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Effect of Drying on Nutritional Composition of Atlantic Sea Cucumber (*Cucumaria frondosa*) Viscera Derived from Newfoundland Fisheries

Yi Liu, Deepika Dave *, Sheila Trenholm, Vegneshwaran V. Ramakrishnan and Wade Murphy

Marine Bioprocessing Facility, Centre for Aquaculture and Seafood Development, Fisheries and Marine Institute, Memorial University of Newfoundland, St. John's, NL A1B 4L6, Canada; Yi.Liu@mi.mun.ca (Y.L.); Sheila.Trenholm@mi.mun.ca (S.T.); Vegneshwaran.Ramakrishnan@mi.mun.ca (V.V.R.); Wade.Murphy@mi.mun.ca (W.M.)

* Correspondence: Deepika.Dave@mi.mun.ca

Abstract: *Cucumaria frondosa* is the main sea cucumber species harvested from Newfoundland waters. During processing, the viscera of sea cucumber are usually discarded as waste. As a matter of fact, sea cucumber viscera are abundant in various nutrients and promising for valorization. In the present study, sea cucumber viscera were pretreated by air drying and freeze drying, and the nutritional compositions of the dried products were investigated, including proximate composition, lipid class, fatty acid profile, and amino acid composition. The dried viscera had similar levels of ash, lipids, and proteins compared to fresh viscera. Both air- and freeze-dried viscera had total fatty acid composition similar to fresh viscera, with high levels of omega-3 polyunsaturated fatty acids (PUFAs) (30–31%), especially eicosapentaenoic acid (27–28%), and low levels of omega-6 PUFAs (~1%). The dried samples were abundant in essential amino acids (46–51%). Compared to air-dried viscera, freeze-dried viscera contained a lower content of moisture and free fatty acids, and higher content of glycine and omega-3 PUFAs in phospholipid fraction. The high content of nutritious components in dried viscera of *Cucumaria frondosa* indicates their great potential for valorization into high-value products.

Keywords: marine by-products; *Cucumaria frondosa*; drying; omega-3 fatty acids; eicosapentaenoic acid; amino acids

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1. Introduction

Sea cucumbers are marine invertebrates belonging to the class Holothuroidea. There are approximately 1700 species of sea cucumbers worldwide, which mainly live on the sea floor [1]. Sea cucumbers contain low content of sugar, fat, and cholesterol, but high content of proteins [2]. They are a source of multiple nutritional components, including vitamins, minerals, collagen, amino acids, and polyunsaturated fatty acids. Sea cucumbers also contain a variety of bioactive compounds, such as triterpene glycosides, chondroitin sulfates, glycosaminoglycans, lectins, bioactive peptides, and glycoprotein [3]. During recent decades, therapeutic and medicinal benefits of sea cucumbers have been intensively studied, due to their anticancer, antioxidant, anti-inflammatory, and antimicrobial properties, and wound healing activities [3–5]. Sea cucumbers have been consumed as a type of tonic food in Asia for thousands of years. The market for sea cucumbers was originally developed in Asia and Indo-Pacific regions. With increased awareness about their nutritional value and benefits, consumption of sea cucumbers has increased drastically worldwide, resulting in a global industry of approximately $1 billion [6].

The Canadian market was started in 1980s from the west coast, and spread to Atlantic provinces after two decades [7]. In Newfoundland and Labrador, the most common sea cucumber species is *Cucumaria frondosa* [8]. Although some sea cucumber species worldwide have been included in the International Union for Conservation of Nature

Red List of Threatened Species, *Cucumaria frondosa* is in a sufficient amount, and it is the main sea cucumber species for exportation in Newfoundland fisheries. Sea cucumbers are eviscerated after harvesting. The viscera (all internal organs, including respiratory track, gonad, and intestines) account for approximately 50% of the total body weight of *Cucumaria frondosa*. Sea cucumber viscera (SCV) have been reported to contain various nutrients such as oligosaccharides, saponins, amino acids, polyunsaturated fatty acids, phenols, flavonoids, and trace metals [9]. Mamelona et al. [10] reported that fresh *Cucumaria frondosa* viscera was abundant in essential amino acids (37% of total amino acids) and polyunsaturated fatty acids (45% of total lipids), especially eicosapentaenoic acid (EPA, 17%). However, unlike by-products of many types of finfish and shellfish, which have been intensively valorized for value addition [11,12], there has not been much research performed on valorization of sea cucumber viscera [13–18]. Currently in Newfoundland, the viscera of *Cucumaria frondosa* are entirely discarded as waste. Considering the various bioactive compounds they contain, more research should be performed to valorize them for the industrial production of high-value nutritional products.

Generally, fishery by-products are not immediately processed into value-added products after their collection from processing facilities, especially in remote areas or during peak period of harvesting. Therefore, a proper method is required to preserve the quality and freshness of the raw materials. Drying is a widely used technique in the food industry to maintain product quality and extend their shelf-life, as microbial contamination and subsequent spoilage of products can be effectively slowed down by reducing moisture content in the products [19]. A number of drying methods have been applied in the food industry based on their advantages and the aimed deliverables, such as fluidized bed drying, vacuum drying, hot air drying, and freeze drying [20]. Hot air drying (convective drying) is a traditional drying method, which removes water by a continuous flow of hot air via heat and mass transfer. Hot air drying is the most commonly used method in industry due to its relatively low equipment cost and simple operation. However, it has been claimed to result in product shrinkage and consequent structure damage. The heat-sensitive nutritional components can be destroyed in the hot atmosphere [20]. Freeze drying (lyophilization) is a process that involves sublimation of ice in the frozen product under low temperature and vacuum [21]. Compared to air drying, freeze drying requires higher investment in equipment installation and operation. However, freeze drying has been reported to be more effective in preserving quality and retaining nutritional properties of products [22–25]. In the present study, *Cucumaria frondosa* viscera were dried using two methods, including air drying and freeze drying, and the dried products were analyzed for nutritional composition. The effect of two drying methods was investigated in terms of dehydrating sea cucumber waste and retaining their nutritional profiles, and the potential of valorization of dried sea cucumber waste was evaluated (Figure 1).

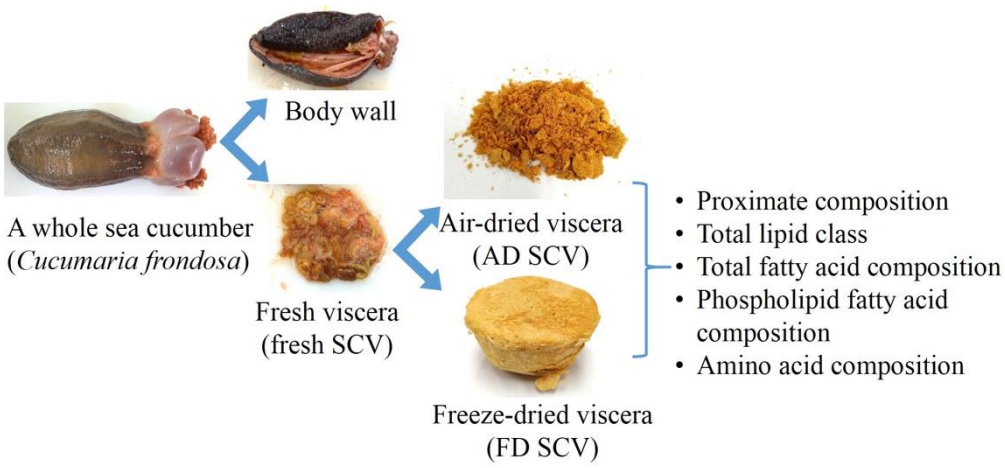


Figure 1. Experimental design of drying sea cucumber (*Cucumaria frondosa*) viscera, analyzing their nutritional profiles.

2. Materials and Methods

2.1. Chemicals

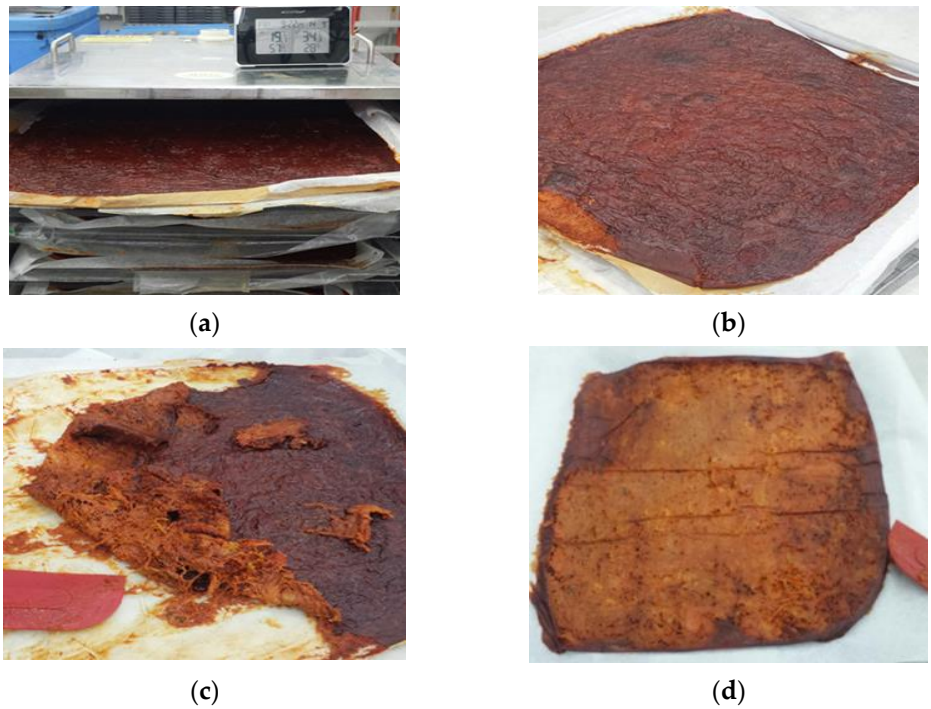
Hexane (≥98.5%), acetanilide (99%), glycine (98.5–101.0%), Kjeltabs Cu−3.5, concentrated sulfuric acid (95.0–98.0%), and concentrated hydrochloric acid (36.5–38.0%) were purchased from Fisher Scientific (Waltham, MA, USA). Nicotinic acid (99.5%) was obtained from Acros Organics (Fair Lawn, NJ, USA). Boric acid (4%) with methyl red/methylene blue indicator was from RICCA Chemical (Arlington, TX, USA). Sodium hydroxide (40%) was from VWR (Radnor, PA, USA). Amino acid standard solution (containing 17 amino acids), acetonitrile (≥99.9%) and N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA, ≥99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade.

2.2. Preparation of Samples

Approximately 10 kg of fresh SCV (sea cucumber viscera) was supplied in August 2018 from a local seafood processing plant in Newfoundland, Canada. The samples were immediately stored at 4–8 °C until further processing.

2.2.1. Air Drying

A portion of the fresh SCV (approximately 3 kg) was spread evenly on parchment paper and placed on trays, which were loaded into a drying oven at 34 °C for 24 h (Figure 2a). Afterwards, the samples were removed from the drying oven and allowed to cool (Figure 2b). The partially dried samples were scraped off the parchment paper and flipped over to expose the undersurface, which was not fully dried (Figure 2c). Then the samples were returned to the drying oven for complete drying (Figure 2d). To prevent surface hardening, the oven temperature was lowered to 27 °C. After 48 h, the samples were removed and reweighed. The air-dried SCV were ground for 2 min in a Ninja blender on high speed to obtain powder with particle size ≤5 mm.



(**a**) (**b**) (**c**) (**d**)

Figure 2. (**a**) Air drying of SCV at 34 °C; (**b**) partially dried SCV after 24 h at 34 °C; (**c**) flipping over the partially dried material; (**d**) SCV after flipped over, ready for 2nd round of drying.

2.2.2. Freeze Drying

A portion of the fresh SCV (approximately 3 kg) was freeze dried using a Labconco 2.5 L Lab Scale freeze dryer. The fresh SCV were frozen in silicone trays at −1 °C overnight. Afterwards, the samples were transferred to freeze drying flasks and weighed. The flasks were loaded onto the freeze dryer and dried between −48 and −52 °C at ≤0.133 mBar for 48 h (Figure 3). After freeze drying, the flasks were removed from the freeze dryer and reweighed. The samples were ground for 2 min in a Ninja blender on high speed to obtain powder with particle size ≤5 mm.

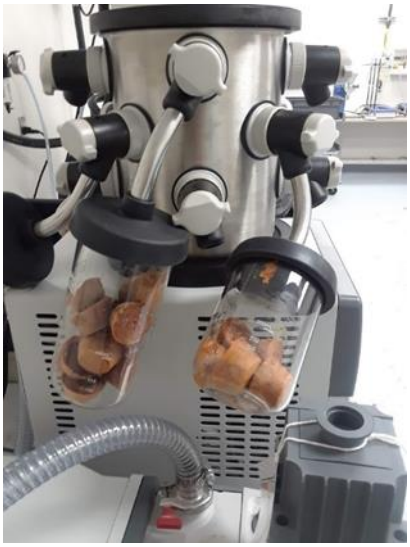


Figure 3. Freeze drying of SCV.

2.3. Proximate Analysis

Proximate analysis was performed on fresh, air-dried, and freeze-dried SCV following the standard method of the Association of Official Analytical Chemists International (AOAC) [26]. Moisture and ash content were determined following AOAC 930.15 and AOAC 938.08. The Kjeldahl method (AOAC 954.01) was applied for protein content analysis and Soxhlet extraction (AOAC 948.15) was applied to determine total lipid content.

2.4. Total Lipid Class and Fatty Acid Composition

Total lipid class, and fatty acid composition of total lipids and phospholipids of fresh and dried SCV were analyzed at Oceans Sciences Centre of Memorial University of Newfoundland (St. John's, NL, Canada).

2.4.1. Lipid Extraction

Lipids in sea cucumber viscera were extracted according to the method by Parrish [27]. An aliquot of approximately 0.25 g sample was weighed in a test tube containing 2 mL of chloroform. Prior to addition of the sample, the test tubes and Teflon® lined caps were rinsed three times with methanol and chloroform, respectively, to remove any lipids. 1 mL of ice-cold methanol, 1 mL of chloroform: methanol (2:1 v/v), and 0.5 mL of chloroform-extracted water were added to the test tube. (Chloroform-extracted water was prepared by adding 1 l of distilled water and 30 mL of chloroform to a separatory funnel. The funnel was manually shaken for 2 min, and the chloroform was allowed to settle and removed from the bottom of the funnel. This procedure was repeated twice to remove any lipids present in the distilled water.) The test tube was then recapped and sonicated for 10 min, followed by centrifugation for 3 min at 3000 rpm. The entire lower organic lipid layer was removed by a double pipetting technique and transferred to a 15 mL lipid-free vial [28]. The double pipetting technique was performed in three steps. Firstly, a 14 cm pipette was

passed through the top aqueous layer in the test tube to the bottom of the test tube, by bubbling air with the pipette bulb to prevent the aqueous layer from entering the pipette. Secondly, the pipette bulb was removed, and a 27 cm pipette was placed inside the 14 cm pipette until it reached the bottom of the test tube. Finally, the lipid layer was extracted using the long pipette and transferred to another lipid-free vial. Each of the short and long pipettes was washed with 3 mL ice-cold chloroform, and the wash was collected. The samples were again sonicated, centrifuged, double pipetted, and the pipettes were rinsed three times as previously described, and all the organic layers were collected together. The collected lipid fraction was evaporated under a gentle stream of nitrogen, sealed with Teflon® tape and stored at −20 °C until use.

2.4.2. Lipid Class Determination

Lipid class was determined using an Iatroscan Mark VI thin-layer chromatograph-flame ionization detector (Iatron Laboratories, Inc., Tokyo, Japan) and silica-coated Chromarods® through a three-step development method [29]. The Chromarods® were calibrated (0.2–20 µg) using lipid standards including: nonadecane (aliphatic hydrocarbon), cholesteryl palmitate (wax esters/steryl ester), 3-hexadecanone (ketone), glyceryl tripalmitate (triacylglycerol), 1,2-dihexadecanoylglycerol (diglyceride), 1-hexadecanol (free aliphatic alcohol), cholesterol (free sterol), 1-monopalmitoyl-rac-glycerol (acetone mobile phase lipids), and 1,2-di-o-hexadecyl-sn-glycerol-3-phosphocholine (phospholipids) purchased from Sigma-Aldrich. The lipid extracts and standards were applied to the Chromarods® and focused on a narrow band using 100% acetone.

Four different solvent systems were used to obtain three chromatograms per rod. The first development system was hexane: diethyl ether: formic acid (99.95:1:0.05). The rods were developed for 25 min, removed from the system for 5 min, and placed again in the system for 20 min for double development. The first chromatograms were obtained by scanning each rod to the lowest point behind the ketone peak. The second development was developed for 40 min in hexane: diethyl ether: formic acid (79:20:1). The second chromatogram was obtained by scanning each rod to the lowest point behind the diglyceride peak. The final development was carried out in two steps. The lipid extract was first developed using 100% acetone for two 15 min periods, and then two 10 min periods in chloroform: methanol: chloroform-extracted water (5:4:1). The third chromatogram was obtained as a complete scan after two double developments. Before each solvent system, the rods were dried in a constant humidity chamber. After each development system, the rods were scanned in the Iatroscan and the data were collected using Peak Simple software (ver 3.67, SRI Instruments, Torrance, CA, USA).

2.4.3. Preparation of Fatty Acid Methyl Esters (FAMEs)

An aliquot of 40 µL lipid extract was transferred to a lipid-free vial, in which 1.5 mL of methylene chloride and Hilditch reagent was added. The Hilditch reagent was prepared by adding 1.5 mL of concentrated sulfuric acid to 100 mL of dry methanol. The sample was capped and vortexed for approximately 5 s, followed by sonication for 4 min. The vial was flushed with nitrogen, capped, sealed with Teflon® tape and heated at 100 °C for 1 h in a VWR drying oven (VWR international, Mississauga, ON, Canada). The vial was cooled to room temperature. Approximately 0.5 mL of a saturated solution of sodium bicarbonate in chloroform-extracted water was slowly added to the vial, followed by the addition of 1.5 mL of hexane and vortexing for 10 s. The top organic layer was removed to a new vial, and the hexane was evaporated with a gentle stream of nitrogen. The fatty acids were re-suspended by adding approximately 0.5 of hexane, sealing the vial with Teflon® tape under nitrogen, and sonicating for an additional 4 min.

2.4.4. Fatty Acid Composition Analysis

Fatty acid composition of the FAME samples was analyzed using a gas chromatograph-flame ionization detector (Hewlett Packard 6890 Series II, Agilent Technologies, Missis-

sauga, ON, Canada) following the method developed by [27]. A ZB wax+ polar capillary column (Phenomenex, Torrance, CA, USA, 30 m in length, 0.32 mm of internal diameter and 0.25 µm of film thicknesses) was used. An aliquot of 10 µL FAME sample was injected directly into the column. The initial oven temperature was set at 65 °C and held for 0.5 min, followed by the ramped temperature of 195 °C at a rate of 40 °C/min and held for 15 min, and further ramped to a final temperature of 220 °C at a rate of 2 °C/min and held for 0.75 min. The flame ionization detector was operated at 260 °C with hydrogen as the carrier gas at a flow rate of 2 mL/min. The injector temperature was started at 150 °C and ramped to a final temperature of 250 °C at a rate of 120 °C/min. The analyte peaks were identified using retention times of standards purchased from Supelco, including 37 component FAME mix, bacterial acid methyl ester mix, PUFA No. 1 and PUFA No. 3. Chromatograms were integrated using Agilent OpenLAB Data Analysis-Build 2.203.0.573.

2.4.5. Separation of Phospholipids

The phospholipids were separated using Strata SI-1 silica tubes (Phenomenex 8B-S012-JDG, Phenomenex, Torrance, CA, USA) with the 12-position vacuum manifold set (Phenomenex AH0-6023, Phenomenex, Torrance, CA, USA). The aspirator was started by pulling a very light vacuum. The column was washed by eluting 2 × 3 mL methanol and 2 × 3 chloroform. Afterwards, 3 mL of a 98:1:0.5 mixture of chloroform: methanol: formic acid was eluted. Once the solvent mixture reached the top of the silica, the stop cocks at the base of the tubes were closed and the sample extract was applied directly to the silica using a long pipette. The sample vial was rinsed with a small amount of chloroform as quickly as possible and the rinsing was applied to the silica. A lipid-cleaned 15 mL vial was located at the end of the column for collection. The stop cocks were opened and once the last of the rinse reached the top of the silica, 8 mL of the 98:1:0.5 mixture of chloroform: methanol: formic acid was eluted through the pipette and all the resulting neutral lipid-containing eluent was collected. Afterwards, the silica gel was rinsed with 2 × 3 mL acetone to recover the acetone mobile polar lipids in a second 15 mL vial. Then the vial containing the acetone mobile polar lipids fraction was replaced with a 40 mL vial. 3 mL of chloroform was passed through the column to return the column to a more neutral polarity. Phospholipids were eluted with 6 mL methanol followed by 9 mL of a mixture of chloroform: methanol: water (5:4:1). The phospholipid fraction was transferred to a 50 mL round bottom flask and dried completely in a flash-evaporator. The lipids were then washed into a 15 mL vial using methanol and chloroform.

2.4.6. Preparation of FAMEs of Phospholipids and Fatty Acid Composition Analysis

The same procedure was followed as described in Section 2.4.3 and Section 2.4.4.

2.5. Amino Acid Analysis

2.5.1. Hydrolysis of Dried SCV

Acidic hydrolysis of dried SCV was carried out following the method by Fountoulakis and Lahm [30]. Ten milligrams of dried viscera sample was reacted with 1 mL 6 N hydrochloric acid at 110 °C for 24 h. The resulting solution was centrifuged at 13,000 rpm for 15 min. The supernatant was collected, and 0.5 mL of the internal standard solution (0.5 mg/mL norleucine) was added. The solution was thoroughly mixed and ready for further analysis.

2.5.2. Derivatization and Measurement of Amino Acids

The derivatization and analysis of amino acids were performed following the method by Stenerson [31]. Fifty microliters of the solution from 2.5.1 was transferred into a 10 mL test tube and completely dried at 70 °C under nitrogen for 5 min. One hundred microliters of MTBSTFA (N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide) was added to the test tube, followed by 100 µL of acetonitrile. The test tube was tightly capped and heated at 100 °C for 2 h. Afterwards, the sample was allowed to cool at room temperature, and

200 µL of acetonitrile was added. The solution was thoroughly mixed and transferred into a GC (gas chromatograph) vial for analysis.

The analysis was carried out using a gas chromatograph-mass spectrometer (ThermoFisher Trace 1300 GC/ISQ-LT MS) equipped with a SLB-5ms, 20 m × 0.18 mm column (internal diameter of 0.18 µm). The inlet temperature was 280 °C. The splitless flow and time was maintained at 100 mL/min and 0.3 min, respectively. The helium flow was 0.5 mL/min. The initial temperature of GC oven was 60 °C, ramped up to 100 °C at a rate of 20 °C/min and held for 1 min, followed by the ramped temperature of 290 °C at a rate of 10 °C/min and held for 3 min, and finally ramped to 340 °C at a rate of 10 °C/min and held for 2 min. The temperature of MS transfer line (the instrumental component where the eluate from the column of the gas chromatograph is transferred to the ion source of the mass spectrometer) and ion source was maintained at 320 °C and 280 °C, respectively. The standards were initially scanned using MS in the range of m/z 40–639. Once the target compounds were identified, the data were exported and analyzed in SIM acquisition mode (Selected Ion Monitoring mode, in which the mass spectrometer is set to only collect data of compounds that possess the selected mass fragments, instead of a wide range of masses).

The amino acid standard solution was diluted in 0.1 N hydrochloric acid at a concentration of 2.5 µmoles/mL of each amino acid, except cystine at a concentration of 1.25 µmoles/mL. Samples for the calibration curve were prepared by taking 10, 20, 30, 40, and 50 µL from the standard solution and diluted using 400 µL 0.1 N hydrochloric acid. The samples were processed and analyzed following the same procedure as described above.

2.6. Statistical Analysis

The proximate composition, lipid class, and fatty acid composition analyses were performed in triplicates. The amino acid analysis was performed in duplicates. The data were analyzed with analysis of variance (ANOVA) at 95% confidence level using Minitab 17.3.1.

3. Results and Discussion

3.1. Proximate Analysis

The proximate composition of fresh (fresh SCV), air-dried (AD SCV), and freeze-dried sea cucumber viscera (FD SCV) are shown in Table 1 and Figure 4. So far, there have been only two studies performed about proximate composition of *Cucumaira frondosa* and its by-products. Mamelona et al. [10] reported 92.3% moisture, 0.7% ash, 2.0% lipids, and 4.5% proteins in fresh viscera of *Cucumaira frondosa* from Québec, QC, Canada. The differences in proximate composition between reported results from their and the present studies are likely due to variation of living conditions of the sea cucumber, such as the habitat and climate, and the harvesting location and time [32,33]. Zhong et al. [34] reported that body wall of *Cucumaira frondosa* from Newfoundland waters contained 87.4% moisture, 2.97% ash, 0.50% lipids, and 8.34% proteins. In comparison to body wall, viscera have similar content of moisture, ash, and proteins, but much higher amount of lipids, possibly due to the better ability of internal organs to store fat.

In comparison to fresh SCV (82.07% moisture), both drying methods removed most water from the fresh sample. However, freeze drying (1.79% moisture in FD SCV) was comparatively more effective than air drying (7.84% moisture in AD SCV). For food materials with high content of moisture (e.g., above 80%), freeze drying might be more effective in removing the high amount of water. Although Tukey Test indicated that the content of ash, lipids, and proteins of the dried samples were statistically different from the fresh SCV (dry weight basis), the actual differences between the dried samples and the fresh SCV were within a range of approx. 0.1% for ash, 0.04–3% for lipids and 1–3% for proteins (Table 1). Considering the large amounts of ash (approx. 12%), lipids (approx. 23–26%), and proteins (approx. 46–48%) these samples contained, the differences are very small. Therefore, the drying methods did not significantly change the proximate composition of fresh SCV except removing water.

Table 1. Proximate composition of fresh, air-dried (AD SCV), and freeze-dried (FD SCV) sea cucumber viscera (wwb: wet weight basis, dwb: dry weight basis).

Parameter	Fresh SCV	AD SCV	FD SCV
	Percent of wet tissue (wwb, Mean ± SD)		
Moisture (%)	82.07 ± 0.03	7.84 ± 0.14	1.79 ± 0.07
Ash (%)	2.14 ± 0.05	11.51 ± 0.14	12.31 ± 0.05
Lipid (%)	4.68 ± 0.01	23.68 ± 0.33	22.77 ± 0.12
Protein (%)	8.65 ± 0.17	42.20 ± 0.29	46.12 ± 0.20
	Percent of dry matter (dwb, Mean ± SD)		
Ash (%)	11.95 ± 0.29 ^{a}	12.49 ± 0.15 ^{b}	12.53 ± 0.05 ^{b}
Lipid (%)	26.12 ± 0.03 ^{a}	25.69 ± 0.36 ^{a}	23.19 ± 0.12 ^{b}
Protein (%)	48.26 ± 0.17 ^{a}	45.79 ± 0.31 ^{b}	46.96 ± 0.20 ^{a,b}

^{a,b} sample means of proximate composition compared by Tukey Test at 95% confidence level was performed for dry matter; means in the same line that do not share the same letter are significantly different ($p < 0.05$).

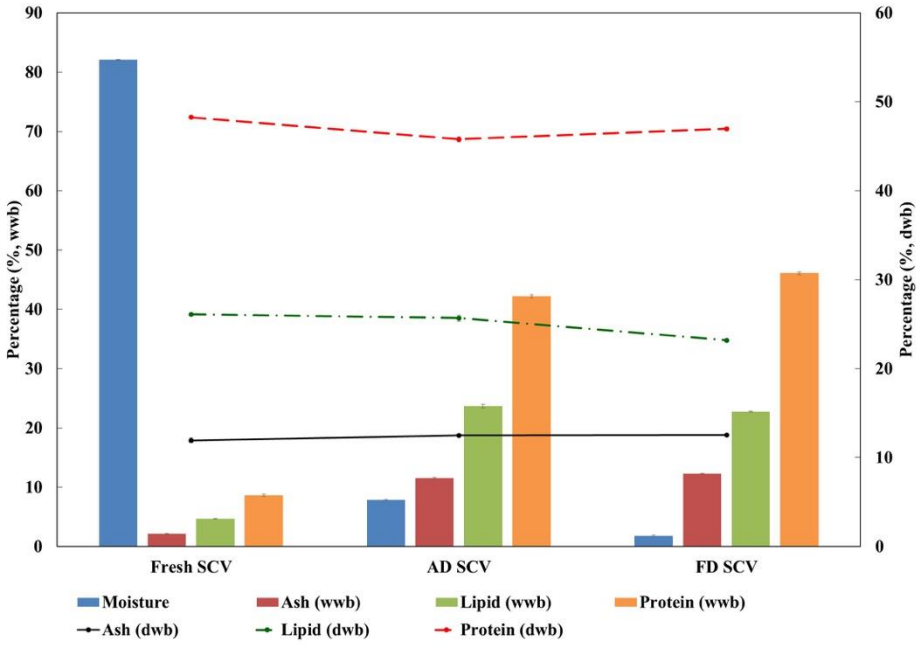


Figure 4. Proximate composition of fresh, air-dried (AD SCV) and freeze-dried (FD SCV) sea cucumber viscera (wwb: wet weight basis, dwb: dry weight basis).

3.2. Total Lipid Class

As indicated in Table 2 and Figure 5, the largest component of total lipids in fresh SCV was triacylglycerols (TAGs) (32.93%). TAGs are composed of one glycerol chain linked to three long-chain fatty acids via esterification. Each TAG molecule usually contains one EPA or DHA. So far, TAGs in plant oil and fish oil are the primary source of omega-3 PUFAs for human consumption. In the present study, after air drying, the TAG content was significantly decreased by 19.73% (13.20% in AD SCV), along with a notable increase of free fatty acids (FFAs) by 21% from fresh SCV (7.75%) to AD SCV (28.75%). After freeze drying, the TAG content was slightly decreased by 6.63%, but the FFA content in FD SCV (6.84%) was similar to fresh SCV (7.75%). FFAs are formed due to hydrolysis of lipids catalyzed by hydrolytic enzymes in raw materials [35]. In the present study, the high amount of FFAs in AD SCV was likely due to hydrolysis of TAGs in fresh SCV resulting from the higher temperature (27 and 34 °C) of air drying for longer drying time (72 h) compared to freeze drying (in the range of −48 ~ −52 °C, 48 h).

Table 2. Total lipid class of fresh, air-dried (AD SCV), and freeze-dried (FD SCV) sea cucumber viscera.

Lipid Class	Fresh SCV (%)	AD SCV (%)	FD SCV (%)
Hydrocarbons	0.05 ± 0.09	0.24 ± 0.28	0.30 ± 0.43
Steryl esters/wax esters	0.10 ± 0.17	0.00 ± 0.00	0.00 ± 0.00
Ethyl esters	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Methyl esters	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ethyl ketones	0.16 ± 0.27	0.00 ± 0.00	0.00 ± 0.00
Methyl ketones	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Glyceryl ethers	26.87 ± 1.93	22.15 ± 0.64	23.14 ± 2.48
Triacylglycerols	32.93 ± 1.96	13.20 ± 0.90	26.30 ± 3.29
Free fatty acids	7.75 ± 1.21	28.75 ± 0.20	6.84 ± 0.36
Alcohols	0.00 ± 0.00	4.01 ± 0.35	0.51 ± 0.72
Sterols	1.92 ± 0.15	1.04 ± 0.18	0.86 ± 0.21
Diacylglycerols	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Acetone mobile polar lipids	3.60 ± 1.70	2.85 ± 2.61	4.91 ± 3.30
Phospholipids	26.63 ± 1.31	27.77 ± 3.20	37.13 ± 8.78

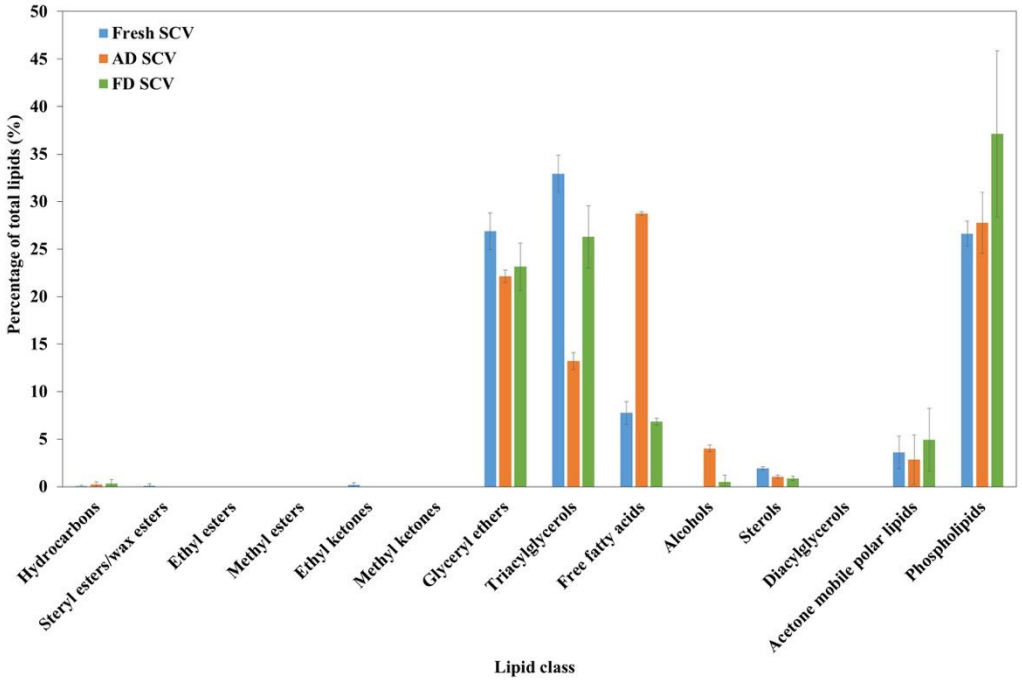


Figure 5. Total lipid class of fresh, air-dried (AD SCV), and freeze-dried (FD SCV) sea cucumber viscera.

In comparison to fresh SCV and AD SCV, FD SCV contained less moisture (Table 1) so they remained in more compact form. Therefore, during lipid extraction, it was more difficult for the organic solvents (chloroform and methanol, see Supplementary Information; for detailed procedure) to penetrate the solid material to extract non-polar TAGs. By comparison, phospholipids (PLs) are amphiphilic due to the hydrophobic fatty acid chains and hydrophilic phosphate moieties in their structures. Therefore, part of PLs in the dried sample might be dissolved in the water added during lipid extraction, which facilitated their extraction into the organic solvents. Consequently, the percentage of extracted TAGs in FD SCV was decreased (26.30%), while extracted PLs was increased (37.13%) compared to fresh SCV (32.93% TAGs and 26.63% PLs). However, both TAG and PL content in FD SCV was higher compared to AD SCV. Since TAGs and PLs are the lipid components of high interest when utilizing marine oil, freeze drying would be more effective for valorization of *Cucumaria frondosa* viscera for nutraceutical products compared to air drying. For drying of other food materials that are abundant in lipids, freeze drying might be selected to prevent

the undesired degradation of TAGs to FFAs during the drying process, and retain the high amounts of TAGs and PLs in the materials.

To the authors' best knowledge, no published reports are available on lipid class of *Cucumaria frondosa* viscera. Gianasi et al. [32] reported that fresh *Cucumaria frondosa* gonads (part of viscera) contained 19.0–22.5% TAGs and 17.0–21.2% PLs, which are consistent with the results in the present study. Vaidya and Cheema [36] reported extremely high content of PLs (75.59%) and low content of TAGs (2.27%) for freeze-dried *Cucumaria frondosa* derived from Newfoundland. However, they did not indicate whether the raw material was obtained from a specific body part or whole sea cucumber. Their reported results are quite different from the present study, possibly because they might have used whole sea cucumber (mixture of different parts) with abundant PLs in some parts. Moreover, lipid class of sea cucumber can be significantly influenced by harvesting season and location.

3.3. Fatty Acid Composition

3.3.1. Total Fatty Acid Composition

As indicated in Table 3 and Figure 6, fatty acid compositions of total lipids in both dried samples were similar to fresh SCV, indicating that both drying methods had no effect on total fatty acid composition of the material. For all three samples, polyunsaturated fatty acids (PUFAs) accounted for the highest fraction (33.01–40.14%), followed by monounsaturated fatty acids (MUFAs, 27.01–28.73%) and saturated fatty acids (SFAs, 25.93–26.97%). The content of omega-3 PUFAs was 30.58–31.65% and omega-6 PUFAs was 1.02–1.26%. The amounts of SFAs, MUFAs, and omega-3 PUFAs in the present study are consistent with the values reported by Mamelona et al. [10] for viscera of *Cucumaria frondosa* in Québec, Canada (26.4% SFAs, 28.2% MUFAs, and 29.5% omega-3 PUFAs), but their omega-6 PUFA content was higher (15.4%), possibly due to different living and harvesting conditions of the sea cucumber.

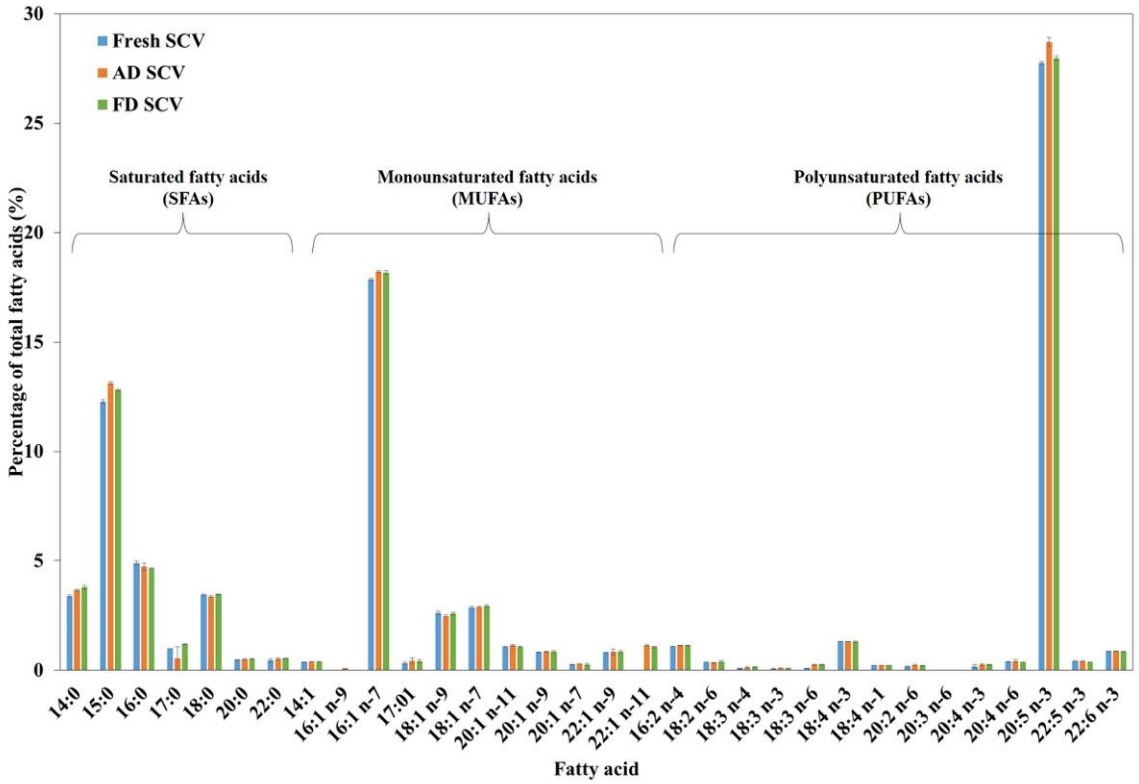


Figure 6. Total fatty acid composition of fresh, air-dried (AD SCV), and freeze-dried (FD SCV) sea cucumber viscera.

Table 3. Total fatty acid composition of fresh, air-dried (AD SCV), and freeze-dried (FD SCV) sea cucumber viscera.

Type	Isomer	Systematic Name	Fresh SCV	AD SCV	FD SCV
Saturated fatty acids (SFAs)	14:0	Tetradecanoic acid	3.39 ± 0.04	3.66 ± 0.04	3.79 ± 0.09
	15:0	Pentadecanoic acid	12.27 ± 0.10	13.12 ± 0.06	12.81 ± 0.04
	16:0	Hexadecanoic acid	4.90 ± 0.09	4.73 ± 0.17	4.66 ± 0.02
	17:0	Heptadecanoic acid	0.99 ± 0.01	0.55 ± 0.54	1.19 ± 0.02
	18:0	Octadecanoic acid	3.45 ± 0.03	3.37 ± 0.05	3.46 ± 0.02
	20:0	Eicosanoic acid	0.48± 0.01	0.50 ± 0.04	0.52 ± 0.01
	22:0	Docosanoic acid	0.45 ± 0.08	0.53 ± 0.06	0.54 ± 0.01
Subtotal (SFAs)			25.93	26.46	26.97
Monounsaturated fatty acids (MUFAs)	14:1	Tetradecenoic acid	0.37 ± 0.00	0.40 ± 0.00	0.40 ± 0.00
	16:1 n-9	cis-7-Hexadecenoic acid	0.00 ± 0.00	0.06 ± 0.01	0.00 ± 0.00
	16:1 n-7	Hexadecenoic acid	17.86 ± 0.05	18.20 ± 0.05	18.16 ± 0.08
	17:1	Heptadecenoic acid	0.33 ± 0.05	0.43 ± 0.14	0.42 ± 0.08
	18:1 n-9	Octadecenoic acid	2.61 ± 0.08	2.47 ± 0.04	2.59 ± 0.06
	18:1 n-7	cis-Vaccenic acid	2.87 ± 0.04	2.89 ± 0.02	2.94 ± 0.06
	20:1 n-11	Gadoleic acid	1.07 ± 0.04	1.14 ± 0.03	1.07 ± 0.03
	20:1 n-9	Eicosenoic acid	0.82 ± 0.03	0.84 ± 0.01	0.85 ± 0.05
	20:1 n-7	Paullinic acid	0.26 ± 0.00	0.30 ± 0.00	0.26 ± 0.05
	22:1 n-9	Erucic acid	0.81 ± 0.01	0.81 ± 0.14	0.77 ± 0.06
	22:1 n-11	Docosenoic acid	0.00 ± 0.00	1.14 ± 0.03	1.07 ± 0.03
Subtotal (MUFAs)			27.01	28.73	28.60
Polyunsaturated fatty acids (PUFAs)	16:2 n-4	Hexadecadienoic acid	1.10 ± 0.00	1.13 ± 0.01	1.13 ± 0.02
	18:2 n-6	Octadecadienoic acid	0.38 ± 0.00	0.34 ± 0.02	0.39 ± 0.05
	18:3 n-4	Octadecatrienoic acid	0.08 ± 0.02	0.13 ± 0.04	0.15 ± 0.00
	18:3 n-3	15-Octadecatrienoic acid	0.06 ± 0.01	0.09 ± 0.02	0.10 ± 0.01
	18:3 n-6	12-Octadecatrienoic acid	0.09 ± 0.02	0.25 ± 0.01	0.25 ± 0.02
	18:4 n-3	6,9,12,15-Octadecatetraenoic acid	1.30 ± 0.01	1.31 ± 0.01	1.31 ± 0.03
	18:4 n-1	Octadeca-9,11,13,15-tetraenoic acid	0.23 ± 0.01	0.23 ± 0.00	0.23 ± 0.01
	20:2 n-6	11,14-Eicosadienoic acid	0.17 ± 0.03	0.24± 0.03	0.22 ± 0.01
	20:3 n-6	8,11,14-Eicosatrienoic acid	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.01
	20:4 n-3	8,11,14,17-Eicosatetraenoic acid	0.18 ± 0.07	0.25 ± 0.05	0.25 ± 0.02
	20:4 n-6	5,8,11,14-Eicosatetraenoic acid	0.39 ± 0.01	0.42 ± 0.07	0.38 ± 0.01
	20:5 n-3	Eicosapentaenoic acid (EPA)	27.76 ± 0.07	28.71 ± 0.20	27.97 ± 0.11
	22:5 n-3	Docosapentaenoic acid (DPA)	0.41 ± 0.03	0.41 ± 0.04	0.38 ± 0.01
	22:6 n-3	Docosahexaenoic acid (DHA)	0.88 ± 0.01	0.87 ± 0.01	0.85 ± 0.00
Other PUFAs			6.53 ± 0.27	5.74 ± 0.18	6.31 ± 0.12
Subtotal (PUFAs)			33.01	40.14	39.91
Total Omega-3 PUFAs			30.58	30.58	31.65
Total Omega-6 PUFAs			1.02	1.02	1.26
Other fatty acids			14.05	4.68	4.52

In the present study, eicosapentaenoic acid (EPA) was the predominant fatty acid (27.76–28.71%), which is in agreement with all reported studies about total fatty acid composition of different parts of *Cucumaria frondosa* [10,32,34,36]. EPA has been proved with a variety of health benefits, such as prevention of inflammation, promotion of fetal development, reduction of cardiovascular risk, and enhancement of cognitive function [37]. Therefore, *Cucumaria frondosa* viscera can be potentially valorized for production of omega-3 PUFA (especially EPA) products.

The content of omega-3 PUFAs, omega-6 PUFAs, and EPA in total lipids of fresh and dried SCV were compared to crude oil extracted from some marine by-products that have been widely commercialized for nutritional supplements [38] (Table 4). In comparison to crude oil from salmon, cod liver, and seal, both fresh and dried SCV have higher amounts of omega-3 PUFAs, and at the same time lower amounts of omega-6 PUFAs. As reported by Simopoulos [39], the omega-6/3 ratio in western diets is as high as 15~20:1. An excessive intake of omega-6 PUFAs might promote pathogenesis of many diseases, such as

inflammation, obesity, cancer, and cardiovascular diseases [39–41]. An increased intake of omega-3 PUFAs can reduce the risks, and a recommended dietary ratio of omega-6/3 is 1~5:1 [42,43]. In fresh and dried SCV, the omega-6/3 ratio is as low as 0.03 and 0.04, respectively, along with high omega-3 PUFA content (above 30%). Furthermore, the EPA content in SCV is significantly higher (27–28%) in comparison to crude oil from other marine by-products (4–9%, Table 4). Therefore, it seems promising to process *Cucumaria frondosa* viscera into omega-3 PUFAs and EPA enriched nutritional products without the concern of excessive intake of omega-6 PUFAs.

Table 4. Comparison of omega-3 PUFAs, omega-6 PUFAs, and EPA in total lipids from the present study and literature.

Species	Total Omega-3 PUFAs (%)	Total Omega-6 PUFAs (%)	Omega-6 /Omega-3	EPA (%)	Reference
Fresh SCV	30.58	1.02	0.03	27.76	The present study
AD SCV	31.65	1.26	0.04	28.71	
FD SCV	30.85	1.24	0.04	27.97	
Crude farmed Atlantic salmon oil	9.93	15.11	1.52	4.63	
Crude seal oil	16.27	2.03	0.12	7.12	Dave et al. [38]
Crude cod liver oil	20.77	2.29	0.11	8.52	
Crude wild Pacific salmon oil	21.18	2.26	0.11	9.54	

3.3.2. Phospholipid Fatty Acid Composition

Phospholipids (PLs) are the main component of cellular membranes of various living organisms, and essential in gene expression, lipoprotein formation, and signaling systems [44]. PLs have been reported with numerous health benefits, including relief of inflammatory reactions, inhibition of certain types of cancer, regulation of blood lipid profiles, improvement of neurological development, enhancement of immunological functions, and reduction of liver diseases [45]. Compared to TAGs, PLs are more biocompatible and bioavailable [46,47]. In the present study, PLs were the most abundant lipid component in both air- and freeze-dried SCV (27.77% in AD SCV and 37.13% in FD SCV, Table 2). Therefore, fatty acid composition of PLs of dried SCV were analyzed and compared to fresh SCV (Table 5 and Figure 7).

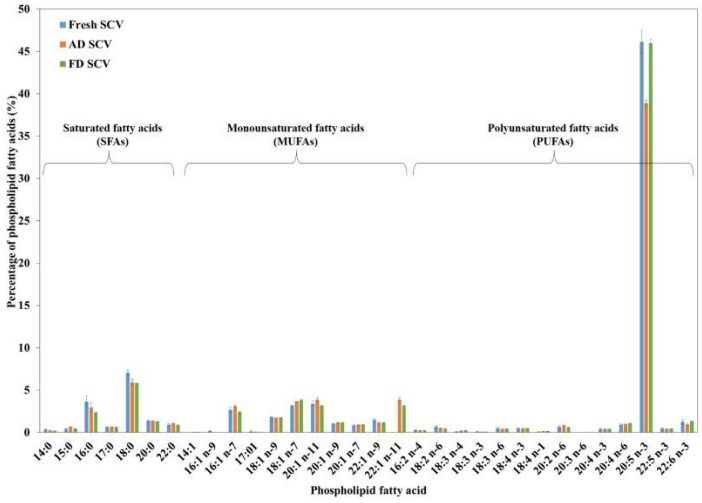


Figure 7. Phospholipid fatty acid composition of fresh, air-dried (AD SCV), and freeze-dried (FD SCV) sea cucumber viscera.

Table 5. Phospholipid fatty acid composition of fresh, air-dried (AD SCV), and freeze-dried (FD SCV) sea cucumber viscera.

Type	Isomer	Systematic Name	Fresh SCV	AD SCV	FD SCV
Saturated fatty acids (SFAs)	14:0	Tetradecanoic acid	0.35 ± 0.11	0.25 ± 0.01	0.18 ± 0.03
	15:0	Pentadecanoic acid	0.44 ± 0.03	0.65 ± 0.03	0.43 ± 0.07
	16:0	Hexadecanoic acid	3.62 ± 0.68	2.96 ± 0.60	2.34 ± 0.13
	17:0	Heptadecanoic acid	0.62 ± 0.03	0.66 ± 0.02	0.64 ± 0.01
	18:0	Octadecanoic acid	7.08 ± 0.32	5.93 ± 0.38	5.81 ± 0.02
	20:0	Eicosanoic acid	1.37 ± 0.12	1.34 ±0.02	1.29 ± 0.03
	22:0	Docosanoic acid	0.93 ± 0.18	1.07 ± 0.03	0.87 ± 0.04
Subtotal (SFAs)			14.41	12.85	11.56
Monounsaturated fatty acids (MUFAs)	14:1	Tetradecenoic acid	0.00 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
	16:1 n-9	cis-7-Hexadecenoic acid	0.19 ± 0.06	0.00 ± 0.00	0.01 ± 0.02
	16:1 n-7	Hexadecenoic acid	2.68 ± 0.29	3.09 ± 0.13	2.40 ± 0.14
	17:1	Heptadecenoic acid	0.12 ± 0.18	0.07 ± 0.00	0.03 ± 0.00
	18:1 n-9	Octadecenoic acid	1.81 ± 0.10	1.69 ± 0.07	1.75 ± 0.03
	18:1 n-7	cis-Vaccenic acid	3.15 ± 0.07	3.63 ± 0.02	3.84 ± 0.08
	20:1 n-11	Gadoleic acid	3.40 ± 0.32	3.86 ± 0.30	3.15 ± 0.07
	20:1 n-9	Eicosenoic acid	1.03 ± 0.02	1.15 ± 0.02	1.16 ± 0.04
	20:1 n-7	Paullinic acid	0.84 ± 0.11	0.91 ± 0.03	0.94 ± 0.03
	22:1 n-9	Erucic acid	1.49 ± 0.14	1.82 ± 0.05	1.76 ± 0.06
	22:1 n-11	Docosenoic acid	0.00 ± 0.00	3.86 ± 0.30	3.15 ± 0.07
Subtotal (MUFAs)			14.71	19.46	17.60
Polyunsaturated fatty acids (PUFAs)	16:2 n-4	Hexadecadienoic acid	0.28 ± 0.05	0.22 ± 0.01	0.25 ± 0.00
	18:2 n-6	Octadecadienoic acid	0.65 ± 0.14	0.48 ± 0.06	0.44 ± 0.03
	18:3 n-4	Octadecatrienoic acid	0.09 ± 0.03	0.20 ± 0.00	0.24 ± 0.01
	18:3 n-3	15-Octadecatrienoic acid	0.10 ± 0.03	0.09 ± 0.01	0.08 ± 0.00
	18:3 n-6	12-Octadecatrienoic acid	0.50 ± 0.11	0.42 ± 0.00	0.42 ± 0.01
	18:4 n-3	6,9,12,15-Octadecatetraenoic acid	0.49 ± 0.03	0.48 ± 0.01	0.52 ± 0.01
	18:4 n-1	Octadeca-9,11,13,15-tetraenoic acid	0.08 ± 0.03	0.14 ±0.01	0.13 ± 0.03
	20:2 n-6	11,14-Eicosadienoic acid	0.67 ± 0.13	0.84 ± 0.02	0.58 ± 0.02
	20:3 n-6	8,11,14-Eicosatrienoic acid	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	20:4 n-3	8,11,14,17-Eicosatetraenoic acid	0.39 ± 0.15	0.40 ± 0.02	0.38 ± 0.02
	20:4 n-6	5,8,11,14-Eicosatetraenoic acid	0.93 ± 0.07	0.97 ± 0.03	1.10 ± 0.02
	20:5 n-3	Eicosapentaenoic acid (EPA)	46.13 ± 1.39	38.88 ± 0.36	45.97 ± 0.50
	22:5 n-3	Docosapentaenoic acid (DPA)	0.46 ± 0.12	0.39 ± 0.01	0.42 ± 0.02
	22:6 n-3	Docosahexaenoic acid (DHA)	1.26 ± 0.27	0.97 ± 0.13	1.32 ± 0.01
Other PUFAs			14.14 ± 4.74	10.46 ± 0.69	8.89 ± 0.27
Subtotal (PUFAs)			52.02	54.93	60.72
Total Omega-3 PUFAs			48.82	48.82	41.21
Total Omega-6 PUFAs			2.75	2.75	2.71
Other fatty acids			18.87	12.77	10.12

For all three samples, the content of PUFAs (52.02–60.72%) in PLs were significantly higher than SFAs (11.56–14.41%) and MUFAs (14.71–19.46%). The amounts of omega-3 PUFAs in PLs of FD SCV (48.69%) were similar to fresh SCV (48.82%), which were slightly higher than AD SCV (41.21%). EPA was the most abundant fatty acid, accounting for 46.13% and 45.97 % in fresh and FD SCV, respectively, and 38.88% in AD SCV, which was relatively lower. Vaidya and Cheema [36] reported similar content of omega-3 PUFAs (49.59%) and EPA (44.4%) in PLs of freeze-dried *Cucumaria frondosa* derived from Newfoundland.

Araujo et al. [48] reported 49.4% omega-3 PUFAs, 2.5% omega-6 PUFAs, and 28.5% EPA for PLs in krill oil. Krill oil is a type of marine oil abundant in PLs, and has been widely commercialized as nutritional supplements. In comparison to fish oil, which is mainly composed of TAGs, omega-3 PUFAs in krill oil can be absorbed by humans more safely and efficiently with low side-effects, due to the high efficiency of PLs to deliver the fatty acid residues into cell membranes [45,49]. The amounts of total omega-3 and 6 PUFAs in PLs of fresh and dried SCV in the present study are quite similar to krill oil, and the EPA

content in SCV is even higher. Therefore, there is a great potential for *Cucumaria frondosa* viscera to be valorized for value-added PL-rich nutritional products.

3.4. Total Amino Acid Composition

In the present study, sixteen amino acids were identified, including eight essential amino acids (EAAs) and eight non-essential amino acids (NEAAs). As indicated in Table 6, the two drying methods afforded similar yields of total EAAs and NEAAs from fresh SCV, with the EAAs/NEAAs ratio of approximately 1. As claimed by Mamelona et al. [50], marine by-products commonly have a ratio of EAAs/NEAAs higher than 0.5, indicating the high quality of marine proteins and their potential as a source of balanced dietary proteins.

Table 6. Amino acid composition of air-dried (AD SCV) and freeze-dried (FD SCV) sea cucumber viscera.

Type	AD SCV (mg/100 g)	AD SCV (%)	FD SCV (mg/100 g)	FD SCV (%)
Essential amino acids (EAAs)				
Histidine	396.18 ± 163.86	1.04	225.99 ± 135.30	0.62
Threonine	3815.16 ± 766.14	10.00	2878.91 ± 377.70	7.95
Valine	64.00 ± 35.21	0.17	62.26 ± 6.95	0.17
Methionine	3100.43 ± 44.01	8.12	2854.84 ± 290.10	7.88
Isoleucine	5938.32 ± 263.54	15.56	5385.95 ± 181.72	14.87
Leucine	295.86 ± 92.82	0.78	185.63 ± 44.80	0.51
Phenylalanine	5841.59 ± 295.02	15.31	5056.60 ± 229.27	13.96
Lysine	224.32 ± 82.05	0.59	223.01 ± 42.83	0.62
Total EAAs	19,675.86	51.57	16,873.19	46.58
Non-essential amino acids (NEAAs)				
Aspartic acid	191.85 ± 22.15	0.50	140.87 ± 5.67	0.39
Glutamic acid	9897.19 ± 4.90	25.93	7926.57 ± 136.11	21.89
Serine	647.22 ± 479.46	1.70	263.47 ± 91.24	0.73
Glycine	1210.25 ± 861.02	3.17	6275.05 ± 98.92	17.33
Alanine	1877.57 ± 270.81	4.92	673.43 ± 342.47	1.86
Proline	4121.35 ± 11.38	10.80	3755.68 ± 184.38	10.37
Tyrosine	407.29 ± 136.74	1.07	196.90 ± 95.39	0.54
Cystine	134.40 ± 4.06	0.35	105.48 ± 9.33	0.29
Total NEAAs	18,487.12	48.44	19,337.45	53.40
EAAs/NEAAs	1.06		0.87	

Glutamic acid was the predominant amino acid in both dried samples (9897.19 mg/100 g in AD SCV and 7926.57 mg/100 g in FD SCV, Table 6 and Figure 8), which agrees with amino acid profiles reported by Mamelona et al. [10] for *Cucumaria frondosa* viscera and Zhong et al. [34] for body wall. The most abundant EAA in both dried samples was isoleucine, followed by phenylalanine. In comparison to AD SCV, FD SCV contained a significantly higher amount of glycine (6275.05 mg/100 g). Heat treatment has been claimed possibly changing compositions of nitrogenous compounds [51]. Similar observations that different drying methods resulted in different amino acid compositions of dried marine materials have been reported by Deng et al. [52] and Kim et al. [53] in their studies about squid and yellow croaker, respectively. As observed in the present study, for drying of food materials that are abundant in glycine, freeze drying might be more effective in retaining the high level of glycine compared to air drying.

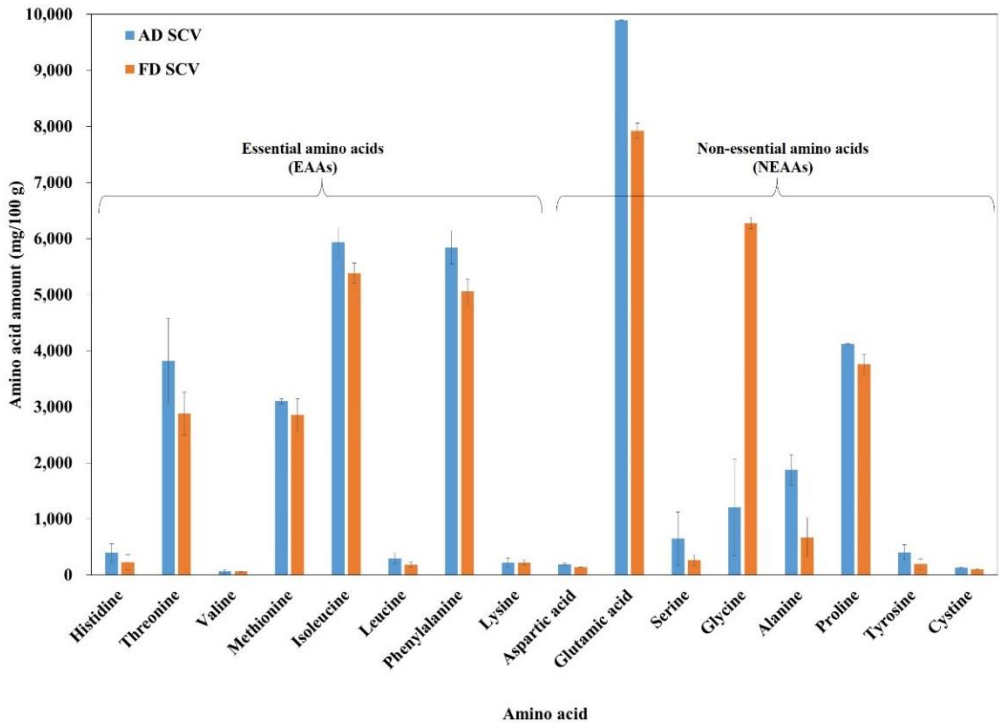


Figure 8. Amino acid composition of air-dried (AD SCV) and freeze-dried (FD SCV) sea cucumber viscera.

Glutamic acid and its derivative glutamine have been reported with various health benefits, including anticancer activity, cell proliferation, wound healing, improvement of protein metabolism, enhancement of immune system, and prevention of bacterial translocation [54,55]. Isoleucine plays a crucial role in formation of hemoglobin, stabilization and regulation of blood sugar, and energy levels [56]. It also promotes muscle recovery after physical exercise and tissue repair after surgery [57]. Phenylalanine is an important precursor for production of tyrosine, which can be converted to proteins and signaling molecules that are closely related to learning ability and stress control [58,59]. Glycine is important for living organisms as it plays the role of a neurotransmitter, and is an essential component in many biological molecules and metabolic reactions [60]. It promotes production and release of interleukin-2 and B lymphocytes in the immune system, thus facilitating phagocytosis [61]. Glycine is crucial in synthesis of glutathione, which is a primary antioxidant enzyme in the human body and promotes generation and activation of natural killer cells [62]. Moreover, glycine has been proved with multiple cytoprotective effects for brain, heart, liver, and kidney [60]. In spite of slightly higher amounts of glutamic acid, isoleucine, and phenylalanine in AD SCV, FD SCV had a significantly higher content of glycine, therefore freeze drying might be more effective in processing *Cucumaria frondosa* viscera for production of functional foods and nutritional supplements.

4. Conclusions

This study creates a paradigm for future research on using drying technology to pretreat sea cucumber viscera for production of value-added products. Compared to air drying, freeze drying was more effective in removing moisture and retaining the nutritional components (TAGs, PLs and glycine) of *Cucumaria frondosa* viscera. The present study indicates the high amounts of nutrients (omega-3 PUFAs and glycine) in dried *Cucumaria frondosa* viscera, and addresses their potential for further processing into functional food, nutraceuticals and pharmaceuticals.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9040703/s1, Experimental methodologies of determination of total lipid class and fatty acid composition of total lipids and phospholipids.

Author Contributions: Conceptualization, Y.L., D.D., and W.M.; methodology, Y.L. and D.D.; validation, S.T. and V.V.R.; formal analysis, Y.L., S.T., and V.V.R.; investigation, Y.L., S.T., and V.V.R.; resources, W.M.; data curation, Y.L.; writing—original draft preparation, Y.L.; writing—review and editing, Y.L. and D.D.; visualization, Y.L.; supervision, D.D.; project administration, D.D.; funding acquisition, D.D. All authors have read and agreed to the published version of the manuscript.

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