

Article

Features and Advantages of Supercritical CO₂ Extraction of Sea Cucumber *Cucumaria frondosa japonica* Semper, 1868

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Abstract: Extraction process of *Cucumaria frondosa japonica* Semper, 1868, which are subspecies of *Cucumaria frondosa* (Gunnerus, 1767), were studied. It was shown that supercritical carbon dioxide extraction of holothuria was more effective than conventional solvent extraction. Step-by-step extraction with carbon dioxide followed by supercritical extraction with the addition of a co-solvent of ethanol can almost double the yields of extracts of triterpene glycosides, styrenes and carotenoids. Moreover, the fraction of triterpene glycosides practically does not contain colored impurities, in contrast to traditional ethanol extraction. The obtained extracts by HPLC in combination with tandem mass spectrometry (HPLC-MS/MS) identified 15 triterpene glycosides, 18 styrene compounds and 14 carotenoids. Supercritical extraction made it possible to obtain extracts with yields superior to conventional hexane and alcohol extracts. Moreover, such an approach with the use of supercritical fluid extraction (SFE) and subsequent profiling of metabolites can help with the study of holothuria species that are not as well studied.

Keywords: sea cucumber; supercritical extraction; *Cucumaria frondosa japonica*; triterpene glycosides; saponins; carotenoids; tandem mass spectrometry

1. Introduction

Oceans are the least studied part of the Earth. Every year, dozens of new compounds are isolated and described from marine hydrobionts around the world. Many of them have different biological activities and many have been used in drug development. Secondary metabolites of the representatives of the class of echinoderms are of great practical and fundamental interest. New secondary metabolites isolated from echinoderms are discovered annually. Many articles have been published describing the

features of the mechanisms of biological action of previously discovered compounds. Of particular interest are compounds isolated from holothurians and sea urchins [1–3].

Sea cucumbers are marine invertebrates that belong to Echinodermata, Holothuroidea. Class Holothuroidea are widely found in the benthic area and deep seas worldwide. Components of sea cucumbers possess different biological activities such as anticancer, antiviral, antioxidant and wound healing. Recently, much attention has been paid to isolation, to determine the structure and study of the properties of sulfated polysaccharides from sea cucumbers [4].

Supercritical fluid solvents represent interesting alternatives for producing high-quality natural food products without toxic residues [5]. The introduction of supercritical fluid extraction (SFE) has led to a novel technology that is being continually developed [6,7]. High-pressure SFE can be used to produce natural thermolabile compounds, leaving no organic solvent residues in food products, which are commonly observed with conventional extraction methods using methanol and hexane [8].

2. Results

2.1. Sterol Compositions Identified in Supercritical CO₂ Extract

Profiling of sterol fractions from the whole-body extract of the sea cucumber using the LC-MS approach allowed numerous new as well as previously isolated triterpene glycosides to be characterized. The total yield of sterol fraction was 114 mg that corresponded to 190 mg/100 g. To identify sterol components, this fraction was hydrolyzed. Alkaline hydrolysis was performed in 2 mL ethanol and 0.15 mL oversaturated KOH solution at 85 °C for 30 min. Then to the reaction mixture, the upper layer was collected and analyzed with HPLC ESI MS-MS. The main sterol components were diatomsterol and cholestanol 20.2 and 13.2% (Table 1).

Table 1. Sterol components identified from the supercritical extract of *C. frondosa japonica* Semper, 1868.

No	Sterol	Molecular Formula	[M+H] ⁺ (m/z)	MS2 (m/z)	Yield, %
1	24-nor-5 α -cholesta-7,22-dien-3 β -ol (Asterosterol)	C ₂₆ H ₄₂ O	371.32	303.26; 275.23; 235.20	1.34
2	24-nor-5 α -cholesta-22-en-3 β -ol (24-nordehydrocholestanol)	C ₂₆ H ₄₄ O	373.34	305.28; 277.25; 237.21	1.48
3	5 α -cholesta-7,22-en-3 β -ol	C ₂₇ H ₄₄ O	385.34	329.28; 303.26; 235.20; 167.14; 143.14	9.26
4	5 α -cholest-22-en-3 β -ol (trans-22-dehydrocholestanol)	C ₂₇ H ₄₆ O	387.35	331.29; 305.28; 237.21; 169.15; 143.14	6.06
5	5 α -cholest-7-en-3 β -ol (lathosterol)	C ₂₇ H ₄₆ O	387.35	331.29; 303.26; 235.20; 167.14; 143.14	3.24
6	5 α -cholestan-3 β -ol (cholestanol)	C ₂₇ H ₄₈ O	389.37	333.31; 305.28; 237.21; 169.15; 143.14	13.20
7	24-methyl-5 α -cholesta-trien-3 β -ol	C ₂₈ H ₄₄ O	397.34	327.26; 299.23; 231.17; 165.12; 141.12	1.94
8	24-methyl-5 α -cholesta-7,22-dien-3 β -ol (diatomsterol)	C ₂₈ H ₄₆ O	399.35	329.27; 303.26; 235.20; 167.14; 141.12	20.22
9	24-methylen-5 α -cholest-7-en-3 β -ol	C ₂₈ H ₄₆ O	399.35	331.29; 235.20; 167.14; 141.12	2.40
10	24-methyl-5 α -cholest-7-en-3 β -ol	C ₂₈ H ₄₈ O	401.37	331.29; 235.20; 167.14; 141.12	1.88
11	24-methyl-5 α -cholest-22-en-3 β -ol (diatomstanol)	C ₂₈ H ₄₈ O	401.37	331.29; 237.21; 169.15; 143.14	2.78
12	24-methylen-5 α -cholestan-3 β -ol	C ₂₈ H ₄₈ O	401.37	333.31; 237.21; 169.15; 143.14	9.28
13	24-methyl-5 α -cholestan-3 β -ol (campestanol)	C ₂₈ H ₅₀ O	403.39	333.31; 237.21; 169.15; 143.14	3.64
14	24-ethyl-5 α -cholesta-trien-3 β -ol (Provitamin D6)	C ₂₉ H ₄₆ O	411.36	327.26; 233.18; 165.12; 140.12	0.64
15	24-ethyl-5 α -cholest-7,22-dien-3 β -ol (Spinasterol)	C ₂₉ H ₄₈ O	413.38	329.28; 235.20; 167.14; 141.12	4.44

Table 1. Cont.

No	Sterol	Molecular Formula	[M+H] ⁺ (m/z)	MS2 (m/z)	Yield, %
16	24-ethyl-5 α -cholest-7-en-3 β -ol (Scottenol)	C ₂₉ H ₅₀ O	415.39	331.29; 235.20; 167.14; 141.12	9.06
17	24-ethyl-5 α -cholest-22-en-3 β -ol (stigmast-22E-en-3 β -ol)	C ₂₉ H ₅₀ O	415.39	331.29; 237.21; 169.15; 143.14	2.66
18	24-ethyl-5 α -cholestan-3 β -ol (sitostanol)	C ₂₉ H ₅₂ O	417.40	333.31; 237.21; 169.15; 143.14	7.82

2.2. Carotenoids Identified in Supercritical CO₂ Extract

During the first step, SFE fatty fraction was isolated. Total yield was 1.1 g. Fourteen carotenoids were identified with a total mass of 71.1 mg (Table 2). The major component of carotenoid fraction was Cucumariaxanthin yielding 33.6 mg, that corresponded to 56.1 mg per 100 g of dry weight. The second main carotenoid was Canthaxanthin, 25.4 mg/100 g. The third main component was Cucumariaxanthin B. Lutein was detected in the smallest amount 0.5 mg/100 g.

Table 2. Components identified from the supercritical extract of *C. frondosa japonica* Semper, 1868.

No	Identity	Molecular Formula	MS (m/z)	MS2 (m/z)	Yield, (mg/100 g)
1	β -Carotene	C ₄₀ H ₅₆	537.5	445.4; 379.4; 346.3; 308.3; 268.3; 224.2; 203.2; 178.2; 133.2; 119.1; 107.1; 95.1	4.2
2	β -Echinone	C ₄₀ H ₅₄ O	551.5	347.3; 265.3; 209.2; 203.2; 157.2; 133.1; 119.1; 95.1; 81.1; 69.1	0.9
3	Canthaxanthin	C ₄₀ H ₅₂ O ₂	565.5	548.5; 413.3; 404.0; 363.3; 307.3; 231.2; 215.2; 203.2; 145.2; 133.2; 95.2; 69.1	25.4
4	(3R)-, (3S)-phoenicoxanthin	C ₄₀ H ₅₂ O ₃	581.5	565.5; 562.5; 488.4; 157.0; 119.0; 105.0; 91.0; 55.0	1.6
5	Lutein	C ₄₀ H ₅₆ O ₂	568.5	550.5; 476.4; 430.4; 367.0; 336.3; 323.3; 175.2; 145.2; 133.2; 107.1; 95.2	0.5
6	Diatoxanthin	C ₄₀ H ₅₄ O ₂	567.5	551.5; 533.5; 459.4; 413.4; 329.3; 263.3; 217.2; 199.2; 175.2; 133.2; 109.2	1.8
7	Alloxanthin	C ₄₀ H ₅₂ O ₂	565.5	547.5; 491.4; 465.4; 411.3; 393.0; 249.2; 209.2; 199.2; 173.2; 157.2; 119.1; 109.2; 81.1	2.3
8	Pectenolone	C ₄₀ H ₅₂ O ₃	581.5	563.5; 315.3; 27.2; 217.2; 199.2; 173.2; 147.2; 119.1; 107.1	2.2
9	(3S,3'S)-7,8-Didehydroastaxanthin	C ₄₀ H ₅₀ O ₄	595.5	577.5; 565.5; 441.6; 425.4; 375.4; 359.4; 165.2	1.7
10	Fucoxanthin	C ₄₂ H ₅₈ O ₆	659.5	641.5; 623.5; 599.5; 581.5; 567.4; 549.4; 489.4; 433.3; 355.3; 239.2; 149.2; 109.2	1.4
11	Fucoxanthinol	C ₄₀ H ₅₆ O ₅	617.5	598.3; 447.4; 285.2; 233.2; 143.0; 109.2; 91.0; 43.0	1.6
12	Cucumariaxanthin A	C ₄₀ H ₅₆ O ₂	568.5	550.2; 476.4; 462.3	56.1
13	Cucumariaxanthin B	C ₄₀ H ₅₈ O ₂	571.1	552.4; 478.1; 464.3	13.7
14	Cucumariaxanthin C	C ₄₀ H ₆₀ O ₂	573.6	556.2; 536.3; 480.5; 466.2	5.1

2.3. Triterpene Glycosides Identified in Supercritical CO₂ Extract

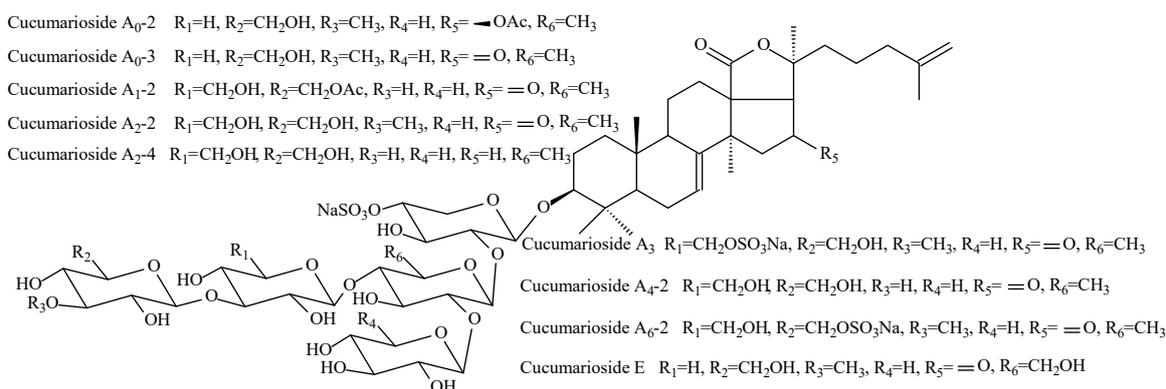
In the second step, SFE extracts were analyzed by HPLC in combination with tandem mass spectrometry (HPLC ESI MS/MS). A total of 15 different triterpene glycosides were identified (Table 3).

Table 3. Triterpene glycosides identified from the supercritical extract of *C. frondosa japonica*.

N ^o	Identity and Retention Time *	Molecular Formula	Adducts	MS (m/z)	MS2 (m/z)	MS3 (m/z)
1	cucumarioside A ₀ -1 27.6 min	C ₆₀ H ₉₃ O ₃₀ SNa	[M _{Na} -Na] ⁻	1325.55	1193.50; 797.20	1017.44; 885.39; 739.34; 665.16; 489.09; 357.05; 211.00
2	cucumarioside A₀-2 30.3 min	C ₆₀ H ₉₃ O ₂₉ SNa	[M _{Na} -Na] ⁻	1309.55	1177.51; 797.20	1001.44; 869.40; 723.34; 665.16; 489.09; 375.05; 211.00
3	cucumarioside A₀-3 30.8 min	C ₅₈ H ₈₉ O ₂₈ SNa	[M _{Na} -Na] ⁻	1265.53	1133.48; 797.20	957.42; 825.37; 679.32; 665.16; 489.09; 375.05; 211.00
4	cucumarioside A ₁ -2 28.4 min	C ₆₀ H ₉₁ O ₃₀ SNa	[M _{Na} -Na] ⁻	1323.53	1191.49; 855.21	987.43; 825.37; 723.17; 519.10; 357.05; 211.00
5	cucumarioside A ₂ -2 28.6 min	C ₅₉ H ₉₁ O ₂₉ SNa	[M _{Na} -Na] ⁻	1296.41	1163.49;	987.43; 827.21; 825.37; 695.17; 679.32; 519.10; 357.05; 211.00
6	cucumarioside A ₂ -3 28.5 min	C ₅₉ H ₉₃ O ₂₉ SNa	[M _{Na} -Na] ⁻	1297.55	1165.51; 827.21	989.44; 827.39; 695.17; 681.33; 519.10; 357.05; 211.00
7	cucumarioside A ₂ -4 29.0 min	C ₅₈ H ₉₁ O ₂₈ SNa	[M _{Na} -Na] ⁻	1267.54	1135.50;	974.45; 813.20; 811.39; 681.15; 665.33; 519.10; 357.05; 211.00
8	cucumarioside A ₂ -5 28.5 min	C ₆₀ H ₉₃ O ₃₁ SNa	[M _{Na} -Na] ⁻	1341.54	1209.50; 813.20	1047.45; 885.39; 739.34; 681.15; 519.10; 357.05; 211.00
9	cucumarioside A ₃ 29.4 min	C ₅₉ H ₉₀ O ₃₂ S ₂ Na ₂	[M _{Na2} -Na] ⁻	1397.48	1265.43; 929.15	1089.36; 825.37; 797.11; 679.32; 621.04; 357.05; 211.00
10	cucumarioside A ₄ -2 29.6 min	C ₅₈ H ₈₉ O ₂₉ SNa	[M _{Na} -Na] ⁻	1281.52	1149.48; 813.20	987.43; 825.37; 681.15; 519.10; 357.05; 211.00
11	cucumarioside A ₆ -2 29.2 min	C ₅₉ H ₉₀ O ₃₂ S ₂ Na ₂	[M _{Na2} -Na] ⁻	1397.48	1265.43; 929.15	987.43; 825.37; 797.11; 679.32; 519.10; 357.05; 211.00
12	cucumarioside A₇-1 27.3 min	C ₅₇ H ₈₇ O ₃₅ S ₃ Na ₃	[M _{Na3} -Na] ⁻	1473.40	1341.35; 1031.09	1063.35; 899.05; 799.35; 659.19; 653.30; 621.04; 357.05; 211.00
13	cucumarioside A₇-2 26.9 min	C ₅₆ H ₈₅ O ₃₅ S ₃ Na ₃	[M _{Na3} -Na] ⁻	1459.38	1327.34; 1031.09	1049.33; 899.05; 785.34; 639.28; 621.04; 357.05; 211.00
14	cucumarioside A ₇ -3 27.1 min	C ₅₇ H ₈₉ O ₃₄ S ₃ Na ₃	[M _{Na3} -Na] ⁻	1459.42	1327.38; 1031.09	1049.37; 899.05; 785.38; 639.32; 621.04; 357.05; 211.00
15	cucumarioside E 34.5 min	C ₅₈ H ₈₉ O ₂₉ SNa	[M _{Na} -Na] ⁻ [M _{Na} +Na] ⁺	1281.52 1327.48	1149.51; 813.22; 1207.51	1105.51; 973.42; 841.41; 679.32; 637.19; 505.2 1075.42; 899.29

*—Bold letters used for those triterpene glycosides that were detected only in SFE fraction.

All of the detected triterpene glycosides were found earlier in this type of holothuria, but this is the first study when it was possible to detect all of these glycosides at the beginning of one complex experiment. The structural formulas of the detected glycosides are shown in Figures 1 and 2.

**Figure 1.** Structure of triterpene glycosides from the supercritical extract of *C. frondosa japonica*.

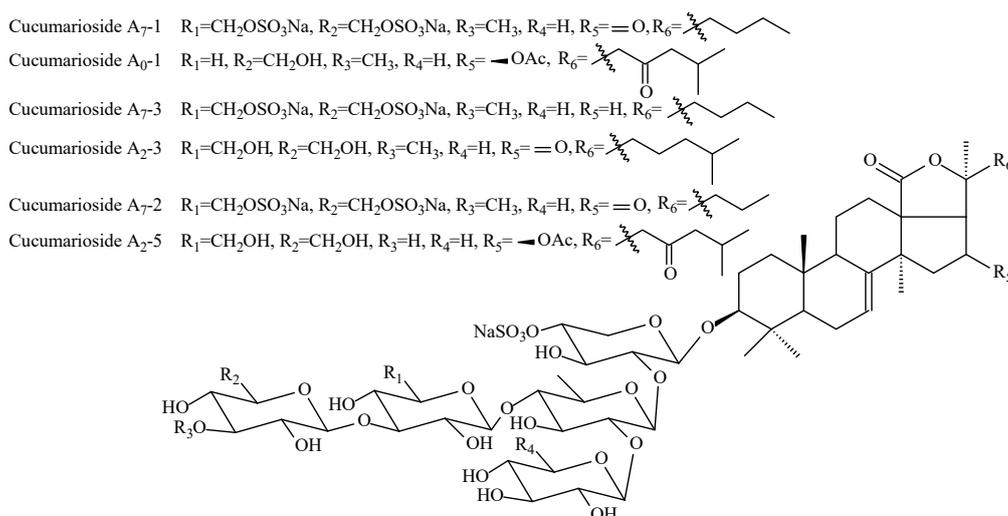


Figure 2. Structure of triterpene glycosides from the supercritical extract of *C. frondosa japonica*.

Total compounds chromatogram of detected triterpene glycosides in negative ion mode shown in Figure 3. The figure shows that the total fraction without preliminary separation has poorly resolved peaks, and although the full qualitative composition can be determined due to the mass spectrometer detector, this does not allow quantitative analysis.

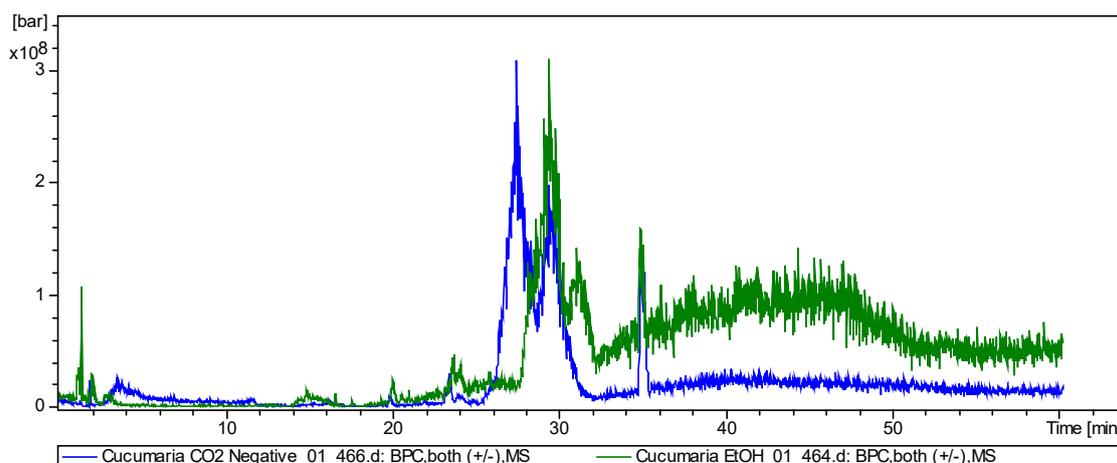


Figure 3. Liquid chromatography–electrospray mass spectrometry (LC-ESI MS) total compounds chromatogram of detected triterpene glycosides in negative ion mode (sulfated, disulfated and non-sulfated glycosides were detected as $[M-Na]^-$, $[M-2Na]^{2-}$ and $[M-H]^-$ ions) in supercritical extract of *C. frondosa japonica*.

3. Discussion

Cucumaria frondosa japonica Semper, 1868 is a subspecies of *Cucumaria frondosa* (Gunnerus, 1767). *Cucumaria frondosa japonica*'s habitat is in the Pacific Ocean but does not occur in the Atlantic Ocean. These two species have very similar metabolites but are slightly modified. Both of these are commercial species of holothuria, are widely used in food and are a source of concentrates for the pharmaceutical industry [9,10]. All this has led to the fact that over the past 40 years, the biochemical composition of this species has been fairly well studied. One of the first works on the biochemistry of this holothurian dates back to 1980. The triterpene glycoside of these holothurians is most well studied, since they are promising substances for medicine [11–13]. Use of supercritical extraction allowed us to increase the yield of the total fraction with triterpene glycosides and for the first time in one complex experiment we

showed all 15 triterpene glycosides present in *Cucumaria frondosa japonica*. For simultaneous qualitative characterization of the present triterpene glycosides, we used the metabolite profiling approach used earlier by other groups for the analysis of triterpene glycosides [14,15]. This approach allows reliable identification of known molecules, even if they are not well separated during HPLC.

The first stage of extraction was carried out at a pressure of 2500 psi, which allows the extraction of almost only hydrophobic nonpolar and weakly polar molecules, such as carotenoids, fats and sterols. This was used to analyze the carotenoids and sterols contained in *Cucumaria frondosa japonica*. Supercritical extraction made it possible to obtain extracts with yields superior to conventional hexane and alcohol extracts. Moreover, such an approach with the use of SFE and subsequent profiling of metabolites can help with the study of holothuria species that are not as well studied as *Cucumaria frondosa japonica*. This approach helps to better study the carotenoid composition and minor components of other types of Echinodermata. Supercritical extraction is a very promising approach and since it avoids unnecessary oxidation of substances, prevents unpleasant odors, improves product quality and yield, and is more profitable to operate.

4. Materials and Methods

4.1. Sea Cucumber

The live specimens of sea cucumber *Cucumaria frondosa japonica* Semper, 1868, were collected in Peter the Great Gulf (Russia, Sea of Japan) in 2019. The body wall of the sea cucumbers, total weight 1450 g, was dissected free of intestine and detritus with scissors, cut into small pieces (about 2 cm long), and stored in a polyethylene bag at $-70\text{ }^{\circ}\text{C}$ until used. Later, sea cucumbers with the total weight of 1450 g, were freeze-dried to 120 g, then the sample was separated into two equal parts, for ethanol extraction and supercritical fluid extraction (SFE).

4.2. Chemicals

Acetonitrile HPLC-grade was purchased from (PANREAC, Barcelona, Spain), formic acid and KOH were purchased from (Sigma-Aldrich, St. Louis, MO, USA). Ethanol was purchased from (LLC ORT "Khimreaktivy", Yekaterinburg, Russia). For all experiments we used deionized water (Siemens Ultra-Clear TWF Water Purification Systems, Günzburg, Germany).

4.3. Supercritical Fluid Extraction

Frozen sea cucumbers were freeze dried and minced. Dry weight was 120 g. Then 60 g was extracted in supercritical CO_2 pressure extraction apparatus (Thar SFC 500, San Diego, CA, USA). Extraction was carried out in two steps, first to get carotenoids and sterols fraction with CO_2 under the pressure of 2500 psi, at a temperature of $60\text{ }^{\circ}\text{C}$, and flow rate was 50 mL/min for liquid CO_2 . flow of 20 g/min. Total yield was 1.1 g or 1.8% from dry weight. The second step was with a flow rate 50 mL/min for liquid CO_2 and 1.00 mL/min for EtOH under the pressure of 7000 psi, at a temperature of $60\text{ }^{\circ}\text{C}$, and a flow of 20 g/min. Yield was 3.4 g and approximately 1.1 g was triterpene glycosides. Another 60 g of dried sea cucumbers were used for usual ethanol extraction.

4.4. Ethanol Extraction

The second part of freeze-dried body with the weight of 60 g was extracted with ethanol. The extraction was carried out at a ratio of 1:2 in a dark container at a temperature of $40\text{ }^{\circ}\text{C}$, with constant alternation on a stirrer for 3 h. Then the extract was filtered off and another 60 mL of ethanol was poured into the precipitate for re-extraction within 3 h. The precipitates were combined and concentrated in a rotary evaporator under vacuum at a temperature of $40\text{ }^{\circ}\text{C}$ and then evaporated under vacuum until a constant mass was yielded. Total yield was 2.7 g.

4.5. High-Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry

The samples and calibration solutions were filtered (0.22 µm Whatman filters) and injected into the chromatographic system (loop volume 20 µL). Reverse-phase HPLC was performed with Shimadzu LC-20 liquid chromatography (Kanda-Nishikicho 1-chrome, Shimadzu, Chiyoda-ku, Tokyo, Japan) equipped with column oven CTO-20A (Kanda-Nishikicho 1-chrome, Shimadzu, Chiyoda-ku, Tokyo, Japan) and UV-VIS detector SPD-20A (Kanda-Nishikicho 1-chrome, Shimadzu, Chiyoda-ku, Tokyo, Japan). The analytical column used was an ZORBAX Eclipse XDB C18 (150 × 4.6 mm, Pore Size: 80, Particle Size: 5.0 µ, Carbon Load: 1%, Reversed Phase C18 (Octadecyl), manufacturer: Agilent Technologies Inc., Wilmington, DE, USA) at 30 °C and the total flow rate 0.22 mL min⁻¹. The gradient elution program with two mobile phases (A, deionized water; B, acetonitrile with formic acid 0.1% v/v) was as follows 0 min 0% B, 25 min 100% B, 60 min 100% B. To study the fraction containing triterpene glycosides, the liquor was previously concentrated, and purified by low pressure hydrophobic chromatography on a Polychrome-I (Polytetrafluoroethylene granules).

ESI-MS and ESI-MSn spectra were recorded using an amaZon SL ion trap (BRUKER DALTONIKS, Bremen, Germany) equipped with an electrospray ion source. ESI MS ionization parameters were optimized as follows: a capillary voltage of 4500 V, end plate bend voltage: 1500V, collision energy: 60 eV, nebulization with nitrogen at 29 psi, dry gas flow of 10 L/min at a temperature of 160 °C. The mass spectra were recorded within *m/z* mass range of 500–2000 in negative and positive ion mode. Product ion mass spectra were recorded in auto MS/MS mode. Identification of compounds was carried out according to the Bruker library and literature data [11,16,17].

5. Conclusions

It was shown that supercritical carbon dioxide extraction of holothuria *Cucumaria frondosa japonica* Semper, 1868 was more effective than conventional solvent extraction. Supercritical extraction is a very promising approach since it avoids unnecessary oxidation of substances, prevents unpleasant odors, improves product quality and yield, and is more profitable to operate. Step-by-step extraction with carbon dioxide followed by supercritical extraction with the addition of a co-solvent of ethanol can almost double the yields of extracts of triterpene glycosides, styrenes and carotenoids. Moreover, the fraction of triterpene glycosides practically does not contain colored impurities, in contrast to traditional ethanol extraction. The obtained extracts by HPLC in combination with tandem mass spectrometry (HPLC-MS/MS) identified 15 triterpene glycosides, 18 styrene compounds and 14 carotenoids. Separation of the triterpene glycoside fraction into sub-fractions by hydrophobic chromatography on a Polychrome-I carrier made it possible to additionally identify 3 minor triterpene glycosides cucumarioside A₇₋₁, cucumarioside A₇₋₂ and cucumarioside E. We managed to isolate all triterpene glycosides, including minor ones, but no new triterpene glycosides were found. This publication is primarily of methodological and technological value. These data will be useful for organizing production facilities for the commercial isolation of valuable products from mountainuria and for studying the composition of triterpene glycosides of not so widespread and well-studied species in order to discover new triterpene glycosides.

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References

1. Carcamo, P.F. Effects of food type and feeding frequency on the performance of early juveniles of the sea urchin *Loxechinus albus* (Echinodermata: Echinoidea): Implications for aquaculture and restocking. *Aquaculture* **2015**, *436*, 172–178. [[CrossRef](#)]
2. Honey-Escandon, M.; Arreguin-Espinosa, R.; Solis-Marin, F.A.; Samyn, Y. Biological and taxonomic perspective of triterpenoid glycosides of sea cucumbers of the family Holothuriidae (Echinodermata, Holothuroidea). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2015**, *180*, 16–39. [[CrossRef](#)] [[PubMed](#)]
3. Tripathi, V.C.; Soyar, S.; Horam, S.; Raj, S.; Arockiaraj, J.; Pasupuleti, M.; Dikshit, D.K. Natural products from polar organisms: Structural diversity, bioactivities and potential pharmaceutical applications. *Polar Sci.* **2018**, *18*, 147–166. [[CrossRef](#)]
4. Gomes, A.R.; Freitas, A.C.; Duarte, A.C.; Rocha-Santos, T.A.P. Echinoderms: A review of bioactive compounds with potential health effects. *Stud. Nat. Prod. Chem.* **2016**, *49*, 1–54. [[CrossRef](#)]
5. Casas, L.; Mantell, C.; Rodríguez, M.; Torres, A.; Macias, F.A.; de la Ossa, E.M. Extraction of natural compounds with biological activity from sunflower leaves with supercritical carbon dioxide. *Chem. Eng. J.* **2009**, *152*, 301–306. [[CrossRef](#)]
6. Herrero, M.; Mendiola, J.; Cifuentes, A.; Ibanez, E. Supercritical fluid extraction: Recent advances and applications. *J. Chromatogr. A* **2010**, *1217*, 2495–2511. [[CrossRef](#)] [[PubMed](#)]
7. Plaza, M.; Herrero, M.; Cifuentes, A.; Ibanez, E. Innovative natural functional ingredients from microalgae. *J. Agric. Food Chem.* **2009**, *57*, 7159–7170. [[CrossRef](#)]
8. Kumar, S.P.J.; Prasad, S.R.; Banerjee, R.; Agarwal, D.K.; Kulkarni, K.S.; Ramesh, K.V. Green solvents and technologies for oil extraction from oilseeds. *Chem. Cent. J.* **2017**, *11*, 7. [[CrossRef](#)] [[PubMed](#)]
9. Jin, J.-O.; Shastina, V.V.; Shin, S.-W.; Joo, Q.X.; Park, J.-I.; Rasskazov, V.A.; Avilov, S.A.; Fedorov, S.N.; Stonik, V.A.; Kwak, J.-Y. Differential effects of triterpene glycosides, frondoside A and cucumarioside A2-2 isolated from sea cucumbers on caspase activation and apoptosis of human leukemia cells. *FEBS Lett.* **2009**, *583*, 697–702. [[CrossRef](#)] [[PubMed](#)]
10. Hossain, A.; Dave, D.; Shahidi, F. Northern sea cucumber (*Cucumaria frondosa*): A potential candidate for functional food, nutraceutical, and pharmaceutical sector. *Mar. Drugs* **2020**, *18*, 274. [[CrossRef](#)] [[PubMed](#)]
11. Aminin, D.L.; Agafonova, I.G.; Berdyshev, E.V.; Isachenko, E.G.; Avilov, S.A.; Stonik, V.A. Immunomodulatory properties of cucumariosides from the edible far-eastern holothurian *Cucumaria japonica*. *J. Med. Food* **2001**, *4*, 127–135. [[CrossRef](#)] [[PubMed](#)]
12. Silchenko, A.; Avilov, S.; Antonov, A.; Kalinovskiy, A.; Dmitrenok, P.; Kalinin, V.; Woodward, C.; Collin, P. Glycosides from the sea cucumber *Cucumaria frondosa*. IV. structure of frondosides A2-4, A2-7, and A2-8, three new minor monosulfated triterpene glycosides. *Can. J. Chem.* **2005**, *83*, 2120–2126. [[CrossRef](#)]
13. Kalinin, V.I.; Silchenko, A.S.; Avilov, S.A.; Stonik, V.A.; Smirnov, A.V. Sea cucumbers triterpene glycosides, the recent progress in structural elucidation and chemotaxonomy. *Phytochem. Rev.* **2005**, *4*, 221–236. [[CrossRef](#)]
14. Popov, R.S.; Ivanchina, N.V.; Silchenko, A.S.; Avilov, S.A.; Kalinin, V.I.; Dolmatov, I.Y.; Stonik, V.A.; Dmitrenok, P.S. Metabolite profiling of triterpene glycosides of the far eastern sea cucumber eupentacta fraudatrix and their distribution in various body components using LC-ESI QTOF-MS. *Mar. Drugs* **2017**, *15*, 302. [[CrossRef](#)] [[PubMed](#)]
15. Bahrami, Y.; Zhang, W.; MM Franco, C. Distribution of saponins in the sea cucumber holothuria lessoni; the body wall versus the viscera, and their biological activities. *Mar. Drugs* **2018**, *16*, 423. [[CrossRef](#)] [[PubMed](#)]
16. Silchenko, A.S.; Kalinovskiy, A.I.; Dmitrenok, P.S.; Kalinin, V.I.; Mazeika, A.N.; Vorobieva, N.S.; Sanina, N.M.; Kostetsky, E.Y. Cucumarioside E from the far eastern sea cucumber *cucumaria japonica* (cucumariidae, dendrochirotida), new minor monosulfated holostane triterpene pentaoside with glucose as the second monosaccharide residue. *Nat. Prod. Commun.* **2015**, *10*, 877–880. [[CrossRef](#)] [[PubMed](#)]

17. Avilov, S.A.; Stonik, V.A.; Kalinovskii, A.I. Structures of four new triterpene glycosides from the holothurian *Cucumaria japonica*. *Chem. Nat. Compd.* **1990**, *26*, 670–675. [[CrossRef](#)]

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