REPORT: DEVELOPMENT OF COLLAGEN EXTRACTION METHODS FROM FISH WASTE

An outline of the collagen extraction methods from Atlantic Blue Fin Tuna (Thunnus thynnus) optimised between October 2019 and March 2020.

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1. INTRODUCTION

1.1. COLLAGEN

Collagen, being a major structural protein in animal skin and bone connective tissue, is the most abundant protein in vertebrates, constituting about 30% of the total proteins (Foegeding, Laneir and Hultin, 1996; Gelse, Poschl, and Aigner, 2003; Singh et al., 2001). Presently, 29 genetically distinct collagen types have been so far identified, with each type differing considerably in the amino acid combination, sequence, structural and functional properties.

Conventionally, collagen has been isolated from the skins of mammalian sources i.e. bovine and porcine with collagen having a broad range of applications primarily in the food, biomedical, pharmaceutical and cosmetic industries owing to its unique properties of biodegradability and weak antigenicity (Ogawa et al., 2004; Kittiphattanabawon et al., 2010). In spite of the extensive applications of mammalian-derived collagen, the outbreak of infections such as transmissible spongiform encephalopathy (TSE), bovine spongiform encephalopathy (BSE), avian influenza and foot-and-mouth disease (FMD) have rendered the reconsideration of such collagen sources in recent years. In addition, collagen of porcine origin is increasingly being rejected due to religious implications. Such reasons have contributed to the need to develop collagen extraction methods from alternative sources. Among such alternatives is the extraction of collagen from marine sources such as fish which provide the desired raw matter for collagen extraction due to the raw material's availability, impose no risk of disease transmission, and creating no religious barriers while offering high collagen yield (Nagai et al., 2001; Jongjareonrak et al., 2005).

1.2. FISH WASTE & THE SITUATION IN MALTA

Globally, fish processing industries generate 50-70% of the original material as by-products (head, skin, bone, scale, offal, viscera) and are often utilised as feedstuff with low value or merely discarded, despite their variety of active components that can be recovered if properly treated, having high-value applications (Kittiphattanabawon et al., 2005). Additionally, the improper by-product disposal can have serious environmental repercussions. Consequently, the optimised use of such by-products especially for the preparation of high value-added products is increasingly being recognised as promising means to mitigate the consequences of disposal while increasing the revenue of producers.

The general waste management options in Malta are rather constrained creating increasing pressures to adopt marine dumping activities. In an effort to assess the dumping activities in Malta in 2005, it was recorded that amongst the waste dumped at sea, including dredge materials and inert geological waste, fish waste arising from fish processing were estimated at 450 tonnes per year and were predicted to increase and exceed 1000 tonnes per year (Axiak, 2005). Recent statistics are however lacking.

The production of fish waste on the Maltese Islands is mainly generated from coastal tuna penning activities which is generally discarded outside territorial waters, however it has been increasingly reported that due to the illegal dumping of such offal, the southern coastline of Marsacala to Delimara has been affected.

In light of this, the consideration of the potential use of fish waste as means of collagen extraction can therefore be explored to alleviate the waste management activities in Malta and the Mediterranean while simultaneously rendering high-value added products and hence offering economic benefits.

1.3. Methods of Collagen Extraction

Collagen extraction from seafood sources involves two main steps, the pre-treatment of raw material and the collagen extraction processes. Prior to pre-treatment, the raw materials are separated from the group of different by-product matter such as skin, scales and bone and in some cases muscle.

1.3.1. Pre-treatment

Pre-treatment involves the cleaning and size-reduction of raw material to facilitate the contaminant removal and maximise the quality of the collagen being extracted. The raw material used for collagen preparation contains non-collagenous lipids, protein and pigment, while bone and scale constitute calcium and other inorganic material. A widely used method for the removal of non-collagenous proteins and pigments from the raw material is alkaline pre-treatment with 0.1 M NaOH. Subsequently, raw materials are effectively demineralised removing calcium and other inorganic matter by the use of EDTA. Devoid of non-collagenous proteins and minerals, the raw material may be then defatted with butyl alcohol treatment (Zhou and Regenstein, 2007; Singh et al., 2012; Pal et al., 2015).

1.3.2. ACID SOLUBILISATION EXTRACTION

The acid solubilisation method has been widely considered as the pioneering collagen method extraction from fish species by various researchers (Duan, Zhang, Du, Yao and Konno, 2009; Liang et al., 2014; Wang et al., 2014; Pal et al., 2015). In this method, collagen is extracted using an acidic solution (most commonly acetic acid but other organic acids such as citric acid, chloroacetic acid and lactic acid may be used) with the resultant collagen being referred to as acid-soluble collagen (ASC). The collagen molecule is rendered a positive charge in acidic condition thereby increasing its solubilisation.

The yield of the extracted collagen has been reported to depend on several factors and parameters, such as species, acid concentration and its ratio to raw material, extraction temperature and time. Generally, fish-derived collagen is achieved at low temperatures (as low as 4 °C) for 24-48 hour. Increasing concentration and proportion of acid, the extraction temperature and time may offer higher yield of collagen, although high temperatures may bring about undesirable collagen degradation.

1.3.3. ENZYMATIC SOLUBILISATION EXTRACTION

Conventionally, collagen is extracted using the acid solubilisation without the aid of an enzyme. However various studies report that the collagen from by-products was not completely solubilised with acid solubilisation extraction alone (Liang et al., Wang et al., Pal et al., 2015). In order to maximise the collagen yield from acid-solubilisation extraction, the enzymatic extraction has been developed incorporating the use of various enzymes namely pepsin, trypsin and collagenase, with pepsin being the most widely used. The resultant pepsin extracted collagen is referred to pepsin soluble collagen (PSC).

Pepsin is able to facilitate collagen extraction following acid solubilisation for various reasons. Firstly, pepsin has the ability to hydrolyse non-collagenous protein with the hydrolysate being easily removed by salt precipitation followed by dialysis to recover the collagen. Pepsin may also hydrolyse collagen telopeptides making the collagenous protein readily soluble in the acid solution thereby resulting in an overall improvement of the extraction efficiency. In addition, the pepsin's hydrolysis may reduce the antigenicity caused by telopeptides in collagen thereby circumventing a significant problem often faced in food and pharmaceutical applications (Duan et al., 2009; Matmaroh et al., 2011; Benjakul et al., 2012; Wang et al., 2014).

1.3.4. COLLAGEN RECOVERY

Following the aforementioned extraction processes, collagen is typically recovered by means of salt precipitation, centrifugation, dialysis and lyophilisation. The extracted collagen is generally precipitated using NaCl in the presence of tris(hydroxymethyl) aminomethane at *p*H 7.5 with salt concentrations adjusted to maximise the removal of impurities and optimise the collagen recovery. The resultant precipitate is then dissolved in a minimal volume of acetic acid and then dialysed to remove salts against distilled water. The collected dialysate is then lyophilised (freeze-dried) and stored at cool temperatures (Pal et al., 2015, Benjakul et al., 2012).

1.4. PRINCIPAL RELATED WORK

A significant body of research on collagen extraction methods from fish species has been carried out where both extractions of acid solubilisation and pepsin solubilisation are explored.

Pioneering studies on acid solubilised collagen have been conducted by Nagai et al. (2000) and generally acid solubilised extraction methods employed by other research bodies are adapted from the methods reported by the latter. Acid solubilised collagen has been extracted from fish scales, skin, bones and fins resulting in varying degrees of yield attained primarily owing to the different hydroxyproline content and the degree of cross-linking in the collagen source. The methods employed by several research groups for the extraction of collagen ranging from 1.35% up to 63.40% yield from different collagen sources is summarised in *Table 1*.

Various studies from Table 1. indicate that the yield from the same sources may substantially vary depending on the tissue parts being treated. The skin collagen yield from bigeye snapper was 10.90% while that of bone collagen of the same species was 1.60%. The authors suggest that this discrepancy in the amount of yield extracted from different parts of the same species is attributed to the higher hydroxyproline content in skin than in bone (Kittiphattanabawon et al., 2005). Similarly, the collagen yielded from skin of grass carp varied from 0.70% in skin and 25.5% in bone (Wang et al., 2014).

Another factor accounting for the differences in yield attained is the fish species which have been reported to have differences in amino acid composition thereby affecting the collagen structure. Kittiphattanabawon et al. (2005) suggest that bone collagen has a higher degree of structural complexity due to the higher extent of hydroxylation of the amino acids. Several fish species have yielded very low amounts of collagen extraction

with yields as low as 1-2% (Kittiphattanabawon et al., 2005; Duan et al., 2009) when relative to the high yields of 50-60% reported (Nagai and Suzuki, 2002; Pal et al., 2015). This may be attributed to the effect of structural cross-linking on the extent of solubility of collagen in acid solutions. The collagen molecule is a helical structure except for the terminal ends of the molecule. Such terminals play a significant role in the cross-linking of the collagen molecule. Collagen solubility increases when collagen molecules are weakly cross-linked at the telopeptide region (Foegeding et al., 1996). Therefore, fish species that render low amounts of collagen yield are expected to have a higher degree of cross-linking relative to species yielding higher amounts of acid-solubilised collagen (Duan et al., 2009).

Table 1. Table of the Principal Literature Reviewed Categorised According to the Types of Fish Species Studied and their % Collagen Yield Attained.

Fish Source	Yield /%	Reference
Skin		
Black Drum	2.30	Ogawa et al., 2004
Sheepshead Seabream	2.60	Ogawa et al., 2004
Rohu	4.13	Hema et al. 2013
Mrigala	4.70	Mahboob, 2015
Dog Shark	8.96	Hema et al. 2013
Bigeye Snapper	10.94	Kittiphattanabawon et al., 2005
Spanish mackerel	13.68	Li et al., 2013
Albacore Tuna*	13.97	Hema et al. 2013
Channel catfish	24.00	Liu et al., 2007
Surf smelt	24.00	Nagai et al., 2010
Grass Carp	25.50	Wang et al., 2014
Yellow Fin Tuna*	27.10	Woo et al., 2008
Amur Sturgeon	37.42	Wang et al., 2014
Carp	41.30	Duan et al., 2009
Rohu	46.13	Pal et al., 2015
Chub Mackerel	49.89	Nagai and Suzuki, 2000
Bullhead Shark	50.10	Nagai and Suzuki, 2000
Japanese Sea Bass	51.40	Nagai and Suzuki, 2000
Catla	63.40	Pal et al., 2015
Spanish mackerel	13.68	Li et al., 2013
Bone		
Bigeye snapper	1.59	Kittiphattanabawon et al., 2005
Carp	1.06	Duan et al., 2009
Grass Carp	0.70	Wang et al., 2014
Spanish mackerel	12.54	Li et al., 2013
Japanese Sea Bass	40.70	Nagai and Suzuki, 2000
Skipjack Tuna*	42.30	Nagai and Suzuki, 2000
Yellow Seabream	40.10	Nagai and Suzuki, 2000
Scales		
Carp	1.35	Duan et al., 2009
Mrigala	3.20	Mahboob, 2015
Catla	3.90	Mahboob, 2015
Grass Carp	16.70	Wang et al. 2014
Red Seabream	37.50	Nagai et al., 2004
Japanese Sea Bass	41.00	Nagai et al., 2004
Sardine	50.90	Nagai et al., 2004
Fin		
Japanese Sea Bass	5.20	Nagai and Suzuki, 2000
Mrigala	5.70	Mahboob, 2015
Muscle		
Leather Jacket	46.48 - 50.24	Muralidharan et al., 2011

*Collagen yield from Tuna species

Pepsin solubilisation extraction methods do not vary greatly except for minor modifications in conditions such as extraction time. Several studies report that limited use of pepsin increase collagen yield from fish by-products (Jongjareonrak et al., 2005, Tamilmozhi et al., 2013; Wang et al., 2014; Liang et al., 2014; Pal et al., 2015; Mahboob 2015) The amount of yield obtained from different species types may be as low as 4% but may range up to over 50%, with the discrepancy often attributed to the differences in species. Some typical studies with a range in yield of pepsin-solubilised collagen include a yield of 4.70% PSC reported from brownstripe red snapper skin (Jongjareonrak et al., 2005), catla skin with a yield of 7.2% (Mahboob, 2015), largefin longbarbel catfish with a yield of 28% (Zhang et al., 2009) and amur sturgeon skin with a yield of 52.80% (Wang et al., 2014).

Some studies suggest that the pepsin solubilised collagen extraction yield was two-fold when relative to acid solubilised collagen. For instance, a yield of 55.92% of PSC from amur sturgeon cartilage was obtained relative to the 27.04% yield of ASC (Liang et al., 2014). However, reversed trends have been also reported where ASC rendered higher yield than PSC as seen in the collagen extraction from grass carp skin conducted by Wang et al., 2014, where yields of ASC and PSC attained where 25.5% and 19.8% respectively. The lower PSC yield was suggested to be due to higher percentage of pepsin being used for longer durations, which results in the complete cleavage of the collagen molecule impairing the triple helix's integrity.

1.5. Knowledge Gap

Although the study of collagen extraction methods from fish species has received significant global attention (as indicated in section 1.4), no studies focus primarily on the development of methods that will be conducive to industrial scale-up. In addition, despite the collagen extraction methods from the *Thunnus* genus already described, studies on collagen isolation from the Atlantic blue fin tuna *Thunnus thynnus* are lacking.

1.6. Remit of the Study

The principal aim of the present study is to implement and optimise collagen extraction methods from non-marketable fish waste of *Thunnus thynnus* that are conducive to industrial scale-up.

2. Materials & Methods

2.1. Selection of Extraction Methods

Collagen extraction methods were reviewed from the literature and selected primarily on the following criteria:

1) the ease with which such methods may be implemented outside the lab environment and hence proposed for industrial scale-up
2) the use of chemical reagents that pose no risks and are accepted for cosmetic production on the basis of the Inventory of Cosmetic Ingredients as amended by Decision 2006/257/EC establishing a common nomenclature of ingredients employed for labelling cosmetic products throughout the EU (*Appendix A*).
3) the least use of highly demanding conditions at which the procedure was carried out (such as high centrifugal speeds or prohibitively costly reagents in return for minimal changes to the purified extract)
4) the highest possible yield.

For such reasons, the methods tested and subsequently optimised are based on the methods described by Nagai and Suzuki (2000), Li et al. (2013) for ASC and PSC from bone and skin tissues and Ogawa et al. (2004) for muscle tissue, following the consultation of the methods provided by BYTHOS (*Appendix B*).

2.2. Raw Material

Fish waste from the processing of Atlantic blue fin tuna was provided from the Malta Aqua Culture Research Centre and stored at -20 °C. Fish samples were thawed at room temperature prior to the extraction procedure. Fish samples were manually skinned and bones were cut using a filleting knife and washed with distilled water.

2.3. Preparation of collagen from muscle, bone and skin

2.3.1. Muscle (ASC)

Acid solubilised collagen (ASC) was extracted using the method of Ogawa et al. 2004 with slight modification. The following procedures for the preparation of acid solubilised collagen were all carried out at ambient temperature except for over-night solution stirring which was carried out at -8 °C and centrifugation at 4 °C.

The muscle tissue was first treated with liquid nitrogen and was grinded cryogenically using mortar and pestle. For deproteinization, the muscles tissue was soaked in 10 volumes of 0.1M NaOH for 48 hours and stirred using a magnetic stirrer. The tissues were then re-soaked in 20 volumes of 0.1M NaOH and stirred for 24 hours. The insoluble components were repeatedly washed with distilled water for neutralisation of the pH and filtered through a cheese-cloth. The alkali-insoluble components were then extracted with 10 volumes of 0.5 acetic acid for four days. The solution was centrifuged at 10000 rpm for 20 minutes at 4 °C and the supernatant was retained. The remaining residue was re-extracted with 10 volumes of 0.5M acetic acid for 4 days. The extract was centrifuged at 10000 rpm for 20 minutes at 4 °C. The supernatants of both extracts were combined and salt-precipitated by the addition of NaCl, giving a final concentration of 0.9 mol/dm^{-3} and the solution was left overnight. The salted-out precipitate was collected by centrifugation at 10000 rpm for 20 minutes. The precipitates were re-dissolved in 10 volumes of 0.5M acetic acid and the procedure of salt-precipitation followed by solubilisation was repeated three more times. The resultant precipitate was dialyzed against 0.5M acetic acid and a sample of the dialysate was freeze dried for further analysis.

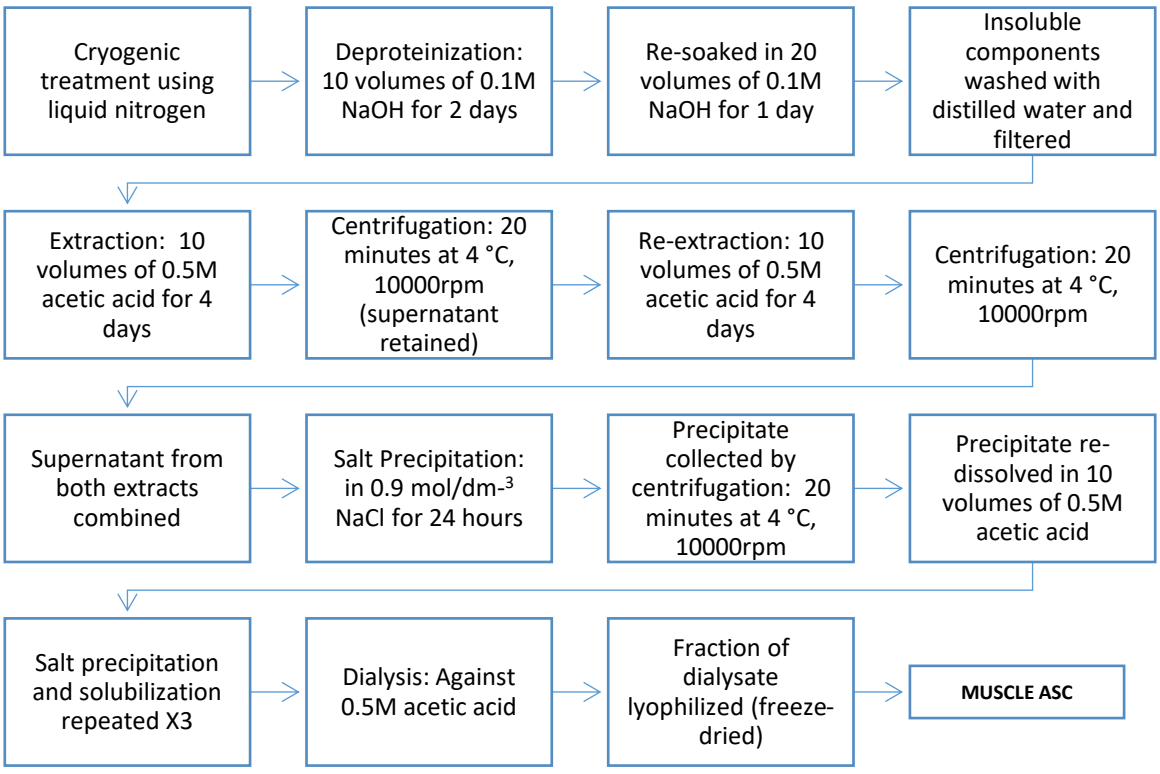

***Figure 1**. Method for Acid Solubilised Collagen Extraction from Muscle Tissue.*

2.3.2. BONE (ASC)

Acid solubilised collagen (ASC) was extracted from bone tissue using the method of Li et al., (2013) with slight modification. The following procedures were all carried out at ambient temperature except for centrifugation which was at 4 °C. 3. The muscle tissue was first treated with liquid nitrogen and was grinded cryogenically using mortar and pestle. For the removal of non-collagenous proteins, the manually cut/shredded bones were treated in 20 volumes of 0.1M NaOH for 48 hours with the solvent being changed every 24 hours. The alkaline-treated bones were washed with distilled water until a neutral pH was reached. For decalcification, the bone tissue was treated with 0.5M EDTA solution at pH 7.5 for 5 days. The residue was defatted in 20 volumes of 10% butyl alcohol for 48 hours and the solvent changed every 24 hours. The defatted bone tissue was washed with cold distilled water and extracted in 10 volumes 0.5M acetic acid for 3 days. The extract was centrifuged for 30 minutes at 4 °C. The mixture of residue and extract (supernatant) was filtered through cheese-cloth and the residue was re-extracted for 3 days and centrifuged. Both supernatants were combined. The undissolved bone residue was set as aside for further treatment. In the presence of 0.5M tris (hydroxymethyl)aminomethane at pH 7.5 the filtrate, NaCl (to a final concentration of 2.6M) was added to the filtrate to salt-precipitate the collagen and left overnight. The resultant precipitate was dialysed against 0.1M acetic acid for 48 hours followed by dialysis against distilled water for 48 hours, with changes in solution every 24 hours. The dialysate was referred to ASC from bone (ASC-B).

2.3.3. BONE (PSC)

The undissolved bone residue following both acetic acid extractions that was previously set aside was then suspended in 10 volumes of 0.5 M acetic acid and 6 mg porcine pepsin (20 units/g residue). The mixture was stirred at -8 °C for 3 days. The PSC from bone (PSC-B) was recovered by the same method used for ASC-B.

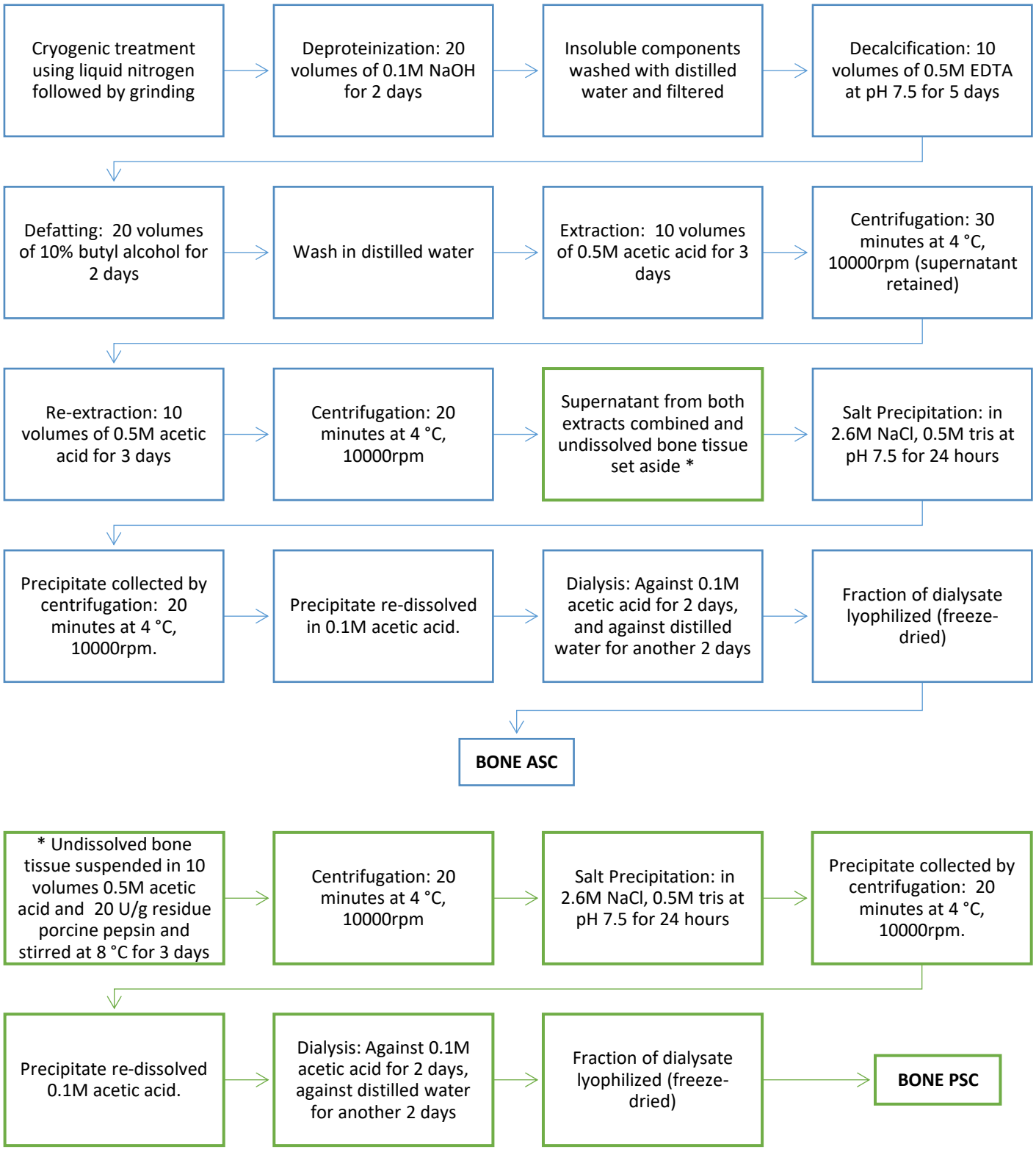

Figure 2. *Method of Acid Soluble Collagen Extraction (Blue) and Pepsin Soluble Collagen (Green) Extraction from Bone Tissue.*

2.3.4. SKIN (ASC)

(*Incomplete extraction: Lab practice interrupted due to COVID-19 situation)*

Acid solubilised collagen (ASC) was extracted from skin using the method of Li et al. 2013 with slight modifications. The following procedures for the preparation of acid solubilised collagen were all carried out at ambient temperature except for centrifugation which was at 4 °C.

For deproteinization, the prepared skin tissue was suspended in 10 volumes of 0.1M NaOH for 48 hours and stirred using a magnetic stirrer. The tissues were then re-soaked in 20 volumes of 0.1M NaOH and stirred for 48 hours and the alkali solution was changed every 12 hours. The insoluble components were repeatedly washed with distilled water until the pH was neutralised and filtered through a cheese-cloth. The alkali-insoluble components were then de-fatted in 20 volumes of 10% butyl alcohol for 2 days. The defatted tissue was then extracted with 15 volumes of 0.5 M acetic acid for 24 hours days. The solution was centrifuged at 10000 rpm for 20 minutes at 4 °C and the supernatant was retained. The remaining residue was re-extracted with 10 volumes of 0.5M acetic acid for 4 days. The extract was centrifuged at 10000 rpm for 20 minutes at 4 °C. The supernatants of both extracts were combined and salt-precipitated by the addition of NaCl, giving a final concentration of 2.6 M in the presence of tris (hydroxymethyl) aminomethane at pH 7.5 and the solution was left was 2 days.

> *Comment: The precipitate that had salted-out was low, presumably due to very low starting skin material that could be skinned from the fins provided, therefore the method was re-done but was carried out till the re-extraction with acetic acid, before lab practice was stopped altogether due to the COVID-19 situation.*

The resultant precipitate was to be collected by centrifugation at 10000 rpm for 30 minutes at 4 °C and dissolved in minimal volumes of 0.5M acetic acid to be then dialysed first against acetic acid for 2 days and then against distilled water for 2 days. A sample of the dialysate was to be then freeze-dried to be sent for further analysis.

2.3.5. SKIN (PSC)

For pepsin-solubilised collagen from skin (PSC-S) the residue that was not dissolved following both re-extraction with acetic acid was to be suspended in 15 volumes of 0.5 M acetic acid and porcine pepsin (20 U/g residue) and the mixture to be stirred for 2 days. Following centrifugation, the filtrate is to be subject to salt precipitation, dialysis and freeze-drying under the same conditions outlined for ASC from skin.

All lyophilised samples of all extracts are to be sent for analysis to determine collagen quality.

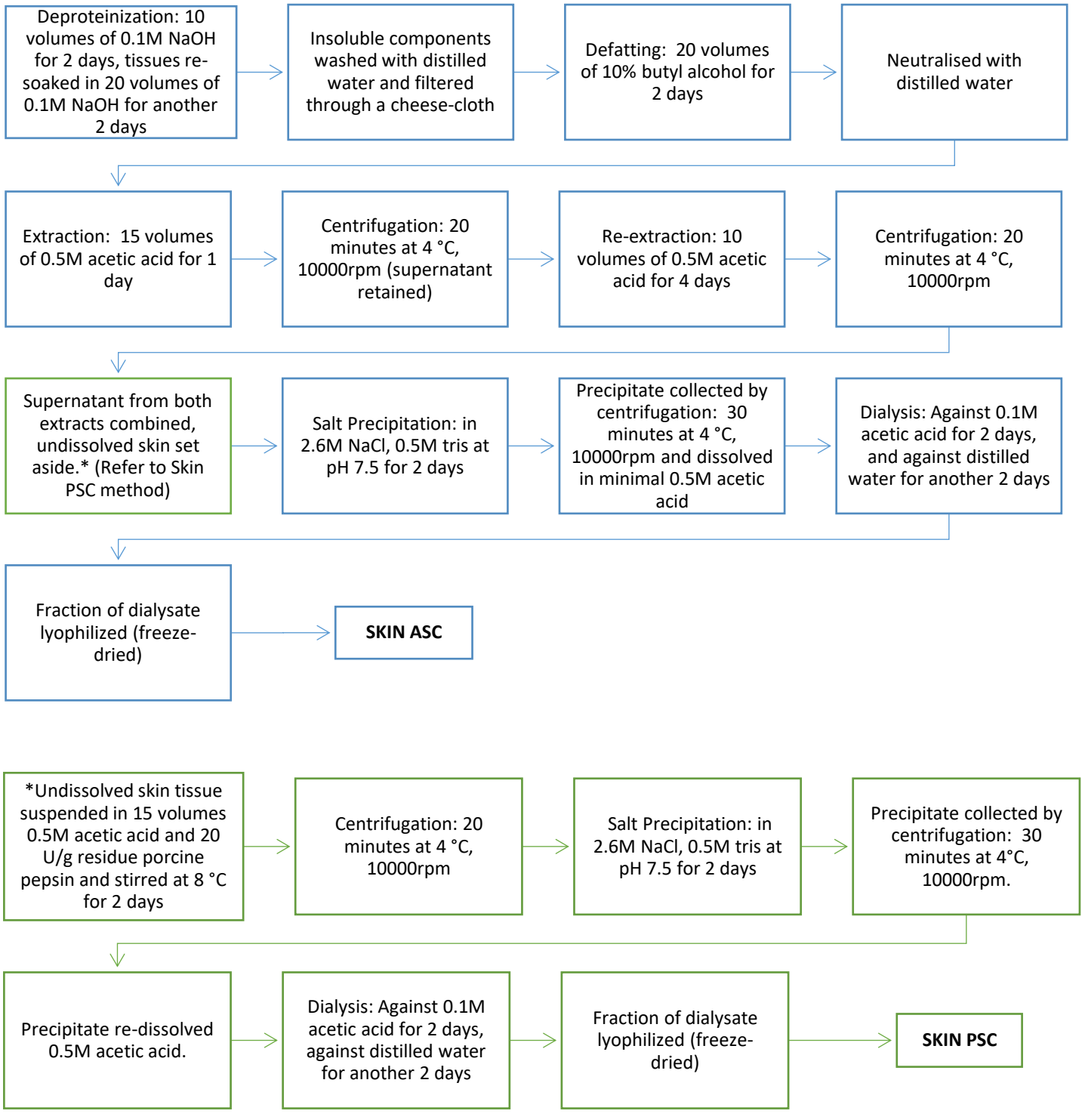

Figure 3. *Method of Acid Soluble Collagen Extraction (Blue) and Pepsin Soluble Collagen (Green) Extraction from Skin Tissue.*

3. RESULTS

Table 2. Table of Results

	Muscle	Bone		Skin
		ASC	PSC	
Starting weight of Sample /g	95	146.2		3.8
Weight of Lyophilized Extract /g	16.3	39	47.6	-
Volume of Extract /ml	32.5	272	50	-
Yield /%	**17.16**	**26.68**	**32.56**	**-**

Acid soluble collagen from muscle and bone were extracted with yield of 17.16% and 26.68% respectively (expressed as a percentage of dry weight). The use of pepsin has increased yield as expected to 32.56% for bone.

The yield achieved from muscle tissue is comparably low when relative to the yield obtained from muscle of *Odonus niger* by Muralidharan et al., (2011) which was within the range of 46.48 - 50.24%.

The acid soluble collagen extracted from bone is low when relative to the 42.30% yield extracted from Skipjack tuna bone (Nagai and Suzuki, 2000), but significantly higher than the percentage yield obtained from grass carp (0.70%) carp (1.06%) bigeye snapper (1.59%) and spanish mackerel (12.54%) (Wang et al., 2014; Duan et al., 2009; Kittiphattanabawon et al., 2005; Li et al., 2013).

The pepsin soluble collagen extracted from bone has increased yield as reported by multiple pioneering studies which report percentage yield increases ranging from 4% up to over 50% depending on the species in question (Jongjareonrak et al., 2005, Tamilmozhi et al., 2013; Wang et al., 2014; Liang et al., 2014; Pal et al., 2015; Mahboob 2015).

(Refer to Appendix C for pictures of extract dialysates)

4. DISCUSSION/FORTHCOMING OBJECTIVES

Pending procedures:

- Pepsin solublisation extraction of muscle from the resultant dialysate of ASC.
- Completion of ASC and PSC from skin
- Protein content determination
- SDS-PAGE
- Once an indication of the quality of collagen is obtained, the methods so far used can be modified to increase yield and improve quality.
    - Factors affecting yield include:
        - ***Temperature****:* Extraction temperatures have been carried out at sub zero temperatures for the most part. Increasing the temperature for up to an optimum level of 27 °C is expected/ suggested to improve yield (Fehng, 2016). However, care must be taken to avoid collagen denaturation due to the thermo-instable nature of collagen owing to its chemical structure.
        - ***Extraction time:*** *Similarly**,** extraction time should not be extended beyond 72 hours (with a change in solution every 12/24hrs)*.
        - ***Concentration of acetic acid:*** *, The concentration of acetic acid used was 0.5M. A positive correlation is suggested between acetic acid concentration and collagen yield up to an optimal concetration of 0.63M, beyond which collagen yield is expected to decrease (Fehng, 2016).*
        - ***Concentration of pepsin****: it is reported that low concentrations with extended extraction time yield higher collagen quality when compared to the use of high pepsin concentrations (*Benjakul et al., 2012)*.*

- Once fully optimised, methods are to be disseminated with San Lucjan.
- Look into other alternatives source species such as jellyfish as proposed during SC meeting (dated 11.02.2020).

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6. APPENDICES

A. ***Hyperlink:*** **Inventory of Cosmetic Ingredients** as amended by Decision 2006/257/EC establishing a common nomenclature of ingredients employed for labelling cosmetic products throughout the EU.

B. Collagen Extraction Protocol Provided by Bythos:

1. COLLAGEN EXTRACTION PROTOCOL

From skin

- washing of the skins with chilled (5°C) water for a period of 10 min
- The skins were then washed with 0.8 M NaCl for three time of 10 min each, followed by rinsing in running water
- Collagen was then extracted using 0.5 M acetic acid solution 1 g of skin per 20 ml of 0.5 M acetic acid
- The collagen solution was then centrifuged and salt solution (0.9 M NaCl) was added to the supernatant to precipitate the collagen
- The precipitated collagen (acid soluble collagen) was separated by centrifugation at 27,000 g for 30 min.
- was re-dissolved in acetic acid and re-precipitated as described above.

2. COLLAGEN EXTRACTION PROTOCOL

From skin

- Mincing skin: 3 mm
- Stirring 2 h at 0° with 0.1 - 0.5 M CH_3COOH, at a ratio of 1:6 - 1:4
- Homogenising at 0°C
- Stirring 24 h 4°C
- Homogenising 2 min 0°C
- Centrifuging 20 min, 10000 g 10°C
- Insoluble material
- Soluble collagen

3. COLLAGEN EXTRACTION PROTOCOL

From Skin, Tissue, muscle

- Washing samples with water at 4°C
- 0.3 % H_2O_2 1:1, 5 min, Disinfection
- Washing with water at 4°C
- Pressing 4°C 3 times:
- Add Citric acid solution 4:1 ph= 2,2
- Extraction for 24 h, 4°C
- Filtration
- Insoluble collagen
- Soluble collagen

4. COLLAGEN EXTRACTION PROTOCOL

From Fish scale

- Pretreating with NaOH to remove Non-collagenous protein
- Washing
- Drying
- Grinding
- Preconditioning Scales with distilled water, citric acid or acid acetic solution
- Extruding
- Drying
- Grinding
- Hydro-extracting
- Lyophilizing
- Collage

5. COLLAGEN EXTRACTION PROTOCOL

From Fish scales

- washed twice in 10 wt% of NaCl solutions
- stirring the solution for 24 h
- Demineralization was achieved with 0.4 mol/l HCl solution (dry scales: solution = 1:15) for 90 min
- washed three times with distilled water for collagen extraction
- All the preparative procedures were performed at 4˚C.
- 0.5 M acetic acid for 2 days
- extracts were centrifuged at 10,000× g for 30 min.
- residues were re-extracted with the same solution for 1 day
- extracts were centrifuged at 10,000× g for 30 min
- supernatants were combined and salted out by adding NaCl to a final concentration of 0.7 M
- precipitated collagens were separated by centrifugation at 10,000× g for 30 min
- re-dissolved in 0.5 M acetic acid to precipitate with NaCl again.
- The resultant precipitates were dialyzed against distilled water and lyophilized
- The residues from the acetic acid extraction were suspended in 0.5 M acetic acid and digested with 0.5% (w/v) pepsin (Sigma p7000) for 72 h.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

- 5% stacking gel and 7.5% resolving gel
- The samples were dissolved in 0.6 M Tris-HCl buffer (pH 6.8) which contained 25% (v/v) glycine, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.1% (w/v) Bromophenol blue
- After electrophoresis, gels were visualized with Coomassie Brilliant Blue R-250.

C. **Other Images**

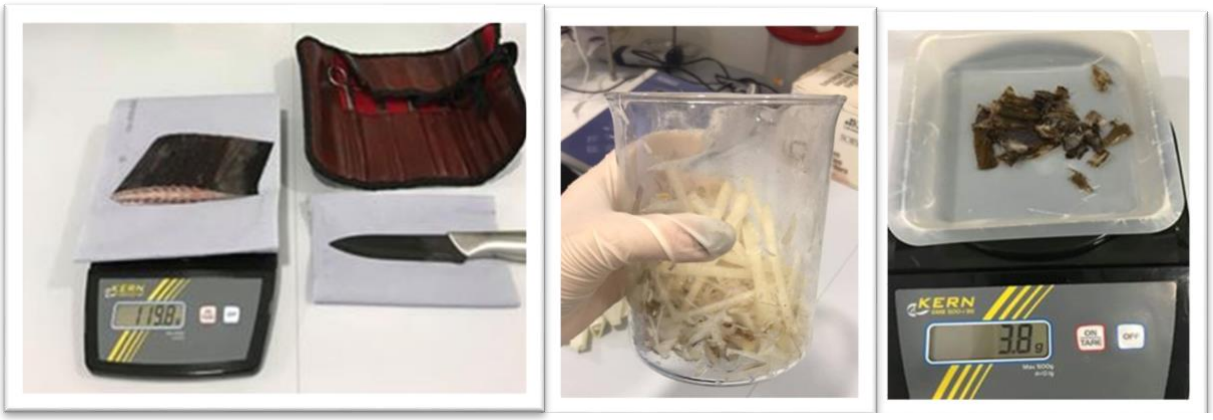

Figure 2. Image of starting raw material. *Left*: fish parts as obtained from source, *Middle*: Bone prior to pre-treatment. *Right: Skin prior to pre-treatment.*

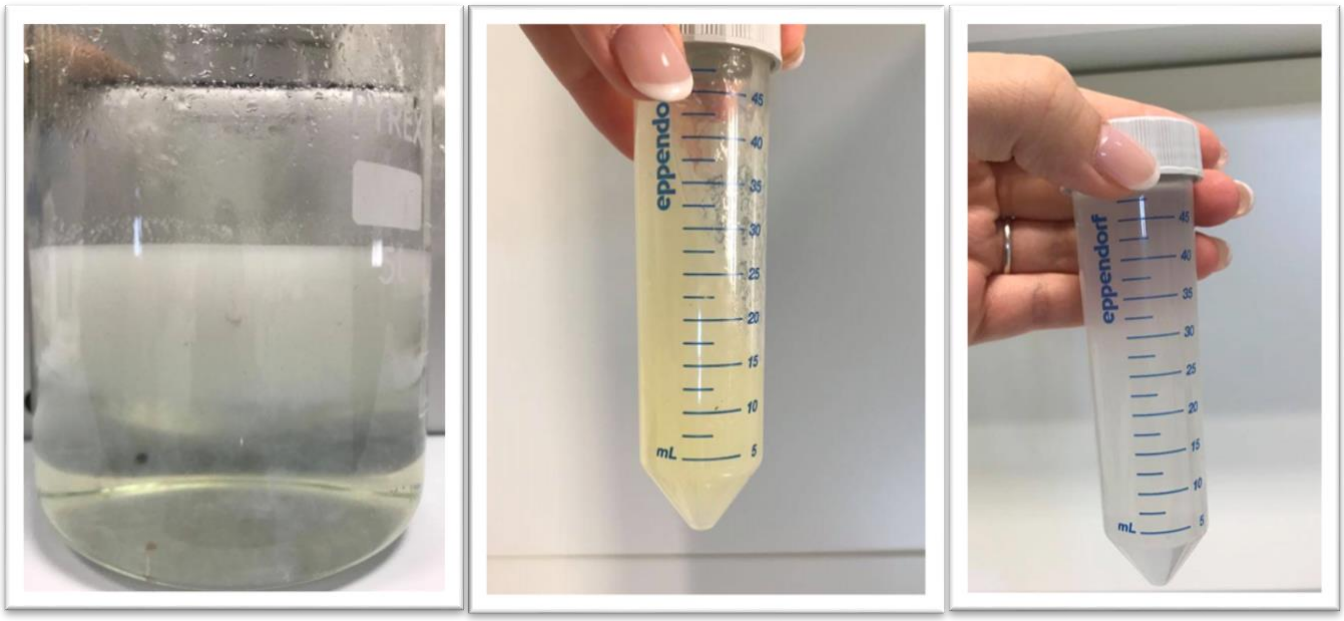

Figure 3 *Left*: Salt precipitation of acid solubilised collagen from bone, *Middle*: Acid solubilised collagen dialysate from muscle, *Right*: Pepsin solubilised collagen dialysate from bone.