Sulfated Chitosan Derived from *Sepia aculeata* Cuttlebone: Extraction, Structural Analysis, and Evaluation of Anticoagulant Activity

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**ABSTRACT:** Sulfated chitosan has garnered significant attention due to its diverse biological activities, including its anticoagulant properties. The main goal of this research work is to make sulfated chitosan from *Sepia aculeata* cuttlebone fragments and test its ability to stop blood clots. Various extraction and purification processes were utilized for separating the polysaccharides from the cuttlebone waste. The extracted polysaccharides were chemically processed by sulfation to improve their anticoagulant activity. Analytical techniques such as FTIR, XRD, and SEM have assessed the structural features of sulfated chitosan. Anticoagulant activity was assessed in vitro utilizing clotting tests that include activated partial thromboplastin time (APTT) and prothrombin time (PT). Sulfated chitosan has potent anticoagulant effects, significantly increasing APTT and PT values when compared to Heparin. The results of this study show that sulfated chitosan can be made from *Sepia aculeata* cuttlebone remnants. The findings suggest that the sulfated chitosan has intrinsic anticoagulant properties. This makes molecules biologically active and have anticoagulant activities. Using cuttlebone waste as a source of sulfated chitosan promotes ecologically responsible disposal while also allowing for the creation of innovative anticoagulant medications.

**Keywords:** *Sepia aculeata*; Anticoagulant; Chitosan; Heparin; Sulfated chitosan.

# Introduction

Chitin is a naturally occurring straight peptide polymer that dissolves in solvents, consisting of β-(1-4)-2-acetamido-2-deoxy-d-glucopyranose monomers. It possesses strong chemical features, biocompatibility, hemo-static action, and wound-healing capabilities [[(Website, n.d.-a)](https://paperpile.com/c/SI3Tjn/HfV7)]. Strong hydrogen interactions inside and between the layers maintain the linkages' opposing orientation at the alpha-form phase [[(Website, n.d.-b)](https://paperpile.com/c/SI3Tjn/dr7o)]. Weak hydrogen bonds within the layers hold the symmetrical arrangement of chitin together. A mix of sequential and opposing connections identifies the crystalline architecture of the less well-defined beta-chitin type [(Muthuswamy Pandian et al., 2022; Ramakrishnan et al., 2023)](https://paperpile.com/c/SI3Tjn/ouez7+CZkyI), [(Merchant et al., 2022)](https://paperpile.com/c/SI3Tjn/VTIhB), [(Sreevarun et al., 2023)](https://paperpile.com/c/SI3Tjn/YZ888).Deacetylated chitin forms chitosan, the next most common polymer on the planet after cellulose. Researchers identified it as a copolymer of (1-4)-2-acet-amido-2-deoxy-b-D-glucan and (1-4)-2-amino-2-deoxy-b-D-glucan. The various architectural amino locations on the C2, C3 primary, and C6 secondary hydroxyl groups orient its functional molecules in several advantageous directions [[(Website, n.d.-c)](https://paperpile.com/c/SI3Tjn/Sgp8)]. Because of its many unique attributes as a naturally occurring beneficial resource, including its antimicrobial capacity, low toxicity, and the ability to degrade, chitosan has received focus from scientists and the industrial group in a variety of sectors, including science and technology, medicines, health and hygiene, products for beauty, the agricultural sector, food quality, and textiles [[(Website, n.d.-d)](https://paperpile.com/c/SI3Tjn/IchC)]. Only aqueous dilute acids effectively dissolved Chitosan, limiting its solubility in water and various naturally occurring solvents. Chitosan's solubility constraint serves as the primary preventative factor, reducing its potency for usage as a pharmaceutical agent. As a result, the creation of better chitosan necessitates more research into structural changes to generate highly water-soluble derivatives that operate across a wide pH range. The novel sulfated chitosan evolved through structural alterations on amino and hydroxyl groups, leading to a wide range of biological activities [(Chokkattu et al., 2023)](https://paperpile.com/c/SI3Tjn/fbsW4), [(Laghari et al., 2023; Ramakrishnan et al., 2023)](https://paperpile.com/c/SI3Tjn/CZkyI+Fj3Pg), [(Muthuswamy Pandian et al., 2022)](https://paperpile.com/c/SI3Tjn/ouez7) Heparin and other polysaccharides are among the numerous drugs developed to treat haemorrhaging-related illnesses. However, in order to minimize haemorrhagic consequences and allergic issues, therapy for anticoagulation, including these drugs, requires continuous monitoring [[(Franchini et al., 2016)](https://paperpile.com/c/SI3Tjn/gFw2)]. Anticoagulants, which treat clotting and other medical conditions, have the fastest yearly growth rate among the top ten therapeutic fields. The increasing population of older individuals and an increased risk of cardiac events drive this growth [[(Fan et al., 2018)](https://paperpile.com/c/SI3Tjn/YZkt), [(Website, n.d.-e)](https://paperpile.com/c/SI3Tjn/p9eg)]. This encourages scientists to work on new, better anticoagulant medicines. Chitosan, a renewable poly-amino saccharide, is ecologically friendly, allergen-free, biocompatible, and recyclable. These features of chitosan include cancer prevention. Its antimicrobial, immune-modulatory, anti-cholesterol emic, and antioxidant properties make it a useful pharmaceutical ingredient and carrier for the development of new therapies. It can also be used to heal wounds and as an organic material [[(“Environmentally Benign, Bright Luminescent Carbon Dots from IV Bag Waste and Chitosan for Antimicrobial and Bioimaging Applications,” 2023)](https://paperpile.com/c/SI3Tjn/o7Vo),[(Website, n.d.-f)](https://paperpile.com/c/SI3Tjn/sT4E),[(M, 2017)](https://paperpile.com/c/SI3Tjn/LO4q)].Chitosan is a polymer with a primary amino group that can undergo structural replacement [[(Website, n.d.-g)](https://paperpile.com/c/SI3Tjn/aBpF)]. Heparin and chitosan have similar architectures [[(Website, n.d.-h)](https://paperpile.com/c/SI3Tjn/W2Bp)]. This property has resulted in the development of various chitosan-based blood thinners [(Adel et al., 2023)](https://paperpile.com/c/SI3Tjn/Zfimu), [(Subramanian & Harikrishnan, 2023)](https://paperpile.com/c/SI3Tjn/7EcUd), [(Solanki et al., 2023)](https://paperpile.com/c/SI3Tjn/eXCFL). Adding sulfate groups to chitosan's composition enhances its blood compatibility and strengthens its anticoagulant properties, as previously noted. As a result, the byproducts of sulfated chitosan offer a promising paradigm for developing new blood thinners. Because sulfated chitosan compounds showed promise as blood thinners, researchers set out to make sulfated forms of chitosan that could stop blood from clotting [[(Website, n.d.-h)](https://paperpile.com/c/SI3Tjn/W2Bp)]. Sulfated chitosan has the same structure as heparin and clearly demonstrates anticoagulant properties [[(Website, n.d.-i)](https://paperpile.com/c/SI3Tjn/rDcc)]. The present study aimed to harvest chitosan from the cuttlefish Sepia aculeata and chemically alter it to produce sulfated chitosan. The physical characteristics of the generated sulfated chitosan were also explored, as well as their pharmacological capabilities, such as their reactive and clotting properties.

# Materials and Methods

## Preparation of cuttlebone powder

The cuttlefish were disassembled and the cuttlebones recovered. Before using them in a larger study, we washed, dried, and finely pulverized them using a pestle and mortar.

## Extraction of chitin and chitosan from cuttlebone of Sepia aculeata

Rinsing the broken cuttlebone is part of the chitin and chitosan extraction process. Cuttlebone were immersed in hot NaOH (2 and 4% w/v) for about 60 min to disperse carbs and amino acids and separate raw chitin. Chitin synthesis needs 4% NaOH [[(*Website*, n.d.-j)](https://paperpile.com/c/SI3Tjn/OREx)]. The containers containing the cuttlebone specimens were withdrawn from the heating surface and left to cool at room temperature for 30 min while the samples were boiling in NaOH [[(*Website*, n.d.-k)](https://paperpile.com/c/SI3Tjn/41Bw)]. *Sepia aculeata* chitin was demineralized, deacetylated, and purified.

## Extraction of Sulfated chitosan from cuttlebone of Sepia aculeata

Sulfated chitosan was produced using 50 ml of DMF. To create viscous chitosan, shake 50 mL of chitosan solution in a DMF-formic acid combination in a 500-ml flask with triple-necked bottoms. After operating at an optimal temperature of 40-60 °C for 1 to 2 h, we incorporated 300 mL of 95% ethanol to precipitate the product. At minimal pressure, it was filtered using a Buchner funnel. After washing with ethanol, the residual material was immersed in purified water. 2 M NaOH was employed to lower the pH to 7-8. The resultant solution was treated with filtered water for 48 h utilizing a dialysis filter with a threshold weight for molecules of 12 kDa. The finished product was then frozen and condensed, yielding sulfated chitosan [[(*Website*, n.d.-l)](https://paperpile.com/c/SI3Tjn/8KRA)].

# Characteristic analysis of sulfated chitosan

## FT-IR spectral analysis of sulfated chitosan

A BRUK-ER'S ALPHA II FTIR spectrometer was used to evaluate the sulfated chitosan isolated from *Sepia aculeata.*

## Scanning electron microscopy (SEM) of sulfated chitosan

The internal structure and external characteristics of sulfated chitosan have been studied utilizing SEM. A thin coating of gold/palladium (40/60) was applied to the sample using the Hitachi Hus-4 vacuum evaporator, which evaporated the mixture instantaneously at 20 volts. The research has been carried out using different magnifying levels with an accelerated potential of 0.5 to 30 kV.

## X-ray diffraction (XRD)

The Shimadzu XRD-6000 was utilized to measure the relationship of XRD intensity with diffraction angle (2θ) and sample position. The specimens' crystal arrangements were discovered, their structural attributes were thoroughly evaluated, and the diameters and positions of the crystallites were computed utilizing the diffraction pattern.

## Anticoagulant potential of sulfated chitosan

The test used heparin sulfate as a reference substance. Various quantities of sulfated chitosan were dissolved in filtered water. A combination of 90 μl human blood plasma, 10 μl of sulfated chitosan (0-2 mg), and heparin sulfate (0-100 μg) has been created [[(*Website*, n.d.-m)](https://paperpile.com/c/SI3Tjn/oeuf)].

## APTT (Activated partial thromboplastin time)

The APTT exam was carried out using a variety of instruments. For one min, 100 μl of plasma was incubated at 37°C with different amounts of sulfated chitosan and heparin sulfate. After adding 100 μl of bovine cephalin, the mixture was kept in incubation at 37°C. After 3 min of the incubation process, the resulting mixture was mixed with 100 μl of pre-heated 0.25 M CaCl2. We subsequently determined the duration of coagulation in comparison to the standard, and stated the level of activity in IU/mg [[(*Website*, n.d.-n)](https://paperpile.com/c/SI3Tjn/y5pV)].

## PT (Prothrombin time)

The technique involved measuring prothrombin time (PT). After administering 200 μl of the thromboplastin reagent, a 100-microliter of plasma with varying amounts of sulfated chitosan was pre-heated for 5 min. At the same time, the bleeding time was documented [[(*Website*, n.d.-m)](https://paperpile.com/c/SI3Tjn/oeuf)].

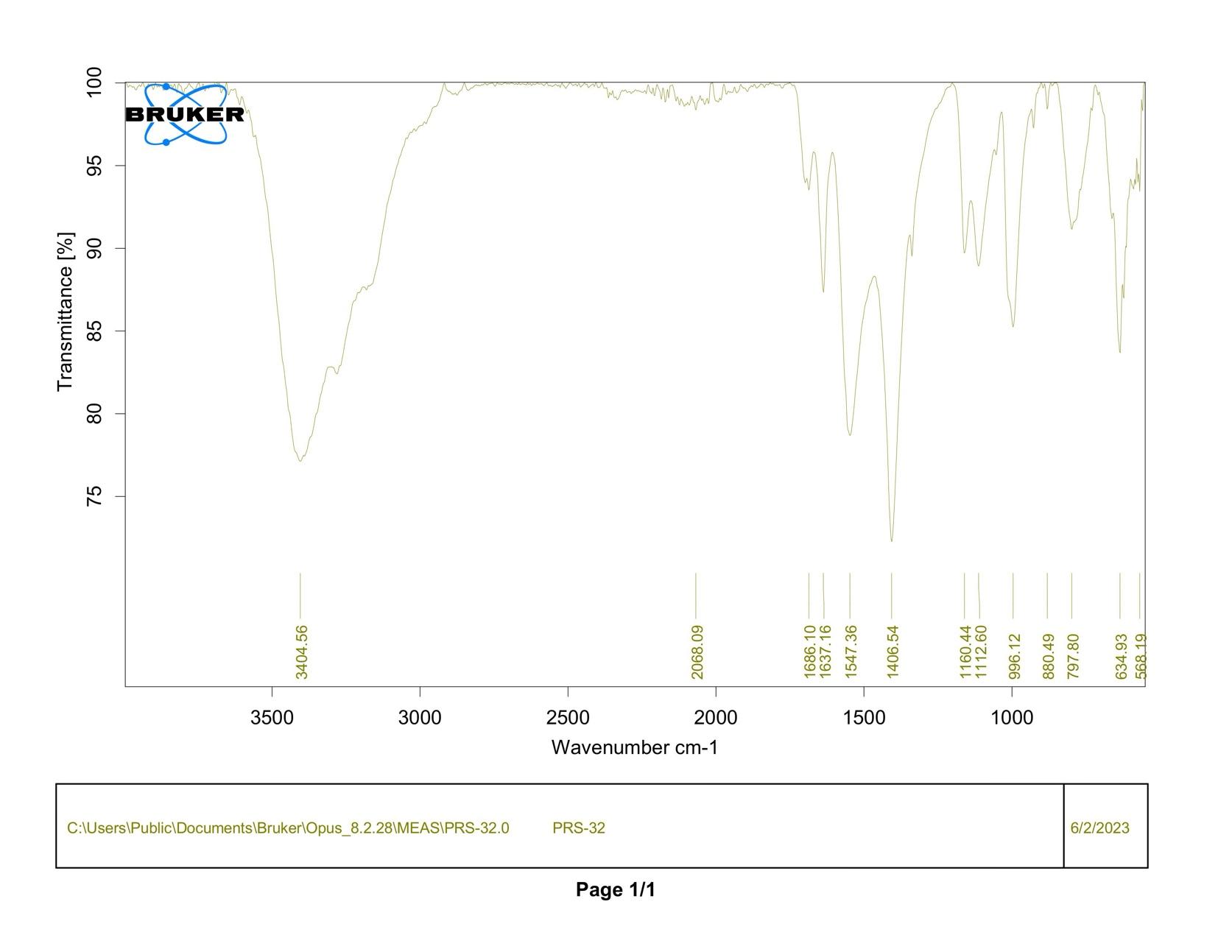
# Results and Discussion

## Production of chitin, chitosan and sulfated chitosan

Researchers discovered that *Sepia aculeata* cuttlebone contained 35.54% chitin and 83.12% chitosan, respectively. The typical production of sulfated chitosan from *S. aculeata* cuttlebone was 85%. In this study, we extracted 35.54% of the chitin present in the *S. aculeata* species. It is generally known that the squid pen produces 3% to 20% chitin [24[(*Website*, n.d.-m)](https://paperpile.com/c/SI3Tjn/oeuf)]. *Loligo lessoniana* and *L. formosana* generated 36.06% and 36.55% of chitin, respectively. *D. sibogae* chitin produced 78.57% of the chitosan [[(*Website*, n.d.-o)](https://paperpile.com/c/SI3Tjn/WgXQ)]. However, the current study utilized 83.12% of the chitosan that *S. aculeata* generated. The lower calcium carbonate level in the *S. aculeata* cuttlebone may have contributed to the increased chitosan synthesis compared to the previous study. Sulfating *S. aculeata* chitosan resulted in 85% sulfation of the polysaccharides. The sulfated polysaccharide synthesis rate was greater than that of *S. pharaonis* [[(*Website*, n.d.-p)](https://paperpile.com/c/SI3Tjn/hWOO)] and *Donax scortum* [[(*Website*, n.d.-q)](https://paperpile.com/c/SI3Tjn/fDed)], but lower than that of *S. lessoniana*.

## FT-IR spectral analysis of sulfated chitosan

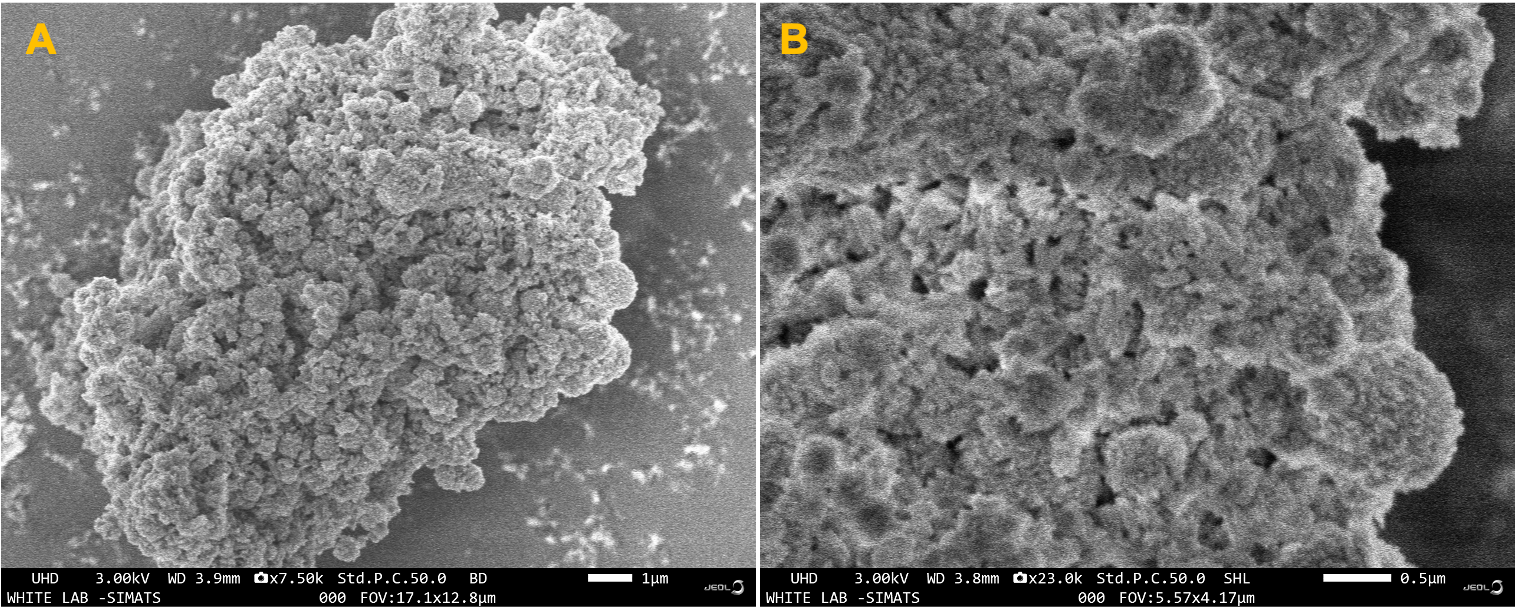
The intensity among the bands appears as 3404.56 cm-1 in Fig. 1. Infrared spectra can show strong, wide lines around 3404.56 cm-1, which proves that hydroxyl groups are present. In this instance, sulfated chitosan links with hydroxyl groups. Chitosan is an organic biopolymer composed of chitin that, when sulfated or treated with an extra sulfate group, gains novel functional categories, such as sulfate esters. The thickening of the bands indicates a powerful hydrogen relationship among the hydroxyl groups, which produces a larger absorbance peak. There were merely mild absorbance bands at 2068.09 cm-1.The FTIR spectrum reveals that the CH2 groups' vibratory stretches manifest as significant absorption bands at 2068.09 cm-1. Stretched C-H resonant frequencies in the CH2 groups created the weakened absorption bands. Stretching tensions of the C=O and CHO bonds cause absorption bands at 1637.16–1406.54 cm−1 wavelength. The C-H bond's changing vibrations produced substantial absorption bands from 3404.56 to 1406.54 cm−1. Polysaccharides have an absorbance coefficient ranging from 1112.60 to 568.19 cm−1, which corresponds to the C-O and C-C link bands(Nikalje et al., 2024) (Chehelgerdi et al., 2023). The FTIR spectrum reveals that the substance consists of chitosan sulfated with hydroxyl and CH2 groups. FTIR is a useful tool in material science and chemistry for identifying functional groups in a wide range of compounds, polymers, and biomolecules. It helps scientists detect and understand the chemical makeup and mechanical characteristics of materials.The hydroxyl groups' translational bending matches the FT-IR spectra at 3474 and 3434 cm−1 and signals at 3500 and 1650 cm−1 [[(*Website*, n.d.-r)](https://paperpile.com/c/SI3Tjn/yfrm)] indicate free hydroxyl groups reacting with hydrogen. The 1345 cm−1 band conforms to both a CO NH distortion and the CH2 group (amide III) generated by the CO NH group. The strong band at 1377 cm−1 indicates uniform deformity of the CH3 group, while the stretching or N-H deformation of amine II is exhibited at 1557 cm−1 [[(*Website*, n.d.-s)](https://paperpile.com/c/SI3Tjn/fJjC)]. The FT-IR spectrum of *S. aculeata* chitin revealed all of the expected chitin peaks. The bands at 2068.09 cm−1 show aliphatic CH stretching, whereas the wide band at 3404.56 cm−1 represents H-bonded OH stretching. The band at 1637.16 cm−1 represents amide stretch in C O. The 1406.54 cm−1 band comes from NH bending. S. aculeata chitin shows ring structural oscillations at 1112.60-634.93 cm−1.The FT-IR spectrum of chitosan cuttlebone shows a band at 3404.56 cm−1, which is caused by NH2 and OH stretching that is hydrogen-bonded. The band at 2068.09 cm−1 indicates the stretching of aliphatic CH. The band at 1636 cm−1 shows amide stretching in C-O. C−O−C's stretching vibration modes are accountable for the bands at 1160.44 and 1112.60 cm−1. FT-IR graph of sulfated chitosan cuttlebone showed a transmittance peak between 3404.56 and 568.19 cm−1. This study uses FT-IR to look at sulfated chitosan (peak at 1160 cm−1) and its absorption bands in the 1161 cm−1 region. The bands show that the N-S-O bond is stretched unevenly and has a similar structure [[(Website, n.d.-t)](https://paperpile.com/c/SI3Tjn/yPpe)]. An extra absorption band at 796 cm−1 implies stretching of C-S-O. The distinct absorption bands indicate that sulfated chitosan contains sulfated residues.[(Aparna et al., 2021; Poornima et al., 2021; Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/SI3Tjn/wseyV+1UOiI+gEUN6), [(Merchant et al., 2022; Pandiyan et al., 2022)](https://paperpile.com/c/SI3Tjn/YCQ3q+VTIhB), [(Chokkattu et al., 2022; Ramamurthy et al., 2022)](https://paperpile.com/c/SI3Tjn/5YFYV+NP7Zn)



**Fig.1**. FT-IR spectrum of Sulfated chitosan from *Sepia aculeata*

## Scanning electron microscopy (SEM)

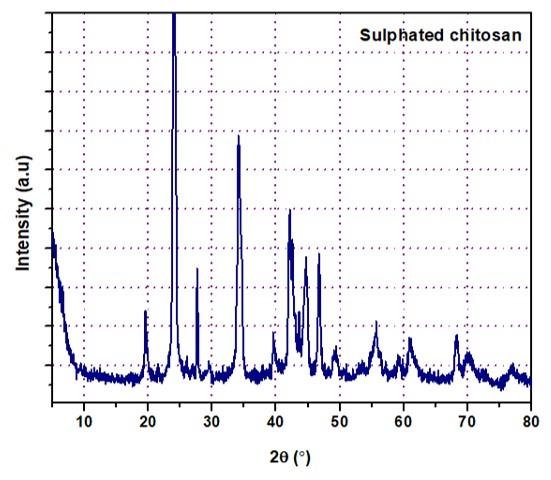
There are clearly visible fibrillar structures in some parts of the sulfated chitosan. The SEM image showed that the sulfated chitosan also had a shape that was almost like a sheet, which is good news for possible biological uses. The pictures (Fig. 2A and 2B) revealed important details about the substance's external topography and molecular composition. The SEM investigation revealed many significant findings, underscoring the unique features of sulfated chitosan. In other regions, the isolated chitosan's extremely permeable structure and flakes-covered sections were visible. Various chitosan components readily display fibrillar structures. The SEM image reveals the almost sheet-like form of sulfated chitosan, which is intriguing for potential biological applications. The unique sheet-like structure of sulfated chitosan suggests its potential for various medicinal applications, thereby creating new avenues for advancement and research in the health care product field. Researchers also found cracking flakes with fibril sequences [[(*Website*, n.d.-r)](https://paperpile.com/c/SI3Tjn/yfrm)] in crab chitosan and cuttlefish *S. aculeata* [[(*Website*, n.d.-u)](https://paperpile.com/c/SI3Tjn/U5Fa)], indicating an increase in quality in specific areas of the chitosan.



**Fig**.2A, 2B. SEM Analysis of Sulfated chitosan *Sepia aculeata*

## X-ray diffraction (XRD)

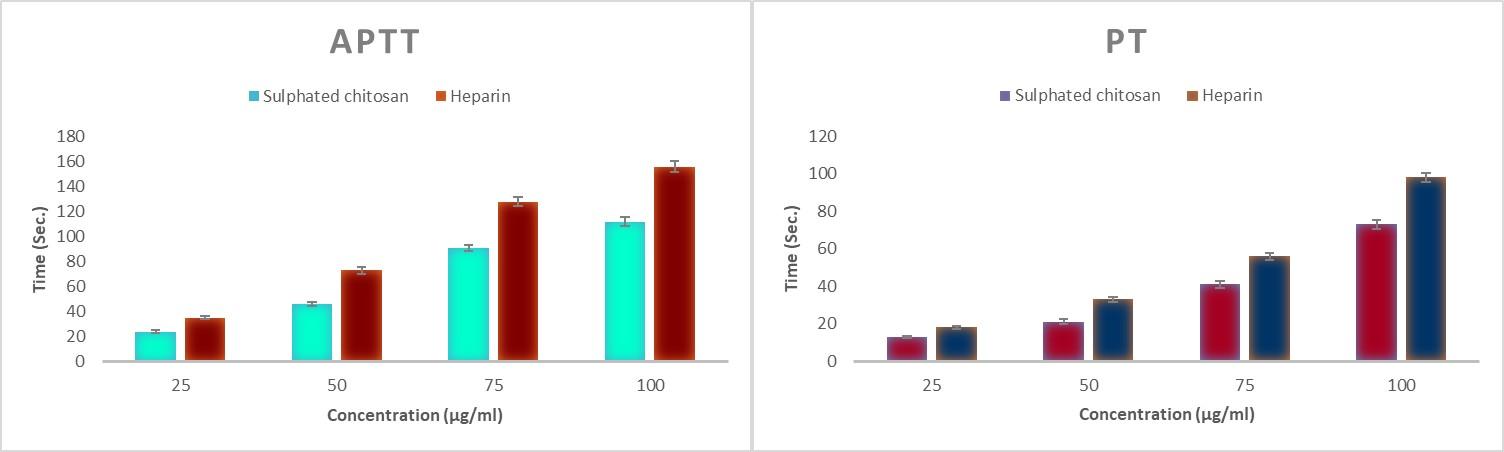
Fig. 3 shows the XRD patterns of sulfated chitosan. The XRD image showed prominent peaks at 2θ = 25° and 2θ = 35°, confirming the existence of certain crystallographic properties. Unlike chitosan, sulfated chitosan displayed two minor peaks around 25° and 35°. Sulfated chitosan had a lesser peak at 2θ = 35°, while chitosan's peak at 2θ = 25° vanished completely. Chitosan frequently exhibits peak dispersal readings in its XRD pattern that match crystallographic areas. The locations and intensities of the peaks revealed information about the molecular arrangement and order within the chitosan framework. The results of our chitosan research closely matched those of Rasti et al. [[(*Website*, n.d.-v)](https://paperpile.com/c/SI3Tjn/Pn2A)]. During X-ray diffraction experiments on sulfated chitosan, the diffraction pattern altered dramatically. The XRD pattern revealed significant peaks at 2θ = 10° and 2θ = 20°, suggesting the existence of certain structural features. In contrast with chitosan, sulfated chitosan showed two small peaks at 2θ of 25° and 35°. In sulfated chitosan, the peak at 2θ = 40° got weaker, whereas the peak at 2θ = 20° vanished totally. These findings show considerable changes in the crystal makeup of sulfated chitosan, providing insight into its unique features and application ability in a range of fields.



**Fig.3**. XRD Analysis of sulfated chitosan *Sepia aculeata*

## Anticoagulant Potential of sulfated chitosan

The results of the prothrombin time (PT) and activated partial thromboplastin time (APTT) tests for sulfated chitosan and heparin levels (µg/ml) are shown in the table 1. Sulfated polysaccharide levels of 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml produced APTT values of 24±1.25 sec, 56±1.75 sec, 91±2.25 sec, and 112±3.52 sec. APTT values for the same heparin levels were 35±1.25 sec, 73±2.75 sec, 128±3.24 sec, and 156±4.53 sec, in that order. Sulfated polysaccharide doses of 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml produced PT values of 13±0.58 sec, 21±1.25 sec, 41±1.70 sec, and 73±2.25 sec. The PT values for comparable heparin levels were 18±0.73 sec, 33±1.25 sec, 56±1.78 sec, and 98±2.25 sec, correspondingly.The sulfated chitosan showed 7.35 IU/mg for APTT and 2.54 IU/mg for PT. Non-fractionated heparin and different types of sulfated chitosan work together with antithrombin III to speed up the inactivation of thrombin [[(*Website*, n.d.-w)](https://paperpile.com/c/SI3Tjn/YVzH)]. However, the present investigation generated sulfated chitosan from the cuttlebone of *S. aculeata*, which exhibited a larger anticoagulant potential (6.45 IU/mg of APTT and 1.98 IU/mg of PT). The anticoagulant effects of sulfated chitosan could potentially stem from the presence of the sulfate group in it. Furthermore, the location of the sulfate group [[(*Website*, n.d.-p)](https://paperpile.com/c/SI3Tjn/hWOO)] and the molecular size of chitosan [[(*Website*, n.d.-m)](https://paperpile.com/c/SI3Tjn/oeuf)] significantly influence the anticoagulant effect of sulfated chitosan. Fig. 4A and 4B demonstrate how concentration effects on clotting speed affect heparin and sulfated chitosan. Clotting time increases as the concentration of sulfated chitosan or heparin rises, demonstrating that these substances are anticoagulants. The current investigation aimed to investigate how architecturally altered sulfated chitosan influences its anticoagulant properties. These results are very important for figuring out what sulfated chitosan might do to stop blood clots and how it compares to heparin, which is known to stop blood clots, in different doses.[(Marya et al., 2022)](https://paperpile.com/c/SI3Tjn/zJqyS), [(Jain & Verma, 2022; Marya et al., 2022)](https://paperpile.com/c/SI3Tjn/zJqyS+QsLLB), [(Wadhwani et al., 2022)](https://paperpile.com/c/SI3Tjn/Pdx2w)



**Fig.** 4A, 4B. *In vitro* anticoagulant activity of Sulfated chitosan from *Sepia aculeata*

**Table 1.** Anticoagulant activity of Sulphated chitosan from *Sepia aculeata*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Concentration**  **(µg/ml)** | **Activated Partial Thromboplastin Time (APTT)** | | **Prothrombin Time (PT)** | |
| **Polysaccharide (Sec.)** | **Heparin**  **(Sec.)** | **Polysaccharide (Sec.)** | **Heparin**  **(Sec.)** |
| 25 | 24±1.25 | 35±1.25 | 13±0.58 | 18±0.73 |
| 50 | 46±1.75 | 73±2.75 | 21±1.25 | 33±1.25 |
| 75 | 91±2.25 | 128±3.24 | 41±1.70 | 56±1.78 |
| 100 | 112±3.52 | 156±4.53 | 73±2.25 | 98±2.25 |

# Conclusion

The results demonstrate that *S. aculeata* is a great source of chitin, which a variety of previously mentioned methods can then convert into chitosan. This application might assist in controlling the waste produced by cuttlefish cuttlebones. Furthermore, we discovered that sulfated chitosan has a broad range of uses in industries such as food and medicine. *S. aculeata* sulfated chitosan, with its strong anticoagulant properties, could potentially replace conventional anticoagulants.

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