POLITECNICO DI TORINO

Department of Applied Science and Technology

Master of Science Course in Chemical and Sustainable Process Engineering

Master of Science Thesis

Collagen isolation from *Cyclopterus lumpus* and *Asterias rubens*: a comparison between different extraction and recovery methods

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March 2022

Summary in Italian

I. Introduzione

Collagene: struttura e biosintesi

Il collagene è la proteina più abbondante nei mammiferi e nell'uomo rappresenta il 20-30% del contenuto proteico totale [1]. Il collagene è una proteina strutturale presente nella matrice extracellulare del tessuto connettivo. La matrice extracellulare consiste in una rete tridimensionale formata da proteine, polisaccaridi e altri componenti e fornisce supporto strutturale ai tessuti, tessuti differenti presentano differenze nella matrice extracellulare. [2]. Il collagene consiste in una macromolecola avente struttura a tripla elica (struttura terziaria) formata da tre α-eliche (struttura secondaria). Ciascuna catena consiste in una catena polipeptidica (struttura primaria) in cui particolarmente frequente è la sequenza $Gly - X - Y$ dove Gly è la glicina – il più piccolo residuo amminoacidico – e X ed Y sono spesso prolina (Pro) e idrossiprolina (Hyp). Il residuo Gly consente la formazione di legami a idrogeno fra residui adiacenti conferendo stabilità e compattezza alla tripla elica mentre Pro e Hyp conferiscono stabilità alla molecola tramite legami a idrogeno che bloccano la rotazione attorno ai legami C-N [3]. In base alla composizione in amminoacidi e al tipo di catene polipeptidiche presenti si distinguono 28 tipi di collagene, alcuni dei quali capaci di formare fibrille (struttura quaternaria) [4]. Le fibrille si aggregano per formare le fibre che costituiscono i tessuti, tessuti ai quali conferiscono forza tensile. In Figura 1 è rappresentata la struttura gerarchica del collagene. Il collagene di tipo I è una molecola eterotrimerica in quanto composta da due catene α_1 e da una catena α_2 ed è in grado di formare fibrille. Il collagene di tipo II è una molecola omotrimerica formata da tre catene α_1 ed è anch'esso collagene di tipo fibrillare.

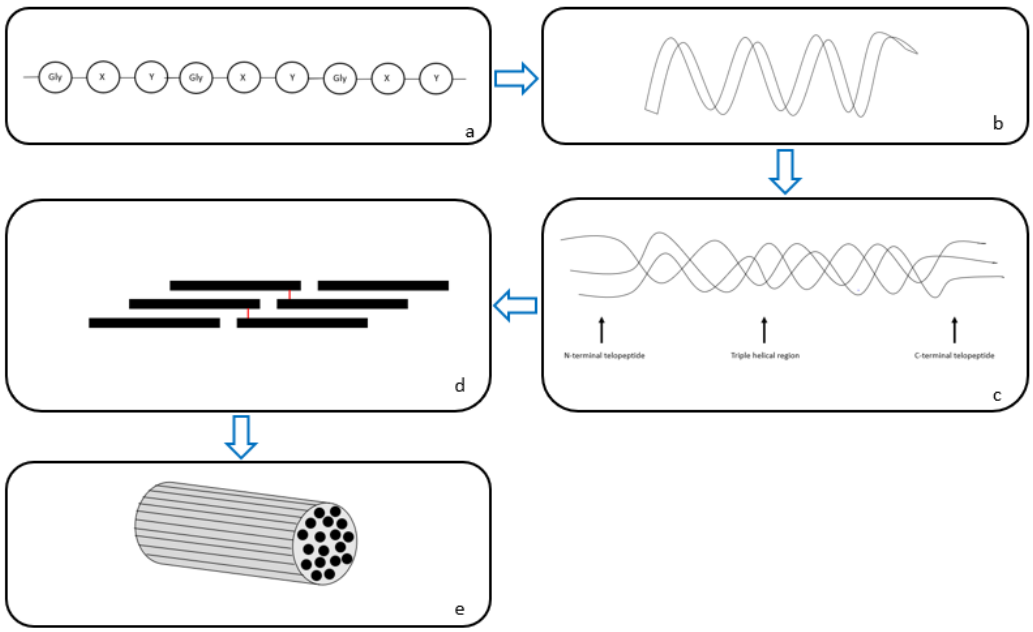

Figura 1. Struttura gerarchica del collagene: catena amminoacidica (struttura primaria) (a), α-elica (struttura secondaria) (b), tripla elica (struttura terziaria) (c), fibrilla (struttura quaternaria) (d), fibra (e).

La biosintesi del collagene di tipo I comincia con la trascrizione in mRNA (acido ribonucleico messaggero) nel nucleo, l'mRNA subisce modifiche post-trascrizionali di splicing (alternativo),

capping all'estremità 5' e poliadenilazione all'estremità 3'. La traduzione dell'mRNA produce le molecole di pre-procollagene contenenti i terminali C- ed N-. In seguito alla rimozione della sequenza segnale e in seguito alle modifiche post-traduzionali (idrossilazione di prolina e lisina) si ottiene il pro-collagene, che viene secreto nello spazio extracellulare dove avviene la rimozione proteolitica dei terminali C- ed N- [5]. La produzione di collagene nel corpo umano diminuisce nel tempo a causa dell'invecchiamento naturale e del foto-invecchiamento, fenomeni che consistono da un lato nella diminuzione della quantità di collagene sintetizzato e dall'altro nella degradazione del collagene esistente [6].

Collagene: fonti di estrazione

Attualmente, la principale fonte di collagene è rappresentata da mammiferi terrestri quali bovini, ovini e suini ma le specie marine -vertebrati e invertebrati- rappresentano un'alternativa valida per più di un motivo. Alcune restrizioni all'uso di collagene estratto da mammiferi sono legate a motivi sanitari, considerata la trasmissibilità all'uomo di alcune malattie quali encefalopatie spongiformi trasmissibili e afta epizootica. Altre restrizioni derivano da motivi religiosi: l'induismo vieta il consumo di carne bovina, l'islam e l'ebraismo vietano il consumo di carne suina. Inoltre, l'estrazione di collagene da specie marine costituisce un'alternativa di grande interesse perché potrebbe contribuire a rendere il mercato del pesce più sostenibile in quanto si potrebbero usare i sottoprodotti e gli scarti della lavorazione del pesce come una risorsa di valore e non un inquinante [7]. In aggiunta a questo, l'utilizzo di specie marine invasive come risorsa di collagene viene considerata una possibilità promettente e conveniente dal punto di vista economico, considerata la pericolosità che queste rappresentano per la biodiversità.

Collagene: domanda di mercato e applicazioni

Nell'ultimo decennio il mercato ha visto un sensibile incremento della domanda di collagene determinato dal crescente utilizzo di questa proteina in diversi ambiti tra cui la cosmetica, le biotecnologie e la nutraceutica. Come riportato nel sito web di Grand View Research [8] la domanda globale di collagene nel 2019 è ammontata a 920.1 tonnellate e nel 2020 il 38.1% del collagene totale prodotto è stato di origine bovina. Contestualmente alla crescente richiesta di collagene, come riportato dalla FAO, il mercato ha visto un aumento di produzione di pesce da pesca e acquacultura e si prevede un andamento crescente [9]. Il collagene viene particolarmente utilizzato in campo biomedico per la realizzazione di scaffold per l'ingegneria tissutale e come drug carrier [10] [11]. Inoltre, la somministrazione orale di collagene e collagene idrolizzato si è rivelata efficace nel migliorare l'aspetto della pelle invecchiata dal tempo e dalle radiazioni [12].

Progetto: scopo e specie coinvolte

Hanno pertanto assunto particolare interesse la ricerca di nuove fonti di collagene e l'ottimizzazione dei processi di estrazione, con l'obiettivo di individuare dei metodi efficienti e competitivi con quelli attuali, tali da garantire una pari o migliore qualità del collagene estratto. Le specie marine coinvolte in questo lavoro sono state *Asterias rubens* (stella marina comune) e *Cyclopterus lumpus* (lompo). *Asterias rubens* è una specie invasiva in quanto è nativa del nord-est Atlantico ma sono stati riportati recenti ritrovamenti di esemplari in prossimità delle coste della Turchia [13]. *Cyclopterus lumpus* è una specie marina sottoutilizzata, sfruttata esclusivamente per la produzione di caviale -che naturalmente riguarda solo le femmine- e come "pesce pulitore" negli allevamenti di salmone in Scozia, Islanda e Norvegia [14].

Pretrattamenti della materia prima, estrazione e recupero del collagene

La materia prima è sottoposta a dei pretrattamenti per rimuovere le sostanze diverse dal collagene, i pretrattamenti sono sintetizzati in Figura 2: a seconda della specie coinvolta alcuni trattamenti possono non essere effettuati.

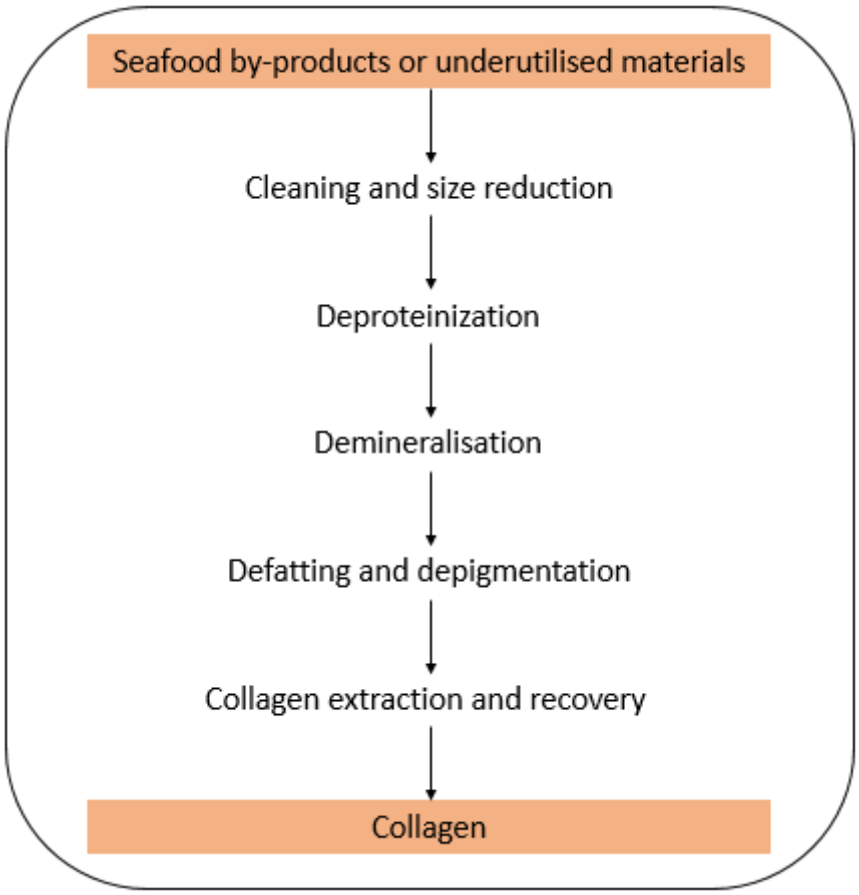

Figura 2.Protocollo di isolamento del collagene da specie marine: pretrattamenti della materia prima e isolamento (estrazione e recupero) del collagene.

La deproteinizzazione è effettuata per rimuovere le proteine non collageniche, solubili in ambiente alcalino. Questo step viene frequentemente condotto utilizzando una soluzione di NaOH. L'effetto di NaOH nello step di deproteinizzazione è stato approfondito nello studio di Liu e colleghi [15].
La demineralizzazione ha lo scopo di allontanare gli ioni come Ca^{2+} ed Mg^{2+}, si utilizza una soluzione di EDTA-4Na avente proprietà chelanti nei confronti degli ioni.
La rimozione dei grassi e degli oli viene effettuata generalmente con soluzione alcolica, la rimozione dei pigmenti con H_2O_2.
Dopo che il materiale è stato liberato da proteine non collageniche, minerali, grassi e pigmenti, il tessuto viene sottoposto all'estrazione di collagene. L'estrazione di collagene in ambiente acido può essere effettuata utilizzando diversi acidi: nel lavoro di Skierka e Sadowska [16] viene confrontata l'efficienza di diversi acidi e l'acido acetico comporta una resa di estrazione del 90%. L'utilizzo di enzimi proteolitici come la pepsina aumenta ulteriormente la resa di estrazione [17]. L'aggiunta di pepsina come biocatalizzatore produce un aumento della resa di estrazione in quanto la pepsina è efficace nella rimozione dei cross-links intermolecolari a livello dei telopeptidi del collagene [16].
Al termine della fase di estrazione si ottiene una soluzione acida contenente le molecole di collagene disciolte. Il salting out è il metodo comunemente utilizzato per recuperare il collagene disciolto nella soluzione acida. Al crescere della concentrazione di sale nella soluzione, la forza

ionica I aumenta e questo determina una riduzione della solubilità delle molecole disciolte nella soluzione. Le macromolecole proteiche, non più solvatate dalle molecole di acqua, interagiscono tra loro, si aggregano ed eventualmente sedimentano. Sali differenti hanno differenti efficienze nella precipitazione delle proteine. La precipitazione isoelettrica rappresenta un metodo alternativo che produce la precipitazione delle molecole di collagene nella soluzione acida. La precipitazione isoelettrica avviene quando il pH della soluzione viene aggiustato al punto isoelettrico (pI) della molecola che si vuole fare precipitare, dunque, è necessario determinare preliminarmente il pI della proteina. Il pI di una proteina è il pH a cui la proteine ha carica netta nulla e dipende dalla presenza di gruppi ionizzabili come gruppi R degli amminoacidi che formano la catena polipeptidica. Al di sopra e al di sotto del punto isoelettrico, le molecole risultano cariche e le forze repulsive determinano la loro solubilità in soluzione, al punto isoelettrico le molecole risultano scariche e interagiscono per interazioni attrattive di tipo idrofobico che le portano ad aggregarsi e a precipitare.
Il salting out richiede un processo di purificazione del collagene ottenuto effettuato per dialisi: la dialisi produce una grande quantità di acqua di scarico e dura fino a quattro giorni. La possibilità di sostituire il recupero per salting out con il recupero per precipitazione isoelettrica è stata esplorata nel lavoro di Lin e colleghi [18].

Sulla base della conoscenza ottenuta da lavori precedenti presenti in letteratura e menzionati sopra, in questo lavoro di Tesi, è stato seguito un protocollo base che è consistito nel pretrattamento della materia prima, nell'estrazione del collagene e nel suo recupero tramite salting out e precipitazione isoelettrica. L'estrazione è stata effettuata in soluzione di acido acetico con e senza aggiunta di pepsina (Figura 3).

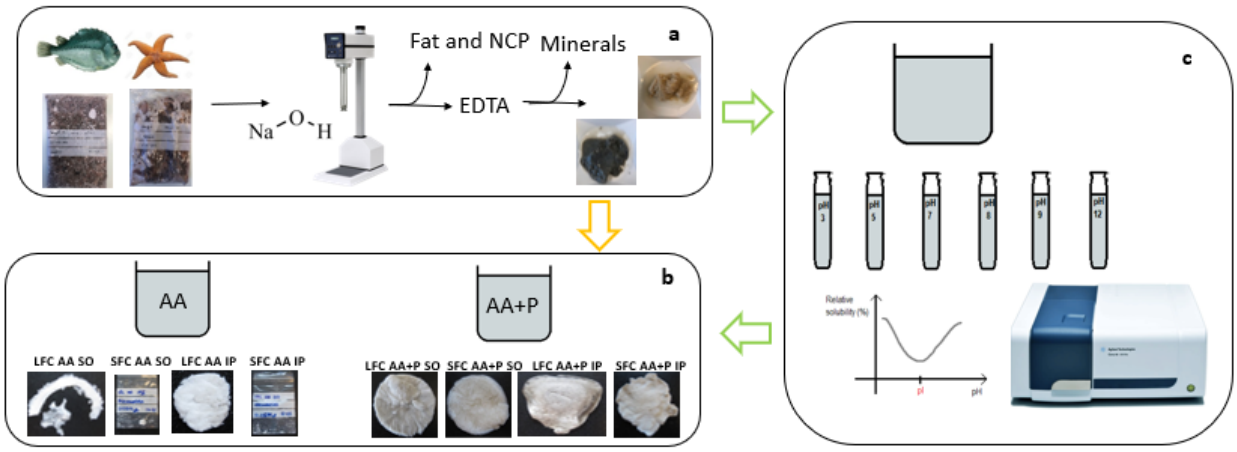

Figura 3. Sintesi schematica del piano di lavoro: pretrattamento delle materie prime (a), estrazione e recupero del collagene (b), determinazione dei punti isoelettrici (c).

II. Materiali e metodi

Pretrattamenti della materia prima ed estrazione del collagene

La materia prima è stata sottoposta ai pretrattamenti che precedono l'estrazione del collagene, con lo scopo di allontanare dai tessuti le sostanze diverse dal collagene (proteine non collageniche, minerali, grassi, pigmenti) per solubilizzazione in soluzione. I pretrattamenti e l'estrazione del

collagene sono stati condotti a 4°C in modo da evitare degradazione del collagene, che avverrebbe a temperature superiori. La carne del lompo è stata precedentemente lavata, tritata e conservata a -80°C, le stelle marine sono state lavate e conservate integre a -80°C. La materia prima è stata scongelata mantenendola in zip bags immerse sotto acqua fredda corrente per circa 30 minuti. In seguito allo scongelamento, la stella marina viene tagliata in pezzi di 0.5-1 cm, il lompo è già tritato.
Per favorire la rimozione successiva di proteine non collageniche e minerali, la dimensione dei tessuti è stata ridotta ulteriormente attraverso omogeneizzazione. La materia prima viene immersa in una soluzione 0.1 M NaOH in rapporto 1:10 (w/v) e viene sottoposta ad omogenizzazione:

- Lompo: 1.30 minuti, 6000 rpm
- Stella marina: 2.30 minuti, 7000 rpm

Dopo l'omogenizzazione, la soluzione è trasferita in bottiglie chiuse per assicurare la sterilità e la deproteinizzazione ha luogo per 30 minuti a 4°C in condizioni di costante miscelazione. In questo modo, le proteine non collageniche, solubili in ambiente alcalino, sono state allontanate dal materiale. Attraverso centrifugazione a 2000xg per 2 minuti a 4°C si ottiene la separazione del supernatante e del precipitato: il supernatante è una soluzione alcalina contenente proteine non collageniche eventualmente recuperabili, il precipitato contiene il collagene desiderato e altre molecole indesiderate. Il precipitato viene lavato con acqua fresca distillata e il pH viene aggiustato al valore neutro.
La demineralizzazione è stata effettuata in presenza di 0.5 M EDTA-4Na come agente chelante per la rimozione di Ca^{2+} ed Mg^{2+} (particolarmente presenti nella stella marina). Non sono state effettuate la rimozione di grassi e pigmenti, ma parte del grasso del lompo risulta rimosso dopo il processo di deproteinizzazione. Per favorire lo scambio di materia, le soluzioni sono state sottoposte a mixing e cambiate ad intervalli prestabiliti. Tra un pretrattamento e l'altro è stata effettuata centrifugazione della soluzione per la separazione della frazione liquida da quella solida contenente il collagene.
L'estrazione di collagene è stata effettuata in ambiente acido dal momento che la proteina risulta avere alta solubilità a pH acidi e, in particolare, si è utilizzata una soluzione 0.5 M di CH_3COOH, risultata particolarmente efficace. L'estrazione del collagene è stata effettuata sia in presenza che in assenza di pepsina.

Determinazione delle curve di solubilità e del punto isoelettrico pI

Preliminarmente al recupero del collagene dalla soluzione acida è stato determinato il punto isoelettrico del collagene. Il punto isoelettrico viene determinato per il collagene di stella marina e lompo ottenuto tramite estrazione in acido acetico con e senza pepsina in modo tale da individuare eventuali differenze tra il pI del collagene della stessa specie estratto in presenza e assenza di pepsina. Il pI viene individuato come pH a cui la solubilità del collagene è minima cioè come pH a cui si ha la massima precipitazione di collagene. Il pH della soluzione principale viene aggiustato ai valori 3.0, 5.0, 7.0, 8.0, 9.0 e 12.0 usando 2 N NaOH, 0.1 N NaOH e 2 N HCl mentre la soluzione è stata continuamente miscelata e mantenuta in ghiaccio. Ad ogni valore di pH, sono prelevati due campioni di 1.5 ml. I campioni sono stati lasciati a 4°C per 15 minuti in modo da consentire l'eventuale precipitazione di collagene. Successivamente è stata effettuata centrifugazione dei campioni e tramite il metodo di Lowry è stata determinata la concentrazione di collagene nelle soluzioni a ciascun pH. A partire dai valori di concentrazione di collagene, la solubilità relativa viene calcolata come il rapporto tra la concentrazione di collagene (µg/ml) ad un certo pH e la massima concentrazione di collagene nel range di pH analizzato.

Recupero del collagene per salting out e per precipitazione isoelettrica

Il recupero del collagene è stato effettuato sia con salting out che con precipitazione isoelettrica. Il salting out è stato condotto aggiungendo in rapporto volumetrico 1:1 una soluzione 5 M di NaCl e 0.1 M di tris(idrossimetil)amminometano alla soluzione di acido acetico in modo da ottenere una concentrazione salina finale di 2.5 M. La precipitazione del collagene comincia contestualmente all'aggiunta della soluzione salina ed è possibile notare in soluzione le molecole di collagene in aggregazione (Figura 4).

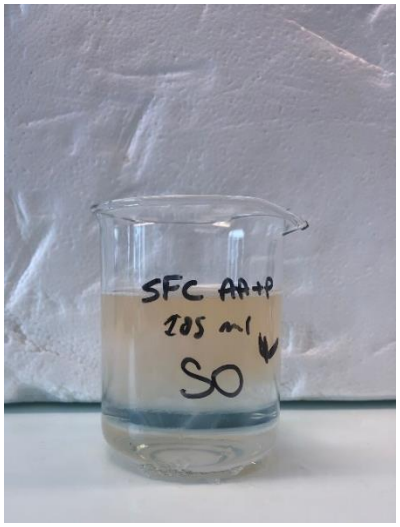

Figura 4. Soluzione acido acetico e pepsina (AA+P) contenente collagene di stella marina (SFC) disciolto durante il salting out (SO): visibile aggregazione di molecole di collagene

La procedura di salting out ha avuto luogo per 30 minuti ed è stata seguita da centrifugazione, il precipitato è stato poi risolubilizzato in una piccola quantità di soluzione 0.1 M di acido acetico e sottoposto a dialisi. La dialisi è durata 72 h e la soluzione è stata cambiata dopo 24 h con 0.1 M CH_3COOH e dopo 48 h con acqua distillata. Al termine della dialisi il collagene viene raccolto e liofilizzato.
Il recupero per precipitazione isoelettrica è stato effettuato aggiustando il pH della soluzione di acido acetico contenente il collagene disciolto al pI precedentemente determinato. La procedura di precipitazione isoelettrica ha avuto luogo per 30 minuti, in seguito è stata eseguita centrifugazione ed il precipitato è stato raccolto e liofilizzato.

Calcolo della resa di estrazione

La resa in collagene viene valutata come rapporto tra il peso di collagene liofilizzato ottenuto e il peso di materia prima utilizzata (sia peso secco che peso umido), si è lavorato con duplicati e si è effettuata una media dei valori ottenuti. La resa di estrazione si è valutata nel caso di estrazione con soluzione di acido acetico e pepsina effettuando il recupero sia per salting out che per precipitazione isoelettrica in modo da confrontare i due metodi di recupero in termini di quantità di collagene estratto.

Caratterizzazione dei campioni di collagene

I campioni di collagene liofilizzato ottenuti tramite estrazione con soluzione di acido acetico e pepsina vengono caratterizzati tramite SDS-PAGE, UV-visibile e FTIR. Si sono valutate anche le caratteristiche organolettiche dei campioni ottenuti (colore, odore e consistenza). Si è scelto di caratterizzare soltanto i campioni ottenuti tramite l'estrazione enzimatica con pepsina in quanto la resa in collagene in assenza di pepsina è molto bassa e, per questo, di scarso interesse dal punto di vista economico e industriale.

Caratterizzazione dei campioni di collagene: elettroforesi su gel con SDS-PAGE

L'elettroforesi su gel di poliacrilammide in presenza di sodio dodecil solfato (SDS-PAGE) come agente denaturante è una tecnica utilizzata per ottenere la separazione delle proteine che compongono il campione in base al peso molecolare. In questo caso la tecnica è stata utilizzata per determinare il tipo di collagene (I, II, ecc.) e per capire se fosse avvenuta degradazione del collagene durante l'estrazione. SDS denatura la tripla elica del collagene e conferisce carica negativa alle catene polipeptidiche. Le catene polipeptidiche sottoposte a una differenza di potenziale migrano sul gel e si separano in base alla differenza di peso molecolare. Sono stati utilizzati gel prefabbricati Biorad di 7.5% poliacrilammide con 12 pozzetti. Il collagene liofilizzato è stato disciolto in una soluzione 5% SDS fino a raggiungere una concentrazione di circa 10 mg/ml, tramite metodo di Lowry si è valutata la concentrazione proteica dei campioni e in base a questa si sono diluiti i campioni con soluzione buffer per ottenere una concentrazione di 2 mg/ml. La soluzione buffer è composta da soluzione buffer Laemmli e 5% (v/v) di β-mercaptoetanolo. I campioni sono stati riscaldati a 95° per 5 minuti, successivamente centrifugati a 5000g per 5 minuti e 7.5 μl di supernatante di ciascun campione sono stati caricati sul gel. Sul gel vengono caricati anche 5 μl di marker (Dual Color Precision Plus Protein™ Prestained Standards). Il buffer di separazione è una soluzione composta da 900 ml di acqua fredda distillata e 100 ml di buffer tris-glycine-SDS. L'elettroforesi viene svolta con una differenza di potenziale di 125 V per 80-85 minuti. In seguito, il gel è stato lavato con acqua e sottoposto a colorazione per 1 h con una soluzione 50% (v/v) metanolo, 7.5% acido acetico e 0.02% Coomassie Brilliant Blue R-250. La decolorazione ha avuto luogo poi per 1 h (cambiando soluzione dopo 30 min) in una soluzione 50% (v/v) metanolo e 7.5% (v/v) acido acetico.

Caratterizzazione dei campioni di collagene: spettroscopia ultravioletta (UV)

La spettroscopia ultravioletta è un'analisi che consiste nel misurare la quantità di radiazione elettromagnetica assorbita da un composto chimico nello spettro ultravioletto, ovvero alle lunghezze d'onda comprese tra il visibile e i raggi X (100 nm – 400 nm). Questa lunghezza d'onda corrisponde alle dimensioni molecolari. L'assorbimento di un fotone UV determina il passaggio della molecola dal ground state allo stato eccitato. I cromofori sono i componenti delle molecole responsabile dell'assorbimento del fotone e ciascun cromoforo ha un picco di assorbimento peculiare. Nel caso delle proteine, i cromofori più comuni sono triptofano e tirosina che producono un picco di assorbimento a 280 nm. Nel caso del collagene il picco di assorbimento caratteristico si trova a 230 nm [19]. 5 mg di collagene sono stati disciolti in 10 ml di 0.5 M CH_3COOH(0.5 g/l), la soluzione è stata centrifugata a 5000g per 10 minuti per ottenere un supernatante chiaro ed è stata valutata l'assorbanza del supernatante tra 190 e 450 nm utilizzando lo spettrofotometro. Per ogni campione di collagene sono state fatte tre letture.

Caratterizzazione dei campioni di collagene: spettroscopia infrarossa a trasformata di Fourier (FTIR)

La spettroscopia FTIR è un'analisi che permette di ottenere lo spettro di assorbimento di una sostanza a lunghezze d'onda nello spettro dell'infrarosso (700 nm – 1 mm). La radiazione assorbita dalla molecola, anche in questo caso, produce l'eccitazione della molecola. Lo spettro di assorbimento della molecola consiste in dei picchi di assorbanza, ciascun picco è associato a un grado di liberà vibrazionale della molecola ed è associabile alla presenza di determinati gruppi nella struttura chimica. In particolare, per il collagene si individuano i picchi relativi ai

gruppi Amide I, Amide II, Amide III, Amide A e Amide B come riportato in altri lavori [20], [21]. I dati sono stati acquisiti posizionando i campioni di collagene liofilizzato sulla cella di cristallo del fotometro e facendo tre letture per ogni campione scannerizzando da $4000\ cm^{-1}$ a $400\ cm^{-1}$.

III. Risultati e discussione

Curva di solubilità e pI

In Figura 5 e in Figura 6 sono riportate le curva di solubilità relativa del collagene estratto rispettivamente da lompo e da stella marina.

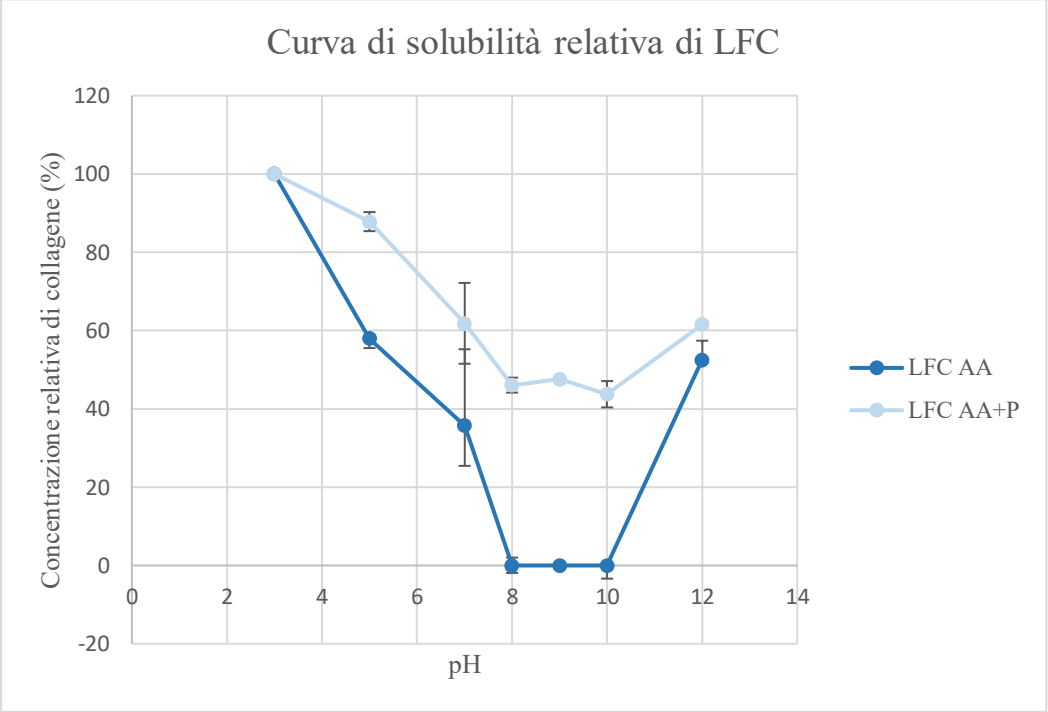

Figura 5. Curva di solubilità del collagene estratto da lompo (LFC) tramite estrazione con soluzione di acido acetico (AA) e di acido acetico e pepsina (AA+P): solubilità relativa (%) in funzione del pH (pH da 3 a 12). Risultati sono media ± deviazione standard di 2 repliche tecniche.

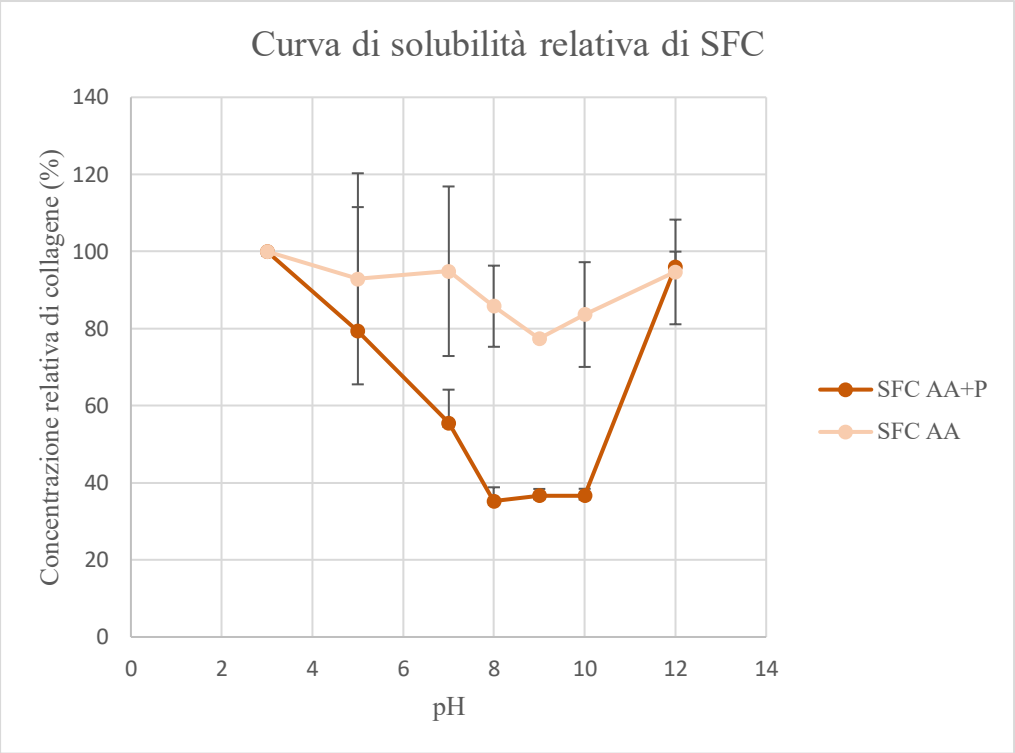

Figura 6. Curva di solubilità del collagene estratto da stella marina (SFC) tramite estrazione con soluzione di acido acetico (AA) e di acido acetico e pepsina (AA+P): solubilità relativa (%) in funzione del pH (pH da 3 a 12). Risultati sono media ± deviazione standard di 2 repliche tecniche.

La massima solubilità relativa si è ottenuta per pH=3 e si è notato che la solubilità è più bassa nel range pH 8-10 mentre è alta a pH acidi. Si è notato, inoltre, un incremento della solubilità a pH superiori al pI. I risultati trovati per il collagene estratto da lompo e da stella marina sono in accordo con quelli trovati in altri lavori [22], [23]. Non si sono evidenziate nette differenze tra le curve di solubilità del collagene estratto in presenza e in assenza di pepsina per cui un singolo pI è stato determinato per ciascuna specie. Il punto isoelettrico determinato per il lompo è stato pI=8, per la stella marina pI=10. Dalle curve di solubilità relativa è stato possibile dedurre il recupero (%) come differenza tra 100% e solubilità relativa al pH scelto: si è trovato che effettuando precipitazione isoelettrica di collagene in soluzione AA+P si ottiene sia per SF che LF un recupero intorno al 60%. Il recupero (%) ha a che vedere esclusivamente con la tecnica di recupero utilizzata mentre la resa in collagene (%) è il risultato dell'intero processo di estrazione per cui dipende da più fattori tra cui il metodo di recupero, il metodo di estrazione e la specie coinvolta.

Resa di estrazione

I risultati di resa percentuale ottenuti sono mostrati in Figura 7 e in Figura 8.

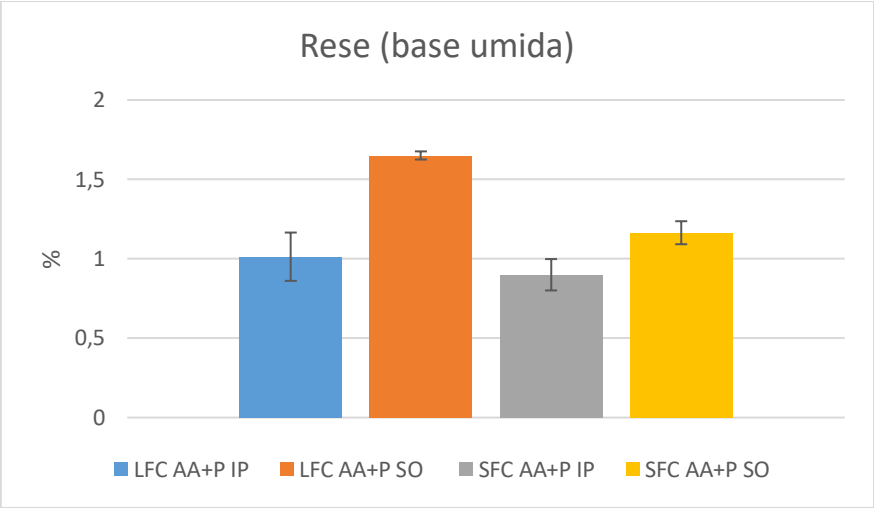

Figura 7. Rese medie in collagene su base umida: collagene estratto da lompo (LFC) e stella marina (SFC) in soluzione di acido acetico + pepsina (AA+P e recupeato tramite precipitazione isoelettrica (IP) e salting out (SO). Risultati ottenuti come media di duplicati in un singolo esperimento di solubilizzazione.

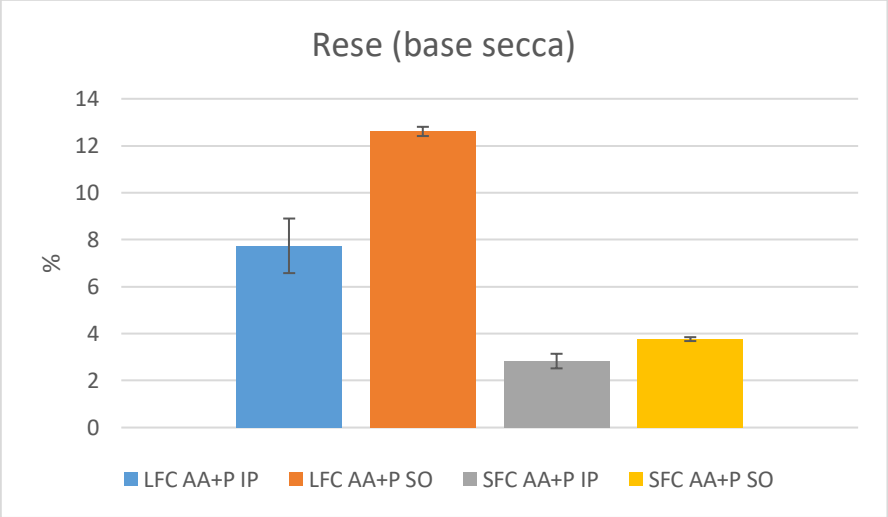

Figura 8. Rese medie in collagene su base secca: collagene estratto da lompo (LFC) e stella marina (SFC) in soluzione di acido acetico + pepsina (AA+P e recupeato tramite precipitazione isoelettrica (IP) e salting out (SO). Risultati ottenuti come media di duplicati in un singolo esperimento di solubilizzazione.

Per il lompo si sono ottenute come rese su base umida 1.0.1±0.152% nel caso di precipitazione isoelettrica e 1.65±0.025% nel caso di salting out, risultati promettenti considerato l'alto contenuto di acqua della materia prima utilizzata (umidità del campione=86.91±0.68%). Questi valori su base secca corrispondono rispettivamente a 7.74±1.162% e 12.61±0.196%. Nel caso di estrazione di collagene da stella marina, la differenza in resa tra i due metodi di recupero è ancora inferiore (su base umida 0.9±0.098% per precipitazione isoelettrica e 1.16±0.072% per salting out, su base secca rispettivamente 2.83±0.31% e 3.76±0.083%). In generale, le rese su base secca sono sempre maggiori rispetto a quelle su base umida e in particolare nel caso del lompo, poiché la materia prima è particolarmente umida, la differenza tra la resa su base secca e su base umida è marcata.

Si è trovato che la resa ottenuta effettuando il recupero per salting out è stata lievemente maggiore di quella ottenuta con la precipitazione isoelettrica sia per il lompo che per la stella marina e che la resa in collagene del lompo è, in ogni caso, maggiore della resa in collagene della stella marina. Si è evidenziato che la differenza in resa tra precipitazione isoelettrica e salting out non è talmente marcata da escludere la precipitazione isoelettrica come un'alternativa valida. Infatti, bisogna tenere in considerazione tra gli svantaggi del recupero effettuato per salting out che il metodo richiede diversi giorni di dialisi per rimuovere il sale dal collagene e questo coinvolge l'utilizzo di una grande quantità di acqua.

Caratterizzazione dei campioni di collagene

I campioni di collagene liofilizzato sottoposti a caratterizzazione sono mostrati in Figura 9.

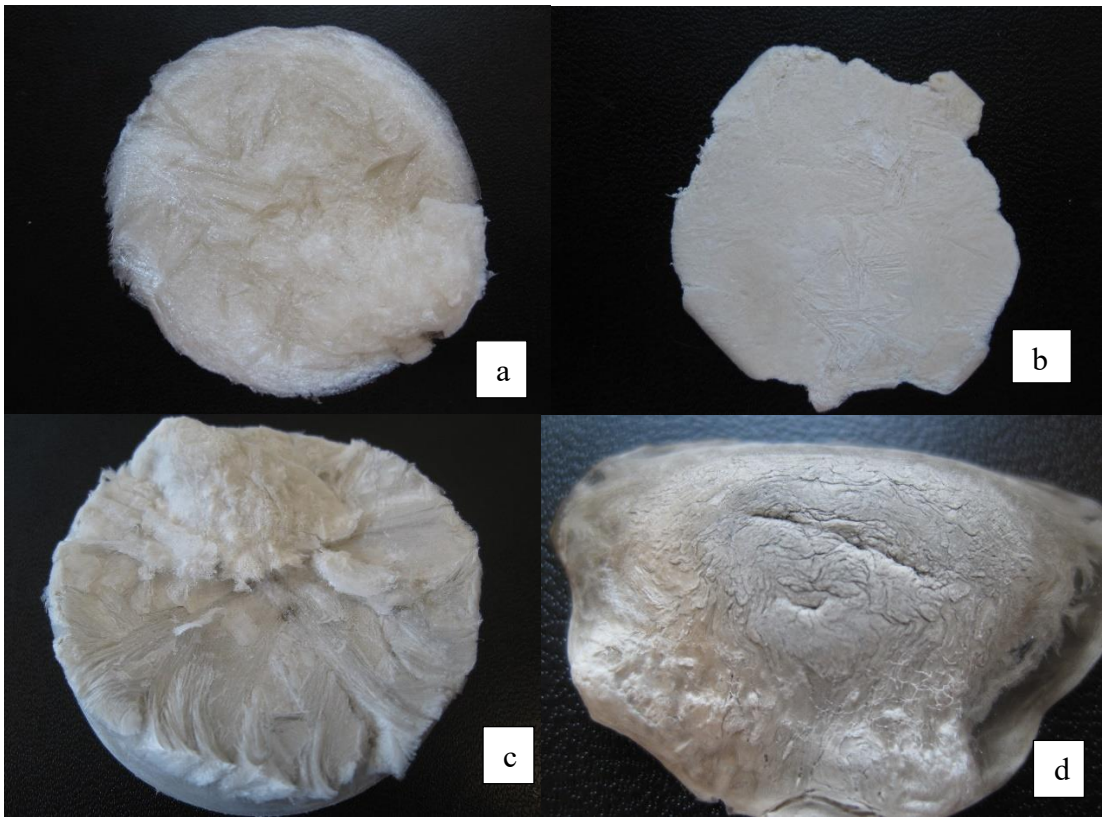

Figura 9. Collagene liofilizzato ottenuto da stella marina (LFC) estratto con soluzione di acido acetico e pepsina (AA+P), recuperato tramite salting out (SO) (a) e precipitazione isoelettrica (IP) (b), collagene liofilizzato ottenuto da lompo (LFC) estratto con soluzione di acido acetico e pepsina (AA+P), recuperato tramite salting out (SO) (c) e precipitazione isoelettrica (IP) (d)

Il collagene ottenuto per salting out presentava una consistenza più morbida e spugnosa e una struttura simil lamellare mentre il collagene ottenuto per precipitazione isoelettrica aveva una consistenza dura. Il collagene estratto da lompo presentava un colore tendente al grigio mentre il collagene estratto da stella marina presentava un colore tendente al bianco. In tutti i campioni di collagene erano percepibile gli odori di pesce e di acido acetico.

Caratterizzazione dei campioni di collagene: pattern elettroforetico del collagene estratto

Il pattern elettroforetico dei campioni è mostrato in Figura 10. Sia per i campioni di collagene da stella marina (SFC) che da lompo (LFC) per entrambi i metodi di recupero si sono individuati due tipi di catena (α_1 a maggior peso molecolare e α_2 a minor peso molecolare) per cui è possibile affermare che in entrambi i casi si tratti di collagene di tipo 1 (rapporto 2:1 tra catena α_1 e catena α_2).

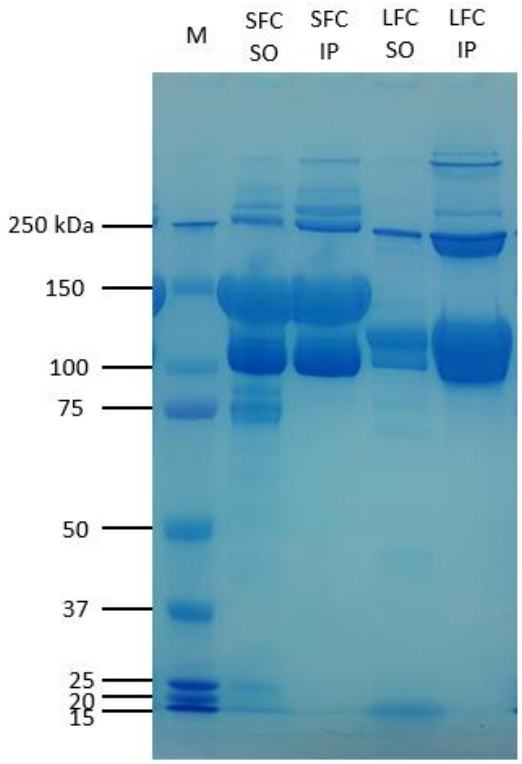

Figura 10. SDS-PAGE: M = marker, LFC SO collagene lompo acido acetico + pepsina salting out, LFC IP = collagene lompo acido acetico + pepsina precipitazione isoelettrica, SFC SO = collagene stella marina acido acetico + pepsina salting out, SFC IP = collagene stella marina acido acetico + pepsina precipitazione isoelettrica. In ogni corsia sono stati caricati 15 µg di collagene.

Si è notato che la catena α_1 del collagene estratto da stella marina ha un peso molecolare superiore rispetto a quella del collagene estratto da lompo (intorno a 150 kDa per SFC e intorno a 120 kDa per LFC). Sia per i campioni di collagene da stella marina (SFC) che di lompo (LFC) si è notata la presenza di una banda intorno a 250 kDa attribuibile a catene di tipo β (le catene β sono formate da due catene α legate tra loro da legami intramolecolari). Nel caso di LFC IP e di SFC IP si è notata anche la presenza di una banda sottile a un pedo molecolare superiore a 250 kDa, attribuibile a catene di tipo γ (trimero formato da tre catene α). Nel caso di collagene recuperato con salting out, sia per LFC che SFC, si è individuata la presenza di bande a basso peso molecolare indicando la presenza di peptidi a basso peso molecolare dovuta a degradazione della proteina. Le bande a basso peso molecolare sono state attribuite a prodotti di degradazione del collagene e non a proteine non collageniche non rimosse nella deproteinizzazione, in quanto tutti i campioni sono stati sottoposti allo stesso processo di deproteinizzazione e se proteine non collageniche fossero presenti allora sarebbero visibile delle bande anche nei campioni ottenuti per precipitazione isoelettrica. Dunque, la degradazione è avvenuta nel caso di recupero per salting out ma non nel caso di recupero per precipitazione isoelettrica. Il pattern elettroforetico dei campioni di collagene ottenuti per precipitazione isoelettrica ha mostrato che questi campioni erano più puri e meno soggetti a degradazione.

Caratterizzazione dei campioni di collagene: spettro UV del collagene estratto

Lo spettro UV dei campioni di collagene è mostrato in Figura 11. L'UV-visibile ha confermato la purezza del collagene ottenuto da LFC ed SFC in quanto tutti i campioni hanno presentato il picco di assorbimento maggiore a 230 nm, tipico del collagene [19].

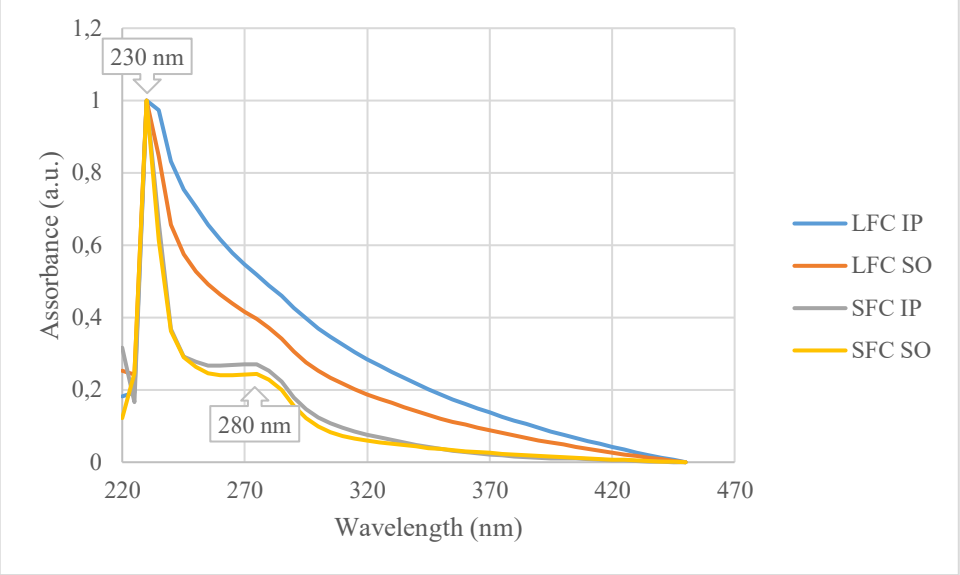

Figura 11. Spettro UV normalizzato rispetto al picco massimo a circa 230 nm dei campioni di collagene da lompo (LFC) e stella marina (SFC) ottenuti tramite estrazione con soluzione di acido acetico + pepsina (AA+P) e recuperati tramite precipitazione isoelettrica (IP) e salting out (SO)

Lo spettro UV del collagene estratto da lompo ha mostrato un picco a 230 nm e alta assorbanza anche nel range 230-300 nm. Lo spettro UV del collagene estratto da stella marina ha presentato un picco appuntito a 230 nm e un secondo picco a 280 nm. Il picco di assorbanza a 280 nm è associato alla presenza di residui amminoacidici aromatici come il triptofano, la tirosina e la fenilalanina che hanno un picco di assorbanza nel range 240-300 nm [24]. Dunque, il picco di assorbanza a 280 nm ha suggerito che il collagene estratto da lompo presentava un contenuto superiore di amminoacidi aromatici rispetto al collagene estratto da stella marina (in cui amminoacidi aromatici sono comunque presenti). Il picco a 280 nm non è stato attribuito alla presenza di amminoacidi aromatici in proteine non collageniche in quanto il pattern elettroforetico ottenuto con SDS-PAGE ha mostrato l'assenza di proteine non collageniche nei campioni caratterizzati.

Caratterizzazione dei campioni di collagene: spettro FTIR del collagene estratto

Nello spettro FTIR dei campioni di collagene sono stati individuati i picchi di assorbanza principali tipici del collagene [25]: amide A, amide B, amide I, amide II e amide III (Figura 12).

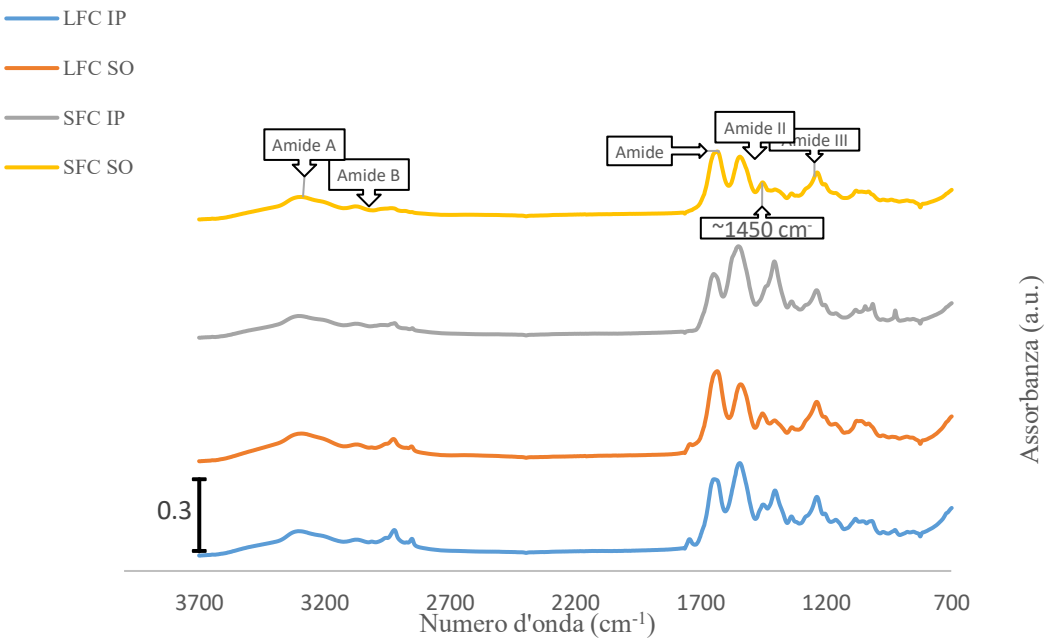

Figura 12. Spettro infrarosso a trasformata di Fourier (FTIR) dei campioni di collagene da stella marina (SFC) e da lompo (LFC) ottenuti per estrazione in soluzione di acido acetico e pepsina (AA+P) e recuperati con precipitazione isoelettrica (IP) e salting out (SO)

La posizione del picco di amide A di SFC IP ed SFC SO erano leggermente differente (3305 cm^{-1} for SFC IP and 3295 cm^{-1} for SFC SO), per LFC IP ed LFC SO la posizione del picco di amide A era molto simile (3305 cm^{-1} e 3303 cm^{-1} rispettivamente). La posizione di amide A ha a che vedere con lo stiramento del legame N-H: il picco relativo allo stiramento di un legame N-H libero avviene a 3400-3440 cm^{-1} e il fatto che il picco di amide A di tutti i campioni si trovi a frequenze minori ha permesso di affermare che N-H è impegnato in legami a idrogeno intramolecolari [26].
I picchi di amide I di LFC IP ed LFC SO erano a 1644 cm^{-1} e 1643 cm^{-1}, quelli di SFC IP e SFC SO a 1646 cm^{-1} and 1633 cm^{-1} rispettivamente. L'assorbimento di amide I è associato allo stiramento del legame C=O [27].
I picchi di amide II di LFC IP e LFC SO sono stati trovati ad aprossimativamente lo stesso numero d'onda (1544 cm^{-1} e 1542 cm^{-1} rispettivamente), mentre anche in questo caso SFC SO presenta il picco ad una frequenza minore rispetto a SFC IP (1542 cm^{-1} e 1548 cm^{-1} rispettivamente). Il picco di assorbimento di amide II è associato al piegamento del legame N-H accoppiato allo stiramento del legame C-N [28].
Nel lavoro di Payne e Veis [29] è riportato che uno shift della frequenza di assorbimento di amide I e di amide II a frequenze più basse è indice di una diminuzione dell'ordine molecolare, cioè un minore contenuto di crosslinks intramolecolari, e questa è una spiegazione plausibile al fatto che SFC SO presenta picchi di assorbimento a frequenze inferiori rispetto a SFC IP.
I picchi di amide B di LFC IP e LFC SO sono stati trovati a 3075 cm^{-1} e 3073 cm^{-1} rispettivamente, quelli di SFC IP e SFC SO a 3068 cm^{-1} e 3077 cm^{-1}. I picchi di assorbimento di amide B a queste frequenze sono collegati allo stiramento asimmetrico di CH_2, Gruppo R nei residui di Gly e Pro, amminoacidi particolarmente presenti nel collagene.
I picchi di assorbimento di amide III di LFC ed SFC sono stati trovati approssimativamente alla stessa lunghezza d'onda (1236 cm^{-1} for IP and 1234 cm^{-1} for SO) e questo picco di assorbanza è associato alla deformazione di N-H e allo stiramento di C-N [30].
Nel caso di LFC IP, LFC SO ed SFC SO si sono individuate dei picchi minori intorno a 1450 cm^{-1}. Secondo quanto affermato nel lavoro di Ahmad and Benjakul [31], rapporto tra l'assorbanza del picco di amide III e l'assorbanza del picco a 1450 cm^{-1} vicino ad 1 indica che la tripla elica

ha mantenuto integra la struttura della tripla elica. Questo rapporto è stato calcolato per LFC IP ed LFC SO (rapporto pari a 1.1) e per SFC SO (rapporto pari a 1.4).
In conclusione, per quanto riguarda i risultati dello spettro FTIR, la presenza e la posizione dei picchi è analoga a quella trovata per collagene estratto da altre specie marine come anguilla [32], carpa nobile [30] e carpa argento [20], permettendo di affermare la presenza di collagene e la presenza di crosslink intramolecolari nel collagene. Inoltre, per quanto riguarda LFC IP, LFC SO e SFC SO è stato possibile affermare che la tripla elica di collagene ha mantenuto la sua integrità durante il processo di estrazione.

IV. Conclusioni

In conclusione, seppure la resa in collagene sia risultata leggermente inferiore nel caso di recupero con precipitazione isoelettrica rispetto al salting out, la caratterizzazione dei campioni ha dimostrato la purezza del collagene estratto con precipitazione isoelettrica, che può quindi rappresentare un metodo di recupero valido alternativo al tradizionale salting out. I vantaggi dell'utilizzo della precipitazione isoelettrica vanno valutati in termini di specificità della tecnica e di risparmio di tempo e materie prime (acqua e reagenti chimici).

Index

1. Introduction

1.1 Background

1.1.1. Collagen

Collagen

Collagen is the most abundant protein in mammals, covering 20%-30% of the total protein body content [1], but it is also present in marine species, especially in skin, bones, scales, fins, swim bladder. It is the main structural entity of protein nature in the extracellular matrix (ECM) of the connective tissue in the body. Similarly to other tissues, connective tissue is formed by cells which have analogue structures and functions, and it consists of the ECM as well. The ECM provides a structural support to the cells and each type of connective tissue has a different type of ECM. The ECM is a three-dimensional network formed by extracellular molecules that are proteins, polysaccharides and other components (Figure 1).

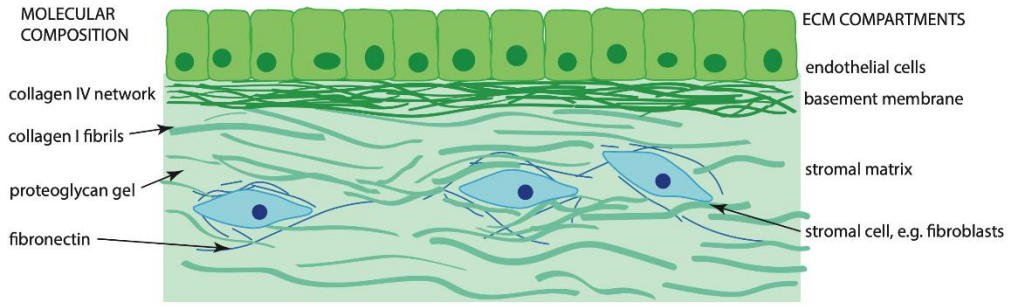

Figure 1. Molecular structure of the extracellular matrix showing the three-dimensional network formed by proteins, polysaccharides and other components [33]

Proteins in the ECM can be both structural proteins such as collagen (arranged into fibrils) and elastin and non-structural proteins such as fibronectin, laminin and tenascin, which are glycoproteins [2]. Fibroblasts are the cells that synthetize the ECM and the collagen. Therefore, the ECM essentially consists of proteins belonging to different families and playing different roles but among them the most abundant proteins are members of the collagen superfamily [5].
Glycosaminoglycans (GAG) are non-branched polysaccharide chains in the ECM, they consist of a repetitive monomer that is a disaccharide unit (Figure 2). They are called GAG because one of the two sugars is an amino-sugar such as N-acetyl-glucosamine or N-acetyl-galactosamine. Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan that is found in the ECM.

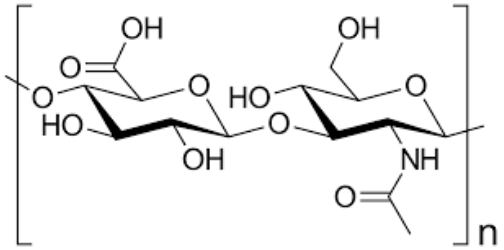

Figure 2. Disaccharide unit of hyaluronic acid (HA), glycosaminoglycan (GAG) composing the extracellular matrix (ECM)

Collagen structure

Collagen is a trimeric macromolecule since it has a triple helix tertiary structure composed of three α-chains (α helices as secondary structure) (Figure 4) formed by around 1014 amino acids with weight around 100 kDa each [34]. The primary structures of the three α-chains slightly differ from each other. The primary structure is peculiar because of the presence of repetitive sequences that are Gly-X-Y where X and Y are amino acids different from glycine (Gly), more often they are proline (Pro) and hydroxyproline (Hyp) (Figure 3).

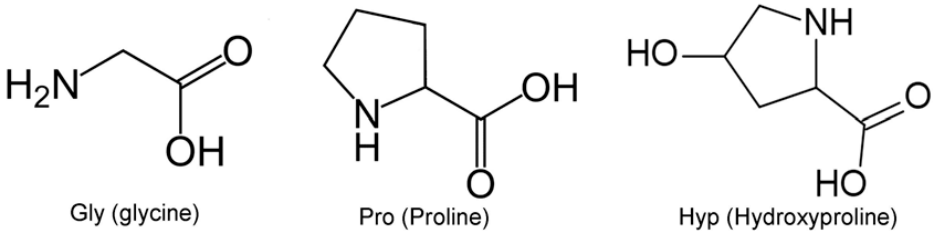

Figure 3. Glycine, Proline and Hydroxyproline amino acidic residues, particularly presents in collagen primary structure

The repetitive unit $(Gly - X - Y)_n$ is characteristic of the collagen super-family. Moreover, the α-chains have a relevant content of essential amino acids (EEA) such as threonine (Thr) and methionine (Met). Essential amino acids are not synthetized by human body, so they need to be introduced through food. The tertiary structure is a triple helix, which is stabilized and packed by hydrogen bonds between adjacent Gly amino acids while Pro and Hyp have a fundamental role in stabilizing the triple helical structure in physiological condition through the formation of hydrogen bonds that block free rotation around the C-N bonds [3]. The three chains are supercoiled around a central axis with all the Gly residues positioned in the center of the triple helix allowing a close packaging along the central axis (glycine is the smallest amino acidic residue) [5].

Figure 4. Type I collagen (Homo sapiens) structure composed of three distinguishable α-helices coiled in a triple helix structure

Types of collagens

Based on the amino acidic composition and on the different α-chains, collagens differentiate into 28 different types. The 28 types of collagens are termed with Roman numerals from I to XXVIII in chronological order of identification. The most common one is the collagen of type I present in skin, bones, tendons, types II and III follows. Over 90% of collagen in human body is of type I. The type I collagen is composed of two identical $\alpha_1(I)$ chains and one $\alpha_2(I)$chain. Type I collagen is characterised by a high content of hydroxyproline amino acids and it is found in tissues such as tendon and bone. Type II collagen is constituted by three $\alpha_1(II)$ chains and it is found in cartilage [4].
Depending on the type of α chains composing it, the collagen macromolecule is homotrimeric -when the three chains are of the same type- or heterotrimeric if different. Type I collagen is heterotrimeric whereas type II is homotrimeric [35]. The dimer formed by two α chains is referred to as β chain and the trimer formed by three α chains as γ chain. Type I collagen shows two different α chains, two different β chains and a single γ chain, the ratio of the different α, β and γ chains in a sample depends on the degree of cross-links left among chains after collagen dissolution [36].

Fibril-forming collagens and their biosynthesis

Collagens can be distinguished based on their structure and on their supramolecular organization so, for instance, we distinguish fibril-forming collagens from not fibril-forming collagen. Fibrillar collagens were the first members of collagen superfamily to be discovered and they are the major products synthesized by connective tissue cells such as fibroblasts. Collagen types I, II, III, V and XI are fibrillar collagens [37]. As mentioned before, molecules belonging to the fibril-forming class can be formed by three equal chains (homotrimers) such as types II, III, VII, VIII, X and others or by two or more different chains (heterotrimers) such as types I, IV, V, VI, IX and XI – actually, I and V can associate into both homotrimers and heterotrimers –.
A single molecule of collagen is called tropocollagen and, in the case of fibrillary collagens, tropocollagen molecules aggregate to form the quaternary structure of the protein. The quaternary structure is a fibril which consists of an ordered and staggered structure formed by the triple helices. Fibrils pack together to form

fibres that constitute different tissues depending on the way the fibrils aggregate, fibres provide tensile strength to the tissues. Depending on the relative composition and amounts of different collagen types that are assembled into fibrils, the tissue will show different properties. Figure 5 shows the hierarchical structure of fibrillary collagen:

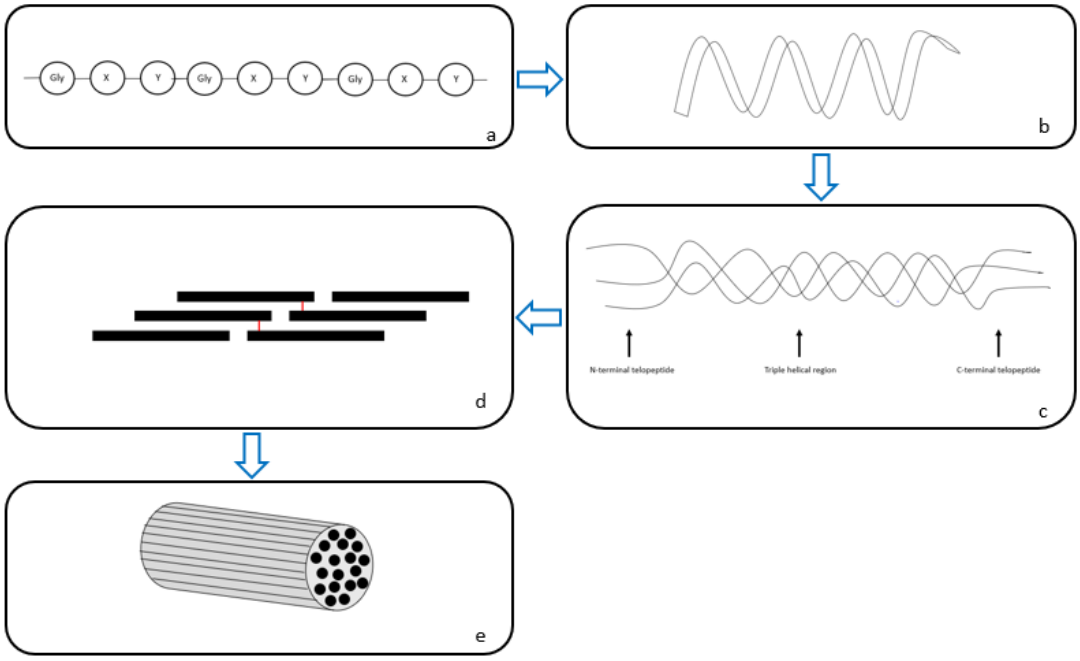

Figure 5. Hierarchical structure of collagen: collagen amino acidic composition (primary structure) (a), α-helix structure (secondary structure) (b), triple helix structure (tertiary structure) (c), fibril structure (quaternary structure) (d), fiber (e).

It is interesting to analyze more in details how the biosynthesis of fibril-forming collagen takes place. The work by Gelse and collaborators [5] focused on type I collagen biosynthesis but a similar mechanism is likely for other types of fibrillar collagens. Collagen genes transcription in cell nucleus depends on the cell type and on a number of factors as well, so different mRNAs of fibrillar collagens can be found. Pre-mRNAs molecules undergo (alternative) splicing, capping at the 5' end and polyadenylation at the 3' end. It has been found that alternative splicing contributes to the generation of different mRNA molecules [38][39]. Once post-transcriptional modifications are ended, mRNA molecules are transported to the endoplasmic reticulum (ER) though the cytoplasm and here they are translated in pre-procollagen molecules in the ribosomes. At this level, pre-procollagen chain contains C- and N-terminal ends. The removal of the signal peptide transforms the pre-procollagen molecules into procollagen molecules. Procollagen molecules are subjected to post-translational modifications at rough ER level. A fundamental post-translational modification is the hydroxylation of proline (Pro) and lysine (Lys) amino acidic residues. In fibril forming collagens, approximately 50% of the proline residues are hydroxylated, so they become Hyp residues. Hydroxylation of these residues confers the ability to form intramolecular hydrogen bonds contributing to the stability of the tiple helix structure and of the fibril structure [5]. So, fibrillar collagens are synthesized as precursors (procollagens) and secreted in the extracellular space, in order to become mature and functional molecules proteolytic removal of the N- and C-terminal propeptides is needed [37].

In fibril-forming collagens, the triple-helix motif represents the major part, but fibrils also contain non-helical portions: the triple helix is flanked by the N- and C-terminal extensions called the N- and C-propeptides respectively. The triple-helical regions are termed Col domains whereas the non-triple-helical regions are termed NC domains (non-collagenous domains).
The cleavage of N- and C-pro-peptides is catalyzed by the procollagen metalloproteinases [40]. After proteolytic cleavage of these terminals, helical domain bordered by telopeptides regions are left that are involved in the covalent cross-linking of collagen molecules and in the linking to other molecular structures of the surrounding matrix. The cleavage of the C-propeptides of type I collagen is an essential step for fibril formation while the function of the N-terminal propeptides in the process of fibril formation is not clearly understood yet [5]. Fibril formation proceeds with the alignment of the processed molecules in a quarter stagger arrangement and fibrils show an alternating light and dark pattern so that they are called banded fibrils. It is unlikely to find fibrils formed by a single type of collagen [35].
Another important type of collagen is the fibril-associated collagen (FACIT): this collagen shows interruptions within the triple helix domain and it interacts with fibrils to form more complex alloys. Collagen types IX, XII, XIV, XVI, XIX, XX, XXI and XXII belong to FACIT group so this is the largest subclass of the collagen superfamily [37].

Collagen production in human body

Damage to human skin is due to either photoaging or natural aging. Photoaging occurs as a consequence to ultraviolet light (UV light) from the sun and natural aging as a consequence of the lasting of time [41]. Photoaging determinates a chronological reduction of collagen quantity in human body because of both a decrease in the production and a degradation of existing collagen. According to Varani and colleagues [6], the reduction of collagen synthesis is due to cellular fibroblast aging and to defective mechanical stimulation in aged tissue. In particular, these phenomena become more and more prominent with the increasing of the age of the individual. It has been highlighted that collagen reduction starts at 18-29 years and that the content of type I procollagen decreased of 68% in old skin considering 80+ years individuals. Degradation of existing collagen is linked to the fact that UV light induces matrix-degrading metalloproteinases in the dermis [42]. The work by Song and colleagues [12] showed that oral administration of collagen peptides from bovine bone to chronologically aged mice could improve the laxity and the skin by increasing skin collagen contend and ratio of type I to type III collagen.

1.1.2. Collagen sources and production methods

Terrestrial species as collagen sources

Collagen is mainly extracted from the discards (skin, bones and hides) of bovine and porcine sources industrially processed. Bovine collagen is obtained by skin and bone of cows, and it is one of the most produced one. As reported in the work by Silvipriya and collaborators [4], nearly 3% of the population is allergic to bovine collagen. Type I collagen is obtained from bovine Achilles' tendon, type

IV is obtained from the placental villi and type II from nasal or articular cartilage. For what concerns porcine, the skin and bones of pigs are utilized, and the collagen thus obtained does not cause allergic response. This work also reports other animal sources used in collagen extraction that are chicken, kangaroo tail, rat tail tendon, duck feet, equine tendon, alligators' bone and skin, birds feet, sheep skin, frog skin and sometimes even humans.

Marine species as potential source of collagen

Collagen can also be obtained from marine species, in particular, from both invertebrates and vertebrates species (fish, jellyfish, sponges, sea urchin, octopus, etc.) [4].
To be more precise about marine collagen, in the book Biological Materials of Marine Origin – Vertebrates [43], *Ehrlich*, defines three main categories of marine collagens that are collagens of invertebrate origin, fish collagens and marine mammal collagens. Marine fish collagens comprehend types I, II, V/XI and XVIII and are of interest from an industrial point of view. *Ehrlich* claims that, even if marine mammal collagens are biomaterial with practical application, the use of marine mammals (whale and dolphin, in particular) should be replaced using more abundant marine species.
It is important to highlight why marine collagen extraction is becoming a priority, but it is also fundamental to underline which are the differences between land-based and marine-based collagens. Certainly, collagen extraction from bovine and porcine sources is linked to issues of various kinds so the extraction from marine species represents a fascinating perspective.
Some limitations to the use of collagen extracted from bovine and porcine sources are related to religions, in fact, Hinduism forbids the consumption of bovine meat, Islam and Hebraism forbid the consumption of porcine meat. According to Pew Research Center data, Hindus, Jews and Muslims made up 39.4% of the global population in 2015 [44].
Other limitations to the extraction of collagen from bovine and porcine sources are linked to the possibility of transmission of diseases such as the bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD). These are some of the reasons that made it necessary to find new sources of collagen and new production methods.
It is interesting to highlight that purified collagen extracted from marine species still has the ability to form fibrils [45]. However, bovine collagen has a high denaturation temperature compared to collagen from marine species, in which the lower content of Hyp produces a reduction of the denaturation temperature [46]. The lower denaturation temperature could affect collagen performances at body temperature. Moreover, fish is an allergenic food so the use of marine collagen in pharmaceutical preparations could result in allergic reactions [47].

Collagen extraction from marine species

In this work marine collagen extraction method will be treated in detail. It consists essentially in two steps, the first step involves the pre-treatment of the raw materials including cleaning, size reduction, deproteinization, demineralisation, defatting and depigmentation (depending on the marine species considered) whereas the second step is the extraction and recovery step [7].

Seafood by-products as a valuable resource

In addition to the mentioned reasons, there is a need to make the seafood market as sustainable as possible since the 50-80% of seafood products are discarded as waste as non-edible by-products causing underutilisation of the raw materials and problems related to the disposing that could lead to pollution [7].

Marine invasive species as a valuable resource

According to National Geographic definition, an invasive species is an organism - animal, plant or microorganism - that is not native to a particular area and it can cause great economic and environmental harm to the new area [48]. Invasive species are usually brought in new areas accidentally because of human activities. This is what happens, for example, to marine species that are carried in the ballast water of ships. In some cases, alien species are introduced intentionally in new environments, such as pets or decorative plants, without anticipating which consequences this will have on the diversity of the pre-existent ecosystem. Invasive species can threat the native wildlife directly becoming predators of the native species and/or indirectly outcompeting the native species for food or other resources [49]. Easy spreading of invasive species is due to the fact that they are not part of the food chain and of the balance of the habitat they happen to be in.

Production of recombinant collagen

An interesting alternative to produce collagen involves the use of biotechnologies. The work by Wang and colleagues [50] focused on the possibility of producing recombinant collagen as an alternative to animal collagen exploiting prokaryotic or eukaryotic organisms. Prokaryotic organisms produce a collagen like protein containing the Gly-X-Y motif, but this collagen is different from human collagen in terms of amino acid type and distribution. Prokaryotes such as *Escherichia coli* are not able to make post-translational modification, and this causes the lack of hydroxyproline in the structure. Rutschmann and colleagues [51] demonstrated that the use of mimivirus hydroxylases could be effective in hydroxylation of proline and lysine in various sequence contexts thus providing a framework for the large-scale production of recombinant collagens. Eukaryotic unicellular organisms like the *Saccaromyces cerevisiae* or *Pichia pastoris* yeasts could be used for collagen production but they are not able to perform the post-translational hydroxylation.
Conversely, eukaryotic multicellular organisms such as tobacco plants can hydroxylate proline residues of collagen, but plant propyl hydroxylases are insufficient for hydroxylating collagen up to the same level of human collagen and that is why tobacco plants should be engineered to produce human propyl hydroxylase. Mammalian cells are able to make post-translational modifications to collagen but the yield of recombinant human collagen is low if compared to that of the cells mentioned above [50].

1.1.3. Collagen and fish market

Collagen market

As reported in Grand View Research website (https://www.grandviewresearch.com/industry-analysis/collagen-market.) the global collagen market demand was estimate at 920.1 tons in 2019. Collagen from bovine source accounted for nearly 38.1% of the total in 2020, others are porcine, poultry and marine sources. Bovine collagen is easily available and cheaper if compared with porcine and marine ones [8]. According to Mordor Intelligence projections, Global Marine Collagen Market is going to grow at a CAGR[1] of 7.35% during the forecast period (2020-2025) [52].

Fish market

According to FAO[2] data, the world fish[3] market produced 172.6 mT in 2017 (92.5 mT from capture fisheries and 80.1 from aquaculture) (Figure 6) and the estimates foresee an increasing trend [53].

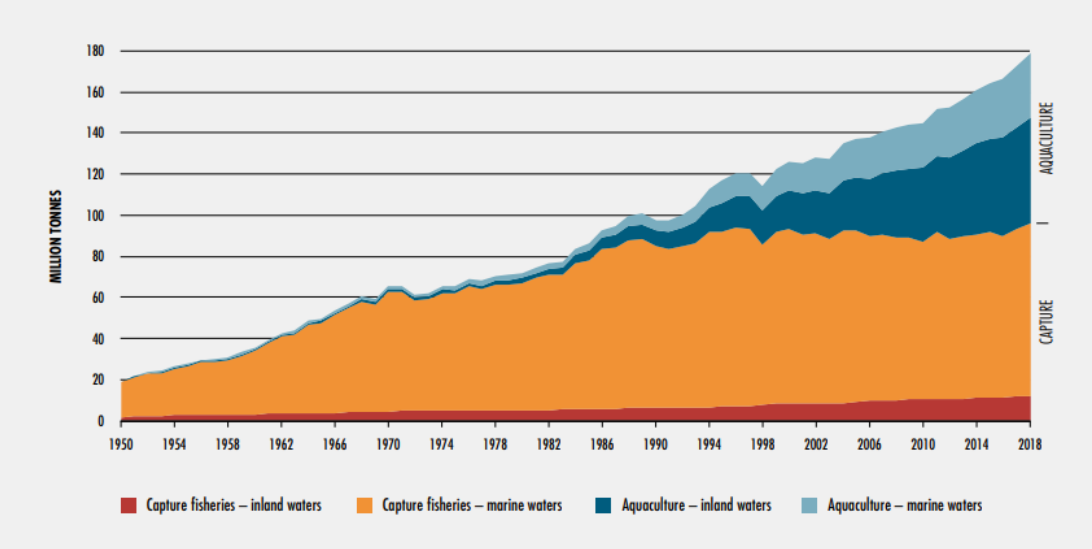

Figure 6. World capture fisheries and aquaculture production (FAO) from 1950 to 2018

[1] CAGR (Compound Annual Growth Rate) is the rate of return that would be required for an investment to grow from its beginning balance to its ending balance, assuming the profits were reinvested at the end of each year of the investment's life span. In this sense, CAGR can be used to calculate returns for anything that can rise or fall in value over time.

[2] FAO (Food and Agriculture Organization of the United Nations) includes the Fisheries and Aquaculture Division (NFI).

[3] The term "fish" comprehends different marine species that are fish, crustaceans, mollusks and other aquatic mammals and reptiles.

1.1.4. Other biomolecules

Collagen is only one of the biomolecules that could be recovered from marine species, others are lipids, minerals, chitin and gelatin.

Chitin

Chitin is a polysaccharide that can be found in exoskeletons of arthropods such as crustaceans (crabs, shrimps, lobsters, etc.). Chitin is the second most abundant polysaccharide resource after cellulose and chitosan can be obtained by deacetylation of chitin with alkaline solution [54]. Chitosan is a biopolymer widely used for many purposes, especially in pharmaceutical and medical uses. Chitin sources involved in the BlueCC [55] project are *Eriocheir sinensis* (Chinese mitten crab), *Callinectes sapidus* (Blue crab), *Paralithodes camtschaticus* (King crab), *Carcinus maenas* (Green crab).

Gelatin

Gelatin is collagen irreversibly hydrolysed and is soluble in water. Gelatin is obtained by denaturing the triple helix in the three constituent α-chains (Figure 7), this partial hydrolysis of the collagen can be activated either by heating the collagen or by a chemical treatment [3]. Therefore, denaturation is an irreversible process that divide the triple helix molecule in its constituent. Gelatin has a molecular weight ranging from 3 to 200 kDa depending on the source and on the process conditions.
Depending on the pre-treatment procedure, two types of gelatin can be obtained: type-A gelatin with acid pre-treatment conditions (isoelectric point at pH 8-9) and type-B gelatin with alkaline pre-treatment conditions (isoelectric point at pH 4-5) [56]. As reported in the work by Coppola and colleagues [57] during hydrolysis the intermolecular bonds in the helix are broken so that the gelatin results as a mixture of single or multistranded polypeptides. Gelatin is a biopolymer that possesses functional characteristics and gelling properties including encapsulating, colloidal stabilisation, crystallisation, thickening, whipping, water holding, film formation, texture enhancer and emulsification so that it can be used in food, pharmaceutical and other allied sectors [7]. There are alternatives to animal-derived gelatin such as Agar Agar and Carrageenan (both obtained by seaweed) or pectin (obtained by fruits) or xanthan gum (obtained from fermentation involving the bacterium *Xanthomonas campestris.*

Collagen hydrolysates

Further hydrolysis can be performed to obtain collagen peptides with a molecular weight going from 300 to 8000 Da, the product obtained is termed as hydrolysed collagen (HC) (Figure 7). These peptides are bioactive and can be obtained by different processes such as chemical or enzymatic treatment or fermentation with proteolytic bacteria. As reported in the work by Pal and collaborators [7], enzymatic hydrolysis using specific proteases is often carried out using both exogenous and/or endogenous enzymes. During enzymatic hydrolysis of collagen and gelatin, the α and β chains are degraded into smaller peptides with a molecular weight of 1-4 kDa. Enzymatic hydrolysis produces a large variety of peptides but

only a few have functional properties, and therefore purification and fractionation are necessary. The peptides are then separated using ultrafiltration and chromatographic techniques such as gel filtration chromatography, reversed-phase chromatography, ion exchange chromatography and size exclusion chromatography.

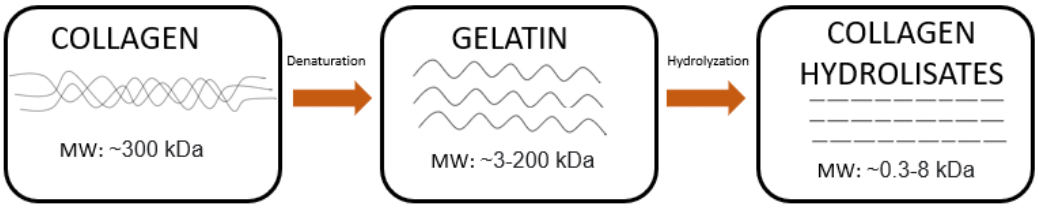

Figure 7. Production of gelatin and collagen hydrolysates from collagen and their respective molecular weights

1.1.5. Applications of collagen and its derivatives

Since this work of thesis is focused on the extraction of marine collagen, it is important to consider which are and could be the applications of marine collagen itself. Nowadays, seafood derived collagen finds application in various fields ranging from pharmaceutical to food, biomedical and cosmetic fields. Moreover, collagen can be used to produce bio-coatings such as casing for meat (i.e. sausages), increasing its durability. In medical and pharmaceutical industries seafood derived collagen is used to develop biomaterials (sponges, films, membranes, gels, scaffolds) used as carriers for proteins, genes and drugs. Collagen is efficient in drug releasing in specific sites and in specific amounts. In biomedical field, collagen is often used for tissue engineering (i.e. wound healing) [7].

Cosmetic field

Due to natural aging and photoaging the skin appearance is modified: the skin becomes thin, dry and wrinkled. As mentioned before, this is due to the decrease of collagen content, and this is why collagen is often present in the formulation of cosmetic products. Collagen is one of the main constituents in cosmetic formulations for skin and hair care since it is a moisturizer and it has excellent ability in softening the skin, marine collagen is particularly interesting because it mainly is of type I as human skin collagen [58].
It is convenient to assume collagen through food or beverages and this is why lately functional food, drink and beverage, dietary supplements and confectionery have been produced. [7] However, collagen is insoluble so hydrolyzed collage is manly applied [59]. Hydrolyzed collagen peptides can be used as dietary supplements to improve the appearance of aged skin. Through denaturation of collagen at temperatures higher than 40°C, the three α chains can be obtained and then the chain are separated. HC can be obtained by enzymatic action of proteolytic enzymes or chemical proteolysis. HC is composed of small peptides with a molecular weight of 3-6 KDa. As reported in the work by León and colleagues [34], HC presents some advantages compared to native collagen since

it maintains some of the properties of collagen (antioxidant and antimicrobial activity, biocompatibility and biodegradability, skin aging control activity, film formation if combined with other biopolymers) but it does not require a multistep extraction procedure and it has a higher solubility and lower viscosity compared to native collagen. HC oral supplementation showed improvement in hydration, wrinkling and elasticity of the skin making HC a performing anti-aging product. [34] The study of Borumand and collaborators [60] showed the positive effect of the daily consumption of Pure Gold Collagen© (a food supplement containing hydrolysed collagen, hyaluronic acid, vitamins and minerals).

Food and nutraceuticals field

Gelatin from both mammals and marine species is widely used in the food industry because of its properties. Gelatin is used as a consistence enhancer and as food stabilizer and for its antimicrobial and antioxidant properties [3]. Gelatin and HC are also used to increase food protein content, indeed HC is used as a functional food ingredients in sausages, beverages and soups for its antioxidant and antimicrobial activity [34].

Biomedical and pharmaceutical fields

Collagen is widely used in the biomedical field, especially in tissue engineering and drug delivery. Tissue engineering (TE) is a branch of biomedical engineering concerned in creating artificial tissues that are structurally and functionally similar to biological ones and that can be used to restore damaged ones in human body (i.e. wound healing). 3D scaffolds have a fundamental role in TE, since they should represent an appropriate environment for cells regeneration. The work by O'Brien [10] explores the quality that a scaffold should have to be suitable for different applications in terms of biocompatibility, biodegradability, mechanical properties consistent with the anatomical site of implantation, porous architecture, cost effectiveness and possibility of scale-up. Typical biomaterials used for the production of scaffolds for TE are ceramics, synthetic polymers and natural polymers such as collagen, various proteoglycans, chitosan and alginate-based substrates [10]. Collagen has been proved to have suitable properties as TE scaffold because of its mechanical properties, in fact, it confers flexibility and stability [61]. In particular, collagen represents a promising material for scaffolds in bone tissue engineering [62].
Collagen is widely used in ophthalmology as graft for corneal replacement, suture material and other applications. Among these applications in the ophthalmology field, a fascinating one is the use of collagen inserts and shields for drug delivery to the cornea or to the corneal surface [11], for instance it was studied as a drug carrier for insoluble antibiotics [63].

1.2. Project

1.2.1. Aim of the project

The aim of the project is to optimize the protocol already commonly employed for collagen extraction using isoelectric precipitation instead of salting out in the collagen extraction step and to study the properties of the collagen recovered with isoelectric precipitation. The two methods of extraction are compared in terms of yields and of functional properties of the two extracted collagens. In the present work two marine species are involved: lumpfish *Cyclopterus lumpus* (LF), and starfish *Asterias rubens* (SF). These species could represent profitable resources for different reasons that are presented in the next chapter.

1.2.2. Involved species

Lumpfish (*Cyclopterus lumpus)*

Lumpfish belongs to the Cyclopteridae family, and it is mainly found in the North Atlantic. The work by Powell and colleagues [14] indicated lumpfish as a "green" alternative for sea-lice control in salmon aquaculture and this is why its use as "cleaner fish" in salmon farming in Scotland, Iceland and Norway is increasing. Female lumpfishes are used as sources of caviar. These uses make lumpfish an underutilised resource since usually the only part of the body that is exploited is the roe to produce caviar and the rest of the body is discarded. Therefore, lumpfish is included in the trial since it is an underutilised resource, despite it is not an invasive species in some ecosystem. Temperature preference of lumpfish originating from the southern and northern parts of Norway is around 6-7 °C [64]. It is important do consider at which temperature the species live because this is the temperature at which collagen of that species is stable and functional.

Starfish (*Asterias rubens)*

Asterias rubens is the common starfish and it belongs to the *Asteriidae* family, that is a class of echinoderms (invertebrates). *Asterias rubens*'s body wall consists of magnesium calcite (that is $CaCO_3$ and $MgCO_3$) ossicles connected by collagenous tissue and muscles [65] and the peculiarity of echinoderms is their peculiar connective tissue (Mutable Collagenous Tissue) able to rapidly modify its stiffness and viscosity [66]. This species is native of the north-east Atlantic but recent findings pointed out that the species is spreading in different locations. The work by Dalgic and colleagues [13] claims that a specimen of *Asterias rubens* was found on 17 February 2009 at 90 m depth at Karasu, Sakarya, Turkey. As reported in the mentioned work, before this identification, this species has been found also in the Sea of Marmara since 1990, in the Bosphorous Strait and in Riva, on the Black Sea coast, where three specimens were collected in 2003. The spreading of this species represents a problem for the Mediterranean mussel, *Mytillus galloprovincialis*, because *A. rubens* is a "keystone predator" for mussel stocks thus affecting the mussel farming industry in many European regions. Moreover, Karhan and colleagues [67] affirmed the possibility of the birth of a competition

between *A. rubens* and a native starfish *Marthasterias glacialis* in the Sea of Marmara.

1.2.3. Pre-treatments of raw materials

Pre-treatments are implemented to remove substances different from collagen and to increase the quality of the recovered collagen. Figure 8 summarises the usual pre-treatment steps during collagen isolation:

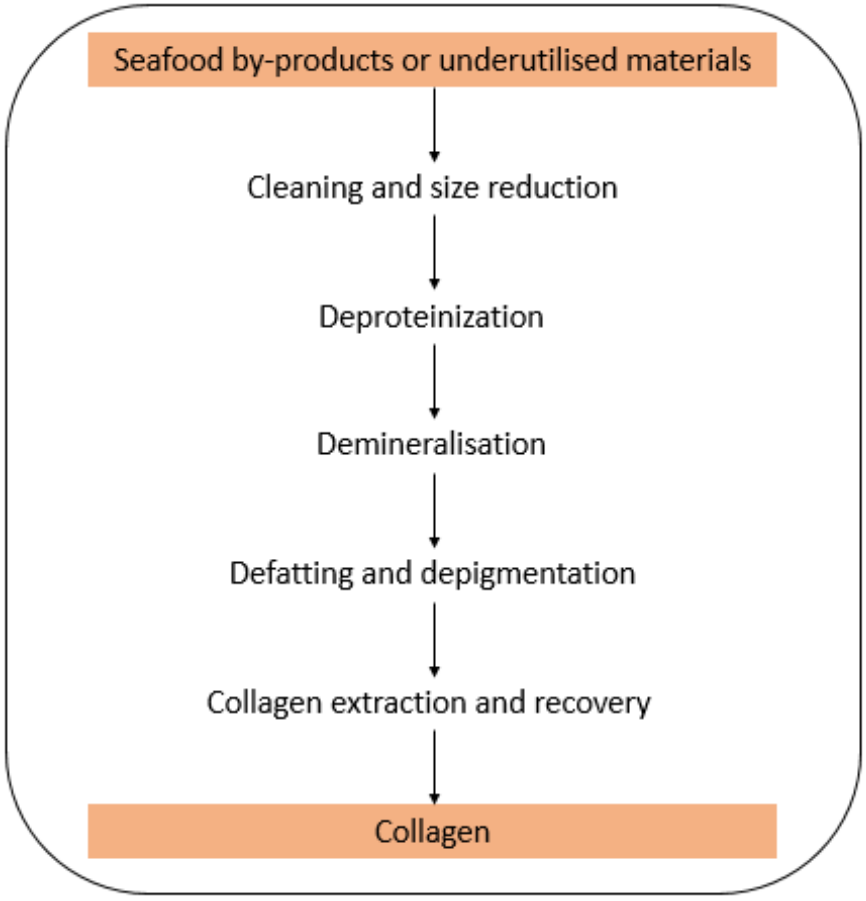

Figure 8. Summary of the collagen isolation from marine species protocol: material pretreatment and collagen isolation (extraction and recovery) steps

Depending on the involved species, some of the steps can be skipped and every step will be performed in the way that is more appropriate for the involved species. More often, also at industrial scale, raw materials are not directly processed but they are first stored in freezers. Cleaning is generally done right after the obtainment of the raw material, before freezing it. Size reduction can be performed before the freezing or once the material is defrosted for processing. Size reduction allows obtaining smaller dimension so that the chemical pre-treatments is enhanced since mass transfer phenomena are facilitated. As the dimension of the material decreases, the ratio surface/volume (S/V) increases and at constant volume the exchange surface is increased. Once the raw material is clean and of the right dimension, chemical pre-treatments are performed. Pre-treatments are usually carried out in series.

Deproteinization

Usually, non-collagenous proteins (NCP) are removed with alkaline pre-treatment using 0.1 M NaOH solution. This procedure exploits the solubility of proteins in basic solutions considering that collagen has low solubility in alkaline solutions, despite it causes a loss of collagen. The work by Liu and colleagues [15] focused on the effects of alkaline pretreatments on the acid-soluble collagen (ASC) from grass carp (*Ctenopharyngodon idella).* To determine the effects of alkaline pre-treatments on collagen extraction five parameters were measured (i.e., total content of proteins removed during the alkaline pretreatment, loss of hydroxyproline during the alkaline pretreatment, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) patterns of proteins removed during the alkaline pretreatment, hydroxyproline content of ASC and SDS–PAGE patterns of ASC). The samples were soaked in 20 volumes of NaOH solution at different concentrations, temperatures and times. NaOH, compared to $Ca(OH)_2$ that is frequently used for pre-treatments, showed the ability of swelling fish skins thus facilitating the acid extraction. 0.05 and 0.1 M NaOH remove non-collagenous proteins after 12 h without causing a significant loss of collagen, but the higher was the NaOH concentration and the higher was the loss of collagen. Also, the highest removal of non-collagenous proteins was observed at 20°C, so the higher was the temperature and the greater was the amount of removed protein, even though at high temperature denaturation occurred [15]. On the other end, alkaline pre-treatment at low temperature is also useful to reduce the endotoxin content making the extracted collagen suitable for biomedical applications [68].

Demineralisation, defatting and depigmentation

Minerals such as calcium ions can be removed using a tetrasodium ethylenediaminetetraacetic acid (EDTA-4Na) solution given the chelating properties of EDTA. The EDTA molecule presents four carboxylic groups and two nitrogen atoms, and it can react with ions such as Na^+, Ca^{2+} and Mg^{2+} to form stable complex through a coordinate covalent bond. Rather than the EDTA itself, the correspondent salt is commonly used, EDTA-4Na (Figure 9) since it is more soluble in aqueous solutions.

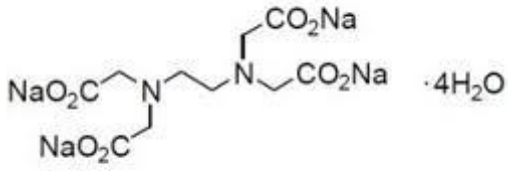

Figure 9. Ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA-4Na) molecule

Fats and grease can be removed with alcoholic solution (isopropyl alcohol or butyl alcohol for instance).
Demineralisation and degreasing are applied only when the amount of minerals and/or fats is relevant, so it depends on the marine specie involved.

Depigmentation can be carried out using H_2O_2 solution, even though this step is not always necessary.
In the work by Xu and colleagues [69] an improved procedure for defatting and depigmentation for southern catfish (*Silurus meridionalis* Chen) is proposed. Southern catfish skin is rich in fat, so degreasing is necessary. Moreover, the colour of catfish skin is marked because of the presence of additional pigments, so the depigmentation step is also necessary. Defatting was conducted using 15% (v/v) isopropyl alcohol solution followed by a treatment with 6% (v/v) non-ionic degreasing agent. Decolouring was carried out using H_2O_2 solution at different concentrations and different pH values. The yield and the purity of extracted collagen were effectively increased by these pre-treatments [69].
Moreover, pre-treatments weaken the fibers so that the extraction collagen is facilitated, and the efficiency of extraction is increased.

1.2.4. Collagen extraction with acetic acid

Collagen extraction from the pre-treated pellet can be done in different ways. A common extraction method involves acid solubilisation of collagen, usually using 0.5 M acetic acid solution to obtain acid-soluble collagen (ASC).
In their work Skierka and Sadowska [16] compared the influence of different acids on the extraction of collagen from Baltic cod (*Gadus morhua).* Extraction was performed at 4°C for 24, 48 and 72 h with 0.15 M HCl and 0.5 M citric, acetic or lactic acid solutions. HCl was the least effective solvent because it gave the lowest yield whereas acetic acid and lactic acid gave up to a 90% yield.
Similarly, Bhuimbar and colleagues [70] performed the extraction from black ruff (*Centrolophus niger)* skin using 0.5 M acetic, citric, hydrochloric, lactic, tartaric, formic and sulfuric acid solutions (1:25 w/v) at 4°C for 24, 48 and 72 h. Lactic acid was found to be the most effective followed by formic, tartaric, acetic and citric acid, inorganic acids showed negligible yield.

1.2.5. Pepsin-aided collagen extraction

Another extraction method is enzymatic solubilisation using proteases. The enzyme can be combined with the acid solubilisation extraction to obtain a higher yield. In this operation non-specific proteolytic enzymes can be used such as papain, ficin, bromelain, trypsin, pancreatin or pepsin [17]. Usually, pepsin from porcine sources is used so the obtained collagen is the pepsin-soluble collagen (PSC). The use of porcine pepsin makes the extracted collagen not suitable for Jews and Muslims. In the work by Ahmad and Benjakul [31] the efficience of pepsin from albacore tuna and from yellowfin tuna was tested.
According to the work by Skierka and Sadowska [16] proteolytic enzymes are efficient in cutting only the non-helical ends of collagen (telopeptides) resulting in an effective removal of intermolecular cross-links. The treatment with acid solution alone is not able to break the strong intermolecular covalent bonds in the telopeptide regions of collagen. The key feature of the pepsin-aided collagen extraction is that the cleavage of peptide bonds occurs in the terminal non-helical region, thus the triple helix structure results intact and the triple helix molecules are able to easily dissolve in the acidic solution [11]. Collagen molecule in which the terminal ends have been cleaved by enzymes is called atelocollagen. Moreover, the telopeptide portion of collagen is immunogenic [71]. Atelocollagen molecules are still able to form fibrils [45]. In more

than one work pepsin-aided collagen extraction is used to obtain PSC after a first acidic extraction is carried out. Essentially, after the acidic extraction using acetic acid, the solution is centrifuged and the supernatant is subjected to salting out to obtain ASC whereas the non-dissolved material is subjected to a second extraction in presence of pepsin to obtain PSC [72], [73]. Acidic extraction followed by pepsin-aided collagen extraction results in a higher total extraction yield. The differences between the ASC and the PSC are covered in detail in different works [74] , [75].
In addition , ultrasound had been reported as a promising way to furtherly increase the efficiency of extraction [76].

1.2.6. Salting out

The most common procedure to obtain collagen precipitation from the acidic solution is salting out. Salting out is a purification technique mainly used to obtain the separation of macromolecules (such as proteins or nucleic acids) from aqueous media. It exploits the fact that a solution containing a high concentration of salt shows a high ionic strength. Ionic strength I of a solution is given by:

$$I = \frac{1}{2}\sum_{i=1}^{N} z_i^2 c_{bi}$$

where c_{bi} is the concentration of ions of type i at the bulk of the solution and z_i is the valence of the ions. The summation is extended to all the ions present in the system. The term ½ considers the fact that both anions and cations are included. For single symmetric electrolyte like NaCl, the ionic strength equals the concentration of the salt because concentration of Na^+ ions is equal to the concentration of Cl^- ions, so in this case ionic strength is easily calculated. High ionic strength in a solution results in reduced solubility of the molecules dissolved therein and because of reduced solubility, molecules aggregate and precipitate. Proteins in water solution arrange in a way that the hydrophobic groups are facing inwards whereas hydrophilic groups are facing outwards so that the protein is solvated by water molecules. Ions such as Na^+ and Cl^- in solutions are solvated by water molecules as well so, as soon as the salt concentration increases, water molecules move from proteins to salt ions. Water molecules interacts more easily with salt ions rather than proteins, that are the least soluble solute, and this means that the number of water molecules available to solvate proteins decreases. When salting out is performed, different salts have different efficiency in making proteins precipitate. The degree of salting out can be attributed to aqueous ionic strength and at low concentrations of salt, the Debye-Hückel theory provides a mathematical link between the activity coefficient and the ionic strength of the aqueous solution [77][78]. At high concentration of salts, specific ion effects should be taken into account and the different efficiency of different salts can be summarized using the Hofmeister series[78][79][80] that for anions is:

$$C_4H_4O_6^{2-} > SO_4^{2-} > HPO_4^{2-} > C_3H_5O(CO_2)_3^{3-} > CH_3CO_2^- > HCO_3^- > CrO_4^{2-} > Cl^- > NO_3^- > ClO_3^-$$

Hofmeister [80] was able to create a separate series for cations and ions using salts with common cation (or anion) and quantifying the salting out effect in salting out. Therefore, the choice of the salt has a relevance when salting out is performed, moreover, every proteins precipitate at a different concentration of salt.

1.2.7. Isoelectric precipitation

In order to use isoelectric precipitation for collagen recovering, the isoelectric point of collagen from each species should be determined preliminary. For what concerns the calculation of the pI of single amino acids, not bound in a polypeptide, it can be determined using pKa that is the negative base-10 logarithm of the acid dissociation constant Ka:

$$pKa = -\log_{10} Ka$$

A single amino acid, not bound in a polypeptide, contains two ionisable group that are an acid group (carboxylic group) and a basic group (amino group) and the pKa is given by the average of the pKa of the two groups that is:

$$pI = \frac{1}{2}(pKa_1 + pKa_2)$$

Where pKa_1 and pKa_2 are the pKa of the two mentioned ionisable groups.
If the amino acid presents as R group an ionisable group (for instance lysine or aspartic acid), pKa is calculated using the Ka of the groups that are present in higher quantity (i.e. in lysine only pKa of amino group is considered). When amino acids are linked in a polypeptide, such as collagen, the pI will depend on the pKa of the R ionisable groups since carboxylic and amino group are engaged in the bonds.
Therefore, each amino acid has its own pI and pI is specific for each protein as well: it is the pH at which the protein has zero net charge, and it is given by the contribution of different R groups of the amino acids composing collagen. Above and below the isoelectric point the proteins are charged and there are repulsive forces between different chains that increase the overall protein solubility in the medium. As soon as the solution pH gets closer to the protein pI, hydrophobic-hydrophobic interactions take place and different chains tend to attract and to aggregate, eventually precipitating. This means that proteins have their lowest solubility at their pI value. The knowledge of the pI can be exploited through different procedures such as electrophoresis, ion exchange chromatography and chromatofocusing.
The salting-out procedure involves a highly concentrated solution so that the recovery process requires a long dialysis process that lasts up to four days in order to assure that the collagen is free from salt and acid. Moreover, salting-out produces a high amount of wastewater. The work by Lin and colleagues [18] focused on the extraction of collagen from bigeye tuna (*Thunnus obesus*) skins and the conclusions were that the yield in the recovery is increase from 14.4% (dry weight) for pepsin-soluble collagen (PSC) obtained through salting out (SO) to 17.7% (dry weight) for PSC obtained through isoelectric precipitation (IP). Moreover, the time required for dialysis was reduced from 4 days for PSC-SO to 2 days for PSC-IP due to the lowest salt concentration. Moreover, SDS-PAGE analysis showed that PSC-IP product is collagen of type I and inductively coupled plasma mass spectrometry (ICP-MS) analysis showed that the product did not contain any heavy metals such as As, Pb, Hg, so the PSC-IP product is a bioactive product with good potential as well as the PSC-SO and it can be used for cosmetic and biomedical purposes. The possibility of using IP has been explored also in the production of collagen from bovine and porcine sources in the work by Tsermoula and colleagues [81]. In this work, collagen is extracted using

both acid and alkali solubilization, at pH 2.0 and pH 11.0 respectively. In any case the pH of the obtained supernatant was brought to pH 5.5 to have collagen sediment [81].

Based on the knowledge gained by the previous works present in literature and mentioned above, in this work of thesis, for the extraction of collagen I followed a basic protocol that can be summarized in some main steps, summarized in Figure 10. First, raw materials were pre-treated through homogenization, then non-collagenous proteins were removed, and demineralization was performed. Collagen extraction was performed in acetic acid solution with and without pepsin to decide whether focus on acidic solubilized collagen or pepsin solubilized collagen in the further steps of the work. Extracted collagen solubility was studied to determine isoelectric point of collagen. Collagen precipitation was performed both via salting out and isoelectric precipitation exploiting the knowledge of the pI of the two samples used. Collagen samples were collected and freeze dried and later analyzed.

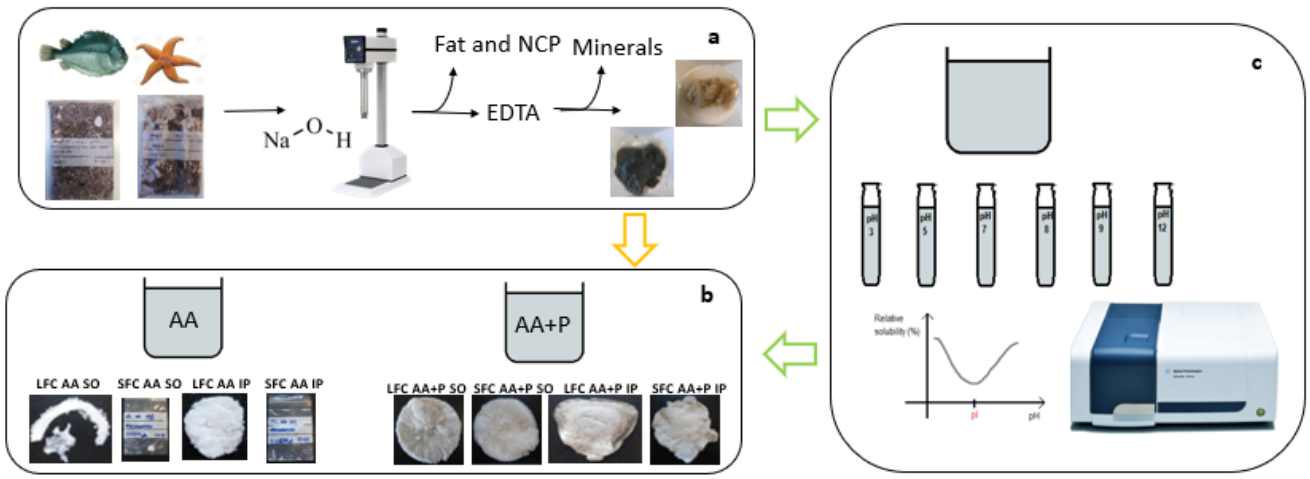

Figure 10. Work summary: Raw material pre-treatments (essentially homogenization, deproteinization and demineralization) (a), collagen acidic extraction with and without pepsin and recovery (b), isoelectric points determination via collagen solubility curves (c).

In the next chapter the used procedure is explained in detail.

2. Material and methods

2.1. Collagen extraction

Thawing

Raw materials were kept in the -80°C freezer inside zip bags until use. Lumpfish (skin and meat mostly) has already been minced and gutted before freezing so it came as a mass of pellets, starfish has been directly frozen, so it presented its intact structure and cutting was needed after thawing. A certain amount of raw material was collected from the main batches using a hammer and paying attention to keep the samples in ice during the operation as much as possible. The weight of the collected material also consisted of a certain quantity of water. The desired amount of raw material was put in sealed zip bags and then defrosted by dipping these bags in a bucket filled with cold running water. Temperature of running water should be around 4°C since higher temperature could degrade collagen contained in the raw materials. The zip bags should be perfectly sealed and totally immersed in water, so some weights were put on them to assure this. This

operation lasted about 30 minutes, but the consistence of raw material was often checked. Figure 11 shows how thawing was performed.

Figure 11. Thawing of raw material (stored at -80°C): raw material inside sealed zip bags immersed in water with cold water running

Lumpfish was already minced so it did not need cutting, starfish needed cutting after defrosting to obtain pieces of 0.5-1 cm. Starfish was cut using a knife on a cutting board. 5g of material were collected and used for the moisture content analysis that was performed to calculate the humidity of the material. This information is needed when the dry basis yield is calculated.

Homogenization

Starfish and lumpfish were subjected to homogenization in order to further reduce the size of the pellet: the smaller is the size and the more efficient is the extraction of components from the raw material. Silverson L5M homogenizer was used. In order to perform homogenization and obtain a homogenous distribution of the pellet, raw material was soaked in 0.1 M NaOH at 1: 10 (w/v) ratio in a beaker filled up to 75% of its volume. A certain amount of solvent (about 50 ml) was kept apart during homogenization, and it is used to rinse the head of the homogenizer once the operation was concluded to remove eventual residual pellets clogged in the head. Starfish and lumpfish need different time and power:

- Lumpfish: 1.30 minutes, 6000 rpm
- Starfish: 2.30 minutes, 7000 rpm

Ramp time did not count. Time and power for the two species have been defined according to the results of previous trials based on a visual and tactile analysis of the product. It has been noted that high homogenization speed produces a jelly pellet in the following steps. Jelly pellet hardly sediments with centrifugation because it does not stick to the wall of the centrifuge bottles, and this makes the collection more difficult. Homogenization was performed using the biggest head available as shown in Figure 12.

Figure 12. Homogenizer working head used to homogenize raw material in NaOH solution

During the operation the product was checked (after 1 min for lumpfish and after 1 and 2 min for starfish) to assure that there was no clogging of the head. Moreover, during homogenization, the beaker was kept in a bucket with ice to assure 4°C and during homogenization the beaker was moved around to improve the efficiency of the performance. The head was kept deep in the solution and attention to the suction was paid especially when the power of the homogenizer was high.

Deproteinization

After homogenization the solutions were transferred from beakers to sealed bottles to ensure sterility and deproteinization was performed using 0.1M NaOH at 1:10 (w/v) ratio at 4°C for 30 minutes under constant stirring. So, when dealing with a high quantity of solution -in the order of litres- stirring was performed using an overhead stirrer and keeping the bucket with the solution in ice, when the amount of solution is lower a magnetic stirrer was used. The stirring, together with the decreased size of the raw material, helps the mass transfer of non-collagenous proteins from the pellet to the solution. Homogenization allowed reducing time needed for deproteinization.
After deproteinization, centrifugation was performed on the sample in order to separate the pellet and the supernatant. The supernatant is an alkaline solution containing non-collagenous proteins (NCP) that could be furtherly recovered. First centrifugation was carried out at 2000xg for 2 minutes at 4°C, then the alkaline solution was removed, and the pellet collected. Pellet was put in a beaker together with chilled distilled water from the cold room (4°C) and the solution was stirred on ice in order to wash the pellet from alkaline solution. Once the pellet was homogeneously distributed in the solution, the pH was adjusted to neutral one (between 7 and 8) using 6M and 2M HCl solution. Then the solution was put in centrifuge bottles and another centrifugation is carried out, at 5000xg for 5 minutes at 4°C.
Lumpfish pellet after deproteinization step was jelly-like and slimy, starfish pellet was grainier because of minerals and fibrous. The colour of starfish pellet was more homogenous whereas lumpfish pellet was grey with black dots on it. During the deproteinization step fats from the lumpfish passed to the alkaline solution and formed a greasy layer on the top of the supernatant shown in Figure 13.

Figure 13. Visible fat layer in lumpfish NaOH solution supernatant after deproteinization

Demineralisation

The deproteinization step was followed by a demineralisation step using 0.5 M EDTA-4Na (pH 7.4) in the ratio 1:15 (w/v). Demineralisation took place for 48 h during which the solution was kept in the cold room (4°C) on the stirrer. Before putting the pellet for demineralisation, the material was separated in small pieces using hands in order not to have a unique big pellet in the solution and to favour the access to the liquid. The solution was changed after 24 h in order to improve the mass exchange of minerals (Ca^{2+}, Mg^{2+}) from the pellet to the solution. After the first demineralisation step, the solution was centrifuged at 5000g for 5 minutes at 4°C, the pellet was separated from the supernatant. Pellet was weighed to calculate the amount of EDTA-4Na solution needed for the second demineralisation step. Based on the weight, EDTA-4Na solution was added in a ratio 1:15 (w/v) and demineralisation was carried out for other 24 h. After that, the solution was centrifuged at 5000xg for 5 minutes at 4°C and the pellet was separated from the supernatant. Pellet was washed with chilled water by stirring it for some time while on ice. Then another centrifugation was carried out at 5000xg for 5 minutes at 4°C. The pellet was collected and weighed.

Extraction

Two types of extraction were performed: one extraction with 0.5 M CH_3COOH 1:15 (w/v) + 1% (w/w) pepsin and the other with only 0.5 M CH_3COOH 1:15 (w/v). One of the aims of the work was to study the effect of pepsin on the extraction yield and on the pI of the extracted collagen. So, the pellet obtained after the pre-treatments was divided in two batches of equal weight and depending on the weight the right amount of acetic acid solution was added. The solutions remained on the stirrer in the cold room (4°C) for 48 hours.After 48 h of extraction, the solutions were centrifuged at 5000g for 10 minutes at 4°C in order to sediment eventual particles. At this point collagen molecules were dissolved in the supernatant and when pepsin was used the pellet was almost completely digested.

2.2. Collagen solubility curves and pI determination

The knowledge of the pI was needed in order to perform recovery using isoelectric precipitation and pI was determined as the pH value at which the collagen solubility in the acetic acid solution was the lowest one. pI was determined for each species, lumpfish (LF) and starfish (SF) and for both acetic acid (AA) solution and acetic acid + pepsin (AA+P) solution in order to investigate difference in the pI depending on the species and on the

type of solution. Collagen concentration in the solution was evaluated using Lowry's method [82]. 50 ml of collagen acidic solution were separated from the main batch and the pH was adjusted to the values of 3.0, 5.0, 7.0, 8.0, 9.0 and 12.0, while the solution was stirred and kept in ice. pH was adjusted using 2 N NaOH, 0.1 N NaOH and 2 N HCl if needed. The initial pH of the acid solution was lower than 3 and the pH at which the solution was found after the extraction is supposed to be the maximum point of solubility of collagen in acid solution. At each point of the pH two samples of 1.5 ml were withdrawn. The samples were collected in Eppendorf tubes in a rack kept in ice during the pH adjustment. The samples were left standing for 15 minutes at 4°C in the cold room to let the collagen particles aggregate and precipitate. The samples were centrifuged for 30 minutes at 15000xg to separate the precipitate from the supernatant. Then, the supernatant was collected and diluted: 900 μl of supernatant were diluted with 100 μl of 1M NaOH, then 50 μl of this 1 ml solution were diluted with 950 μl of 0.1 M NaOH. Reagent C is a 1:100 (v/v) mixture of reagent B (4% (w/v) $CuSO_4 \cdot 5H_2O$) and reagent A (2.0% (w/v) Na_2CO_3, 0.4% (w/v) $NaOH$, 0.16% (w/v) Na-K-tartrate, 1% (w/v) SDS). 3 ml of reagent C were added to each sample and then after 30 minutes of incubation 0.3 ml of phenol reagent (1:1 (v/v) mixture of Folin-Ciocalteu's phenol reagent and distilled water) were added. Samples were immediately vortexed with a time of incubation of 45 minutes, then the absorbance of each sample was measured at 660 nm using a spectrophotometer. Bovine serum albumin (BSA) solution at 2 mg/ml concentration was used to build through serial dilutions the standard curve ($R^2 \approx 0{,}987$) (Figure 14).

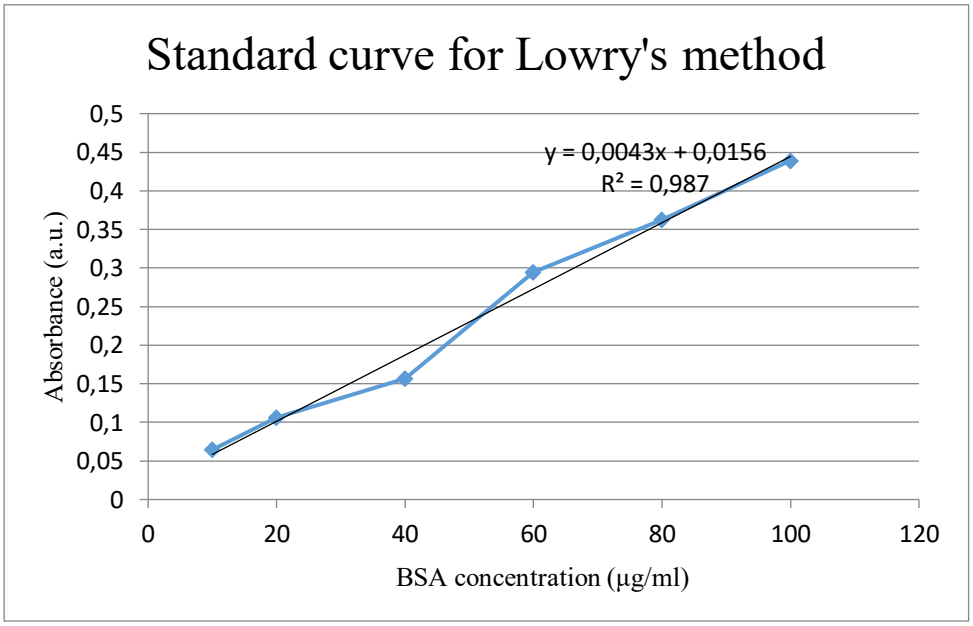

Figure 14. Standard curve used for protein determination according to Lowry's method (standard curve obtained using serial dilutions starting from a 2.0 mg/ml bovine serum albumin (BSA) solution.

Collagen absolute solubility, that is the protein concentration (µg/ml) at each pH, was calculated through average absorbance using the BSA standard curve in Figure 14. Through linearization of the BSA standard curve the following equation was obtained:

$$y = 0.0043x + 0.0156$$

where x is the BSA concentration (µg/ml) and y is the absorbance (a.u.). Absorbance of the collagen samples was measured at each pH using a spectrophotometer and the absolute solubility of collagen at each pH was calculated as follows:

$$x = \frac{y - 0.0156}{0.0043}$$

Relative solubility was calculated as the ratio between protein content at each pH and the maximum protein content in the chosen pH range. pI was determined as the pH with the lowest relative solubility in the range of pH between 3 and 10. A minimum in the solubility coincides with a maximum recovery via isoelectric precipitation at that pH.

2.3. Collagen recovery

Collagen recovery was performed in two different ways, so the two main batches were divided in two batches of similar weight.

Salting out

Salting out was performed using a solution made of 5 M NaCl and 0.1 M tris(hydroxymethyl)aminomethane that was mixed to the acetic acid solution in a ratio 1:1 (v/v) so that the final concentration of NaCl salt was 2.5 M. Once the salt solution was added to the acid solution collagen precipitation started and, in some cases, it was possible to properly see the formation of an aggregation in the upper part of the solution as shown in Figure 15.

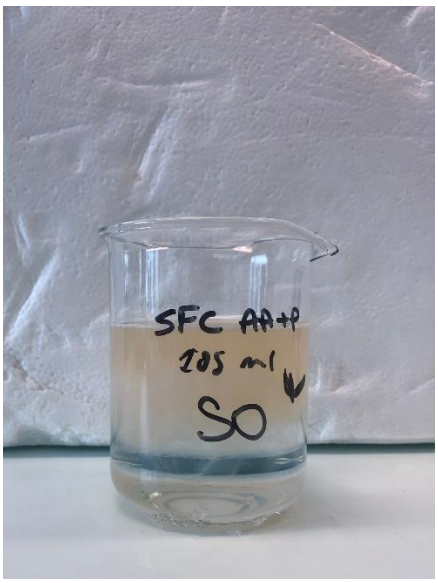

Figure 15. Starfish collagen acetic acid + pepsin (SFC AA+P) solution after salting out (SO): visible aggregation of collagen molecules.

The solutions were left standing for 30 minutes, then they were centrifuged for 1h at 15000xg at 4°C. The pellet –if present- was collected from the centrifuge bottles and subjected to dialysis to remove salt from the collagen. Collagen was dissolved in a small amount of 0.5 M CH_3COOH using a magnetic stirrer: the aim was to divide the pellet in smaller pieces in order to increase the efficiency of the dialysis. The acid solution was poured in dialysis bag and the dialysis bags were dipped in 0.1 M CH_3COOH solution and left at 4°C in the cold room. Figure 16 shows the dialysis set-up.

:

Figure 16. Dialysis setup used to remove salt from collagen recovered via salting out (SO): dialysis bags are sealed using clamps and rubber bands and immersed in the solution in a plastic beaker.

The solubilized pellet was kept in the dialysis bag clamping the bag using plastic clips, the sealing was furtherly assured using rubber bands. The dialysis bag was completely dipped in the solution. After 24 h the solution was changed with 0.1 M CH_3COOH, after 48 h the solution was changed with distilled water and after 72 h the collagen from the dialysis bag was collected and put in plastic containers that were put in the freezer at -80°C.

Isoelectric precipitation

To recover collagen using isoelectric precipitation, the pH of the acidic solutions was adjusted to the found pI using 6N NaOH and 2N NaOH solution, maintaining the solution on the stirrer and on ice. Once the pH corresponding to the protein pI was reached, the solution was left standing for 30 minutes at 4°C in order to let the collagen particles interact and aggregate to form a pellet. In some cases, it was possible to see that the solution became blurry because of the formation of the pellet (Figure 17).

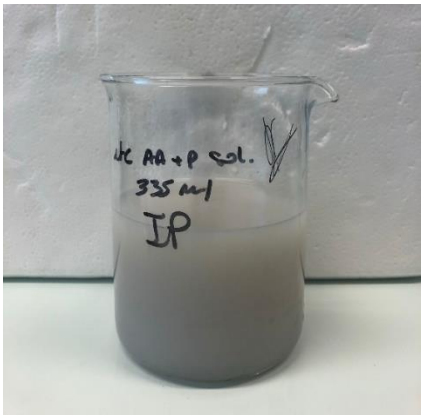

Figure 17. Lumpfish collagen acetic acid + pepsin (LFC AA+P) solution after isoelectric precipitation (IP): blurry solution because of collagen molecules aggregation.

The solution was centrifuged at 15000g for 1h at 4°C to obtain the collagen precipitation. The precipitated collagen was collected from the centrifuge bottles and put in plastic containers then stored at -80°C.
The supernatant obtained after centrifugation of lumpfish collagen acetic acid (LFC AA) solution subjected to isoelectric precipitation was collected and stored in the cold room at 4°C. It was noticed that 24 hours after the first centrifugation other fibres of collagen aggregated in the supernatant of LFC AA solutions so a second centrifugation at 15000g and 4°C was performed for 1 h. Figure 18 shows how the LFC AA IP supernatant looked after 24 h after the first centrifugation.

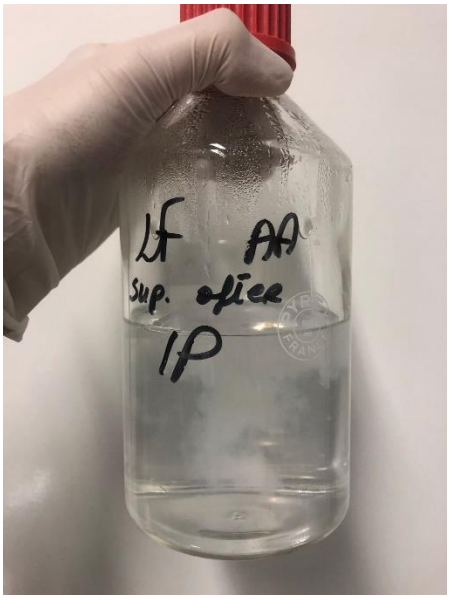

Figure 18. Lumpfish acetic acid (LF AA) supernatant after 24 h from isoelectric precipitation (IP): visible aggregation of collagen molecules.

2.4. Samples freeze-drying

Collagen samples were collected from the -80°C freezer and put in the food grade freeze dryer. Freeze dryer worked at -46°C and under vacuum condition, freeze-drying lasted for 24-48 hours depending on the sample. The freeze dryer works in such a way that ice is removed from the samples by sublimation so that the ice does not melt in water before evaporation. In this way, the quality of the sample (texture, color, smell, etc.) is maintained throughout the procedure.

2.5. Extraction yield determination

Since solubility of collagen in absence of pepsin was low, the next step was to perform the acidic extraction of collagen using pepsin. The yield of pepsin-aided acidic collagen extraction was calculated for lumpfish and starfish using both types of recovery methods (isoelectric precipitation and salting out) to establish the most suitable extraction method for each species. Yield was calculated in an appropriate trial performed in technical duplicates starting from 100g of raw material. After defrosting of raw material, 5g were stored on ice and used for moisture content measurement, since this value is necessary to calculate yield on a dry basis. A small portion of material (0.5-1 g) was spread as much as possible on a plate, which was inserted in the moisture content analyzer. Heat allowed the water evaporation from the samples and the loss of weight was used to determine the moisture content (MC) of the samples. Defrosted raw material was pretreated according to the protocol described in the methods section (chapter 2.1). Collagen extraction was performed using an acetic acid solution containing pepsin (AA+P solution) and, once the extraction was concluded, the solution was divided in 4 batches of approximately equal volume (that is, equal weight), of which 2 were used for isoelectric precipitation (IP) and 2 for salting out (SO). Then, collagen was recovered from each batch, freeze dried and weighed. The entire experimental work plan followed is schematized in Figure 19.

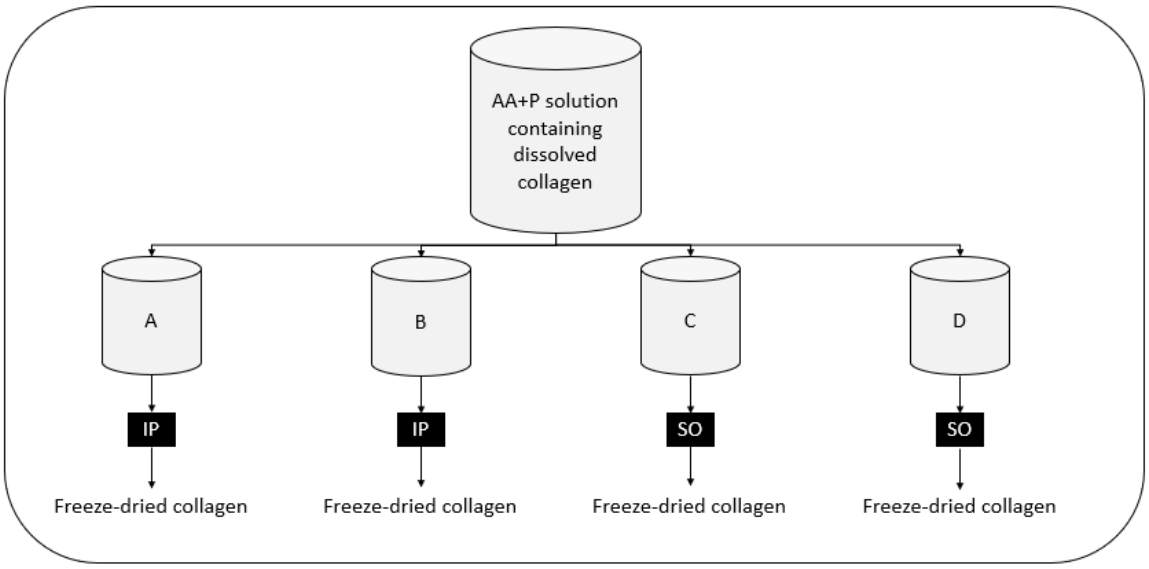

Figure 19. Collagen yield calculation work plan.

At this point, both the yields on wet and dry basis were calculated as follows:

$$Yield\ (wet\ basis, \%) = \frac{weight\ of\ freeze\ dried\ collagen\ from\ batch\ X}{wet\ weight\ of\ raw\ material} \cdot \frac{total\ weight\ of\ AA + P\ solution}{weight\ of\ AA + P\ solution\ of\ batch\ X} \cdot 100$$

$$Yield\ (dry\ basis, \%) = \frac{weight\ of\ freeze\ dried\ collagen\ from\ batch\ X}{dry\ weight\ of\ raw\ material} \cdot \frac{total\ weight\ of\ AA + P\ solution}{weight\ of\ AA + P\ solution\ of\ batch\ X} \cdot 100$$

Yield was calculated for each batch and then average yields and standard deviations were calculated for each sample.

2.6. Characterization of collagen samples

Collagen characterization was performed only on samples obtained from the extraction with acetic acid and pepsin since the amount of collagen obtained without pepsin was negligible making the acetic acid extraction not of interest from an industrial point of view. Thus, SDS-PAGE, UV-Visible and FTIR analyses were performed on the following samples of collagen:

- LFC AA+P IP (hereafter termed as LFC IP)
- LFC AA+P SO (hereafter termed as LFC SO)
- SFC AA+P IP (hereafter termed as SFC IP)
- SFC AA+P SO (from now termed as SFC SO)

These analyses were performed to assess the qualities and the properties of the extracted collagens as detailed in Table 1.

Table 1. Analyses performed to characterize collagen samples: technique and purpose.

Technique	Collagen characterization
SDS-PAGE	To define collagen type and detect degradation products
UV spectroscopy	To evaluate collagen purity
FTIR spectroscopy	To study collagen structural properties

2.6.1. SDS PAGE

Protein gel electrophoresis with SDS-PAGE was the method used to characterize the purity of the extracted collagen in order to understand which type of collagen (I, II, etc) was extracted and to understand if degradation of collagen occurred during the extraction. The different collagen chains are separate based on their size exploiting the fact that the speed of migration of proteins negatively charged by SDS subjected to an electric field only depends on their molecular mass. This means that the higher is the molecular weight of the protein and the shorter will be its migration path on the gel. Moreover, this method allows either the determination of the type of collagen or spotting differences between same type of collagen from different sources. 7.5% precast polyacrylamide gels with 12 wells from Bio-Rad were used. Freeze dried collagen samples were dissolved in 5% (w/v) SDS solution reaching a concentration of about 10 mg/ml. Dissolution occurred stirring the sample at room temperature for a couple of hours. From previous trials of SDS-PAGE analysis emerged the need of adjusting the pH of lumpfish and starfish collagen samples obtained through salting out (LFC SO and SFC SO), so the pH of these solutions was adjusted to neutral pH. As soon as collagen was totally dissolved, collagen concentration in each solution was calculated using Lowry's method [82]. After that, sample solutions were mixed with buffer solution in order to obtain a concentration of 2 mg/ml, results from Lowry's are used to calculate the quantity of buffer solution needed to dilute the solution to the desired concentration. Buffer solution is composed of Laemmli sample buffer and 5% (v/v) β-mercaptoethanol. Laemmli buffer is a 0.5 M Tris-HCl, 5% (w/v) SDS, 20% (v/v) glycerol, pH 6.8 solution. Samples were heated at 95°C for 5 minutes using a heater bloc and then cold down. Samples were centrifuged at 5000g for 5 minutes and the supernatants were loaded onto the gels. 5 μl of marker (Dual Color Precision Plus Protein™ Prestained Standards) and 7.5 μl of samples, corresponding to 15 μg of protein, were loaded on each lane. Running buffer was prepared with 900 ml of cold distilled water and 100 ml of tris-glycine-SDS buffer (buffer was diluted 10X). The running buffer was kept on ice before being put in the cast and during the running the whole machine was kept in a bucket with ice, this avoids heating during the electrophoretic run. The gel was run at 125 V for 80-85 min. After that the gel was removed from the plastic plates, it was rinsed with water and stained for 1 hour in a staining solution made of 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid. Then gel de-staining was performed in a solution made of 50% (v/v) methanol and 7.5% (v/v) acetic acid (changing solution after 30 minutes, 1 h in total). The gel was left in water in the fridge overnight.

2.6.2. UV spectroscopy

Ultraviolet spectroscopy analysis consists in the measurement of the quantity of electromagnetic radiation having ultraviolet wavelength (100—400 nm) that is absorbed by a chemical compound. This quantity is expressed as absorbance that is the inverse of transmittance. Absorption of an UV photon makes the molecule to pass from the ground state to an excited state at higher energy. Cromophores are the components of the molecules responsible for the photon absorption and for

the energy change. In proteins, the most common amino acids behaving as cromophores are tryptophan and tyrosine that result in an absorption peak at 280 nm.
In our case, the UV spectrum of collagen samples was used to determine the purity of the collagen by evaluating the maximum absorption peak at 230 nm, that is the characteristic value for collagen absorption peak [19].
5 mg of freeze-dried collagen samples were dissolved in 10 ml of 0.5 M CH_3COOH (0.5 g/l) and left stirring at 4°C until collagen dissolution. As soon as the collagen was dissolved, the solutions were put in falcon tubes and centrifuged at 5000g for 10 minutes at 4°C in order to sediment as pellet eventual undissolved material and to obtain a clear supernatant. The absorbance of the supernatant was assessed between 190-450 nm using a spectrophotometer. Zero and baseline were obtained taking the reading with 0.5 M acetic acid solution (as blank); for each tested collagen solution tested, three readings were taken. The absorption spectrum of each sample was obtained averaging the results of the three different readings and normalizing it with respect to the maximum absorbance value in the spectrum. The four absorption spectra were charted together allowing qualitative comparison among them.

2.6.3. FTIR spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used to obtain an infrared absorption spectrum of the freeze-dried collagen samples. This technique measures the quantity of light that the sample absorbs in the range of infrared wavelength going between 2.5 μm and 15 μm (that is 4000 cm^{-1} to 400 cm^{-1} in terms of wavenumber). FTIR can be used to study the secondary structure of proteins, which deals with proteins' conformation in the space and their stability. When a radiation in the infrared range invests a molecule, absorption occurs and in the case of proteins, this reflects into a vibrational transition of the molecule. Depending on the mass of the vibrating atoms and on the type of bond, absorption determines a different position of peak in the spectrum [83]. Polar bonds play a role in infrared radiation absorption and amino acids bond each other through peptide bonds - that are polar bonds-. A peptide bond is an amide bond occurring between two amino acids, its formation is at the bases of a polypeptide chain assembly. Therefore, in the peptide bond (Figure 20), the amino group of one amino acid is linked to the carboxyl group of another amino acid and this is how the primary structure of a protein is formed.

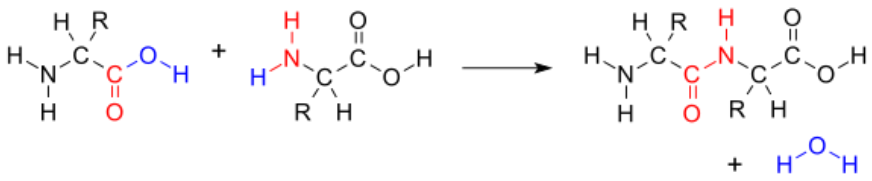

Figure 20. Amide/peptide bond formation between two amino acids.

Amide bonds can be involved in hydrogen bonds, usually exposed to water when proteins are in aqueous solutions. Moreover, amide bonds can form hydrogen bonds within the polypeptide chain itself and this is how the secondary structure of the protein forms. Periodic secondary structure types are α-helix and β-sheet. In the former, the hydrogen bond takes place between two successive turns of the

helix, whereas in the latter the hydrogen bond takes place between two parallel – or antiparallel - strands. A FTIR spectrum of a protein sample shows the characteristic absorption bands of the protein, for instance the Amide I and Amide II bands. Amide I band represents stretching vibration of the C=O bond of the amide, Amide II band the bending vibrations of the N-H bond. Both C=O and N-H are involved in the hydrogen bonds among amide bonds in the secondary structure of the protein so Amide I and Amide II bands give information about the quantity of secondary structure of the protein [84]. Amide I and Amide II are not the only amide bands that appear in the absorption spectrum, others are III, A and B bands. Amide III is linked to the combination of N-H bending and C-N stretching vibration with small contributions from the C=O in plane bending and the C-C stretching vibration. Amide A band is linked to the stretching vibration of N-H and it is usually part of a Fermi resonance in which the second component absorbs in the range 3100-3030 cm^{-1} and this is Amide B [83].
FTIR technique was used to individuate the wavelengths of the absorption peaks of Amides A, B, I, II and III in collagen extracted samples. In fact, IR spectra were used to compare the different collagen samples and to detect eventual changes in the chemical structures of samples produced by different recovery methods. In this sense, we were able to compare the absorption peaks of LFC IP with LFC SO and SFC IP with SFC SO. Data were acquired by positioning the freeze dried collagen samples on the crystal cell of the photometer and taking three readings for each sample scanning from $4000\ cm^{-1}$ to $400\ cm^{-1}$ at a rate of around $2\ cm^{-1}$ per point at room temperature (25°C).

3. Results and discussion

3.1. Solubility curves and pI

The absolute solubility curves of lumpfish (LFC) and starfish collagens (SFC) extracted with acetic acid (AA) solution and with acetic acid + pepsin (AA+P) solution are shown in Figure 21 and Figure 22, respectively. Results were obtained from a single solubilization experiment, working with technical duplicates. Raw data are reported in Appendix A. However, similar solubility curves were already found in previous lab trials.
Absolute solubility, corresponding to collagen concentration (expressed as concentration of extracted collagen in solution μg/ml), was calculated as reported in the material section (see paragraph 2.2). Absolute solubility was higher in acidic solution containing pepsin as biocatalyst for both LFC and SFC, as shown in Figure 21 and Figure 22. Absolute solubility of LFC AA at pH 8, 9 and10 was so low that it was approximated with 0 (Figure 21).

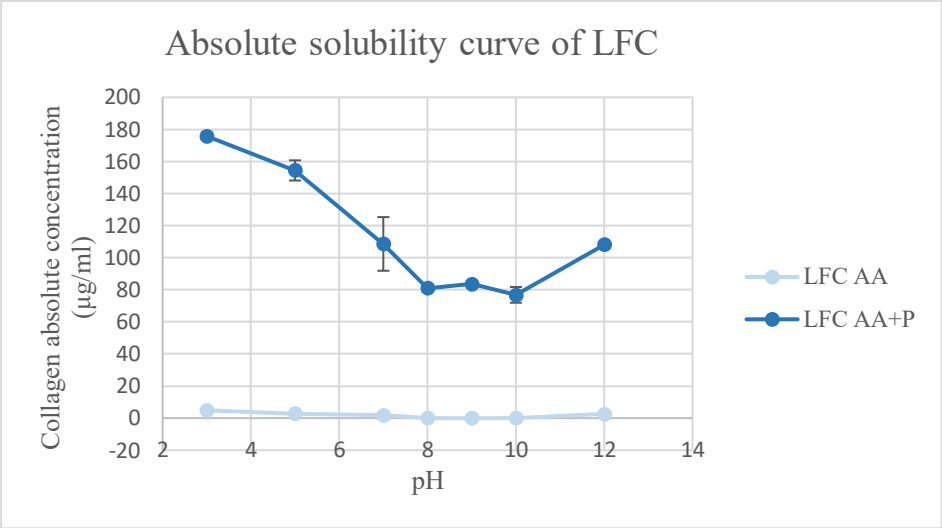

Figure 21. Lumpfish collagen (LFC) extracted with acetic acid (AA) and acetic acid + pepsin (AA+P) solutions solubility curves: collagen concentration (µg/ml) as a function of pH (pH tested in the range between 3 and 12) (results are averages ± standard deviations of 2 replicates).

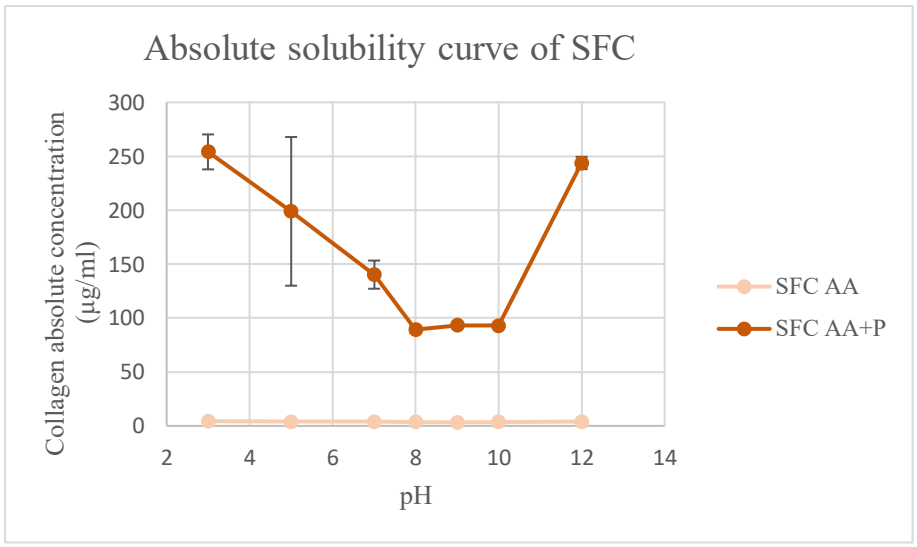

Figure 22. Starfish collagen (SFC) extracted with acetic acid (AA) and acetic acid + pepsin (AA+P) solutions solubility curves: collagen concentration (µg/ml) as a function of pH (pH tested in the range between 3 and 12) (results are averages ± standard deviations of 2 replicates).

Absolute solubility curves were used to assess that collagen extraction was more effective using pepsin as a biocatalyst. Collagen absolute concentration depends on the treated sample quantities, but since we started from the same quantity of material for the two different extractions (AA and AA+P solutions) for either starfish or lumpfish, the comparison of the absolute solubility curves within each sample material gave a first idea of the fact that collagen solubility in absence of pepsin was lower. This result was in agreement with the results from the work by Ahmad and Benjakul [31], in which collagen extraction yield was lower in absence of pepsin because acetic acid alone was not able to readily solubilize the triple helical structure, especially by breaking the crosslinks at the telopeptide region. In this case pepsin was able to cleave the intermolecular bonds at the telopeptide regions, resulting in a higher concentration of extracted collagen in the AA+P solution compared to the AA. However, the efficacy of pepsin in extracting collagen might depend on the fish species, on the collagen composition and configuration, on the amount of pepsin used, etc. [85].

Relative solubility, corresponding to collagen relative concentration, was calculated as reported in the material section (see paragraph 2.2), using collagen absolute concentration (Figure 21 and Figure 22). The relative solubility curves of lumpfish (LFC) and starfish collagens (SFC) extracted with acetic acid (AA) solution and with acetic acid + pepsin (AA+P) solution are shown in Figure 23 and Figure 24 as average ± standard deviations of two technical replicates. Raw data are reported in Appendix A. LFC relative solubility at pH 8, 9 and 10 in the case of only acetic acid (AA) solution was so low that it was approximated with 0, meaning that collagen precipitation was very effective at those pH values (Figure 23).

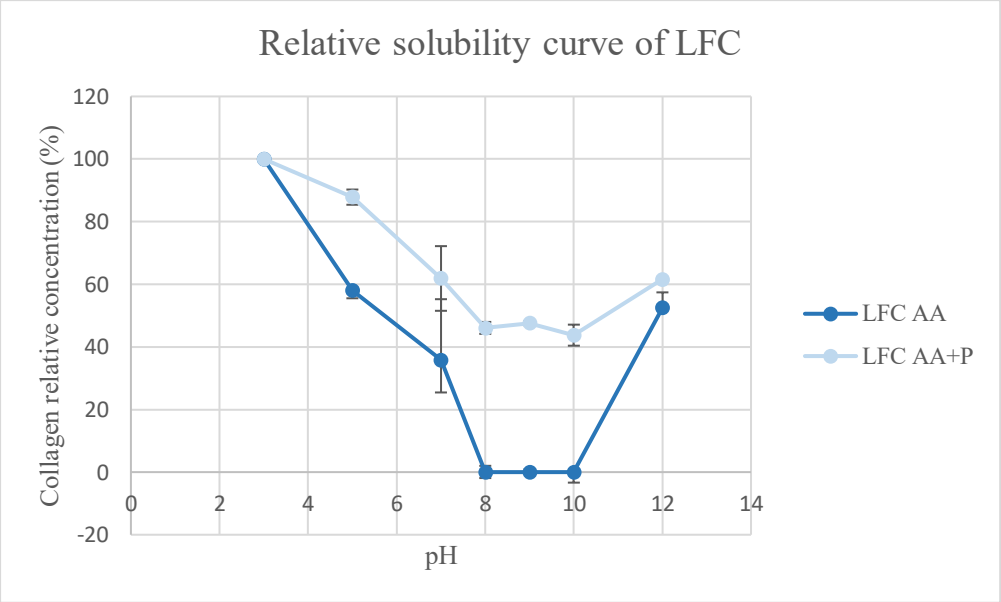

Figure 23. Lumpfish collagen (LFC) extracted with acetic acid (AA) and acetic acid + pepsin (AA+P) solutions solubility curves: relative solubility (%) as a function of pH (pH tested in the range between 3 and 12) (results are averages ± standard deviations of 2 replicates).

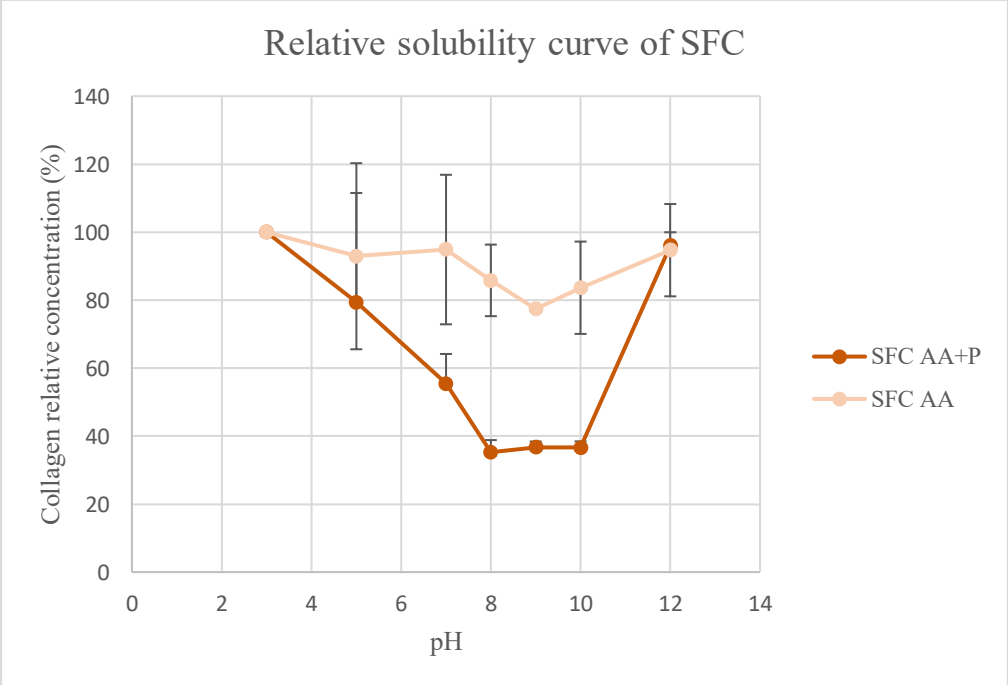

Figure 24. Starfish collagen (SFC) extracted with acetic acid (AA) and acetic acid + pepsin (AA+P) solutions solubility curves: relative solubility (%) as a function of pH (pH tested in the range between 3 and 12) (results are averages ± standard deviations of 2 replicates).

Relative solubility curves were used to determine the effect of pH on collagen solubility in the acidic solution and to determine the isoelectric points of extracted collagens. Both lumpfish and starfish collagen were highly solubilized at acidic pH, showing maximum solubility at pH=3. In general, collagen protein showed the highest solubility at acidic pH, a lower solubility in the range of pH between 7 and 10 and then a slightly increment at high pH. For what concerns the determination of the isoelectric point (pI) of the collagen, that corresponds to the pH value at which the lowest solubility was observed, no significant differences were detected comparing the solutions with and without pepsin from the two species (see Figure 23 and Figure 24). As a result, a single pI value was determined for each species, which was independent from the pepsin addition.
Below and above the pI, higher solubility of collagen molecules is due to the repulsive forces caused by total net charges of the molecules whereas at pI net charges are zero and there are hydrophobic-hydrophobic interactions that results in attractive interactions [22].
Thus, the solubility curves allowed determining the pI for the collagen extracted from the two sources with values of 8 for LFC and 10 for SFC.
Relative solubilities of lumpfish collagen at pH 8, 9 and 10 (Figure 23) were very similar to each other, so the lowest value of pH (i.e., 8) was chosen as pI for the collagen extracted by this source for further process optimization reasons. Indeed, after solubilizing collagen in the acetic acid solution, the pH has to be increased up to the pI value using basic solutions and going up to a pH=10 would require a higher quantity of chemicals.
pH=10 was chosen as starfish collagen (SFC) isoelectric point (pI). This choice was based either on the similar relative solubility values observed at pH 8, 9 and 10 in Figure 24, and also on results from preliminary trials (not shown here), where at pH 10 the solution showed visible aggregation of collagen molecules.
Collagen pI values determined for lumpfish and starfish are in agreement with the results obtained by Jongjareonrak and collaborators that reported pI=7 for collagen from Brownstripe red snapper (*Lutjanus vitta)* [22]. Something similar was found in the work by Kittiphattanabawon and colleagues [23] concerning the solubility curve of collagen obtained from the skin and bone of bigeye snapper (*Priacanthus tayenus*), and as well as in the work by Ahmed and colleagues [86] for collagen extracted from the by-products of bigeye tuna (*Thunnus obesus*).
Relative solubility curves considering AA+P treatment for both species (Figure 23 and Figure 24) allowed also estimating the recovery (%) of collagen from the solution In this regard, it is important to make a distinction between the recovery and the yield of extraction, which is described in detail in the following paragraph. Recovery depends only on the recovery method used, while the yield of extraction depends on all the isolation steps, and it gives an information about how efficient the whole adopted extraction process is. In other words, the yield assesses how much collagen can be isolated starting from a certain quantity of sourcing material following a specific process protocol, while recovery (%) assesses how much of the starting amount of collagen dissolved in the acidic solution can be recovered at the end of this step. It should be noted that not all the collagen dissolved in the acidic solution precipitates when the pH of the solution reaches the protein pI. Therefore, recovery (%) can be calculated as the difference between 100% and the relative solubility at the chosen pH. Thus, for lumpfish collagen extracted with acetic acid and pepsin (Figure 23), at pH = 8, the relative solubility was around 40-45% meaning that the recovery was about 55-60%. This means that, when IP was performed on the AA+P solution containing lumpfish collagen, 55-60% of the collagen precipitated whereas 40-45% of the collagen remained in the solution. For starfish (Figure 24) recovery was about 65% meaning that isoelectric precipitation could be really efficient for starfish collagen producing a high recovery rate.

In conclusion, solubility curves determination showed that collagen extraction was more efficient when pepsin-aided acidic extraction was performed and that isoelectric precipitation produced a fine recovery. Moreover, a single isoelectric point (pI) independent on the type of extraction (i.e., AA or AA+P solution) was determined for collagen extracted from each species. pI=8 was determined for LFC and pI=10 for SFC.

3.2. Yield of extraction

Extraction yield was calculated in the case of pepsin-aided acidic extraction for both recovery methods (IP and SO). In order to calculate yield on dry basis, the knowledge of the raw material moisture content was needed. Moisture content of LF and SF raw material and collagen extraction yields for LFC SO, LFC IP, SFC SO and SFC IP were calculated as reported in 2.5. Raw data used to calculate MC are shown in Appendix B and raw data used to calculate the yield are shown in Appendix C. Moisture content (MC) accounted for 68.23±2.61% for SF raw material and for 86.91±0.68% for LF raw material (values are averages ± standard deviations of three technical replicates). MC values were used to determine the dry weight of the starting raw materials, that were 31.77 g for lumpfish and 13.09 g for lumpfish.
Yield results, either on wet or dry basis, respectively shown in Figure 25 and Figure 26, highlighted that for both extraction methods lumpfish appeared to be a good collagen source, displaying a higher collagen yield, compared to starfish.

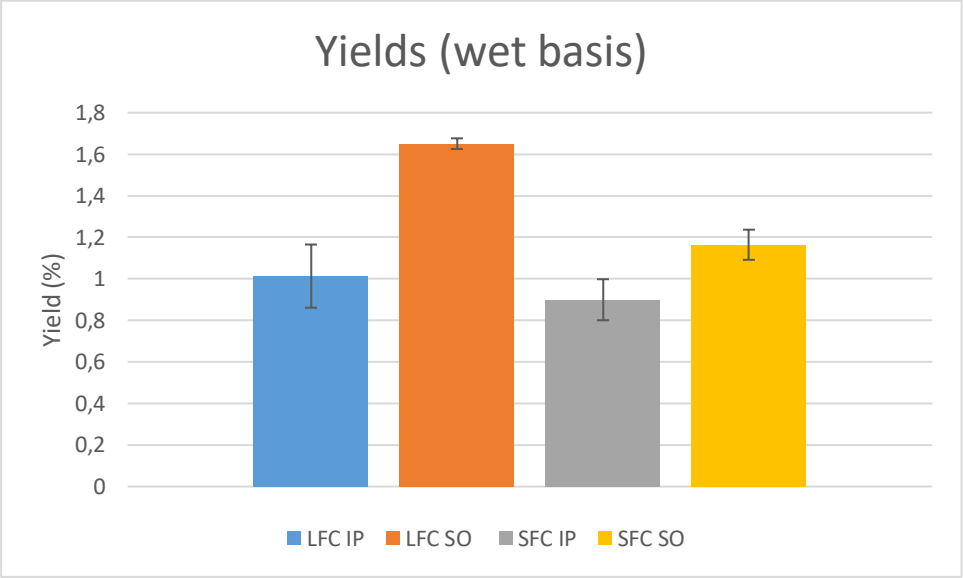

Figure 25. Lumpfish (LFC) and starfish (SFC) collagen yields on wet basis obtained via isoelectric precipitation (IP) and salting out (SO) (results are averages ± standard deviations of 2 technical replicates for each treatment).

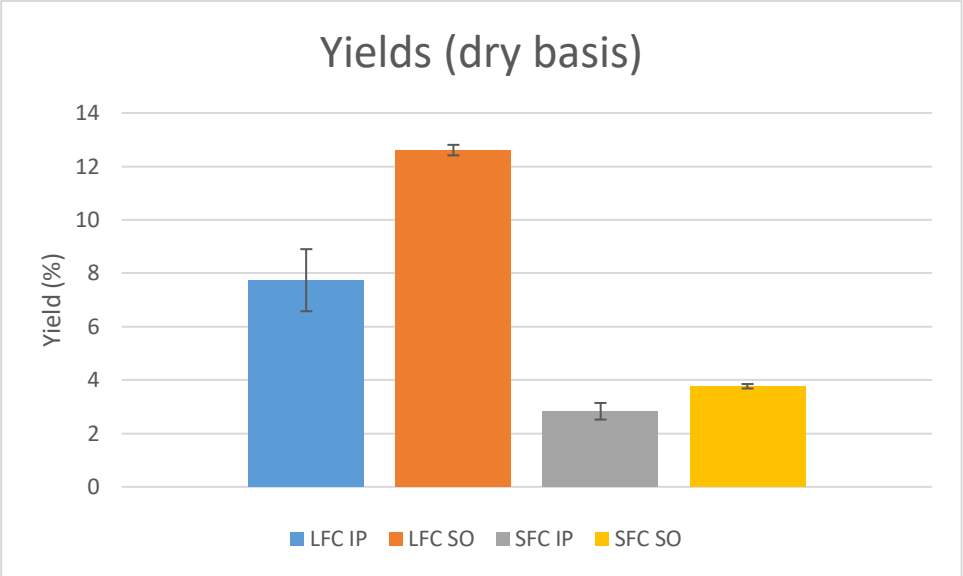

Figure 26. Lumpfish (LFC) and starfish (SFC) collagen average yields on dry basis obtained via isoelectric precipitation (IP) and salting out (SO) (results are averages ± standard deviations of 2 technical replicates for each treatment).

For what concerns lumpfish, yield on wet basis in the case of isoelectric precipitation (IP) was 1.01±0.15% and in the case of salting out (SO) was 1.65±0.03% (Figure 25). On a dry basis these results correspond to a 7.74±1.16% yield for IP and 12.61±0.20% for SO (Figure 26). In the case of starfish collagen extraction, the difference between IP and SO yield was even lower. Indeed, the yield on wet basis for SFC IP was 0.9±0.10% and for SFC SO was 1.16±0.07% (Figure 25). On a dry basis these results correspond to a 2.83±0.31% yield for IP and 3.76±0.08% for SO (Figure 26). Lumpfish moisture content (MC=86.91±0.68%) was greater compared to that of other species, while that of starfish (MC=68.23±2.61%) was on average [32][87] and this resulted in lumpfish yields on dry basis being greater than the starfish yields (Figure 26). Yields on wet basis of starfish and lumpfish collagen obtained via isoelectric precipitation are close to each other (SFC IP 0.9±0.10% and LFC IP 1.01±0.15%) and this was in agreement with the fact that absolute solubility (Figure 21 and Figure 22) and recovery (%) (Figure 23 and Figure 24) were similar for the AA+P solutions.

However, for both lumpfish and starfish salting out recovery resulted in higher yield compared to precipitation via pH adjustment to isoelectric point. The work of *Lin et al.* on Bigeye Tuna skin showed a moderate increase of the yield when isoelectric precipitation was performed instead of salting out, passing from 14.14% to 17.17% on dry weight [18]. The yields in my thesis work are lower compared to the ones found in the above mentioned work, but it is important to consider that in the work by Lin et al. the collagen extraction was performed on the skin only, in which collagen is the main constituent. In fact, in previous studies collagen extraction involved only the skin tissues [22], [68] and often the skin showed higher yields compared to other tissues [23].

Thus, lumpfish yield results were promising considering the high moisture content of the raw material. Even if the yields obtained with recovery via salting out were higher compared to those obtained via isoelectric precipitation, the difference was not remarkable, suggesting that IP is a valid alternative to SO. Especially when considered on an industrial scale, isoelectric precipitation results in time, chemicals and water saving.

3.3. Characterization of collagen samples

Characterization was performed on the collagen samples extracted with acetic acid + pepsin solution and recovered via isoelectric precipitation and salting out, that are LFC AA+P IP, LFC AA+P SO, SFC AA+P IP and SFC AA+P SO. Hereafter the nomenclature of the samples will not include "AA+P" in the name for brevity. The characterized collagen samples are shown in Figure 27. Starfish freeze-dried collagen extracted with acetic acid and pepsin solution (SFC), recovered via salting out (SO) (a) and isoelectric precipitation (IP) (b), lumpfish freeze-dried collagen extracted with acetic acid and pepsin solution (LFC), recovered via salting out (SO) (c) and isoelectric precipitation (IP) (d)Figure 27.

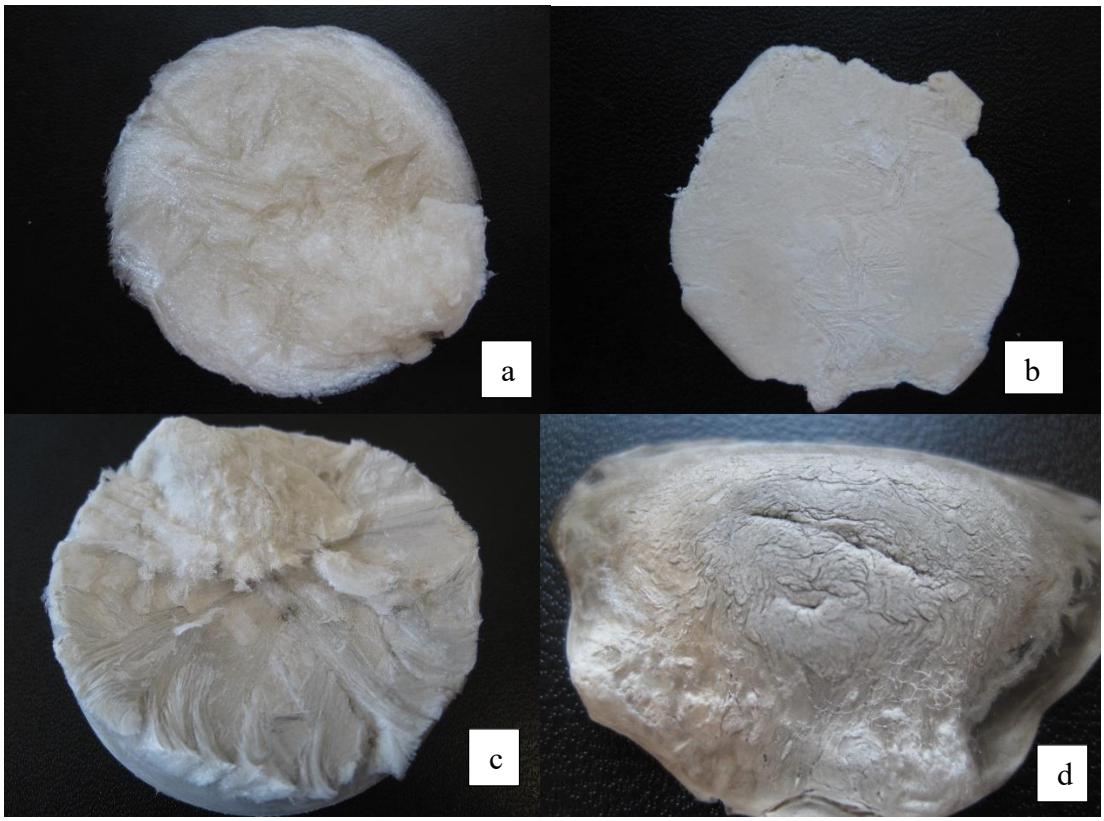

Figure 27. Starfish freeze-dried collagen extracted with acetic acid and pepsin solution (SFC), recovered via salting out (SO) (a) and isoelectric precipitation (IP) (b), lumpfish freeze-dried collagen extracted with acetic acid and pepsin solution (LFC), recovered via salting out (SO) (c) and isoelectric precipitation (IP) (d).

Collagen recovered using salting out (SO) appeared soft and spongy, showing a lamellar-fibrillar structure, whereas collagen recovered using isoelectric precipitation appeared hard (Figure 27). This suggested that centrifugation on the acidic solution, after pH adjustment to the pI should be carried out at a lower speed and/or for less time (i.e., speed was 15000g and time was 1 h). This difference in the samples structure was such that collecting little samples was more complex in the case of collagen recovered with IP and dissolution of those samples took more time compared to the piece of samples collected from the SO samples. Moreover, it was possible to assess that lumpfish collagen (LFC) retained its greyish colour whereas starfish collagen (SFC) lost almost all the colour during the extraction (starting raw material was pale orange) and appeared white (Figure 27). Both the samples slightly smelled of fish and acetic acid. Organoleptic characteristics must be taken into account because they can really affect the quality of the product depending on the application.

3.3.1. Electrophoretic pattern of extracted collagen by SDS-PAGE

The electrophoretic pattern of collagen obtained with different recovery methods from lumpfish and starfish is shown in Figure 28.

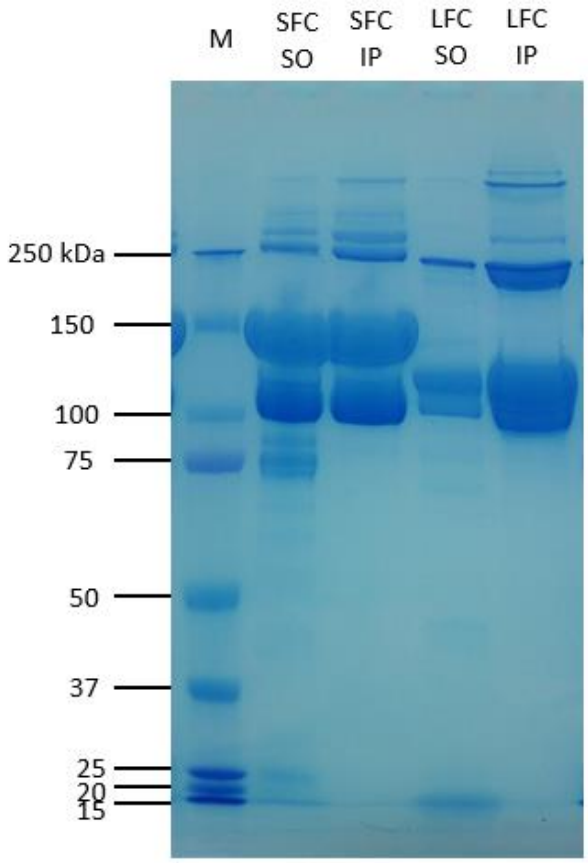

Figure 28. SDS-PAGE pattern of protein marker (M), starfish collagen from salting out (SFC SO), starfish collagen from isoelectric precipitation (SFC IP), lumpfish collagen from salting out (LFC SO), lumpfish collagen isoelectric precipitation (LFC IP). On each lane loaded 15 µg of extracted collagen.

Electrophoretic patterns of collagen extracted from the same species with a different recovery method (i.e. IP or SO) were similar. Independently on the recovery method used, both LFC and SFC electrophoretic pattern showed the presence of two major bands, attributable to collagen α chains according to their molecular weights. For the interpretation of the SDS-PAGE results, it should be noted that collagen from different sources could have different amino acid composition and this can lead to differences in the molecular weight observed for the main protein bands [17].
The SFC SO and the SFC IP patterns showed the presence of two different types of α chains in the range 100-150 kDa which were recognized as α_1 and α_2 chains. The α_1 chain of starfish collagen has higher molecular weight (closer to 150 kDa) compared to the α_2 chain (closer to 100 kDa). Similar results were found for collagen from *Asterias amurensis* [88] and *Acanthaster planci* (crown-of-thorns starfish) [89], which are invasive species of starfish as well. The LFC SO and the LFC IP patterns showed the presence of two different types of α chains in the range 100-150 kDa, recognized as α_1

and α_2 chains. In the case of LFC, α_1 chain of collagen was found at around 120 kDa and α_2 chain at a lower molecular weight (closer to 100 kDa). Similar results were found for collagen extracted from other species of fish such as unicorn leatherjacket [31], Baltic cod (skin) [16], bighead carp [21].
It was noted that, while α_2 chains of LFC and SFC showed a similar molecular weight (around 100 kDa), the α_1 chains showed a slight difference in molecular weight (around 120 kDa for LFC and around 150 kDa for SFC). As mentioned above, difference in the molecular weight of the α chains are attributable to difference in the primary structure.
Since two different types of α chains (α_1 and α_2) were detected and since they appeared to be in a 2:1 ratio, it was confirmed that both collagens extracted (LFC and SFC) are collagens of type I with a $[\alpha_1(\mathrm{I})]_2\alpha_2(\mathrm{I})$ configuration [90]. Indeed, collagen of type I consists in a triple helix formed by two α_1 chains and one α_2 chain, coiled in the triple helical structure and packed among themselves through intramolecular bonds. Further bands can be observed in the collagens electrophoretic pattern, and these were attributable to the existence of intramolecular bonds between different α chains. Both the SFC and the LFC samples showed the presence of β chain (dimer) at around 250 kDa, indicating the presence of intramolecular bonds between two α chains. β chains retained their integrity in LFC and SFC independently on the recovery method used. In the case of LFC IP and SFC IP, it was possible to notice a thin band at a higher molecular weight (>250 kDa) that can be attributable to γ chain (trimer) formed by three α chains. Therefore, both IP and SO allowed maintaining the integrity of some β chains and IP allowed maintaining the integrity of some γ chains as well.
Therefore, the extraction with acetic acid + pepsin (AA+P) solution determined the cleavage of the triple helix at the telopeptide regions, thus breaking the β and γ chains into α chains (most abundant components according to electrophoretic pattern in Figure 28), but pepsin digestion was not total since β and γ chains were observed in the electrophoretic pattern. It was noticed that the recovery method affected the electrophoretic pattern of extracted collagen. Indeed, salting out (SO) produced higher collagen degradation compared to isoelectric precipitation (IP). Not only γ chains are not observed in the LFC SO and SFC SO lane, but LFC SO and SFC SO also showed the presence of low molecular weight bands. The presence of bands at a low molecular weight (<100 kDa) was indicative of the presence of degraded polypeptides, which were absent in the corresponding samples coming from isoelectric precipitation. This evidence suggested that degradation of collagen probably happened during the performance of salting out and not during isoelectric precipitation. The presence of low molecular weight products was more evident in the SFC SO lane than in the LFC SO lane because, even if all the lanes were loaded with the same amount of sample (i.e., 15 μg of collagen), the LFC SO lane appeared underloaded, probably due to a lower solubilization efficiency in SDS of the LFC SO sample. Low molecular weight products in SFC SO and LFC SO lanes could be either degradation products or non-collagenous proteins (NCP). They were more likely degradation products generated by collagen degradation occurred during salting out recovery method since LFC IP and SFC IP did not show the presence of low molecular peptide, indicating that deproteinization step was effective in removing NCP. Indeed, all the samples were subjected to the same deproteinization step, thus, if NCP proteins were not totally removed, bands attributable to their presence should have appeared in both lanes (i.e., IP and SO) of a single species. However, it should be pointed out that deproteinization step could have worked differently on the tissues from the two different species.
Therefore, electrophoretic pattern of the collagen samples showed that both LFC and SFC are collagen of type I since two major bands attributable to α chains were visible

in each lane at a molecular weight in the 100-150 kDa range. For all the samples, a further band was visible at around 250 kDa, which was recognized as β chain (dimer formed by two α chains). For LFC IP and SFC IP, a further thin band was visible at a molecular weight higher than 250 kDa and this could be attributable to γ chains (trimers formed by three α chains). SFC SO and LFC SO lanes showed the presence of low molecular products that were recognized as collagen degradation products. In conclusion, collagens obtained with IP (LFC IP and SFC IP) showed higher purity and less degradation extent of the collagen structure, since no degradation products were detected, and dimers and trimers of collagen were found. This indicated that salting out recovery method produced degradation of the extracted collagen whereas isoelectric precipitation did not.

3.3.2. UV spectra

The normalized ultraviolet spectra of LFC and SFC at wavelengths between 220 nm and 450 nm is shown in Figure 29. Lumpfish and starfish collagen obtained with isoelectric precipitation and salting out showed maximum absorption peaks at 230 nm (Figure 29). As reported in the work by Pal and collaborators [19], the triple helix of collagen has its maximum absorption peak at 230 nm, which is probably due to the presence of $C = O$, $COOH$, $CO - NH_2$ groups in the chains that form the triple helix [32].

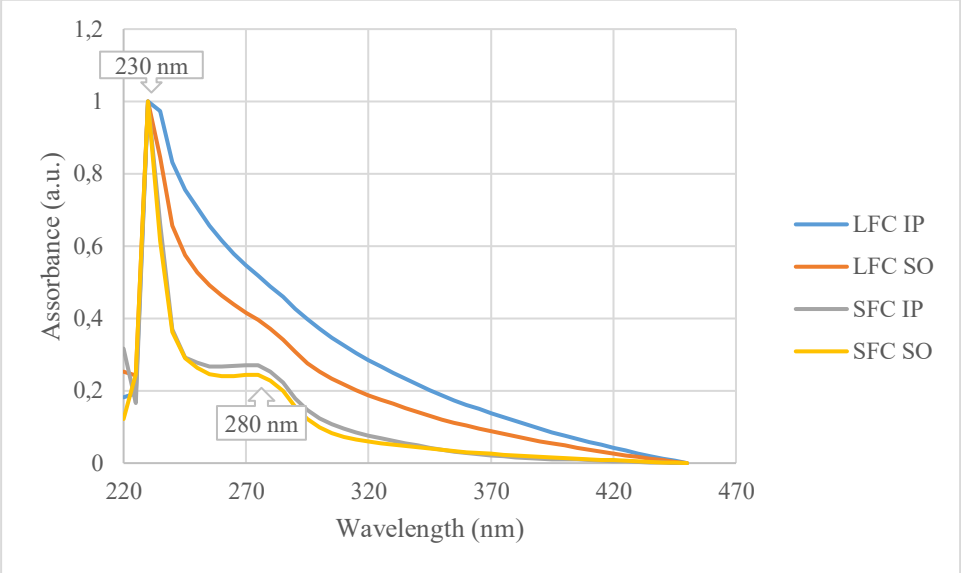

Figure 29. Ultraviolet (UV) absorption spectrum of lumpfish (LFC) and starfish (SFC) collagens extracted with acetic acid and pepsin (AA+P) solution and recovered using both isoelectric precipitation (IP) and salting out (SO) normalized to the maximum peak at around 230 nm.

Similar spectroscopic results were found in other studies performing collagen extraction from skin of balloon fish [87], skin of squid [85], skin of Nile tilapia [90], marine eel-fish [75]. Thus, this molecular "signature" can be used to determine the purity of the collagen using UV spectroscopy. In the case of SFC (both IP and SO) spectrum, the major peak at 230 nm was really sharp and a secondary evident peak at 280 nm was found indicating the presence of aromatic amino acids in the protein structure [70]. 280 nm is the usual maximum absorption wavelength for proteins indicating the presence of aromatic residuals such as tyrosine, tryptophane and phenylalanine in the amino acidic

chain, in fact the absorption wavelength in the UV range for tyrosine is 275 nm and for phenylalanine is 258 nm, in general aromatic side chains absorb in the 240-300 nm range [24].
Starfish collagen (SFC) UV spectrum was very similar to other UV spectra found for marine collagens in other works in literature which were previously mentioned. Indeed, usually the number of aromatic residues in the primary structure is low so the peak at 280 nm is not as much evident as the one at 230 nm and often it is not present.
In the lumpfish UV-spectra, the peak is wider (especially the LFC IP one) and no further evident peaks of absorption were detected in the range between 260-280 nm, despite a broad extended absorption in the spectrum.
This suggested a higher content of aromatic amino acids in the LFC compared to the SFC. Further amino acid analysis should be performed to assess the presence of aromatic amino acids in starfish and lumpfish collagen samples and the eventual amount of aromatic residues in their primary structure. Indeed, this high absorbance in the 240-300 nm range could not be attributed to aromatic amino acid of eventual non-collagenous proteins (NCP) in the sample since the electrophoretic pattern of LFC IP and SFC IP samples (Figure 28) showed that those samples consisted of collagen. As a matter of fact, there were no other bands attributable to other proteins in LFC IP and SFC IP lanes. Nevertheless, the electrophoretic pattern of collagen samples obtained via salting out, especially SFC SO, showed the presence of other bands but those bands were attributed to degradation products since all the samples were subjected to the same pretreatments and extraction method, thus deproteinization worked in the same way for all the samples.
Therefore, collagen samples UV spectrum allowed to assess that the LFC and SFC samples contain pure collagen and that LFC contained a higher amount of aromatic amino acids. This should be confirmed with a successive amino acids analysis.

3.3.3. FTIR spectra

FTIR spectra of collagen samples are shown in Figure 30 and the wavenumbers of the major absorption peaks are reported in Table 2. Amide groups and their absorption peak wavenumber in the Fourier transform infrared (FTIR) spectra of lumpfish (LFC) and starfish (SFC) collagen extracted with acetic acid and pepsin (AA+P) solution and recovered using both isoelectric precipitation (IP) and salting out (SO).

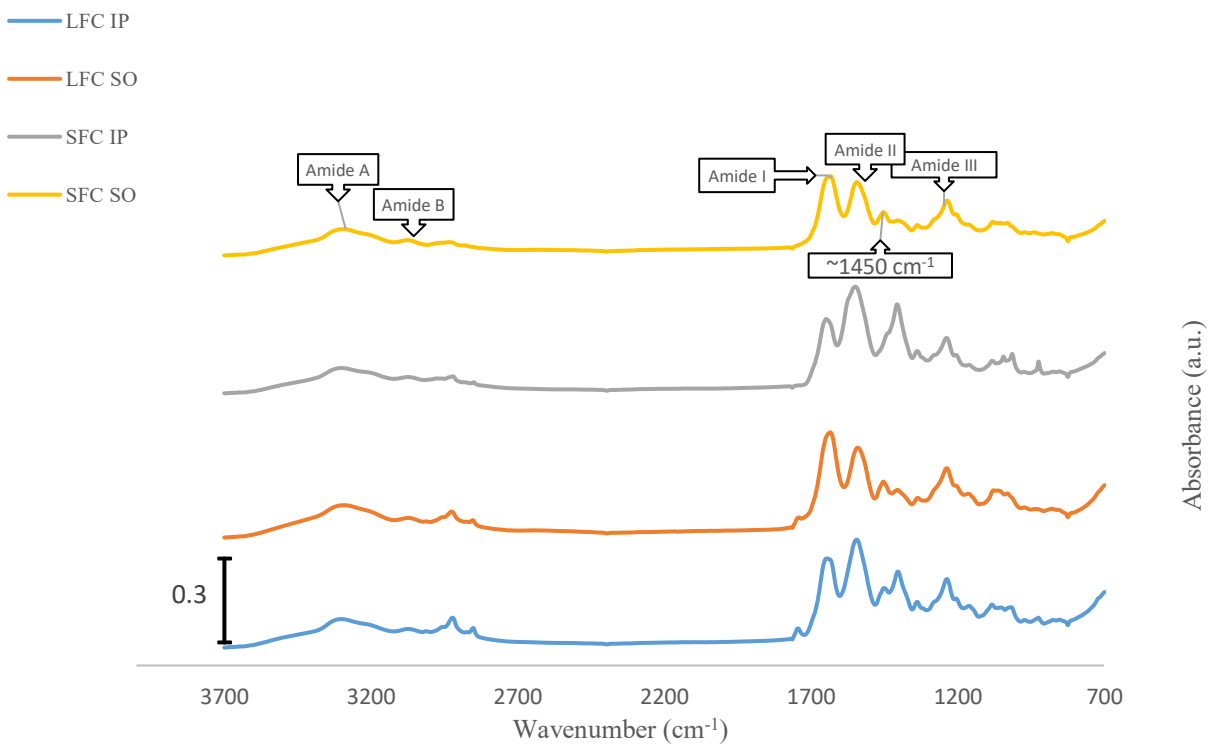

Figure 30. Fourier transform infrared (FTIR) spectra of lumpfish (LFC) and starfish (SFC) collagens extracted with acetic acid and pepsin (AA+P) solution and recovered using both isoelectric precipitation (IP) and salting out (SO). Major peaks of absorption assigned to Amide A, Amide B, Amide I, Amide II and Amide III groups are indicated.

Table 2. Amide groups and their absorption peak wavenumber in the Fourier transform infrared (FTIR) spectra of lumpfish (LFC) and starfish (SFC) collagen extracted with acetic acid and pepsin (AA+P) solution and recovered using both isoelectric precipitation (IP) and salting out (SO).

Region	Wavenumber of the absorption peak			
	LFC IP	LFC SO	SFC IP	SFC SO
Amide A (stretching NH)	3305 cm^{-1}	3303 cm^{-1}	3305 cm^{-1}	3295 cm^{-1}
Amide B (asymmetrical stretching CH2)	3075 cm^{-1}	3073 cm^{-1}	3068 cm^{-1}	3077 cm^{-1}
Amide I (stretching C=O)	1644 cm^{-1}	1643 cm^{-1}	1646 cm^{-1}	1633 cm^{-1}
Amide II (bending NH)	1544 cm^{-1}	1542 cm^{-1}	1548 cm^{-1}	1542 cm^{-1}
Amide III (deformation NH, stretching CN)	1236 cm^{-1}	1234 cm^{-1}	1236 cm^{-1}	1234 cm^{-1}

Amide A and amide I bands are linked to stretching vibrations of covalent bonds, that is the change of length of bonds, whereas amide II is associated with the bending vibration of covalent bond, that is the change of angle in the bond as shown in Figure 31.

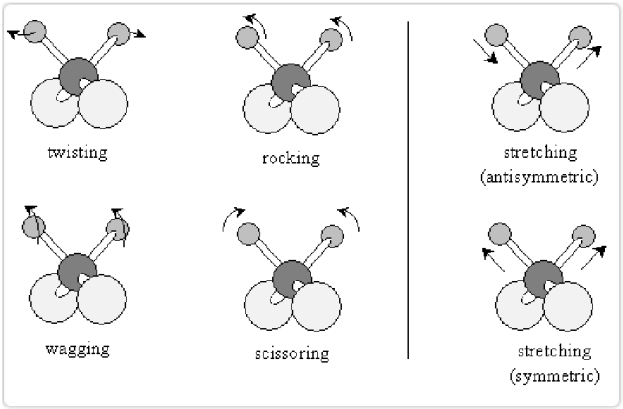

Figure 31. Stretching and bending vibration modes

Amide A bands of SFC IP and SFC SO slightly differed between each other ($3305\ cm^{-1}$ for SFC IP and $3295\ cm^{-1}$ for SFC SO) (Figure 30) indicating the presence of differences in the secondary structure of starfish collagen extracted with the two different methods. On the other hand, absorption peaks of amide A for LFC IP and LFC SO were found approximately at the same wavenumber (i.e., 3305 cm^{-1} and 3303 cm^{-1}) (Figure 30). Amide A band is linked to the $N-H$ stretching vibration: a free $N-H$ stretching vibration occurs in the range of $3400-3440\ cm^{-1}$ whereas the stretching vibration of a $N-H$ bond involved in a hydrogen bond in the protein structure presents absorption peak at a lower frequency, usually $3300\ cm^{-1}$ [26]. This displays that $N-H$ bonds in the structures of extracted collagens are involved in hydrogen bonds, packaging the triple helix structure. SFC SO collagen showed the Amide A absorption peak at even a lower wavenumber probably indicating the major presence of hydrogen bonds.

The amide I bands of LFC IP and LFC SO were at $1644\ cm^{-1}$ and $1643\ cm^{-1}$, the amide I bands of SFC IP and SFC SO at $1646\ cm^{-1}$ and $1633\ cm^{-1}$ respectively. Therefore, the amide I absorption peak of starfish collagen recovered via salting out was at a lower frequency compared to the starfish collagen obtained via isoelectric precipitation. This absorption peak of amide I is associated with the stretching vibrations of the $C=O$. The frequency of the stretching vibration depends both on the strength of the hydrogen bond to the carbonyl oxygen and on the environment determined by the local conformation of the protein [27].

Amide II peaks for LFC IP and LFC SO were found approximately at the same wavenumber ($1544\ cm^{-1}$ and $1542\ cm^{-1}$ respectively), whereas there was a slight difference for the amide II absorption peaks of starfish collagen ($1548\ cm^{-1}$ and $1542\ cm^{-1}$ respectively), so the peak for SFC SO was at lower frequency compared to the one of SFC IP). According to the work by Krimm and Bandekar [28] amide II absorption peak is associated with $N-H$ bending vibration coupled with $C-N$ stretching vibration.

In the work by Payne and Veis [29] a shift of the amide I and amide II bands to lower frequency is associated with a decrease in the molecular order. This could be an explanation for the slight difference in the wavenumbers found in the case of SFC SO. In fact, SFC SO showed amide I and amide II peaks at lower frequencies compared to SFC IP (amide I bands of SFC IP and SFC SO at $1646\ cm^{-1}$ and $1633\ cm^{-1}$ respectively, amide II bands of SFC IP and SFC SO at $1548\ cm^{-1}$ and $1542\ cm^{-1}$ respectively). Amide A of SFC SO was found at a lower frequency compared to SFC IP, as well. Therefore, SFC SO presented a higher molecular disorder meaning a lower presence of intermolecular cross-links [91] and this is in agreement with the fact that

SDS PAGE of SFC SO presented a higher content of low molecular polypeptides compared to the SFC IP (Figure 28).
Amide B peaks of LFC IP and LFC SO were found at $3075\ cm^{-1}$ and $3073\ cm^{-1}$ respectively, amide B peaks of SFC IP and SFC SO at $3068\ cm^{-1}$ and $3077\ cm^{-1}$. Amide B peaks of absorption at these frequencies are linked to the asymmetrical stretch of CH_2, residual groups from Gly and Pro [91]. Gly and Pro amino acids are particularly present in collagen primary structure, thus these positions of the amide B peaks are a confirmation of the presence of collagen as well, together with the presence of all the other found peaks.
Amide III peaks of absorption of LFC and SFC were found at approximately the same wavelength ($1236\ cm^{-1}$ for IP and $1234\ cm^{-1}$ for SO). Amide III peak is associated with $N-H$ deformation and $C-N$ stretching vibrations [30].
Minor peaks around $1450\ cm^{-1}$ were observed for LFC IP, LFC SO and SFC SO samples (Figure 30). The ratios between the amide III and the $1450\ cm^{-1}$ bands were evaluated for LFC IP, LFC SO and SFC SO and reported in Table 3.

Table 3. Absorbance of Amide III band and ~$1450\ cm^{-1}$ band ratios for lumpfish (LFC) and starfish (SFC) collagen extracted with acetic acid and pepsin (AA+P) solution and recovered via both isoelectric precipitation (IP) and salting out (SO).

	Amide III band	~$1450\ cm^{-1}$ band	Ratio
LFC IP	$1236\ cm^{-1}$	$1450\ cm^{-1}$	1.1
LFC SO	$1234\ cm^{-1}$	$1448\ cm^{-1}$	1.1
SFC IP	$1236\ cm^{-1}$	-	-
SFC SO	$1234\ cm^{-1}$	1452	1.4

According to the work by Ahmad and Benjakul [31], a value for this ratio around 1 is indicative of the integrity of the collagen triple helical structure. From ratios calculated from FTIR results shown in Table 3 it was possible to assess that lumpfish collagen extracted via isoelectric precipitation and salting out conserved its integrity (ratios close to 1) while SFC SO partly lost the integrity of the structure. In fact, the position of SFC SO amide I and amide II peaks showed that SFC SO structure presented a lower extent of intramolecular bonds compared to the other three samples.
In conclusion, in all the tested extracted collagen samples main absorption bands were found in the interval between 1600-1800 cm^{-1}, 1470-1570 cm^{-1}, 1250-1350 cm^{-1} and 3300-3500 cm^{-1} , attributable respectively to Amide I, Amide II, Amide III and Amide A according to Ji and collaborators [25]. Amide B peaks were found for every species at around 3070 cm^{-1}. These characteristic absorption bands are in agreement with FTIR results found for collagens from marine eel-fish [32], bighead carp [30] and silver carp [20], confirming the presence of collagen in the extracted solutions. In addition, LFC IP, LFC SO and SFC SO absorption peaks positions and absorbance values allowed assessing the integrity of the collagen structure and the presence of intramolecular bonds meaning that the extraction procedure adopted did not break the triple helix structure.

4. Conclusions

In the last decades, collagen market has seen an increment of the demand due to the growing variety of its applications, especially as a biomedical material for tissue engineering and drug delivery or as a food supplement for cosmetic purpose. Therefore, the possibility of extracting collagen from fish market by-products, underutilised and invasive marine species on an industrial scale is really fascinating. In this work of thesis, collagen from lumpfish (*Cyclopterus lumpus*) and starfish (*Asterias rubens*) was successfully extracted and characterized.
The aim of this work of thesis was to investigate the effectiveness of isoelectric precipitation as a recovery method in collagen isolation protocol and to assess if it is a valid alternative to salting out. From a broader point of view, the aim was to implement a new protocol for collagen extraction from marine species as much productive and efficient as possible. The extraction consists in a series of successive steps that need optimization, among which one is the recovery. During my work of thesis, I had the opportunity to try both the acidic extraction and the pepsin-aided acetic extraction. From the results obtained, I assessed that for the species involved in this trial the presence of pepsin significantly increases the yield of extraction.
In order to furtherly optimize the protocol, it could be useful to track collagen content during the isolation steps to understand which step need further optimization. Collagen content in solutions can be assessed by measuring the hydroxyproline content since Hyp is a peculiar amino acid in collagen.
However, once it was confirmed that acidic extraction in presence of pepsin as biocatalyst was more effective compared to the acidic extraction without pepsin, salting out and isoelectric precipitation were compared in term of yields. Performing isoelectric precipitation on the samples required the preliminary determination of the isoelectric points of the samples. Isoelectric points were determined for both species for the two extraction methods (with and without pepsin). Acidic and pepsin-aided acidic extraction produced collagen with analogous relative solubility curves so for each species a single value of isoelectric point was determined, independent from the type of extraction. Collagen showed the lowest solubility in the pH 8-10 range for both species and pI=8 was determined for lumpfish and pI=10 for starfish. These values of pI were successively used to perform isoelectric precipitation.
Recovery via salting out led to a higher yield compared to recovery via isoelectric precipitation but, especially for what concerns starfish, the yield difference was not remarkable, so isoelectric precipitation could be a valid alternative to salting out in collagen recovery from an acidic solution. Collagen yield of lumpfish was higher compared to collagen yield of starfish and, considering the high moisture content of the raw material, yield results were outstanding. Comparing the two recovery methods, the isoelectric precipitation showed some strengths in terms of:

- specificity of the isoelectric point for the collagen of the involved species
- less time (no need to perform dialysis after the recovery) and minor quantity of chemicals needed with respect to more traditional salting out procedure
- production of minor quantity of wastewater (dialysis requires high quantities of water since the solution is periodically changed)

In addition, collagen samples obtained with pepsin-aided acidic extraction and with the two different recovery methods were characterized. The performed analyses showed that there was no evident difference between collagen recovered with the two different methods. SDS-PAGE, FTIR and UV-spectroscopy analyses revealed that extracted collagen was of type I for both species and that isolation occurred retaining the integrity of the triple helix.

In future, collagen recovered via isoelectric precipitation could be further characterized investigating thermal properties (i.e. via DSC) and fibril formation properties in order to its suitability in many industrial applications.

5. Appendix A

Appendix A contains raw data used to obtain absolute and relative solubility curves of lumpfish and starfish collagen in the acidic solution. Absolute and relative concentrations in Figure 21, Figure 22, Figure 23, Figure 24 are average ± standard deviations of 2 technical replicates (I, II). Absorbance of a 0.1M NaOH solution was used as blank (corresponding to a reading value of 0,037). Average absolute and relative concentrations of LFC AA at pH 8-9-10 were adjusted to 0 (Figure 21 and Figure 23).

LFC AA						
pH	Abs I	Abs II	Abs I - abs blank	Abs II - abs blank	Absolute concentration (µg/ml)I	Absolute concentration (µg/ml) II
3	0,0735	0,0738	0,0365	0,0368	4,860465116	4,930232558
5	0,0647	0,0649	0,0277	0,0279	2,813953488	2,860465116
7	0,0572	0,0631	0,0202	0,0261	1,069767442	2,441860465
8	0,0528	0,0522	0,0158	0,0152	0,046511628	-0,093023256
9	0,0505	0,0502	0,0135	0,0132	-0,488372093	-0,558139535
10	0,0493	0,0491	0,0123	0,0121	-0,76744186	-0,813953488
12	0,0643	0,063	0,0273	0,026	2,720930233	2,418604651
LFC AA+P						
pH	Abs I	Abs II	Abs I - abs blank	Abs II - abs blank	Absolute concentration (µg/ml)I	Absolute concentration (µg/ml) II
3	0,8014	0,8154	0,7644	0,7784	174,1395349	177,3953488
5	0,6974	0,7357	0,6604	0,6987	149,9534884	158,8604651
7	0,5704	0,4687	0,5334	0,4317	120,4186047	96,76744186
8	0,4076	0,3937	0,3706	0,3567	82,55813953	79,3255814
9	0,4124	0,4117	0,3754	0,3747	83,6744186	83,51162791
10	0,3979	0,3681	0,3609	0,3311	80,30232558	73,37209302
12	0,5176	0,5185	0,4806	0,4815	108,1395349	108,3488372

SFC AA						
pH	Abs I	Abs II	Abs I - abs blank	Abs II - abs blank	Absolute concentration (µg/ml) I	Absolute concentration (µg/ml) II
3	0,0734	0,0689	0,0364	0,0319	4,837209302	3,790697674
5	0,0679	0,0709	0,0309	0,0339	3,558139535	4,255813953
7	0,0691	0,0706	0,0321	0,0336	3,837209302	4,186046512
8	0,0689	0,0678	0,0319	0,0308	3,790697674	3,534883721
9	0,0686	0,0653	0,0316	0,0283	3,720930233	2,953488372
10	0,068	0,0678	0,031	0,0308	3,581395349	3,534883721
12	0,0703	0,0696	0,0333	0,0326	4,11627907	3,953488372
SFC AA+P						
pH	Abs I	Abs II	Abs I - abs blank	Abs II - abs blank	Absolute concentration (µg/ml) I	Absolute concentration (µg/ml) II
3	1,1941	1,0956	1,1571	1,0586	265,4651163	242,5581395
5	0,6983	1,1174	0,6613	1,0804	150,1627907	247,627907
7	0,6159	0,6953	0,5789	0,6583	131	149,4651163
8	0,4263	0,4467	0,3893	0,4097	86,90697674	91,65116279
9	0,458	0,448	0,421	0,411	94,27906977	91,95348837
10	0,4562	0,4481	0,4192	0,4111	93,86046512	91,97674419
12	1,118	1,0834	1,081	1,0464	247,7674419	239,7209302

LFC AA		
pH	Relative concentration (%) I	Relative concentration (%) II
3	100	100
5	57,89473684	58,01886792
7	22,00956938	49,52830189
8	0,956937799	-1,886792453
9	-10,04784689	-11,32075472
10	-15,78947368	-16,50943396
12	55,98086124	49,05660377
LFC AA+P		
pH	Relative concentration (%) I	Relative concentration (%) II
3	100	100
5	86,11111111	89,55165181
7	69,15064103	54,54902989
8	47,40918803	44,71683272
9	48,05021368	47,07656004
10	46,11378205	41,36077609
12	62,09935897	61,07760881

SFC AA		
pH	Relative concentration (%) I	Relative concentration (%) II
3	100	100
5	73,55769231	112,2699387
7	79,32692308	110,4294479
8	78,36538462	93,25153374
9	76,92307692	77,91411043
10	74,03846154	93,25153374
12	85,09615385	104,2944785
SFC AA+P		
pH	Relative concentration (%) I	Relative concentration (%) II
3	100	100
5	56,56592203	102,0901246
7	49,34734998	61,62032598
8	32,73762593	37,7852349
9	35,51467367	37,90987536
10	35,35698642	37,91946309
12	93,33333333	98,83029722

6. Appendix B

Appendix B contains raw data used to calculate lumpfish (LF) and starfish (SF) raw materials moisture content (MC) (%). MC was determined as average ± standard deviations between 3 technical replicates.

	Lumpfish (LF) moisture content (MC) (%)	Starfish (SF) moisture content (MC) (%)
I	87,45	65,99
II	87,14	67,61
III	86,14	71,11

7. Appendix C

Appendix C contains raw data used to calculate collagen extraction yield from acidic solution using isoelectric precipitation (IP) and salting out (SO). Yield was determined as average ± standard deviations between 2 technical replicates for each species and each recovery method.

	Weight of freeze-dried collagen (g)	Wet weight of raw material (g)	Dry weight of raw material (g)	Total weight of AA+P solution (g)	Weight of AA+P solution of batch X (g)
SFC IP A	0,2457	100	31,77	157	39,8
SFC IP B	0,2103	100	31,77	157	39,8
SFC SO C	0,2821	100	31,77	157	37,6
SFC SO D	0,3081	100	31,77	157	39,8
LFC IP A	0,2285	100	13,09	527,1	133,02
LFC IP B	0,2828	100	13,09	527,1	133,02
LFC SO C	0,4054	100	13,09	527,1	128,04
LFC SO D	0,412	100	13,09	527,1	133,02

8. Figures

9. Tables

10. Abbreviations and acronyms

AA = acetic acid
ASC = acid-soluble collagen
BSA = bovine serum albumin
C = collagen
ECM = exracellular matrix
EDTA-4Na = tetrasodium ethylenediaminetetraacetic acid
ER = endoplasmatic reticulum
FACIT = fibril-associated collagens with interrupted triple helices
FTIR = Fourier transform infrared spectroscopy
GAG = glycosaminoglycan
HA = hydrolyzed collagen
HC = hydrolyzed collagen
ICP-MS = inductively coupled plasma mass spectrometry
IP = isoelectric precipitation
LF = lumpfish
MC = moisture content
NC = non-collagenous
NCP = non-collagenous proteins
P = pepsin
PSC = pepsin-soluble collagen
RNA = acido ribonucleico
SDS-PAGE = sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SF = starfish
SO = salting out
TE = tissue engineering
UV = ultraviolet

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12. Acknowledgements

This project was conducted as part of the European BlueCC project that studies the commercial exploitation of collagen and chitin obtained from marine sources. The project is led by Nofima – the Norwegian Institute of Food, Fisheries and Aquaculture Research.
This work, in particular, was carried out at the Division of Food and Nutrition Science of the Department of Biology and Biological Engineering at Chalmers University of Technology (Göteborg, Sweden) and was co-funded by the Erasmus+ programme of the European Union (number of agreement 577 /072020).

I would like to express my sincere gratitude to my supervisors Mehdi Abdollahi of Chalmers University of Göteborg and Cristina Pagliano of Politecnico of Torino. Prof. Abdollahi gave me the opportunity to explore such a particular and fascinating field and he kindly followed and supported me during this journey. Thank you for all the suggestions and for trusting me. Prof. Pagliano helped me through the development of the thesis work and the results analysis, always encouraging me and constantly guaranteeing her availability.

Moreover, I want to offer my special thanks to Dr Naveen Kumar Vate of Chalmers University, who helped me in my first lab experience and who followed me during the experiments.

Thanks to Chalmers University of Technology for hosting me and for providing me with all the lab equipment I needed and thanks to the “marine group” of the Division of Food and Nutrition Science, meeting all of you was such an honor and I will never forget the time spent in the lab together.

Finally, I am grateful for all the love and the care coming from my family and my friends. You make my heart full, and you are the driving force that always had and forever will push me forward. Thank you for your constant and essential presence in my life.

Ithaka

As you set out for Ithaka
hope your road is a long one,
full of adventure, full of discovery.
Laistrygonians, Cyclops,
angry Poseidon—don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare excitement
stirs your spirit and your body.
Laistrygonians, Cyclops,
wild Poseidon—you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Hope your road is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you enter harbors you're seeing for the first
time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind—
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to learn and go on learning from their scholars.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have
fooled you.
Wise as you will have become, so full of
experience,
you'll have understood by then what these
Ithakas mean.

C.P. Cavafy

Ιθάκη

Σὰ βγεῖς στὸν πηγαιμὸ γιὰ τὴν Ἰθάκη,
νὰ εὔχεσαι νἆναι μακρὺς ὁ δρόμος,
γεμάτος περιπέτειες, γεμάτος γνώσεις.
Τοὺς Λαιστρυγόνας καὶ τοὺς Κύκλωπας,
τὸν θυμωμένο Ποσειδῶνα μὴ φοβᾶσαι,
τέτοια στὸν δρόμο σου ποτέ σου δὲν θὰ βρεῖς,
ἂν μέν᾽ ἡ σκέψις σου ὑψηλή, ἂν ἐκλεκτὴ
συγκίνησις τὸ πνεῦμα καὶ τὸ σῶμα σου ἀγγίζει.
Τοὺς Λαιστρυγόνας καὶ τοὺς Κύκλωπας,
τὸν ἄγριο Ποσειδώνα δὲν θὰ συναντήσεις,
ἂν δὲν τοὺς κουβανεῖς μὲς στὴν ψυχή σου,
ἂν ἡ ψυχή σου δὲν τοὺς στήνει ἐμπρός σου.

Νὰ εὔχεσαι νά ῾ναι μακρὺς ὁ δρόμος.
Πολλὰ τὰ καλοκαιρινὰ πρωϊὰ νὰ εἶναι
ποὺ μὲ τί εὐχαρίστηση, μὲ τί χαρὰ
θὰ μπαίνεις σὲ λιμένας πρωτοειδωμένους·
νὰ σταματήσεις σ᾽ ἐμπορεῖα Φοινικικά,
καὶ τὲς καλὲς πραγμάτειες ν᾽ ἀποκτήσεις,
σεντέφια καὶ κοράλλια, κεχριμπάρια κ᾽ ἔβενους,
καὶ ἡδονικὰ μυρωδικὰ κάθε λογῆς,
ὅσο μπορεῖς πιὸ ἄφθονα ἡδονικὰ μυρωδικά.
Σὲ πόλεις Αἰγυπτιακὲς πολλὲς νὰ πᾷς,
νὰ μάθεις καὶ νὰ μάθεις ἀπ᾽ τοὺς
σπουδασμένους.

Πάντα στὸ νοῦ σου νἄχῃς τὴν Ἰθάκη.
Τὸ φθάσιμον ἐκεῖ εἶν᾽ ὁ προορισμός σου.
Ἀλλὰ μὴ βιάζῃς τὸ ταξείδι διόλου.
Καλλίτερα χρόνια πολλὰ νὰ διαρκέσει.

Καὶ γέρος πιὰ ν᾽ ἀράξῃς στὸ νησί,
πλούσιος μὲ ὅσα κέρδισες στὸν δρόμο,
μὴ προσδοκώντας πλούτη νὰ σὲ δώσῃ ἡ Ἰθάκη.
Ἡ Ἰθάκη σ᾽ ἔδωσε τ᾽ ὡραῖο ταξίδι.
Χωρὶς αὐτὴν δὲν θἄβγαινες στὸν δρόμο.
Ἄλλα δὲν ἔχει νὰ σὲ δώσει πιά.

Κι ἂν πτωχικὴ τὴν βρῇς, ἡ Ἰθάκη δὲν σὲ γέλασε.
Ἔτσι σοφὸς ποὺ ἔγινες, μὲ τόση πείρα,
ἤδη θὰ τὸ κατάλαβες ἡ Ἰθάκες τί σημαίνουν.

Κ.Π. ΚΑΒΑΦΗΣ