



Seafood Pilot Plant



Aquaculture Facility



Bioprocessing Pilot Plant



Bioprocessing Laboratory

## Utilizing Byproducts of Atlantic Sea Cucumber towards High-Value Nutraceutical Applications

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## **Utilizing Byproducts of Atlantic Sea Cucumber towards High-Value Nutraceutical Applications**

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## EXECUTIVE SUMMARY

Sea cucumber (SC) is a valuable seafood, particularly in Asia, priced both as an exotic and costly delicacy and its medicinal and tonic properties. The body wall and muscle bands of SC, the most traded form, are harvested and sold off, while the aquapharyngeal bulbs (flowers) are separated from the whole SC as byproducts or low-valued products. The internal organs or viscera, including intestines, gonads, respiratory trees and water inside the body cavity, constitute about 50% of the whole SC weight but are often discarded as waste. This disposal leads to resource wastage and environmental contamination due to the presence of heavy metals, especially arsenic content (Muhsin et al., 2023). Flowers and viscera of *Cucumaria frondosa* are rich in nutrients and bioactive compounds (omega-3, sulphated polysaccharides, phenolics), which have shown the potential to mitigate chronic diseases, boost immune systems and exhibits significant biological and pharmacological activities, including anticancer anti-inflammatory and anti-ageing effects (Al Shemali et al., 2016; Hossain et. al., 2020; Liang et al., 2022; Tangrodchanapong et al., 2020). Those are excellent sources of essential amino acids, vitamins, and minerals and require no fortification to develop high-quality nutraceutical products for pets or humans (Hossain et. al., 2023; Liu et al., 2021). However, further research is necessary to analyze their nutritional and bioactive properties. The client (Green Seafoods Ltd.) proposed to analyze commercially processed SC byproducts and evaluate their qualities to develop high-value pet nutraceuticals.

SC flowers and viscera were collected from the commercially processed SC on day 1 (CPSC-1) and day 3 (CPSC-3), and from manually processed live (Live-SC) and revived (Rev-SC) SC. The Live-SC were separated immediately after the catch and stored in the seawater to release their stress, while Rev-SC were collected after 24 hours of vessel hold and revived in running seawater. Byproducts were subjected to heat treatment (boiling or steaming) and dried using hot-air or freeze-drying systems to study nutritional and bioactive properties. The results showed that the nutritional composition of SC flowers and viscera varied. The results showed that the nutritional composition of SC flowers and viscera varied. Live-SC and Rev-SC flowers contained higher protein (>60% dry weight basis, dwb) and lower lipid (<3.8%) levels compared to commercially processed flowers (>50% protein, >20% lipids, dwb). Boiling enhanced protein content (>70%) across all samples while reducing ash content. For lipids, boiling increased levels in Live-SC and

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Rev-SC but decreased them in commercially processed samples. Phospholipid content was higher in boiled CPSC-3 flowers than in steamed ones, making boiled byproducts suitable protein supplements for pets.

SC viscera contained over 50% protein and higher lipid content (>20% dwb) than flowers. Heat treatment concentrated protein content in commercially processed viscera, with boiling and steaming yielding similar results. However, steaming reduced lipid content in Live-SC and CPSC-3 viscera. Lipid composition analysis revealed that SC viscera (15.8–33.5 mg/g) had higher total lipids than flowers (7.8–14.3 mg/g). Polyunsaturated fatty acids (PUFAs), particularly omega-3 eicosapentaenoic acid (EPA), were predominant in both flowers (25.9–33.5%) and viscera (22.5–25.0%), offering numerous health benefits for human or pets.

SC byproducts were also rich in minerals (calcium, sodium, potassium, magnesium) and trace elements (zinc, manganese, selenium). Boiling reduced major elements except calcium in Live-SC, while zinc and manganese levels remained stable. Vitamins A, B1, B3, and B5 were higher in steamed flowers, whereas boiling reduced water-soluble vitamins. Heavy metals, including arsenic, chromium, and lead, were present below regulatory limits, with boiling further reducing their concentrations. This project analyzed microbial activity and heavy metal content in byproducts. Microbial contamination can cause spoilage and health risks to pets, with recommended limits of  $10^6$  CFU/g in pet food (Kukier et al., 2012; Serhan et al., 2022). While fresh SC viscera had higher microbial activity but remained within acceptable levels. Toxic heavy metals like chromium, cadmium, lead, mercury, and tin were below the maximum tolerable limit (MTL) for pets like cats and dogs. Although the arsenic level was close to the MTL, boiling reduced it by >15% in SC flowers. Steamed byproducts retained more phenolics compared to boiled ones, as steaming preserved water-soluble components. These findings highlight the potential of SC flowers and viscera as nutraceutical ingredients rich in protein, lipids, essential minerals, vitamins, and bioactive compounds.

In summary, SC byproducts, particularly from Live-SC and commercially processed samples, show promise for pet nutraceutical applications. The nutritional quality of commercially processed flowers, with their high protein and lipid composition, makes them an excellent candidate for high-value product development.

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## ACRONYMS/ABBREVIATIONS

CASD: Centre for Aquaculture and Seafood Development

CPSC-1: Commercially processed sea cucumbers on day 1

CPSC-3: Commercially processed sea cucumbers on day 3

DHA: Docosahexaenoic

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

dwb: Dry weight basis

EPA: Eicosapentaenoic acid

FD: Freeze-drying

Live-SC: Live sea cucumbers

MTL: Maximum tolerable limit

MUFA: Mono-unsaturated fatty acids

OD: Hot-air drying in oven dryer

PUFA: Polyunsaturated fatty acids

Rev-SC: Revived sea cucumbers

SC: Sea cucumber

SFA: Saturated fatty acids

wwb: Wet weight basis

## 1 BACKGROUND AND INTRODUCTION

Sea cucumbers (SCs) are a high-value seafood in Southeast Asia, prized for their nutritional and health benefits. The Atlantic species *Cucumaria frondosa* is particularly noted for its biological and pharmacological properties, including anticancer effects and benefits for hyperglycemia, inflammation, Alzheimer's, aging, and stress (Al Shemali et al., 2016; Attoub et al., 2013; Liang et al., 2022; Ma et al., 2012; Tangrodchanapong et al., 2020). Its compounds, including eicosapentaenoic acid (EPA), saponins, and chondroitin sulfate, aid in diabetes treatment by reducing serum glucose and improving insulin resistance (Han et al., 2019; Zhang et al., 2020).

Green Seafoods Ltd. processes thousands of tons of live SCs annually, generating a few hundred tons of raw flowers (aquapharyngeal bulbs) as byproducts and viscera as waste materials. Flowers are dried and exported at very low prices. Despite this, flowers contain more nutrients, bioactive compounds (phenolics, flavonoids, carotenoids), and antioxidant and antidiabetic activities compared to body walls (Hossain et al., 2023; Senadheera et al., 2021, 2022). SC flowers have significant potential to produce high-value functional foods and nutraceuticals for both pets and humans, as they are rich in essential amino acids, vitamins, minerals, and antioxidants without needing fortification (Hossain et al., 2023; Liu et al., 2021). Disposal of viscera waste incurs high costs and poses environmental risks if not managed properly (Babji et al., 2020). However, dried SC viscera are rich in protein (>50%), lipids (~22%), and bioactive compounds like polysaccharides, saponins, chondroitin sulphates etc. (Babji et al., 2020; Liu et al., 2021). The high omega-3/omega-6 ratio in viscera lipids can help mitigate conditions like obesity, inflammation, cancer, and cardiovascular diseases (Liu et al., 2021; Simopoulos, 2008, 2016). Hydrolysis of SC viscera yields valuable protein hydrolysates and oils with high concentrations of vitamins and antioxidants, which can be used in developing nutraceuticals for pets and humans (Babji et al., 2020). SC viscera exhibited high antioxidant and antimicrobial activities and functional roles in reducing hypertension (Tripoteau et al., 2015; Yan, Tao, & Qin, 2016).

Pet nutraceuticals are fortified with vitamins, minerals, chondroitin sulphate, glucosamine, omega-3, herbs, or amino acids, providing diet completeness to promote pet wellness, including immune support, joint health, cognitive behaviour and reduced microbial infections and inflammation (Kruzer, 2015). Pet nutraceuticals have become increasingly popular due to growing awareness about their benefits, increasing pet ownership and rising pet care expenditures. The global veterinary dietary supplement market was valued at USD 1.6 billion in 2020 and is expected to reach USD 2.5 billion by 2028, growing at a CAGR of 8.2%. SC byproducts, rich in protein, omega-3, and vitamins, can significantly contribute to pet health when used in nutraceutical, pharmaceutical, or fortifying products.

The client requested CASD's assistance in developing high-value products from SC flower and viscera byproducts. This includes evaluating their nutritional quality, functional properties, and antinutritional components and exploring their potential for pet and human nutraceuticals or cosmeceuticals. While SC byproducts can be used in human nutritional powders, their production is more strictly regulated than pet products. Research is needed to identify regulatory requirements for developing pet nutraceuticals.

## 2 SCOPE AND PURPOSE

### 2.1 Scope of Project

The project's initial focus is on SC byproducts for pet nutraceuticals, with potential applications in human products and cosmeceuticals.

The scope of the project is to evaluate nutritional values, bioactive compounds and antinutritional qualities of the low-valued SC byproducts from commercial processing industries, providing Green Seafoods Ltd. with the information needed to develop high-value products from these byproducts.

### 2.2 Purpose of Project

This project aims to assist the client in developing a new nutraceutical product from SC byproduct, especially for pets. By evaluating the nutritional, antinutritional, and bioactive properties of these byproducts, the client can determine their potential for high-value product development.

## 3 OBJECTIVES

The overall objective of the proposed project is to develop high-value products from SC byproducts (flower and viscera) for veterinary dietary supplements/pet nutraceuticals. This will provide the Client with access to the global markets with novel Newfoundland products.

Specific objectives are below:

1. To perform a scientific assessment of the production technology, raw materials, active ingredients and applications of nutraceuticals (mainly for dogs and cats).
2. To evaluate the nutritional composition of flowers and viscera byproducts generated and stored at the client's processing plant and compare them with freshly processed byproducts.
3. To assess the pretreatment (boiling & steaming) effects on the byproducts' quality.
  - I. To compare the nutrition and bioactive qualities of the byproducts dried using freeze-drying and air-drying systems.
  - II. To develop fine powder products from the selected pretreatments and drying processes.
  - III. To generate a detailed nutritional profile, including amino and fatty acid compositions for the selected products.
  - IV. To estimate astaxanthin carotenoid levels in the products.
  - V. To evaluate the antinutritional components (heavy metals, microbial contaminations) in the final products.
4. To provide recommendations for potential product applications such as nutraceuticals, veterinary supplements, aquafeeds or cosmetics, based on the nutritional composition.

## 4 MATERIALS AND METHODS

The byproducts used for this project were from the Marcel Angle III vessel, which caught the SC at 3Ps French Zone. The fisherman landed the SC at Grandbank Harbour, NL, on December 7, 2023, after 24 hours of last dragging. Around 30 kg of SC were separated in perforated trays (**Figure 1**) immediately after the last catch. Those were released in a 250 L insulated fish tub filled with seawater after about 24 hours. This set of SC was live and active in the seawater and designated as live SC (Live-SC) or control. Another 30 kg of SC was collected from the vessel hold after 24 hours of the last catch and released in seawater in a separate 250 L tub. Those were designated as revived SC (Rev-SC). Due to snowy weather, Live-SC and Rev-SC were stored overnight in a truck at -4°C. After 20 hours, both samples were transported to Winterton, NL, and kept in the client's processing plant under running seawater for five days to revitalize the SC. Rev-SC faced the vessel hold hydrostatic pressure and stress from SC fluid in the hold. Live-SC were free from a hold-stressed environment, and they were revitalized quickly. The main truck for commercial processing arrived after 68 hours.

### 4.1 Sea cucumber processing and byproduct collection

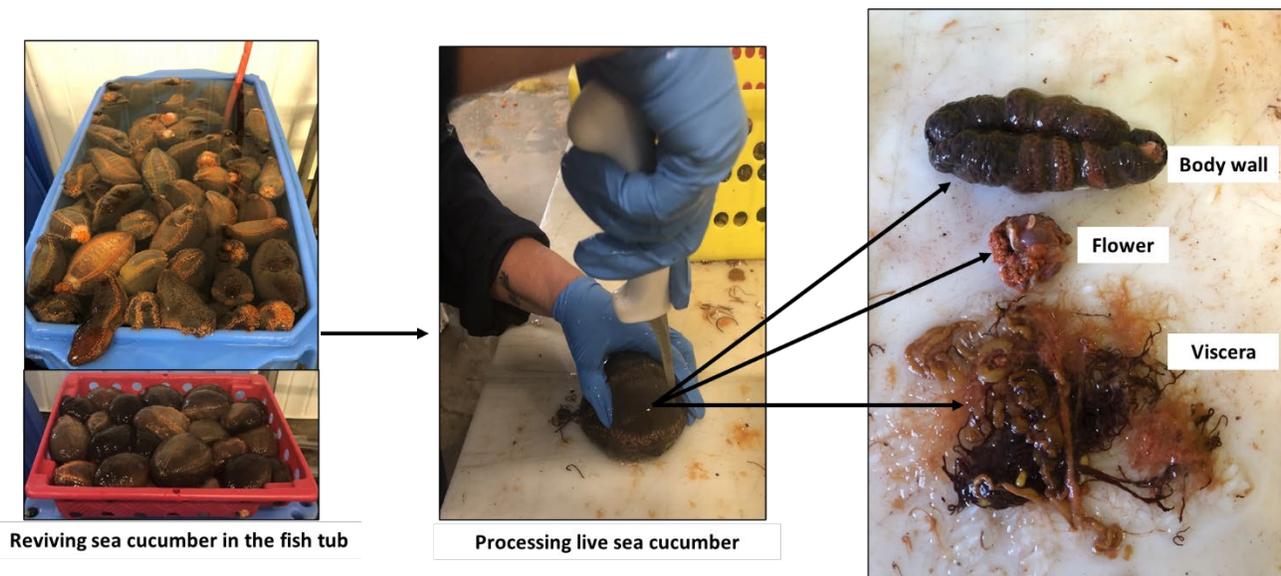
After 116 hours of the last fishing, the client began processing SC in their commercial plant, separated flowers following the cocoon cut system and stored them in steel baskets. Using a vacuum system, they removed viscera from the body wall, and the accumulated viscera were collected in the vacuum tanks. SC processing from the same vessel was continued for three days. The client's workers collected 7 kg of flowers from the basket and 7 kg of viscera from the vacuum tank on day 1 of processing. Those were vacuum-packed in 1 lb bags and designated as CPSC-1 samples. In the same way, flower and viscera were collected and vacuum-packed on day 2 and day 3, which were designated CPSC-2 and CPSC-3 samples, respectively.

100 Live-SC and Rev-SC were processed manually on day 2 and day 3, respectively, by slitting longitudinally from the base of the flower to the anus (**Figure 1**). Their viscera and flowers were separated and vacuum-packed. All samples were stored at -20°C at the client's plant before transferring them to MI. Four flower and four viscera samples were used for this project (**Table 1**). All samples were stored at -20°C until lab analysis.

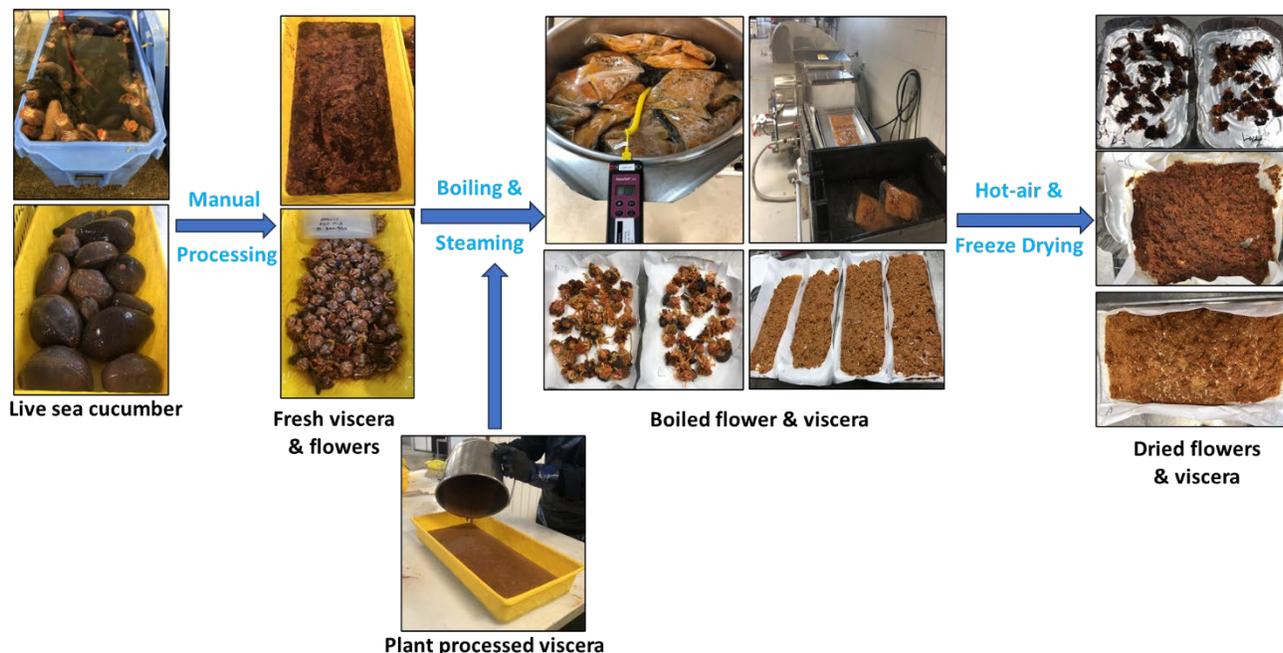
**Table 1. Description of byproduct sampling from Atlantic sea cucumbers used for the project**

Sample name	Procedure of sampling
Live sea cucumbers (Live-SC)	About 30 kg SC from the last drag was placed in a perforated stack & nest trays at the fishing vessel. Those were released in seawater in a 250 L insulated fish tub after 24 hours of fishing and then under-running seawater at the client's SC processing plant for four days.
Revived sea cucumbers (Rev-SC)	About 30 kg SC was collected from the vessel hold where SC faced hydrostatic pressure and stress from SC fluid for 24 hours and transferred to seawater in a 250 L insulated fish tub. Those were placed under running seawater at the client's SC processing plant for 5 days.

Commercially processed sea cucumbers on day 1 (CPSC-1)	On day 1 of commercial processing (122 hours of last fishing), the client line workers collected approximately 7 kg of flowers from the storage basket and viscera from vacuum tanks.
Commercially processed sea cucumbers on day 3 (CPSC-3)	On day 3 of processing (170 hours of last fishing), the client line workers collected approximately 7 kg of flowers from the storage basket and viscera from vacuum tanks.



**Figure 1. Reviving and manually eviscerating sea cucumber byproducts.**



**Figure 2. Processing, applying heat treatments and drying sea cucumber byproducts.**

## 4.2 Pretreatment and drying

Frozen SC flowers and viscera were thawed overnight in a chill room (4 °C) and then placed under cold water for about 2 hours until all ice pellets melted. The thawed samples were used for heat treatment at the pasteurization temperature ( $\geq 85$  °C) for 1 minute using a steam kettle and a Laitram steam cooker. Due to the high liquid content, boiling the viscera in open water was not feasible as it would wash away lipids and water-soluble bioactive compounds. Instead, the viscera were heat-treated in vacuum packages. As a trial basis, several packs of viscera were submerged in hot ( $98 \pm 2$  °C) water in a steam kettle, and the time and temperature of the sample inside the bags were recorded (**Figure 2**). The heat treatment time was determined from the trial experiments, which was around 15 minutes. Then, the final viscera samples were heat-treated in hot ( $98 \pm 2$  °C) water in a steam kettle for 15 minutes (Table 2). Similarly, several bags of viscera were heat treated in a Laitram steam cooker at 98 °C and 100% steam to determine the time when the temperature of viscera reaches  $\geq 85$  °C. Steaming at 98 °C and 100% steam for 7 minutes achieved the target temperature for viscera. Flowers were heat-treated for 7 minutes in the steam kettle at  $98 \pm 2$  °C to reach  $\geq 85$  °C and 4.5 minutes under live steam in the Laitram steam cooker. Heat-treated samples were dried in a hot-air drying system at 60 °C for 48 hours and in a freeze-drying system for 50 hours (**Table 2**).

**Table 2. Heat treatments and drying parameters applied to the sea cucumber byproducts**

Byproduct types	Pretreatment and drying		Byproduct temperature (°C)	
	Parameters	Duration	Initial	Final
Flowers	Boiling at 98±2 °C	7 min	10	≥85
	Steaming at 98 °C and 100% steam	4.5 min	10	≥85
	Oven drying (60±8°C)	48 h		
	Freeze drying at 50±5 °C under vacuum of 0.7-0.8 mbar	50 h		
Viscera	Boiling at 98±2 °C	15 min	10	≥85
	Steaming at 98 °C and 100% steam	7 min	10	≥85
	Oven drying (60±8°C)	48 h		
	Freeze drying at 50±5 °C under vacuum of 0.7-0.8 mbar	50 h		

### 4.3 Byproduct grinding

Thawed SC was minced for 1 min in a Ninja Blender (Shark Ninja Operating LLC, Ville St. Laurent, QC, Canada) at high speed. Moisture content was measured using AOAC 930.15 methods (AOAC, 2012) at 105 °C. Approximately 200 g of mince from each sample was dried overnight at 105 °C and then ground into a fine powder (~0.5 mm particle size) using a coffee grinder for the proximate analysis. Heat-treated dried byproducts were first ground in a Ninja Blender for 30 seconds and then further ground with a coffee grinder. The resulting fine powder was used for laboratory analysis.

### 4.4 Laboratory analysis

The freshness, microbial activity and nutritional quality (proximate, lipid class, fatty acid and amino acid compositions) of thawed SC byproducts (flowers and viscera) from Live-SC, Rev-SC, CPSC-1 and CPSC-3 samples were determined. The nutritional quality of heat-treated dried SC byproducts, including vitamins, minerals, and other functional properties such as phenolic and astaxanthin content and antioxidant activity, was determined. The detailed procedures are in Appendix A.

1. The microbial activity in all thawed byproducts and heat-treated viscera from CPSC-3 were analyzed by standard plate count at the Avalon Laboratories, St. John's, NL, following their official laboratory method MFHBP-18.
2. Proximate analysis was performed on thawed, heat-treated, air-dried, and freeze-dried SC byproducts following the standard method of the Association of Official Analytical Chemists International (AOAC) (AOAC, 2012). Moisture and ash content were determined following AOAC 930.15 and AOAC 938.08. The Kjeldahl method (AOAC 954.01) was used for protein content analysis and the Soxhlet extraction (AOAC 948.15) was used for total lipid content.
3. Astaxanthin content analysis of heat-treated air-dried and freeze-dried SC byproducts was determined following Jaramillo et al., (2018).

4. Total lipid class and fatty acid composition of lipids in thawed and heat-treated air-dried and freeze-dried SC byproducts were analyzed at the Ocean Science Centre (OSC) of Memorial University of Newfoundland (St. John's, NL, Canada) following Parrish and Wells (2021).
5. Amino acid analysis of heat-treated dried SC byproducts carried out following Liu et al., (2021).
6. The phenolic of heat-treated air-dried and freeze-dried SC byproducts was extracted following Carletti et al., (2022). The total phenolic content in the extract was determined using the Folin-Ciocalteu reagent. The antioxidant activity of the extract was assessed as DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity, according to Hossain et al., (2022).
7. Mineral and trace element analysis for the pretreated dried SC byproduct powder was performed at the Research & Productivity Council (RPC), Fredericton, NB, following their routinely used methods.
8. Vitamin analysis of the heat-treated dried SC byproducts was carried out by the Health & Nutrition Laboratory of SGS Canada Inc. (Burnaby, BC) using their standard methods.

#### 4.5 Statistical analysis

Statistical analysis was performed for the proximate composition among the processing states. Proximate tests were carried out in triplicate, and the data were presented as mean  $\pm$  standard deviation. The data were processed using a one-way analysis of variance (ANOVA), and mean separations were analyzed using Tukey's HSD test using R-Studio.

## 5 RESULTS AND DISCUSSIONS

### 5.1 Survival rates of sea cucumbers

The survival rate of the SC under running seawater at the Client's plant differed between Live-SC and Rev-SC. Of the 116 Live-SC, four were dead, resulting in a survival rate of 96.6%. In contrast, 20 out of 140 Rev-SC were dead, giving a survival rate of 85.7%. Surviving SC were active, anchored, and moved their flowers, responding by retracting their tentacles when touched.

### 5.2 Perform a scientific assessment on pet nutraceuticals (Objective 1)

#### 5.2.1 Production technology

Pet nutraceuticals combine nutritional and pharmaceutical substances in feed to support normal body function and combat nutritional diseases (Kruzer, 2015). Used as alternative medicine, they promote animal health and longevity. Despite the lack of stringent production regulations and the legal requirements for veterinary supplements, the popularity of pet nutraceuticals has risen over the years. Thermal processes, such as baking and extrusion, are key in enhancing the nutritional quality, palatability, and shelf life of pet products. Kępińska-Pacelik et al., (2023) evaluated baked and extruded nutraceutical dog snacks prepared with different flours and insect meals. They found that dogs preferred the baked snacks, which also contained higher crude fat levels but lower carbohydrates and trace elements than extruded snacks. While extrusion eliminates harmful microbial activity, it can reduce nutritional quality by denaturing proteins, oxidizing lipids, and lowering digestibility.

The nutritional and safety qualities of pet foods for dogs and cats vary based on the species, development processes and production regulations. Leiva et al., (2019) analyzed 226 dog foods and 25 cat foods, developed using different thermal processes and evaluated based on Good Manufacturing Practices (GMP) according to Central American Technical Regulation. The study found that 100 g dog foods contained  $20.3 \pm 4.7$  g protein,  $12.9 \pm 2.5$  g fat,  $8.4 \pm 1.9$  g ash, and  $1.9 \pm 0.7$  g calcium, while cat foods contained  $29.0 \pm 4.6$  g protein,  $12.3 \pm 3.3$  g fat,  $9.3 \pm 2.3$  g ash, and  $1.7 \pm 0.8$  g calcium per 100 g. Key trace elements in dog foods included calcium ( $2470 \pm 1270$  mg), iron ( $274.0 \pm 1.5$  mg), and selenium ( $2.1 \pm 1.8$  mg) per 100 g. The European Pet Food Industry Federation (FEDIAF), recommends minimum levels of protein (21.0 g), fat (5.5 g), calcium (0.58 g), and other nutrients in pet foods (FEDIAF, 2024) (Appendix B). The Association of American Feed Control Officials (AAFCO) recommends fat concentrations of 8.5 g/100 g pet food for young dogs or puppies (AAFCO, 2019). The FEDIAF recommends the minimum microbial activity limits to be  $5 \times 10^4$  CFU/g (FEDIAF, 2017). Products with higher concentrations of nutritional or pharmaceutical substances can be used as nutraceuticals for pets if deemed safe for consumption.

Nutraceutical products for pets are the result of a rigorous research and development process which guarantees the quality and effectiveness of the products for particular purposes. Kruzer (2015) reviewed several safety and efficacy studies on the ingredients of pet nutraceuticals in various animal species. They stated that veterinary supplement requirements varied based on the animals' body system and species. Raw ingredients of pet nutraceuticals are selected based on its bioavailability, safety, and efficacy in specific species. When those ingredients are put into a finished pet product and claimed as nutraceuticals, other aspects, such as label claims, contaminants, purity, and shelf life, also need to be considered (Kruzer, 2015). Pet nutraceutical production regulation is different than the pharmaceutical drug manufacturing process. Drug manufacturing is strictly regulated by the Drug Regulation authorities like the Food and Drug Administration (FDA) in the USA and Health Canada in Canada. So, when a consumer purchases a pharmaceutical drug for humans or animals, it automatically includes all the benefits and regulations approved by the authority, which include product ingredients, effective dosages, purity and contaminants, side effects, and safety concerns. Since veterinary supplements and nutraceutical products are not pharmaceutical drugs, manufacturers have no legal obligation to obtain or provide information that would otherwise be discovered during the FDA or Health Canada drug approval process (Kruzer, 2015). This can be surprising to pet owners, who may assume and trust a product that often looks like a pharmaceutical drug and claims it will help their pet. Some manufacturers not only use ingredients that have been researched but also take voluntary steps to help ensure their finished products are shelf stable, pure, and free of contaminants and meet label claims (Kruzer, 2015).

There is limited published information on the production technology for pet nutraceuticals. Integricare (Oakville, ON), a Canadian pet supplement company, published a comprehensive guide on manufacturing pet supplements, key ingredients, and quality standards (Integricare, 2023). Pet dietary supplements are typically processed foods made from raw materials sourced from animals and plants, combined with concentrated nutrients to address deficiencies in vitamins, proteins, and minerals, thereby promoting pets' health. Manufacturers must adhere to specific regulations before releasing pet nutraceuticals. If Food and Drug Authorities recommend safety standards, manufacturers must comply to avoid misbranding or confiscation. Before producing pet nutraceuticals, manufacturers should consider the following:

### Premarket approval

The food supplements and nutraceuticals approved as safe by the Food and Drug Regulation Authority can be used as feed additives, including ingredients like grains, forages, and other vitamins and minerals. Products not approved as animal food additives nor GRAS (generally recognized as safe) will be prohibited from being legally marketed.

### Claims and labelling

Pet nutraceuticals must have clear and understandable labels that contain relevant information about specific details that guarantee the safety of their consumption. The labels should contain other essential information, including the brand and product name, purpose statement, ingredient list, nutritional facts, directions for usage, and proper dosage.

#### 5.2.2 Applications of nutraceuticals

Most of the pet nutraceuticals' target ingredients are protein, omega-3 fatty acids, probiotics, vitamins, chondroitin, glucosamine and methylsulphonylmethane (MSM) (Kruzer, 2015). Glucosamine, Omega-3 fatty acids, antioxidants, MSM, etc. are the main compounds that have well been accepted and adopted by commercial ventures for the prevention of osteoarthritis, fatty liver and oxidative stress in pets (Rafiqi et al., 2016). Chondroitin and glucosamine help form cartilage, muscle, and ligaments in the joint and relieve joint pain, and MSM is a good anti-inflammatory agent for pets. Major nutrients in pet foods are protein and fats, fatty acids including linoleic acid, eicosapentaenoic (EPA), docosahexaenoic (DHA), arachidonic acid (cats), essential amino acids, major minerals (Ca, K, Na, Mg) and trace elements (Fe, Cu, Mn, Se, Zn), vitamins and vitamin-like substances (choline).

Veterinarians primarily recommend nutraceuticals for preventative purposes and/or due to client interest. In dogs and cats, the most popular nutraceuticals are used for joint and digestive health problems, followed by cognition, skin/coat and heart issues (Finno, 2020). In cats, nutraceuticals remain high in demand for controlling hairball disease, followed by urinary tract infections and kidney disease (Finno, 2020). Omega-3 fats are essential for coat development and maintenance. Those are found in marine life and contain anti-inflammatory and anti-plastic properties. The fatty acid supplements with EPA and DHA – from fish oils like salmon and anchovy are well documented and used extensively in pet food and nutraceuticals. Vitamin B1 (thiamine), magnesium, and essential amino acid tryptophan have behavioural effects on pets.

There are a few examples of pet nutraceuticals using marine-sourced ingredients (**Table 3**). These products act as antioxidants and may help with cancer support. Glucosamine and methylsulphonylmethane in marine animals like green lip mussels and SC are used to treat arthritis and joint pain. The products containing those bioactive compounds restore mobility, remove limping or trembling, and reduce inflammation. The omega-3 fatty acids of marine sources are used for inflammatory conditions in dogs and cats.

**Table 3. A short list of companies that produced pet nutraceuticals using marine-sourced ingredients**

Company	Product name	Marine source ingredients	Health benefits claimed by the company
Pet wellness Direct	<u>VetSmart Formulas</u>	<ul style="list-style-type: none"> <li>• Green-lipped mussel</li> <li>• Sea cucumber</li> </ul>	<ul style="list-style-type: none"> <li>• Restores mobility and reduces inflammation</li> <li>• Therapeutic for joint pain, arthritis, and hip dysplasia</li> <li>• Remove limping or any trembling</li> </ul>
Ocean Pride Fisheries	<u>Panasea pet food topper</u>	<ul style="list-style-type: none"> <li>• Dried sea cucumber flower</li> </ul>	<ul style="list-style-type: none"> <li>• Beneficial for inflammation and muscle protein synthesis</li> <li>• Protects against oxidation by free radicals.</li> </ul>
VetRxDirect	<u>MegaFlex</u>	<ul style="list-style-type: none"> <li>• Sea cucumber</li> <li>• Shellfish</li> </ul>	<ul style="list-style-type: none"> <li>• Alleviate day-to-day pains</li> <li>• Aid in rebuilding cartilage of dogs and cats</li> <li>• Maintain flexible joints</li> </ul>
Coastside Resouces	<u>Vetri-Lox</u>	<ul style="list-style-type: none"> <li>• Sea cucumber lipids</li> </ul>	<ul style="list-style-type: none"> <li>• Boost joint health</li> </ul>
Sky Health International Ltd.	<u>Petrum 360</u>	<ul style="list-style-type: none"> <li>• Canadian sea cucumber</li> </ul>	<ul style="list-style-type: none"> <li>• Helps with joint pains, immunity</li> <li>• Energy</li> <li>• Has anti-inflammatory and antioxidant agents that help cancer support, and longevity</li> </ul>

### 5.3 The nutritional composition of SC byproducts (Objective 2)

#### 5.3.1 Proximate composition of thawed SC flowers

The moisture content and other proximate composition (dry weight basis [dwb]) of the thawed flowers and viscera are shown in **Table 4** and **Table 5**, respectively. Protein content was highest in flowers ranging from 60-65% (dwb) (**Table 4**), with variations among SC processed under different conditions. Commercially processed SC flowers had significantly higher moisture and lipid content, but lower ash and protein compared to Live-SC and Rev-SC flowers. Lipid content in CPSC-1 and CPSC-3 was 5-6 times higher than in Live-SC and Rev-SC. The commercially processed flowers had a higher proportion of viscera attached (**Figure 3**). Meanwhile, minimum viscera were present in the flowers of Live-SC and Rev-SC, which were processed manually, and the viscera were separated entirely from the flowers. Viscera generally store more lipids than body walls or flowers (Liu et al., 2021), and that could be the reason for the higher lipid content in commercially processed SC.

**Table 4. Proximate composition (dwb) of thawed flowers of Atlantic sea cucumber (n=3).**

Processing states	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
Live-SC	83.9±0.0 <sup>d</sup>	20.4±0.2 <sup>b</sup>	65.5±0.0 <sup>a</sup>	3.8±0.4 <sup>c</sup>
Rev-SC	85.0±0.4 <sup>c</sup>	21.2±0.1 <sup>a</sup>	65.0±0.3 <sup>a</sup>	3.6±0.3 <sup>c</sup>
CPSC-1	86.7±0.1 <sup>a</sup>	17.7±0.0 <sup>d</sup>	62.2±0.1 <sup>b</sup>	20.1±0.9 <sup>b</sup>
CPSC-3	85.9±0.1 <sup>b</sup>	19.0±0.0 <sup>c</sup>	61.3±1.7 <sup>b</sup>	22.3±0.5 <sup>a</sup>
p-value	<0.001	<0.001	<0.01	<0.001

Data are expressed as mean ± SD from triplicate determination. Different letters in the same column show significant differences ( $p < 0.05$ ) among processing states.

### 5.3.2 Proximate composition of thawed SC viscera

The proximate composition in the viscera differed from flowers and varied across the processing conditions (**Table 5**). As compared to the flowers, viscera had less protein (48.8-52.2% dwb) and higher lipid (21-25.7% dwb) contents. The viscera of Live-SC had significantly higher moisture (85%), ash (16.4% dwb) and lipid (25.7% dwb) compared to those of CPSC-1 (moisture 84.4%; ash 15.6% dwb and lipid 21.3% dwb) and CPSC-3 (moisture 84.3%; ash 15.7% dwb and lipid 21.0% dwb) ( $p < 0.001$ ) (**Table 5**). There was no significant difference in the protein content in the viscera of the SC processed at different conditions or states. Since the viscera had a higher lipid content, Live-SC and Rev-SC showed high lipid in the viscera.

The proximate composition of the thawed SC viscera in this study aligns with prior findings for *C. frondosa* (Liu et al., 2021) with similar moisture (82%), protein (48.3% dwb), and lipid (26.1% dwb) levels. However, ash content (14.8-16.4% dwb) was slightly higher than previously reported (11.9% dwb). Mamelona et al., (2010) reported higher moisture (92.3%) and less ash, protein and lipid than the current study. This difference might be due to the differences in the location or season when the SC was harvested (Abuzaytoun et al., 2022; David & MacDonald, 2002; Grant et al., 2006; Muhsin et al., 2023). The SC of the current study was collected from the 3Ps French zone, and the source of the Mamelona et al., (2010) was unclear but likely a local processing plant.

**Table 5. Proximate composition of thawed viscera byproducts of Atlantic sea cucumber (n=3)**

Processing states	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)
Live-SC	85.0±0.0 <sup>a</sup>	16.4±0.0 <sup>a</sup>	51.0±4.0 <sup>a</sup>	25.7±1.8 <sup>a</sup>
Rev-SC	83.5±0.1 <sup>c</sup>	14.8±0.0 <sup>c</sup>	48.8±0.3 <sup>a</sup>	26.9±0.1 <sup>a</sup>
CPSC-1	84.4±0.0 <sup>b</sup>	15.6±0.0 <sup>b</sup>	52.2±0.2 <sup>a</sup>	21.3±0.1 <sup>b</sup>
CPSC-3	84.3±0.1 <sup>b</sup>	15.7±0.1 <sup>b</sup>	50.7±1.1 <sup>a</sup>	21.0±0.1 <sup>b</sup>
<b>p-value</b>	<0.001	<0.001	NS	<0.001

Data are expressed as mean ± SD from triplicate determination. Different letters in the same column show significant differences ( $p < 0.05$ ) among processing states.



**Figure 3. Manually processed flowers from Rev-SC (A) and commercially processed flowers from day 3 CPSC-3 (B)**

## 5.4 Pretreatment and drying effects on nutritional and bioactive quality (Objectives 3 & 4)

### 5.4.1 Proximate composition of boiled and steamed SC byproducts (Objective 3-I)

The moisture content of the heat-treated (boiled and steamed) flowers differed between air-drying and freeze-drying systems. The moisture was higher in the hot-air-dried flowers than in freeze-dried ones, either treated by boiling or steaming (**Figure 4**). Steam-treated flowers had higher ash content, while boiled flowers contained more protein across all samples (Live-SC, Rev-SC, CPSC-1, and CPSC-3). Hot-air-dried flowers from Live-SC and CPSC-3 had higher lipid content than their freeze-dried counterparts. Overall, Live-SC and Rev-SC flowers had higher ash content, whereas CPSC-1 and CPSC-3 flowers had higher lipids. Heat treatment reduced lipid content by half in CPSC-1 and CPSC-3 flowers, from 20.1% and 22.3% dwb (non-treated) to 10.0% and 9.3% dwb, respectively) (**Table 5**) (**Figure 4**). Open boiling of the flowers in the vegetable bags likely caused lipid loss from the extra viscera attached to the flowers of commercially processed SC. The steamed flowers of CPSC-1 and CPSC-3, and boiled flowers of CPSC-3 and Live-SC have a higher content of lipids when dried in hot air than their freeze-dried counterparts. Liu et al., (2021) similarly reported higher lipid content in hot-air dried byproducts than in the freeze-dried ones

Boiling treatment increased protein levels (>70%) and lowered ash content in all SC flowers. Flowers were heat-treated in the open water which caused leaching ash and concentrated protein in the samples. The boiled SC flower byproducts have high protein more than three times the minimum level of protein in pet food recommended by the FEDIAF (FEDIAF, 2024). They could be used as protein supplements for pets. Commercially processed flowers are also a good source of lipids.

The proximate composition of the boiled, steamed and hot-air-dried viscera was different from freeze-dried viscera. The moisture content was higher in the hot-air-dried viscera when those were steam-treated. The moisture content of the boiled hot-air dried viscera of Live-SC was similar to that of the freeze-dried sample (**Figure 5**). Ash and protein content were at comparable levels in the heat-treated viscera, while the steamed hot-air dried viscera of Live-SC and Rev-SC were higher than freeze-dried viscera. In the previous studies, Liu et al., (2021) reported that air-dried viscera had ash 12.5%, protein 45.8% and lipid 25.7% and freeze-dried viscera had ash 12.5%, protein 46.9% and lipid 25.2% which were a little different from the current study. The heat-treated and dried SC viscera had higher ash and protein and less lipid compared to the Liu et al., (2021) study. The extra level of heat treatment applied to the viscera might cause the lipid to decline, as was reflected in the flowers in the current study. Bechtel et al., (2013) reported a similar level of protein (50.3%) and higher lipids (22.2%) in the dried viscera of commercially harvested red SC (*Parastichopus californicus*) in Alaska. The FEDIAF recommended minimum levels of protein and fat as 21.0 g and 5.5 g in 100 g of pet food (FEDIAF, 2024). The pretreated dried SC viscera have high protein more than twice and fats more than three times the minimum level recommended for pet food. The dried viscera could be used as protein and fat supplements for pets.

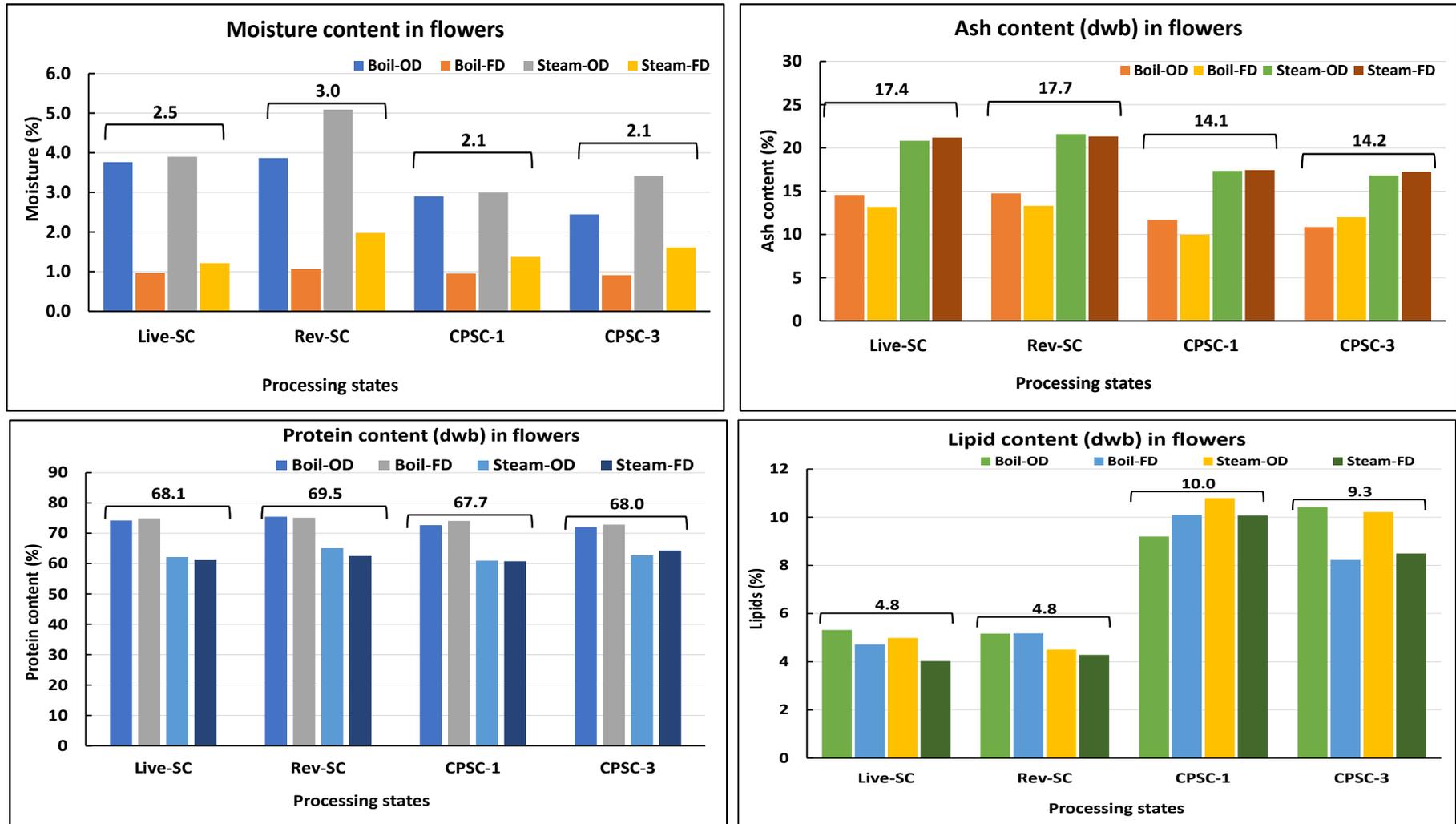


Figure 4. Proximate composition (dwb) of the heat-treated and hot-air or oven (OD) dried and freeze (FD) dried sea cucumber flowers

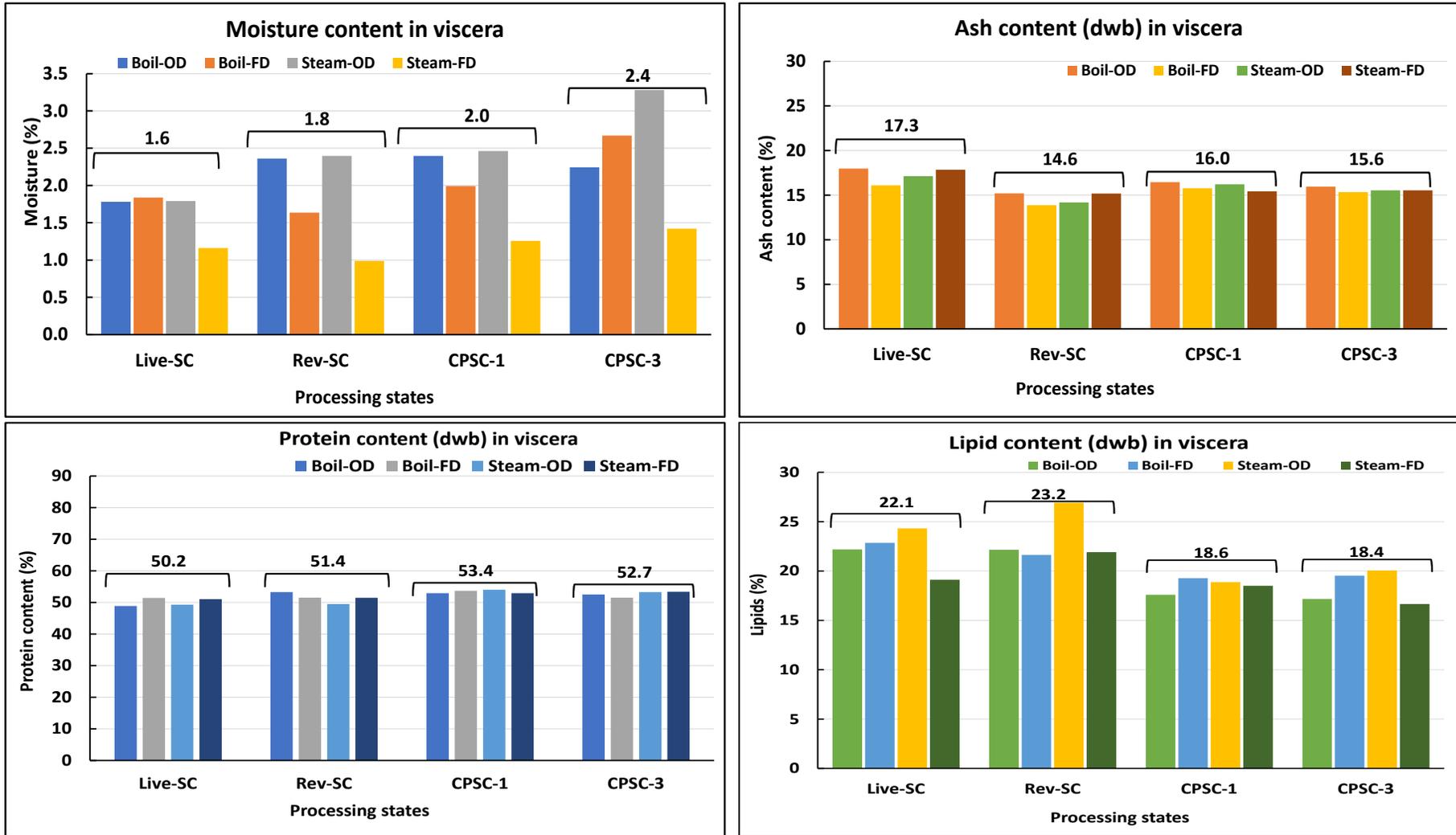


Figure 5. Proximate composition (dwb) of the heat-treated and hot-air or oven (OD) dried and freeze (FD) dried sea cucumber viscera

#### 5.4.2 Phenolic content of boiled and steamed SC byproducts Objective 3-I)

The effect of heat treatment and drying on the phenolic content of SC byproducts from Live-SC and CPSC-3 was evaluated in **Table 6**. Steamed flowers had higher total phenolic contents than boiled ones, likely because steaming retains water-soluble compounds, while open boiling in vegetable bags may wash out phenolics. This aligns with Hossain et al., (2023), who reported 241.4 mg GAE/100 g free phenolics content in the freeze-dried non-treated flowers of the same species. The total phenolic content of viscera from Live-SC and CPSC-3 ranged from 158.5 to 193.5 mg GAE/100 g and 157.3 to 191.7 mg GAE/100 g, respectively. Phenolic contents were higher in the steamed viscera under both drying methods. The functional properties of the phenolic compounds are that they are antioxidant, anti-inflammatory, and anticancer (Hossain, et al., 2020). The phenolic content in the SC flower and viscera could be a good source of health benefits for the animal.

**Table 6. Phenolic content in heat-treated byproducts of sea cucumber processes at different states**

Parameters	Flowers				Viscera			
	Hot-air drying		Freeze drying		Hot-air drying		Freeze drying	
	Boiled	Steamed	Boiled	Steamed	Boiled	Steamed	Boiled	Steamed
Live-SC	136.2±4.1	202.5±3.3	182.5±5.2	272.3±2.3	182.9±0.9	193.5±6.4	158.5±6.6	192.6±0.7
CPSC-3	165.6±6.9	238.9±4.0	173.9±2.4	236.8±4.0	191.7±13.7	173.0±6.8	157.3±2.2	180.6±6.9

Mean ±SD (n=3)

#### 5.5 Development of fine powder products from selected pretreatments and drying processes (Objective 3-II)

Fine powder product development is part of the next phase.

#### 5.6 Nutritional profile of heat-treated SC by-products (Objectives 3-III)

##### 5.6.1 Amino acid composition in SC byproducts

The laboratory work was temporarily paused due to health and safety issues. Laboratory activities have since resumed, and amino acid composition is currently being analyzed.

##### 5.6.2 Lipid class in SC byproducts

The lipid content measured in thin-layer chromatography is presented in **Table 7**. Total lipids determined in this technique were low for all the byproduct samples compared to those estimated by the Soxhlet method (**Table 4** and **Table 5**). However, total lipids determined by both methods were higher in SC viscera (15.8-33.5 mg/g) than in flowers (7.8-14.3 mg/g) (**Table 7**). Live-SC and Rev-SC flowers had lower lipids compared to the commercially processed flowers (CPSC-1 and CPSC-3). Phospholipid was the most abundant lipid class in the SC flowers processed at different conditions or states followed by triacylglycerols and glyceryl ethers (**Table 7**). The phospholipids were higher in the flowers of Live-SC (61.7%), and Rev-SC (59.9%), compared to the commercially processed CPSC-1 (38.5%) and CPSC-3 (39.5%). Free fatty acids were lower in Live-SC and Rev-SC flowers but higher in commercially processed

flowers, indicating lipid degradation due to poor handling, prolonged storage, or processing conditions. The presence of free fatty acids signals byproduct deterioration (Angerosa, Campestre, & Giansante, 2006).

The phospholipid in the flowers was higher compared to the viscera. In contrast, glyceryl ethers, triacylglycerols, and free fatty acids were higher in the viscera than in the flower of the SC of the same processing states. In the SC viscera, phospholipid was the highest lipid class, followed by triacylglycerols and glyceryl ethers. Triacylglycerols serve mainly as an energy store, and they accumulate in the internal organs like the liver and gut walls of animals. Abuzaytoun et al., (2022) reported higher triacylglycerols than phospholipids in the viscera of SC, which changed with the growing seasons, with the highest triacylglycerols in the summer.

**Table 7. Lipid classes in the thawed byproducts of sea cucumbers processed in different states\***

Parameters	Flower				Viscera			
	Live-SC	Rev-SC	CPSC-1	CPSC-3	Live-SC	Rev-SC	CPSC-1	CPSC-3
<b>Total lipids (mg/g ww)</b>	<b>11.8</b>	<b>7.8</b>	<b>14.3</b>	<b>13.3</b>	<b>26.9</b>	<b>15.8</b>	<b>29.5</b>	<b>33.5</b>
<b>Lipid class (%)</b>								
Hydrocarbons	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Steryl esters	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Ethyl esters	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ethyl ketones	1.8	1.6	0.4	1.1	1.5	0.9	0.7	0.7
Methyl ketones	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glyceryl ethers	3.4	3.6	17.4	17.0	18.9	10.8	23.4	29.2
Triacylglycerols	14.0	15.9	28.1	24.6	24.3	18.4	21.7	25.5
Free fatty acids	4.9	4.5	6.0	8.2	9.2	10.7	7.5	8.3
Alcohols	1.2	1.5	0.9	0.9	1.1	1.9	1.3	1.0
Sterols	6.1	7.5	4.8	4.9	3.3	4.8	3.7	3.8
Diacylglycerols	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Polar lipids	6.9	5.4	3.7	3.8	4.7	4.3	2.2	4.3
Phospholipids	61.7	59.9	38.6	39.5	37.0	48.1	39.3	27.3

\*Lipid class analysis was conducted by the Department of Ocean Sciences, MUN and are reported on a dwb.

The total lipids increased about >6 times in the heat-treated flowers of Live-SC, and that was >8 times in flowers of CPSC-3 (**Table 8**). The total lipids were 67.1 mg/g in boiled and 66.6 mg/g in steamed hot-air dried flowers (**Table 8**), and that was 11.8 mg/g in thawed or non-treated flowers of Live-SC (**Table 7**). In contrast, the boiled flowers of CPSC-3 had 106.4 mg/g of total lipids, and steamed flowers had 115.4 mg/g, which was much higher than the non-treated flowers (13.3 mg/g).

The heat treatment increased the total lipids in both Live-SC and CPSC-3 viscera (**Table 8**). Phospholipid was also the most abundant lipid class in the heat-treated Live-SC and CPSC-3 flowers, which was a little higher than in the non-treated counterparts. The phospholipid class was lower in the heat-treated viscera than in the heat-treated flowers. The phospholipid levels did not change when the flowers were heat treated. Moreover, the free fatty acids were lowered in those viscera, meaning that heat treatment and drying improved the byproduct quality for the lipid class. Bimbo (1998) claimed that the allowable limit of free fatty acids is 1-7% for crude fish oil that will be refined for food or pharmaceutical purposes. The free fatty acid level of the heat-treated byproducts was within that limit. The phospholipids in the heat-treated viscera were higher than the previous reports of Liu et al., (2021). This could be due to SC's different growing locations and processing methods, such as the viscera being dried without boiling or steaming. Phospholipids help maintain healthy heart function, blood pressure, and circulation in pets. Due to anti-inflammatory properties, phospholipid-bound omega-3s can help relieve joint discomfort, support joint health and boost the immune system in aging pets (Magalhães et al., 2021). The triacylglycerols serve mainly as an energy store in animals. The viscera having higher triacylglycerols can be a good source of energy for pets.

**Table 8. Lipid classes in heat-treated hot-air dried SC flowers and viscera**

Parameters	Flower				Viscera			
	Live-SC		CPSC-3		Live-SC		CPSC-3	
	Boiled	Steamed	Boiled	Steamed	Boiled	Steamed	Boiled	Steamed
<b>Total lipids (mg/g)</b>	<b>67.1</b>	<b>66.6</b>	<b>106.4</b>	<b>115.4</b>	<b>301.0</b>	<b>293.8</b>	<b>207.6</b>	<b>131.8</b>
<b>Lipid class (%)</b>								
Hydrocarbons	0.1	0.0	0.1	2.7	0.2	0.0	0.0	0.0
Steryl esters	0.0	0.0	0.2	0.0	0.0	0.0	0.1	0.0
Ethyl esters	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ethyl ketones	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Methyl ketones	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glyceryl ethers	2.7	3.5	15.4	20.0	23.3	33.9	19.0	7.4
Triacylglycerols	13.2	14.5	13.5	16.7	26.9	31.6	31.0	15.5
Free fatty acids	2.5	2.4	5.7	6.1	1.7	3.0	8.2	5.4
Alcohols	1.2	1.0	3.0	4.2	3.7	2.2	2.4	0.8
Sterols	3.1	2.4	4.6	4.5	1.1	2.2	2.8	1.0
Diacylglycerols	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Polar lipids	12.0	12.3	7.5	7.4	9.1	3.0	4.6	19.5
Phospholipids	65.1	64.0	49.9	38.5	33.9	24.1	31.8	50.4

### 5.6.3 Fatty acid composition of SC byproducts

The fatty acid composition is an essential indicator of SC byproducts. The total fatty acids were higher in the viscera, ranging from 14.6 mg/g in CPSC-1 to 25.7 mg/g in Rev-SC, than in flowers, which ranged from 5.2 mg/g in Rev-SC to 11.2 mg/g in CPSC-1 (**Table 9**). Those were higher in the flowers of commercially processed SC (CPSC-1 and CPSC-3) than in Live-SC and Rev-SC. In contrast, fatty acids were higher in the viscera of Live-SC and Rev-SC than in commercially processed SC (CPSC-1 and CPSC-3). Polyunsaturated fatty acids (PUFAs) accounted for the highest fraction among the major fatty acid classes, followed by mono-unsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) in all the byproducts (flower and viscera) studied (**Table 9**).

**Table 9. Summary of the fatty acid composition in the thawed byproducts of sea cucumbers processed at different states\***

Fatty acid types	Flower				Viscera			
	Live-SC	Rev-SC	CPSC-1	CPSC-3	Live-SC	Rev-SC	CPSC-1	CPSC-3
<b>Total FA (mg/g)</b>	7.2	5.2	11.2	9.9	20.2	25.7	14.5	18.9
SFAs (%)	11.5	11.7	10.9	11.8	12.5	11.6	12.9	14.2
MUFAs (%)	25.0	25.4	28.6	28.4	30.7	29.7	30.4	30.4
PUFAs (%)	55.0	54.7	42.4	42.5	37.7	36.1	39.2	39.6
PUFA: SFA ratio	4.77	4.69	3.90	3.60	3.03	3.11	3.04	2.79
Omega-3 FA (%)	36.8	36.4	31.0	30.2	28.7	27.4	29.6	29.5
Omega-6 FA (%)	15.9	15.7	8.6	9.3	5.7	5.2	6.1	6.4
Omega-6 : Omega-3	0.43	0.43	0.28	0.31	0.20	0.19	0.21	0.22
DPA (%)	0.25	0.25	0.25	0.22	0.22	0.20	0.23	0.23
DHA (%)	0.92	0.87	1.07	0.97	1.23	1.23	1.16	1.32
EPA (%)	32.6	32.1	27.0	25.9	24.1	22.5	25.0	24.4
DHA : EPA	0.03	0.03	0.04	0.04	0.05	0.05	0.05	0.05

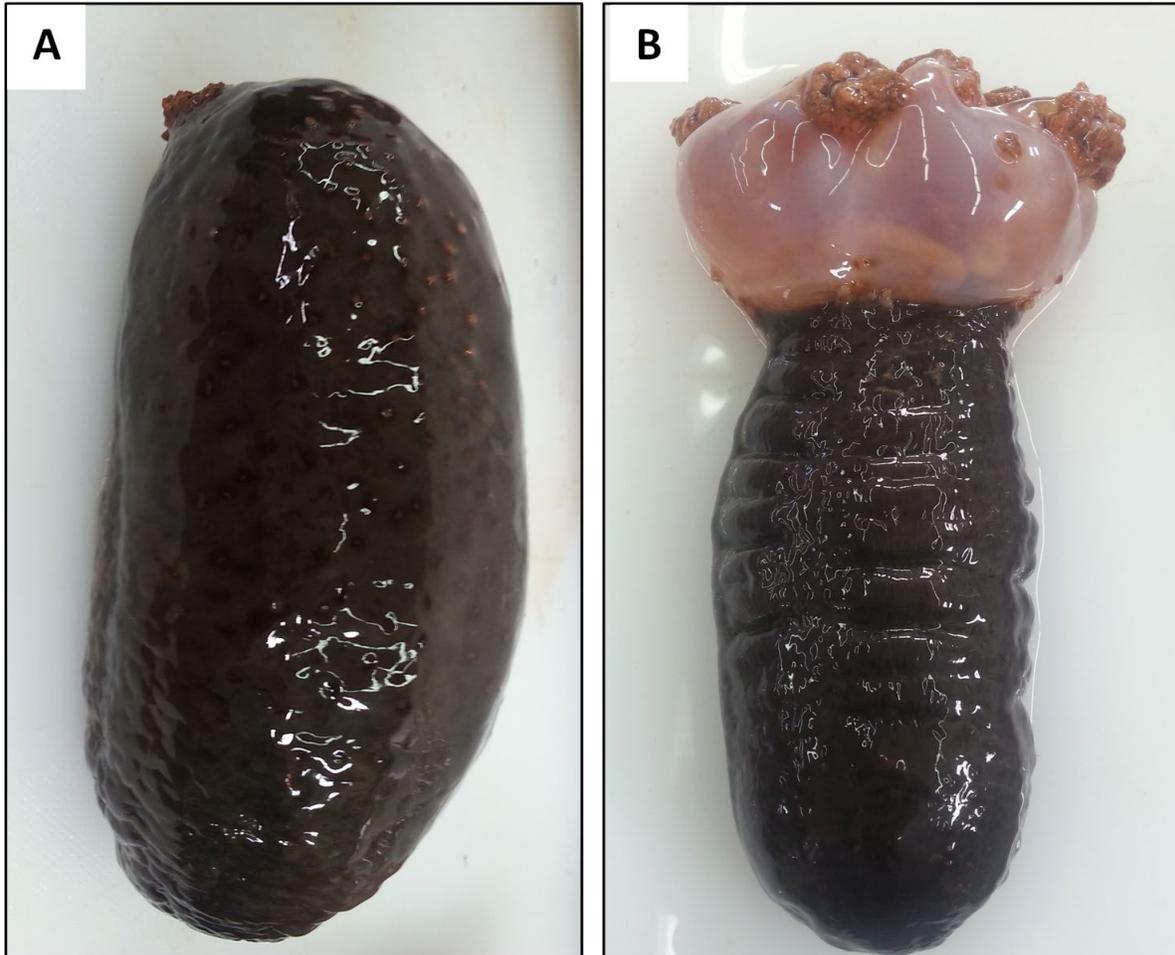
SFAs: Saturated fatty acids; MUFAs: Mono-unsaturated fatty acids; PUFAs: Poly-unsaturated fatty acids; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid

\*Fatty acid compositional analysis was conducted by the Department of Ocean Science, MUN and are reported on a dwb.

PUFAs were higher in the flowers of Live-SC and Rev-SC than those in CPSC-1 and CPSC-3. Whereas PUFAs in viscera of SC processed under different conditions were at similar levels. The MUFA levels were very stable among the byproducts of SC processed at different conditions. The MUFAs and PUFAs contents in the current study were comparable with the values reported by Liu et al., (2021) in viscera of the same SC species, which were higher than reported by Babji et al., (2020). The PUFA:SFA ratio is an important factor in maintaining a healthy balance of fats in a pet's diet. The ideal ratio for most animals is to have more PUFAs (omega-3s and omega-6s) than SFAs. A decreased ratio of SFA and an increased ratio of PUFA, especially with higher omega-3s (EPA/DHA), will decrease inflammation (Burron et al., 2024). This is beneficial for pets with conditions like arthritis or other inflammatory diseases. The SC byproducts had a

PUFA: SFA ratio of >2, which is good for pet diets. Interestingly, the Omega-3 Eicosapentaenoic acid (EPA), an essential fatty acid, was the major polyunsaturated fatty acid. EPA corresponds to approximately 22.5-25% in viscera and 25.9-33.5% in flowers, which is high and valuable, especially from SC byproducts. Among others, EPA, together with docosahexaenoic acid (DHA), has been associated with the treatment of several non-neoplastic diseases in dogs and cats like inflammatory skin disorders, cardiovascular conditions, renal disease and osteoarthritis (Magalhães, et al., 2021). The ratio of EPA to DHA is important in maintaining pet health, especially for older pets. Both EPA and DHA are part of the omega-3 fatty acids, but they are quite different in their effects on the body, and with the right ratio of both, many benefits can be achieved. EPA is best known as an anti-inflammatory and may be utilized to reduce the signs of arthritis, reduce body inflammation, and ensure joint health in aged pets (Raditic & Gaylord, 2020). DHA is crucial for neurological health and for the structure and function of the brain, which is critical in aging animals with cognitive dysfunction (Raditic & Gaylord, 2020).

Live-SC were active, anchoring, climbing, and moving their flowers, indicating a stress-free condition and serving as the control group. In contrast, CPSC-3 were mostly dead, flattened, and had their aquapharyngeal bulbs or flowers filled with gut materials, representing the most stressed or dead SC (**Figure 6**). Heat-treated byproducts (flower and viscera) from these two groups were analyzed to assess the effects of heat treatment and drying on the bioactive components and functional properties of those byproducts. The total fatty acids were higher in the boiled (46.4 mg/g) and steamed (46.5 mg/g) hot-air dried flowers (**Table 10**) than in the thawed or non-treated (7.2 mg/g) flowers of Live-SC (**Table 9**). Similarly, boiled (71.9 mg/g) and steamed (75.5 mg/g) flowers of CPSC-3 had higher total fatty acids than thawed flowers (9.9 mg/g) of CPSC-3. While heat treatment and drying increased total fatty acids, levels of SFA, MUFA, and PUFA remained relatively stable. Omega-3 contents increased by approximately 3% in treated Live-SC flowers.



**Figure 6. Live sea cucumber (A) and most stressed or dead SC (B)**

Heat treatment and drying increased total fatty acid composition about 10 times the non-treated viscera of Live-SC. That was >5 times for the CPSC-3 without apparent changes in the SFA, MUFA and PUFA amount. Omega-3 and Omega-6 amounts in the heat-treated viscera were at a similar level as the non-treated viscera of Live-SC and CPSC-3. The SFA, MUFA, and PUFA amount, including omega-3 in the heat-treated viscera, were consistent with the previous studies carried out in hot-air and freeze-dried viscera of *C. frondosa* (Mamelona et al., 2010). Liu et al., (2021) also reported that hot air- and freeze-dried viscera had a total fatty acid composition similar to fresh viscera, with high levels of omega-3 PUFA (30-31%), especially eicosapentaenoic acid (27–28%). The EPA content in SC flowers (26.6-36.9%) and viscera (23.3-24.9%) was higher in comparison to crude oil from other marine byproducts of salmon, cod and seal (4–9%) (Liu et al., 2021). Therefore, the heat treatment seems promising in concentrating fatty acids in the byproduct and enriching nutritional quality.

**Table 10. Summary of the fatty acid composition of heat-treated hot-air dried SC flower and viscera\***

Fatty acid types	Flower				Viscera			
	Live-SC		CPSC-3		Live-SC		CPSC-3	
	Boiled	Steamed	Boiled	Steamed	Boiled	Steamed	Boiled	Steamed
<b>Total FA (mg/g)</b>	<b>46.4</b>	<b>46.5</b>	<b>71.9</b>	<b>75.5</b>	<b>211.2</b>	<b>216.1</b>	<b>155.1</b>	<b>89.9</b>
SFAs (%)	10.0	10.3	9.6	10.2	12.3	12.1	13.9	13.6
MUFAs (%)	20.9	26.3	28.8	27.1	30.9	30.2	30.7	30.9
PUFAs (%)	65.6	58.5	46.8	42.5	39.3	36.7	40.0	40.2
PUFA: SFA ratio	6.5	5.7	4.9	4.1	3.2	3.0	2.9	3.0
Omega-3 FA (%)	40.2	39.2	32.9	30.1	29.7	27.4	29.5	29.7
Omega-6 FA (%)	17.6	16.8	11.4	9.2	5.5	5.7	6.5	6.4
Omega-6 : Omega-3	0.44	0.43	0.35	0.31	0.19	0.21	0.22	0.22
DPA (%)	0.25	0.13	0.23	0.22	0.21	0.19	0.21	0.22
DHA (%)	1.00	0.93	0.92	0.97	1.25	1.15	1.32	1.29
EPA (%)	36.9	35.3	29.4	26.6	24.9	23.3	24.5	24.7
DHA : EPA	0.03	0.03	0.03	0.04	0.05	0.05	0.05	0.05

SFAs: Saturated fatty acids; MUFAs: Mono-unsaturated fatty acids; PUFAs: Poly-unsaturated fatty acids; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid

\*Fatty acid compositional analysis was conducted by the Department of Ocean Science, MUN and are reported on a dwb.

#### 5.6.4 Major and trace element contents

Heat-treated byproducts of SC contained variable concentrations of major (calcium, sodium, potassium and magnesium) and essential trace (copper, iron, manganese, selenium, zinc) elements (**Table 11**). Flowers and viscera showed high calcium, sodium, potassium and magnesium content, as expected in SC products and byproducts (Mamelona et al., 2010; Muhsin et al., 2023; Ramalho et al., 2020). The sodium, potassium and magnesium content of boiled-dried flowers of Live-SC and CPSC-3 were less than the steamed flowers. Major elements were higher in the viscera of the current study than in the viscera of the Mamelona et al., (2010) study. The major elements calcium contributes to healthy bones and teeth, muscle building and function, a strong heart, and a healthy nervous system in all animals, including pets. Potassium regulates the activity of blood cells and muscles, especially the heart muscle, which maintains fluid balance in the body, regulates blood pressure, and acts as an enzyme activator (Muhsin et al., 2023).

Regarding essential trace elements, the byproducts showed values generally comparable to or higher than previous studies on the hot-air-dried SC coproducts of the same species (Ramalho et al., 2020). However, copper was higher in the SC viscera of the Mamelona et al., (2010) study than the viscera of the current study. This difference might be due to the differences in the SC source. Ramalho et al., (2020) reported high levels of calcium (1350 mg/kg), selenium (2.3 mg/kg), and zinc (63.5 mg/kg), and very high levels of

iron (88.0 mg/kg), potassium (20,000 mg/kg) and sodium (19,000 mg/kg) in the air-dried coproducts of SC collected from a commercial processing line at Cloridorme, QC. Those values in the current study were generally comparable to or higher than Ramalho et al., (2020) studies.

### 5.6.5 Vitamin contents

The contents of fat-soluble and water-soluble vitamins in SC byproducts are shown in **Table 12**. Vitamin E and D in the SC byproducts were below detectable limits. SC viscera showed higher beta carotene than flowers, while commercially processed (CPSC-3) flowers had higher vitamin A (beta carotene) than the flowers of Live-SC. At the same time, only the boiled flowers of Live-SC had vitamin A above detectable limits (10.1µg/100 g).

Water-soluble vitamins from SC viscera were richest in B3 (niacin), followed by vitamin B5 (pantothenic acid) (**Table 12**). The steam-treated flowers of CPSC-3 had higher levels of vitamins B1 (thiamin), B3 and B5 compared to the Live-SC flowers treated either by boiling or steaming. The reverse trend was observed for vitamin B3 (niacinamide), which was higher in Live-SC flowers than CPSC-3 flowers under the same heat treatment. Water-soluble vitamins analyzed in the present study are crucial nutrients in various physiological reactions. In the previous studies, Mamelona et al., (2010) reported much higher vitamin E levels (194.7 µg/100 g) in the SC viscera. However, thiamin, niacin and pantothenic acid levels were higher in the SC viscera in the current study than in their studies, which could be due to differences in the source of SC. The consumption of a relatively small amount of SC viscera may satisfy the nutritional needs for several vitamins A and B in both animal and human diets, which support the immune system, strengthen bones, heal wounds, turn food into energy, and repair cellular damage (Babji et al., 2020).

## 5.7 Estimate of astaxanthin in the products (Objective 3-IV)

The laboratory work was temporarily paused due to health and safety issues. Laboratory activities have since resumed and astaxanthin content is currently being assessed.



**Table 11. Mineral contents of heat-treated hot-air dried flowers and viscera byproducts collected from live (Live-SC) and commercially processed sea cucumbers on day 3 (CPSC-3)\***

Trace elements (mg/kg)	Flower				Viscera	
	CPSC-3		Live-SC		Live-SC	CPSC-3
	Boiled	Steamed	Boiled	Steamed	Boiled	Boiled
Calcium	26400.0±461.9	17300.0	29500.0	28000.0	2390.0	4580.0
Sodium	15300.0±923.8	34100.0	20100.0	36400.0	44000.0	39300.0
Potassium	6490.0±404.1	13900.0	8070.0	15400.0	24600.0	18400.0
Magnesium	5790.0±184.8	6390.0	6490.0	7370.0	6110.0	5170.0
Strontium	151.0±3.5	120.0	172.0	182.0	40.7	51.3
Iron	92.5±1.7	130.0	57.0	59.0	109.0	181.0
Zinc	88.4±2.0	82.8	71.9	60.8	108.0	120.0
Aluminum	34.9±0.8	59.8	2.3	2.5	9.8	99.5
Boron	14.6±0.0	26.6	17.8	28.6	32	28.2
Uranium	6.5±0.1	7.45	5.56	5.9	9.61	25.4
Manganese	5.6±0.2	4.8	5.4	5.7	1.8	2.9
Barium	5.4±0.2	5	3.5	3.6	0.9	6.8
Rubidium	2.4±0.0	5.08	3.03	6.04	8.2	6.49
Selenium	2.1±0.1	2.5	1.9	2.3	1.9	2.4
Copper	1.6±0.1	2.4	1.4	2.1	3	2.9
Nickel	1.5±0.1	1.9	1.1	1.2	0.5	0.7
Molybdenum	1.5±0.0	1.61	0.81	0.77	0.83	2.29
Lithium	0.6±0.0	0.82	0.73	0.99	0.75	0.72
Vanadium	0.4±0.0	0.6	0.4	0.4	0.4	0.7
Cobalt	0.3±0.0	0.29	0.23	0.23	0.21	0.27
Lead	0.1±0.0	0.18	0.13	0.16	0.18	0.22
Bismuth	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
Antimony	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Beryllium	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Silver	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Tellurium	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Thallium	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02

Mean ±SD (n=2); < 0.02: below determination limit

\*Analyses conducted by the Research and Productivity Council (Fredericton, NB).

**Table 12. Vitamin contents in heat-treated hot-air dried flowers and viscera byproducts collected from live (Live-SC) and commercially processed SC on day 3 (CPSC-3) and Mamelona et al., (2010) study\***

Vitamins (µg/100 g)	Flowers				Viscera		
	Live-SC		CPSC-3		Live-SC	CPSC-3	<i>(Mamelona et al., 2010)</i>
	Boiled	Steamed	Boiled	Steamed	Boiled	Boiled	Non-treated
Vitamin A (retinol)	10.1	<6.0	<6.0	<6.0	<6.0	<6.0	1.5
Vitamin A (beta carotene)	92.3	82.0	136.3	129.0	206.3	224.6	
Vitamin E (alpha-tocopherol)	<1.4	<1.4	<1.4	<1.4	<1.4	<1.4	194.7
Vitamin D3 (cholecalciferol)	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	
Vitamin D2 (ergocalciferol)	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	
Vitamin D	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	
Vitamin B1 (thiamin)	55.4	74.5	62.2	109.3	181.4	44.7	43.9
Vitamin B2 (riboflavin)	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	
Vitamin B3 (niacine)	141.2	240.5	262.4	756.0	1124.4	1434.4	670.4
Vitamin B3 (niacinamide)	311.9	653.0	248.2	324.4	294.8	203.7	
Vitamin B5 (pantothenic acid)	170.6	302.3	225.5	512.2	1157.2	913.4	315.7

\*Analyses were conducted by the Health & Nutrition Laboratory of SGS Canada Inc. (Burnaby, BC)

## 5.8 Evaluation of antinutritional components in the final products (Objective 3-V)

### 5.8.1 Heavy metal contents

Heavy metal content and microbial activity in the byproducts were analyzed as the antinutritional components. Arsenic, chromium, cadmium, lead, mercury and tin are treated as toxic or heavy metals when they exceed the recommended limit for human or other animal consumption. Regarding non-essential or toxic trace metals (arsenic, chromium, cadmium, lead, mercury and tin), their levels were low in SC flowers compared to viscera (Table 13). However, those were in the same ranges as reported in previous studies in SC and other fish products (Gajdosechova et al., 2020; Mamelona et al., 2010). The

lead, cadmium, and mercury concentrations were well below the maximum tolerable limits (MTL) in the pet food (FDA, 2011; NRC, 2005). Total arsenic was close to the maximum tolerable limits. Arsenic is generally not considered an essential nutrient for higher animals or one of the most toxic nutrients for pets (NRC, 2005). The majority of arsenic found in sea plants and fish is organic and virtually non-toxic in nature (NRC, 2005). According to NRC references, 30 mg/kg is a reasonable MTL for arsenic for dogs and cats. The FDA recommended 12.5 mg/kg as MTL for the domestic animals (FDA, 2011).

**Table 13. Heavy metal contents of heat-treated hot-air dried flower and viscera of live (Live-SC) and commercially processed sea cucumbers on day 3 (CPSC-3)\***

Elements (mg/kg)	Flower				Viscera		Maximum tolerable limit (mg/kg) (FDA, 2011; NRC, 2005)
	Live-SC		CPSC-3		CPSC-3		
	Boiled	Steamed	Boiled	Steamed	Boiled	Steamed	
Total arsenic	7.0	8.7	7.1±0.2	8.4	7.5	10.5	12.5
Chromium	2.0	2.1	3.0±0.1	4.6	1.7	2.2	10.0
Cadmium	1.21	1.52	1.5±0.0	2.48	3.19	4.08	10.0
Lead	0.13	0.16	0.1±0.0	0.18	0.18	0.22	10.0
Mercury	0.02	0.02	0.02±0.0	0.03	0.03	0.03	0.27
Tin	< 0.02	0.05	< 0.02	0.03	< 0.02	< 0.02	

Mean ±SD (n=2); < 0.02: below determination limit

\*Analyses conducted by the Research and Productivity Council (Fredericton, NB).

### 5.8.2 Microbial activity

Raw materials, particularly those of animal origin, carry a microbial load detected in the early stages of the feed production chain. Examining the microbiological characteristics of pet food and nutraceuticals is essential to safeguard the health and safety of animals, pet owners, and the surrounding environment. The spread of microorganisms in feed can lead to undesirable changes, resulting in spoilage and posing potential health risks to pets (Hadid et al., 2024). Although no stringent regulations specify a maximum permissible limit of bacterial and fungal contamination in pet food or nutraceuticals (Kazimierska et al., 2021; Leiva et al., 2019), Kukier et al., (2012) and Serhan et al., (2022) recommended that total aerobic microbial count in pet food should not exceed 10<sup>6</sup> CFU/g. The total aerobic microbial count in the thawed SC flowers and viscera was far less than the recommended levels (Kukier et al., 2012; Serhan et al., 2022) (Table 14).

The viscera of CPSC-3 had the highest microbial activity (2.1 x 10<sup>5</sup> CFU/g) among all the byproducts studied. Therefore, the byproducts were heat-treated and dried using hot-air and freeze-drying systems. The microbial activity was less in the heat-treated air-dried viscera of CPSC-3 than in the non-treated one. The total aerobic microbial count was 95 CFU/g in the boiled and hot-air-dried CPSC-3 viscera, while that was

<5 CFU/g in the steamed and dried ones. The microbial activity in the heat-treated viscera was even less than the total aerobic microbial counts in commercial dog foods available on the European market (Kazimierska, Biel, Witkowicz, Karakulska, & Stachurska, 2021).

**Table 14. Microbial analysis of the thawed flowers and viscera collected from live (Live-SC), revived (Rev-SC), and commercially processed sea cucumbers on day 1 (CPSC-1) and day 3 (CPSC-3)**

Sample types	Flowers (CFU/g)	Viscera (CFU/g)
Live-SC	$2.8 \times 10^2$	$2.0 \times 10^3$
Rev-SC	$2.0 \times 10^3$	$5.1 \times 10^4$
CPSC-1	$1.8 \times 10^3$	$3.7 \times 10^4$
CPSC-3	$1.3 \times 10^3$	$2.1 \times 10^5$

CFU: colony forming unit

## 6 CONCLUSIONS

The nutritional and bioactive qualities of SC flowers and viscera make them promising raw materials for high-value products, including pet nutraceuticals. Live-SC and Rev-SC flowers contain higher protein (>60% dwb) and lower lipids than commercially processed flowers, which have substantial levels of both protein (>50% dwb) and lipids (>20% dwb). Boiling concentrated protein levels (>70%) and reduced ash content across all samples, while phospholipid levels were higher in boiled CPSC-3 flowers compared to steamed ones. Boiled byproducts are suitable as protein supplements, while commercially processed flowers have high protein and lipids.

SC viscera, especially from Live-SC and Rev-SC, contain >50% protein and >20% lipids (dwb), with commercially processed viscera showing similar protein levels but lower lipids. Both boiling and steaming improved the nutritional quality of viscera, with minimal differences in protein and ash content. However, steaming reduced total lipids in Live-SC and CPSC-3 viscera.

SC flowers and viscera are rich in macro-elements (calcium, sodium, potassium, magnesium) and trace elements (zinc, copper, iron, manganese, selenium). Boiling reduced some macro-elements and trace elements (iron, copper, selenium) in flowers, likely due to leaching into the cooking water, but zinc and manganese were retained. Boiled flowers had higher vitamin A, while steamed flowers contained more B vitamins (B1, B3, B5).

Heat treatment reduced microbial activity, concentrated lipids, and preserved fatty acid profiles (phospholipids, PUFA, omega-3). Omega-3-bound phospholipids provide health benefits for pets, supporting cardiovascular health and promoting healthy skin and coats (Magalhães, et al., 2021).

SC byproducts are also a natural source of calcium, potassium, vitamin Bs, and phenolic compounds, known for their antioxidant, anti-inflammatory, and anticancer properties. The combination of proteins, lipids, vitamins, minerals, and phenolics in SC byproducts highlights their potential as raw materials for pet nutraceuticals.

## 7 RECOMMENDATIONS

SC flowers and viscera are excellent sources of nutrients and bioactive compounds, including fatty acids (especially omega-3), vitamins, minerals and phenolics. These compounds can be developed into potential animal nutritional supplements and feed additives to stimulate growth, promote good health, boost the immune system, and provide physiological advantages over conventional pet foods (Muhsin et al., 2023). The potential utilization of SC byproducts as a natural source of nutrients is promising. Based on the findings of this project, the following recommendations are proposed:

1. **Sulphated Polysaccharides and Saponins:** Among the various components, sulphated polysaccharides and saponins in the SC body walls are reported for their numerous bioactivities, including anticoagulant, anti-inflammatory, antitumor and immunomodulatory activities for animals (Cao, et al., 2017). Their estimation and characterization are recommended.
2. **Market Potential:** The data from this project can be used to assess market interest in SC byproducts as raw materials for commercial pet nutraceuticals or to develop the Client's own product.
3. **Toxicology Testing:** it is recommended that dried SC byproducts be tested for potential toxicity or sensitivity reactions in animals.
4. **Nutrient Bioavailability:** The bioavailability of nutrients and bioactive compounds is crucial for nutraceutical applications. Therefore, the digestibility of the powder should be tested either in vivo or in vitro.
5. **Liquid Extracts or Hydrolysates:** Developing SC liquid extracts or hydrolysates may enhance bioavailability and functional properties. These should be analyzed for their bioactive effects.
6. **Heat Treatment:** Heat treatment concentrates nutritional components and bioactive compounds, improves handling, and extends shelf life. Boiling is particularly effective for enhancing nutrition in SC flowers while boiling and steaming have similar effects on the viscera as on pet nutraceuticals.
7. Although the arsenic level is a bit high, it was not specified as organic or inorganic. It is crucial to characterize inorganic and organic arsenic and lower the inorganic arsenic level in SC byproducts.

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## APPENDIX A - DETAILED LABORATORY ANALYSIS PERFORMED FOR THE PROJECT

### a. Microbial activity

The microbial activity in all thawed byproducts and heat-treated viscera from CPSC-3 were analyzed by standard plate count at the Avalon Laboratories, St. John's. NL, following their official laboratory method MFHBP-18.

### b. Proximate analysis

Proximate analysis was performed on thawed, heat-treated air-dried, and freeze-dried SC byproducts following the standard method of the Association of Official Analytical Chemists International (AOAC) (AOAC, 2012). Moisture and ash content were determined following AOAC 930.15 and AOAC 938.08. The Kjeldahl method (AOAC 954.01) was used for protein content analysis and the Soxhlet extraction (AOAC 948.15) for total lipid content.

### c. Astaxanthin analysis

The astaxanthin content was determined following Jaramillo et al., (2018) with few modifications. Approximately 0.25 g of minced SC byproducts was mixed with 10 ml of isopropanol by vortexing for 30 seconds. After 10 min, the mixture was homogenized with 10 ml hexane. The extract was collected by centrifugation at 3000 rpm and 10 °C for 10 minutes. Extraction was repeated two more times with 8 ml of solvent, and the pooled extract was centrifuged to remove solids if any. The extract was rinsed with an equal volume of 2% sodium chloride using a separatory funnel. The upper pigmented layer was collected and dehydrated using 2-3 g of anhydrous sodium sulfate followed by drying under N<sub>2</sub> at 55-60 °C. It was re-dissolved with 5 ml hexane and absorbance was measured at 470 nm.

The astaxanthin content was calculated using the following equation (Sindhua & Sherief, 2011):

$$\text{Astaxanthin} \left[ \frac{\mu\text{g}}{\text{g}} \right] = \frac{A \times D \times 10^6}{100 \times G \times d \times E}$$

Where,

A: Absorbance at  $\lambda_{470}$

D: Volume of extract in hexane (ml)

10<sup>6</sup>: Dilution multiple

G: Sample weight (g)

d: Cuvette width (1 cm)

E: Extinction coefficient at  $\lambda_{470}$  (2100 for astaxanthin)

### d. Total lipid class and fatty acid composition

The total lipid class and fatty acid composition of lipids in thawed and heat-treated air-dried and freeze-dried SC byproducts were analyzed at the Department of Ocean Science (OSC) of Memorial University of

Newfoundland (St. John's, NL, Canada). Lipid classes were separated by thin-layer chromatography (TLC) on silica gel-coated Chromarods and detected and quantified by flame ionization detection (FID) in an automatic FID scanner following the method developed by Parrish and Wells (2021). The total lipid was calculated by taking the sum of all lipid classes. The fatty acid composition of each sample was analyzed using gas chromatography with an FID, as described by Parrish and Wells (2021).

#### **e. Amino acid analysis**

Hydrolysis of dried SC byproducts and derivatization and measurement of amino acids of heat-treated dried SC byproducts were carried out following Liu et al., (2021).

#### **f. Total phenolic content and antioxidant activity**

The total phenolic content of heat-treated air-dried and freeze-dried SC byproducts was determined following Carletti et al., (2022) with few modifications. About 1 g fine SC byproduct powder was homogenized with 96% ethanol at a ratio 1:25 (w/v) using FisherBrand homogenizer 850 at 24,000 speed for 30 sec twice. The mixture was incubated in an Incubator shaker (Thermo-Scientific Max Q 6000, Marietta, Ohio, USA) overnight at 125 rpm speed at room temperature. The extract was collected after centrifugation at 3,000 g for 5 min. Centrifugation was repeated two more times with a final round at 5000 rpm. The extract was stored at -20 °C until polyphenol or antioxidant activities were determined.

Total phenolic content was determined in the byproduct extracts using the Folin-Ciocalteu reagent with a protocol adapted from Carletti et al., (2022). 600 mL of double distilled water and 150 ml of diluted (1:1) Folin-Ciocalteu reagent were added to 100 µL of extract and incubated in the dark for 5 min at room temperature after vortexing. Subsequently, 750 mL of 2% sodium carbonate was added, vortexed and incubated in the dark for 90 min. Absorbance was then detected at 750 nm using a Hach DR 6000 spectrophotometer. The calibration curve was prepared by using serial dilutions (0.012, 0.025, 0.05, 0.1, 0.2, 0.3 mg/ml) of gallic acid standard stock solution (1mg/ml). Total polyphenol content was estimated in triplicates and calculated from the gallic acid calibration curve as milligrams of gallic acid-equivalent (GAE) phenolic compounds per 100 g of byproduct powder (mg GAE/100 g).

The DPPH radical scavenging activity was assessed according to Hossain et al., (2022). The activity was expressed as milligrams of Trolox equivalents (TE) per 100 g of dried byproduct powder (mg TE/100 g) on a percentage basis, relative to the negative control (water) using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = (A_b - A_s) / A_b * 100$$

where,  $A_b$  = Absorbance of blank (water) and  $A_s$  = Absorbance of sample.

#### **g. Mineral and trace element analysis**

The mineral analysis for the pretreated dried SC byproduct powder was performed at the Research & Productivity Council (RPC), Fredericton, NB, following their routinely used methods. Individual samples were subjected to acid digestion using microwave-assisted digestion in nitric acid (SOP IAS-M26). The resulting solutions were analyzed for all the elements except mercury by inductively coupled plasma mass spectrometry (ICP-MS) following RPC's SOP IAS-M01 method. Mercury was analyzed by cold vapour atomic absorption spectroscopy following RPC's SOP IAS-M52 & SOP IAS-M53 methods.

#### **h. Vitamin analysis**



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The Health & Nutrition Laboratory of SGS Canada Inc. (Burnaby, BC) carried out the vitamin analysis of the heat-treated dried SC byproducts using their standard methods. Beta carotene, vitamin A (retinol), and E (alpha-tocopherol) were determined using AFVAN-SLMF-0013 method, vitamin D3 (cholecalciferol), D2 (ergocalciferol) and D using AFVAN-SLMF-0025, and vitamin B1 (thiamin), B2 (riboflavin), B3 (niacin and niacinamide), and B5 (pantothenic acid) using AFVAN-SLMF-0023.



**APPENDIX B-RECOMMENDED MINIMUM LEVELS OF NUTRIENTS FOR COMPLETE DOG FOODS AND CATFOODS. UNIT PER 100 G DRY MATTER (FEDIAF, 2024)**

Nutrient	Unit	Dogs		Cats	
		Adult	Early growth/ reproduction	Adult	Early growth/ reproduction
<b>Proteins</b>	<b>g</b>	<b>21.00</b>	<b>25.00</b>	<b>33.30</b>	<b>28.00/30.00</b>
Arginine	g	0.60	0.82	1.30	1.07/1.11
Histidine	g	0.27	0.39	0.35	0.33
Isoleucine	g	0.53	0.65	0.57	0.54
Leucine	g	0.95	1.29	1.36	1.28
Lysine	g	0.46	0.88	0.45	0.85
Methionine	g	0.46	0.35	0.23	0.44
Methionine + cystine	g	0.88	0.70	0.45	0.88
Phenylalanine	g	0.63	0.65	0.53	0.50
Phenylalanine + tyrosine	g	1.03	1.30	2.04	1.91
Threonine	g	0.60	0.81	0.69	0.65
Tryptophan	g	0.20	0.23	0.17	0.16
Valine	g	0.68	0.68	0.68	0.64
Taurine				0.08	0.06
<b>Fats</b>	<b>g</b>	<b>5.50</b>	<b>8.50</b>	<b>9.00</b>	<b>9.00</b>
Linoleic acid (ω-6)	mg	1.53	1.30	0.67	0.55
Arachidonic acid (ω-6)	g	-	30.00	8.00	20.00
Alpha-linolenic acid (ω-3)	g	-	0.08	-	0.02
EPA + DHA (ω-3)	g	-	0.05	-	0.01
<b>Minerals</b>					
Calcium	g	0.58	1.00	0.53	1.00 g
Phosphorus	g	0.46	0.90	0.35	0.84 g
Potassium	g	0.58	0.44	0.80	0.60
Sodium	g	0.12	0.22	0.10	0.16
Chloride	g	0.17	0.33	0.15	0.24
Magnesium	g	0.08	0.04	0.05	0.05
<b>Trace elements</b>					
Copper	mg	0.83	1.10	0.67	1.00
Iodine	mg	0.12	0.15	0.17	0.18
Iron	mg	4.17	8.80	10.70	8.00



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Manganese	µg	0.67	0.56	0.67	1.00
Selenium (wet diets)	µg	27.00	40.00	35.00	30.00
Selenium (dry diets)	mg	22.00	40.00	28.00	30.00
Zinc	mg	8.34	10.00	10.00	7.50
<b>Vitamins</b>					
Vitamin A	IU	702.00	500.00	444.00	900.00
Vitamin D	IU	63.90	55.20	33.30	28.00
Vitamin E	mg	4.17	5.00	5.07	3.80
Vitamin B1 (Thiamine)	mg	0.25	0.18	0.59	0.55
Vitamin B2 (Riboflavin)	mg	0.69	0.42	0.42	0.32
Vitamin B5 (Pantothenic acid)	mg	1.64	1.20	0.77	0.57
Vitamin B6 (Pyridoxine)	µg	0.17	0.12	0.33	0.25
Vitamin B12 (Cyanocobalamin)	mg	3.87	2.80	2.35	1.80
Vitamin B3 (Niacin)	µg	1.89	1.36	4.21	3.20
Vitamin B9 (Folic acid)	µg	29.90	21.60	101.00	75.00
Choline	µg	189.00	170.00	320.00	240.00