standard solutions of sodium hyaluronate. After many experiments, it has been possible to achieve consistent peaks, with a retention time of 8 min for all the standard solutions (Figure 3.26), using the methodology described before.

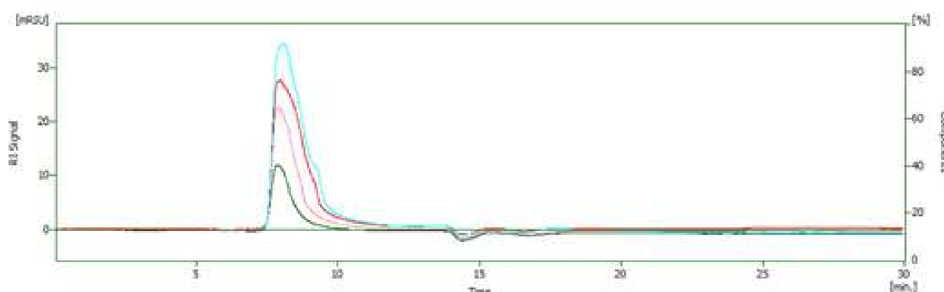

Figure 3.26 - HPLC chromatograms of standard solutions. Green - 0.25 mg/mL; Rose - 0.50 mg/mL; Red - 0.75 mg/mL; Blue - 1.0 mg/mL.

Since the flow rate used was 0.6 mL/min instead of 1 mL/min, it makes sense that retention time was around 8 min instead of 5 min, as reported in the mentioned work. At this point, it is possible to state that this methodology is capable of detecting sodium hyaluronate.

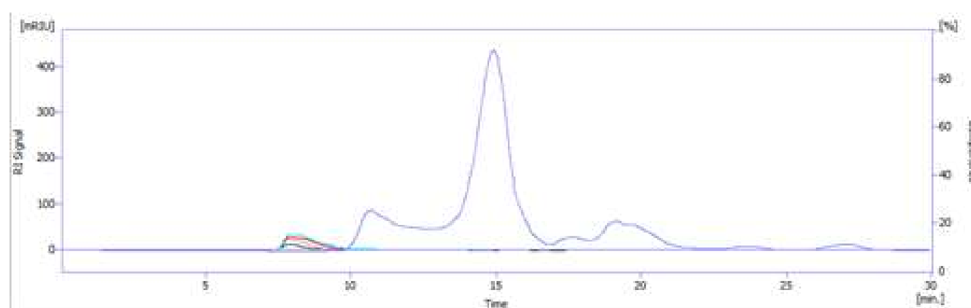

Figure 3.27 - HPLC chromatograms of standard solutions and 1st supernatant from 3rd extraction methodology. Green - 0.25 mg/mL; Rose - 0.50 mg/mL; Red - 0.75 mg/mL; Blue - 1.0 mg/mL; Violet – Supernatant.

When comparing the chromatograms for the standard solutions with the chromatogram for the first supernatant obtained by the third extraction methodology (Figure 3.27), it becomes clear that this analysis isn't appropriate for the determination of HA in supernatants. Chromatogram for the supernatant shows any peak with a retention time of 8 min and four major peaks with a bigger retention time, meaning that what was being detected had lower

molecular weight than the commercial sodium hyaluronate. Many analyses were carried out with the same supernatant and with others to prove the inefficacy of this technique in the detection of HA. Figure 3.28 compares the chromatogram for the first supernatant obtained by the third extraction methodology with the chromatogram for the first supernatant obtained by the second extraction methodology, to prove that supernatants followed the same tendency.

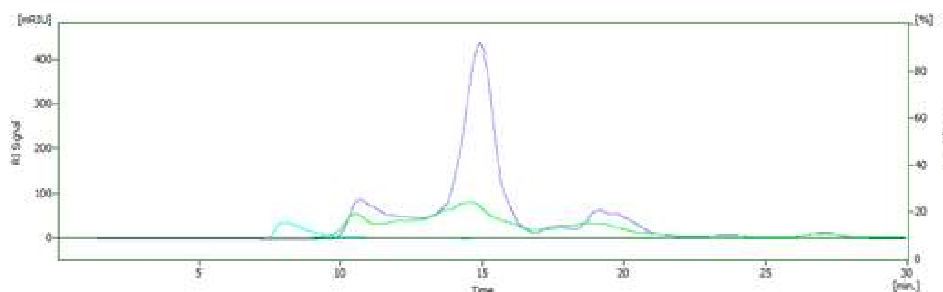

Figure 3.28 - HPLC chromatograms of standard solution and 1st supernatants from 2nd and 3rd extraction methodologies. Blue - 1.0 mg/mL; Green - Supernatant from 2nd methodology; Violet – Supernatant from 3rd methodology.

By the herein presented results, this method of analysis proved not suitable to detect HA and was therefore abandoned.

4. Lyophilized powders characterization

4.1. Yield of lyophilisation

The yield of lyophilisation is presented in Table 3.2. For yield determination, it was considered an initial comb's mass of 464 g. Based on Table 3.1, the dry weight is thus 142 g.

Table 3.2 - Yield of lyophilisation.

Acetic Acid Extraction #	Lyophilized powders weight (g)	Total weight (g)	Yield (%)
First	6.7094		
Second	2.3019	11.3576	8.00
Third	2.3463		

4.2. Total collagen determination: hydroxyproline quantification

Total collagen determination was performed using the supernatants obtained by the third extraction methodology. The standard curve used to calculate the collagen content of the supernatants is shown in Figure 3.29.

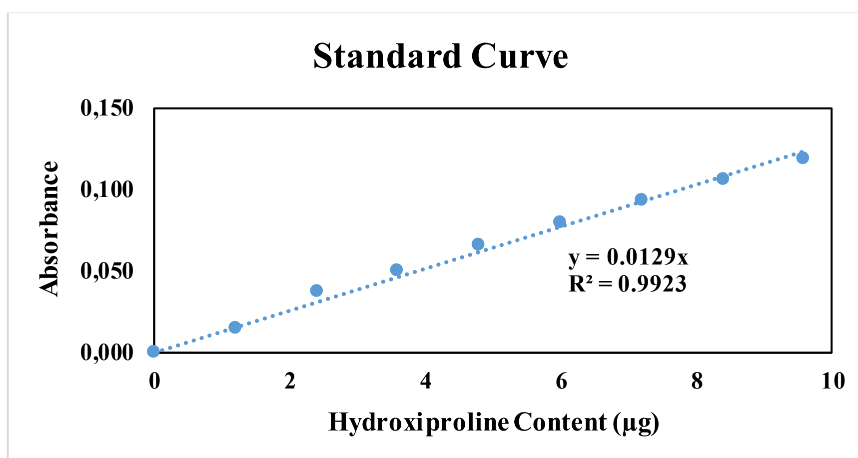

Figure 3.29 - Standard curve for total collagen determination.

Total collagen content was calculated for the three supernatants obtained by the third extraction methodology, as presented in Table 3.3.

Table 3.3 - Total Collagen Determination.

Supernatant	Absorbance	Sample Weight (g)	Hydroxyproline content (µg)	Hydroxyproline content (g/100g)	Collagen content (g/100g)
First	0.054	0.1107	4.186	3.781	30.251
Second	0.053	0.1017	4.109	4.040	32.319
Third	0.083	0.1123	6.434	5.729	45.835

Considering the values of Table 3.2, the total collagen content per supernatant was 2.0297 g (1st supernatant), 0.7440 g (2nd supernatant) and 1.0754 g (3rd supernatant). This means that this extraction methodology could extract a total of 3.8491 g of collagen from 142 g of dried chicken combs. Overall, the yield of collagen extraction was 2.71 %, considering the dry weight of 142 g. In terms of comparison between the extracted collagen quantity with the initial comb's protein content, the extraction yield was 17.88 %.

There are no papers or references on collagen extraction from chicken combs. When comparing with reports of collagen extraction from chicken feet [201], the extraction yield on

the basis of wet weight is seven times lower than the reported, although they used a sample to solvent ratio of 1:80 (w/V). Moreover, results from this characterization are in complete disagreement with FPLC analyses. Based on this technique, it has been said that first supernatants had the highest content of collagen, but total collagen determination showed the exact opposite – third supernatant had the highest content, followed by the second and then the first. Now knowing that extraction yield was so low, it is plausible to refute the conclusions made based on FPLC analyses, because more collagen could be extracted from the combs. Nevertheless, an explanation for this contradictory results is that with increasing concentration of collagen in the supernatant, collagen aggregation would be more prone to happen, thus clogging the filters, used previously to FPLC analyses, more quickly. This means that less collagen was passing through the filters when the supernatant was more concentrated, resulting in lower intensity collagen peaks on FPLC chromatograms.

4.3. Total hyaluronic acid determination: Carbazole method

The standard curve used to calculate HA content is shown in Figure 3.30.

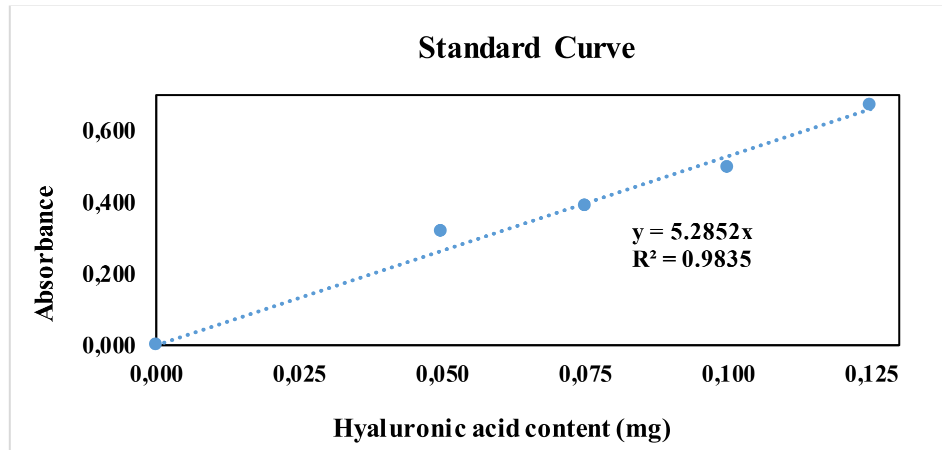

Figure 3.30 - Standard curve for total hyaluronic acid determination.

HA content was calculated for the supernatants obtained by the third extraction methodology. Results are presented in Table 3.4.

Table 3.4 - Total hyaluronic acid determination.

Supernatant	Absorbance	Sample Weight (mg)	HA content (mg)	HA content (g/100g)
First	0.275	7.6	0.052	0.685
Second	0.401	10.6	0.076	0.716
Third	0.320	6.8	0.061	0.890

HA – hyaluronic acid.

These results are coincident with the ones obtained for the total collagen content, being the third extract the one with the highest HA content. This proves that it is possible to extract HA together with collagen, by a simple procedure.

The overall quantity of HA extracted from 142 g of dried chicken combs was 0.083 g, resulting in a yield of 0.06 % in terms of dry weight, or 0.018 % in the basis of washed combs weight. One of the first reports on HA extraction [32] states a 0.6 % yield in the basis of dry weight, using sodium acetate for extraction, while a more recent report using a similar methodology states a yield of just 0.1 % in the basis of frozen combs weight. When using enzymes for extraction, reports state 1.34 % yield [1] or 0.77 % and 0.91 % yields [3] in terms of dry weight. Nevertheless, when comparing to extraction with sodium acetate, this result is not so distant. Possibly, most of the collagen was not extracted from the combs, together with HA. If this has been the case, it means that the extraction methodology needs to be further optimized in order to achieve better extraction yields. Regardless these facts, these results prove that it is possible to extract HA from chicken combs using AA, rendering the later as a novel extracting agent for HA.

4.4. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of lyophilized powders obtained by the third extraction methodology are presented in Figure 3.31.

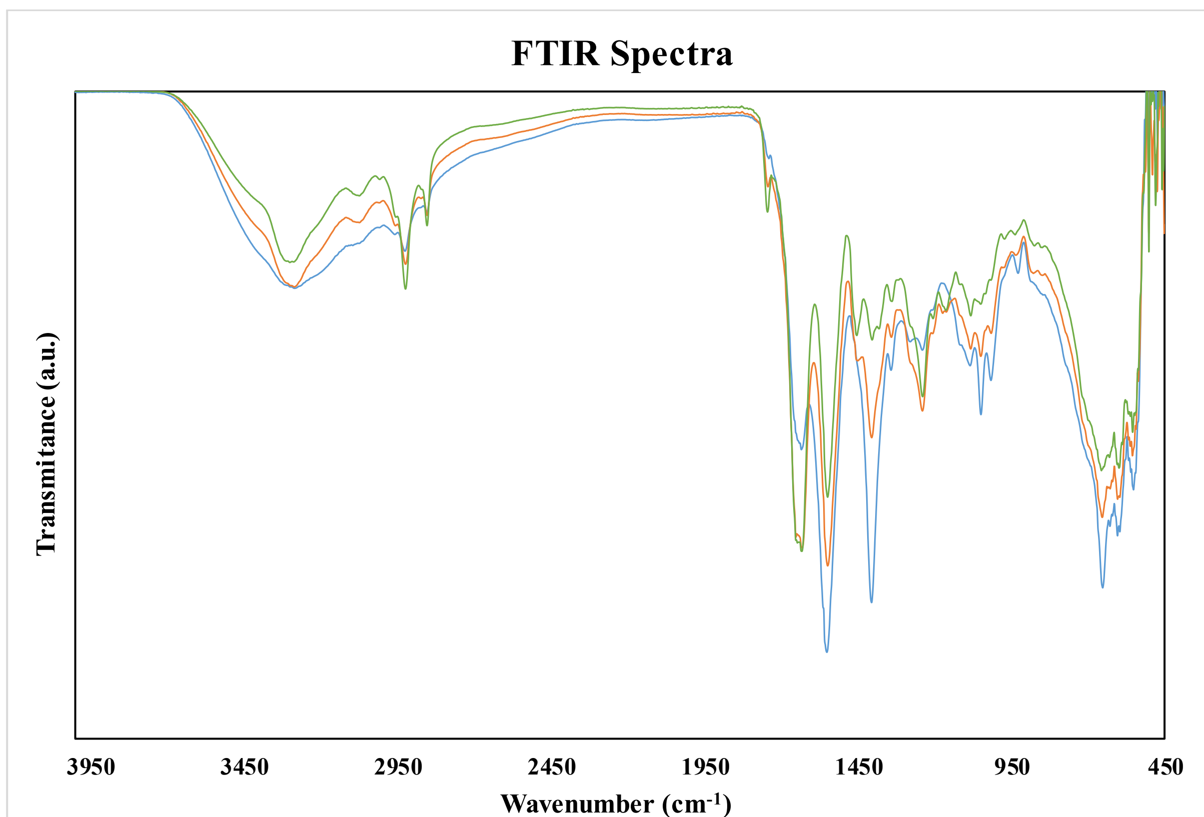

Figure 3.31 – FTIR spectra of lyophilized powders from 3rd extraction. Blue – 1st powder; Orange – 2nd powder; Green – 3rd powder.

All the spectra are similar, which means there are no loss of major chemical bonds neither formation of new ones between AA extraction steps. Differences in band intensity may be attributed to analysis procedure since spectra intensity is too low.

These FTIR spectra resemble collagen FTIR spectre, as can be seen in Figure 3.32. They exhibit the characteristic peaks of amides A, B, I, II and III.

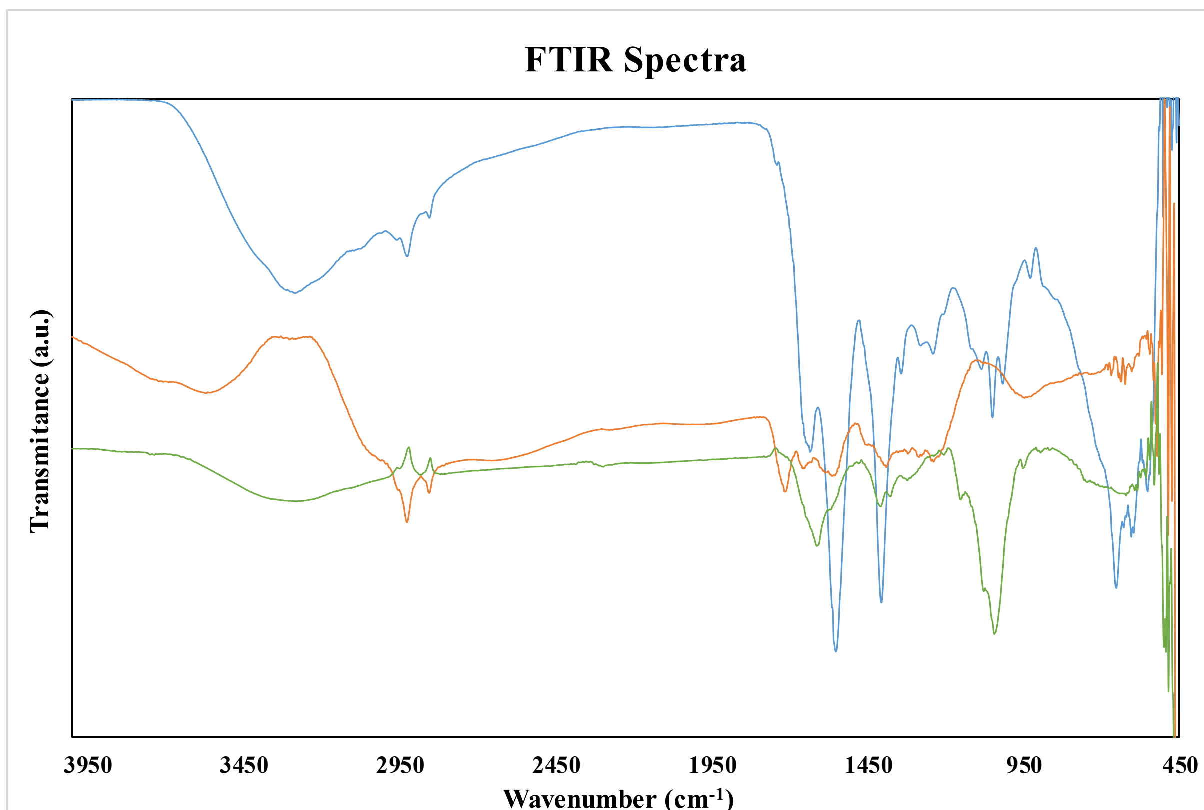

Figure 3.32 - Comparison between FTIR spectra of lyophilized powder, native collagen solution and sodium hyaluronate. Blue - 1st lyophilized powder; Orange - Native collagen solution; Green - Sodium hyaluronate.

Amide A is associated with N-H stretching vibrations and normally occurs in the wavenumber range 3400-3440 cm^{-1}. But, in lyophilized powders spectra, it appears at 3284 cm^{-1}, meaning that N-H group is involved with hydrogen bonds in the peptide chain [200]. This shift to lower frequencies could also be due to the detection of OH groups in HA, that normally occurs in the range of 3200-3550 cm^{-1} [44]. In fact, as can be seen in Figure 3.32, this band is much more coincident with the sodium hyaluronate band, but since collagen is the uppermost compound in lyophilized powder, the band couldn't be attributed to HA. Amide B is represented by two bands at 2927 cm^{-1} and 2856 cm^{-1}, that can be attributed to CH_2 asymmetrical stretching [200].

Amides I, II and III have characteristic bands that have the same wavenumber range in collagen and HA [75], [200], [264]. Amide I characteristic wavenumber is in the range of 1600-1700 cm^{-1} [200], appearing at 1633 cm^{-1} for the lyophilized powder, at 1650 cm^{-1} for native collagen and an almost no clear band at 1650 cm^{-1} for sodium hyaluronate. In collagen, this band represents C- or O- stretching vibrations, while on sodium hyaluronate it represents C=O stretching vibration. Moreover, sodium hyaluronate has a major band at 1610 cm^{-1} corresponding to the C=O stretching vibration of acid groups [264]. Amide II is associated with

N-H bending vibration coupled with C-N stretching vibration, occurring at a wavenumber range of 1500-1600 cm^{-1} [75], [200]. On lyophilized powder spectra, it appears at 1550 cm^{-1}, while on collagen and sodium hyaluronate spectra it appears at 1555 cm^{-1} and 1567 cm^{-1}, respectively. Amide III is normally responsible for N-H deformation and C-N stretching vibrations and usually occurs at 1220-1320 cm^{-1} [200]. For lyophilized powder spectra, there are two prominent bands at 1280 and 1240 cm^{-1} and the same is observed on native collagen spectre. On the other hand, on sodium hyaluronate spectre there is only one band at 1320 cm^{-1} that corresponds to amide III.

Collagen spectre also has a band at 1455 cm^{-1} that corresponds to pyrrolidine ring vibration of proline and hydroxyproline [265], but it is not present on lyophilized powder spectra.

On sodium hyaluronate spectre, a band appears at 1409 cm^{-1} that corresponds to C-O bond of carboxyl acid, at 1150 cm^{-1} corresponding to C-O-C bond, at 1062 cm^{-1} corresponding to exocyclic C-O bond and C-C bonds and at 1041 cm^{-1} corresponding to C-OH bond as described by Villetti *et al.* [264]. Lyophilized powder spectra present similar bands with sodium hyaluronate spectre at the range of 1000-1150 cm^{-1}, while native collagen spectre doesn't have any of these bands. This could mean that lyophilized powder spectra have characteristic bands of both collagen and HA, confirming that it was possible to extract both collagen and HA from chicken combs.

4.5. Differential Scanning Calorimetry (DSC)

DSC thermograms, presented in Figure 3.33, are representative of the three measurements, since standard deviation was too high for all lyophilized powders analyses. The enthalpies for each peak were calculated using the DSC software and are presented in Table 3.5.

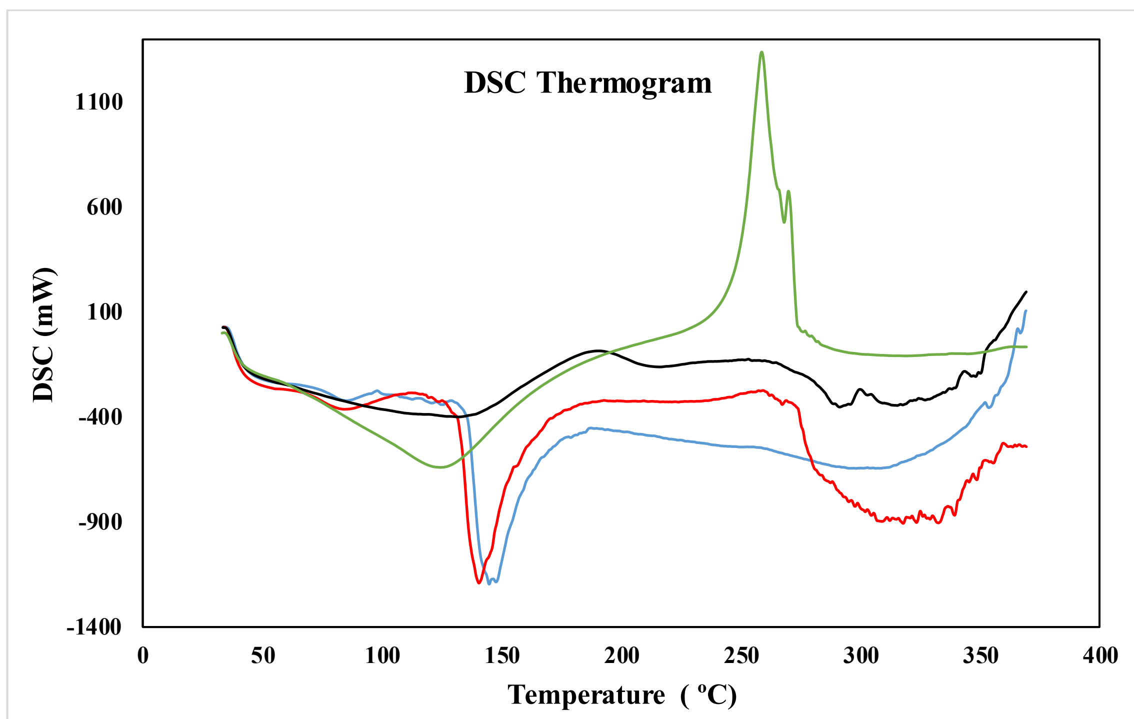

Figure 3.33 - Comparison of DSC thermograms of lyophilized powders from 3rd extraction and sodium hyaluronate. Blue - 1st lyophilized powder; Red - 2nd lyophilized powder; Black - 3rd lyophilized powder; Green - Sodium hyaluronate.

Table 3.5 - DSC thermograms peaks and calculated enthalpies.

Sample Thermogram	Peak maximum (ºC)	Enthalpy (J)
First lyophilized powder	132.88	-100.57
	237.20	0.455
	281.46	-71.50
Second lyophilized powder	128.88	-102.15
	243.76	3.55
	300.78	-145.42
Third Lyophilized powder	121.19	-16.16
	201.86	-7.00
	237.77	1.01
	274.96	-7.59
	298.45	-15.70
Sodium hyaluronate	113.82	-94.08
	244.11	120.26

As can be seen in Figure 3.33, the thermograms from the lyophilized powders have broad exothermal peaks with their maximum at 132.88 ºC, 128.88 ºC and 121.19 ºC on the first,

second and third lyophilized powder, respectively. These peaks are connected with the collagen secondary structure transition from the triple helix to a randomly coiled conformation [266]. According to Pietrucha [266], this transition into a randomly coiled conformation is preceded by the release of loosely bound water and by the breakage of inter and intramolecular hydrogen bonds. This process should be endothermic, but the results given by the DSC are a sum up of the overall processes happening at a given temperature and time. A similar phenomenon – evaporation of moisture –, also happens for HA at around 150 ºC [264], [267]. This is represented by the exothermal peak with a maximum at 113.82 ºC on the thermogram of sodium hyaluronate. Again, although the evaporation process is endothermic, the overall process was measured with a negative enthalpy by the DSC equipment. These peaks suggest that lyophilized powders' collagen and HA together are more resistant to heating than sodium hyaluronate, since it is required a high temperature in order to evaporate the water content. A more plausible explanation could be that lyophilized powders have more water content than commercial sodium hyaluronate.

On sodium hyaluronate thermogram, a major endothermal peak is observed with a maxima at 244.11 ºC, corresponding to the degradation of this polysaccharide [267]. Although barely visible, lyophilized powders thermograms also have endothermal peaks close to this temperature, supporting the evidence of the presence of HA, but in minor quantities since the enthalpy associated with these peaks is minimum when compared to sodium hyaluronate's degradation peak.

Broad exothermal peaks with maximum around 274-300 ºC, observed on lyophilized powders thermograms, could be the transition into viscous-flow that characterizes the beginning of thermal destruction of collagen [268]. The transition into a liquid state might explain why the overall enthalpy is negative, since there is breakage of atomic bonds.

Chapter 4: General Conclusions

Based on the obtained results, it was possible to confirm that both HA and collagen were effectively extracted from chicken combs, using a simple acidic extraction. The optimized extraction methodology allowed to extract HA and collagen with yields of 0.06 % and 2.71 % in terms of dry weight, respectively. These yields are lower than those obtained in other extractions methods. Nevertheless, the performance of a higher number of AA extractions can be tested in the future, in future by-products batches to improve the extraction yields, but always taking into account the maintenance of collagen and HA stability. Actually, the number of extractions performed in this experimental work, i.e. 3 times, took into account this premise. The chemical characterization by FPLC, FTIR and DSC confirmed the identity of the compounds when comparing with the commercial HA and collagen type I. Also, no signs of degradation were found by the characterization techniques used.

The extraction costs were accounted for 54.69 €/kg of combs processed, in what refers to consumables, namely acetone and AA 96 %. The cost of distilled water and electricity, used for grinding, refrigeration and lyophilisation process, were not considered at such a small scale. The income from selling these lyophilized powders would be 99.9 € from HA (ca. 555 €/g in Sigma-Aldrich) and 219 € from collagen (collagen type I from bovine Achilles tendon ca. 26.85 €/g in Sigma-Aldrich), performing a total of 318.9 €/kg of combs processed. This income took into consideration a final consumer's selling and that lyophilized HA and collagen meet the required standards. These facts sustain the potential of exploitation of a conjoint extraction of HA and collagen.

Chapter 5: Future perspectives

In the future, some adjustments can be done to the optimized process in this experimental work to increase the yields of extraction when performing the industrial scale-up. Hence, this scale-up needs to be further studied in order to estimate the improvement approaches and if these ones increase the yields. In the first stages of extraction, it would be important to select a better method to chop the combs, in order to achieve a better delipidation process. The better grinded chicken combs will certainly give raise to higher yields owing to the better penetration of the organic solvents. An increment of AA extractions to the methodology followed by determination of total HA and total collagen content would be important to find what is the limit number of AA consecutive extractions that could/should be performed. These two simple improvements should raise the extraction yields and turn the process more profitable at industrial scale.

The extracted HA and collagen, should be subjected to a deeper characterization such as the determination of the molecular weight of the extracted macromolecules, by polyacrylamide gel electrophoresis or by mass spectrometry. Mass spectrometry analyses would be also made in order to identify the collagen type. Nuclear magnetic resonance spectroscopy would be also performed providing detailed information about the molecules' structure.

In order to prove the non-toxicity, tests would be performed to guarantee that the extraction process do not introduce toxicity.

Finally, development of a hydrogel containing HA and collagen would be relevant to demonstrate as proof of concept a matrix to carry these molecules with synergic effects to be applied in biomedical and cosmetic fields.

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