Bull. Korean Fish. Soc. 9(3), 185—193, 1976 韓水誌9(3), 185—193, 1976

The Origin of Molluscs Sterol (1)

The Sterol Compositon of Bivalves and Snails

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연체동물의 스테롤의 기원에 관하여 (1)

二枚貝와 卷貝의 스테롤組成의 差異

趙 鏞 桂*·金 容 巖*

패류의 스테롤 조성은 복잡하나 이들 스테롤의 유래(由來)에 관한 연구는 없다. 이들 스테롤의 기원에 관한 연구로서 우선 2매패(二枚貝)인 개량조개와 북방조개, 권패(卷貝)인 전복, 함수산(鹹水産) 소라의 스테롤 조성을 조사한 결과 다음과 같은 결과를 얻었다.

1. 각 시료의 스테롤의 함량을 보면 2매패인 개량조개 및 북방조개가 총지질에 대하여 각각 12.0%, 11.8%로서 권패인, 전복의 16.2% 소라의 15.3% 보다 약간 낮았다.
2. 15% $AgNO_3$-Silica Gel HF254 TLC 상에서 조성이 복잡한 스테롤을 상호 분리할 수 있었다.
3. 2매패인 개량조개 및 북방조개의 스테롤 조성은 C—26의 스테롤인 22—트란스—24—놀 코레스타—5.22—디엔—3—β을 이 각각 3.0%, 3.9%, 22—데하이드로 코레스테롤이 6.7%, 10.2%, 콜레스테롤이 39.0%, 48.6%, 브라시카스테롤이 14.0%, 13.8%, 24—메치렌코레스테롤이 10.0%, 11.9%, 스티그마스스테롤이 2.4%, 소량, β—시토스테롤이 10.5%, 11.9%, 프코스테롤 4.3%, 소량이었고, 북방 대합에는 구조 미상의 스테롤이 소량 함유되어 있었다.
4. 권패인 전복 및 소라에는 코레스테롤이 각각 98.0%, 97.5%였고, 22—데하이드로 코레스테롤, 브라시카스테롤이 미량 함유되어 있다. 또 전복에는 이외 24—메치렌코레스테롤, 프코스테롤이 미량 함유되어 있었다.
5. 2매패에는 코레스테롤이 50% 미만인데 대하여 권패에는 코레스테롤이 98% 정도로, 2매패와 권패의 스트롤조성상에 차가 현저히 보였다.

Introtuction

The occurrence and distribution of the sterols in animals were particulary reviewed by Bergmann[1]. The presence of the sterol in marine mollusks is interesting in the view of chemotaxonomy and comparative biochemistry, although the origin and biochemical roles of them are not clarified. In the earlier studies by nearly Japanese workers[2,3,4], however, the separation of closely structurally related sterols was not completely accomplished.

Mollusks contain very complex and interesting sterol mixtures[5]. Until the introduction of refined techniques such as gas-liquid chromatography, and more recently the combined use of GLC and Mass spectrometry[6], many of sterols were inseparable and thus not detected as pure components. Recently, Kritchevsky *et al.* [7] showed by GLC that a clam contained C-26 sterol(4.8%), 22-dehydrocholesterol(7.9%), cholesterol(36.9%), brassicasterol(14.1%), 24-met-

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hylenecholesterol(20.2%) and C-29 sterol(16.2 %). Idler *et al.*[8] isolated 22-trans-24-norcholesta-5, 22-dien-3β-ol from scallop *Placopecten magellanicus* Gmelin. Furthermore, Yasuda[9] demonstrated by GLC that small amounts of iso-fucosterol were present in the husked Japanese little neck, *Tapes japonicus* Deshayes.

On the other hand, ealry studies of the gastropoda sterols indicated cholesterol as the principal sterol of all species with the exception of *Buccinum uundatum*, which contained principally 7-dehydrocholesterol[10]. Tsujimoto *et al*[11]. found cholesterol and small amounts of the pelecypoda sterol "conchasterol" in ear top shell. Toyama *et al.*[12] found cholesterol in *Turbo cornutus*, *Lumella coronata corensis*, and *Monotonta labio*, and Tanaka *et al.*[2] identified a tetra-unsaturated sterol $\Delta^{5,7,22,25}$ -cholestatetraenol, as well as cholesterol and clionasterol and clionasterol in *Tonna luteostoma*, and choleserol and 2,2% $\Delta^{5,7}$-sterols in *Littoria brevicula*[14]. From the above results, the sterol composition reported in the early papers should be reinvestigated by more sensitive methods, especially on the presence of minor sterols and it is necessary to study the origin of the sophiscated sterols in molusks whether they are biosynthesized from lower carbon units or food-chained from the sterols in their feed.

It is well known that crustaceans lack of ability[15,16,17] to synthesize sterols de novo. Salaque[18] demonstrated the oyster had no ability to synthesize sterol from acetate, with isotope tracer techniques. It is very interesting to classify mollusks according to their presence or absence of ability to synthesize sterols from acetate or mevalonate.

The present investigation is undertaken to clarify the sterol constituents of the several marine mollusks, which are not yet shown distinctly, and the authors engage in this experiment as part of studies of the taxonomical significance on the sterol composition between pelecypoda and gastropoda. In this study, the sterols of the pelecypoda, *Mactra sulcataria*, *Spisula sachalinensis*, and the gastropoda, *Haliotis discus hannai* Ino, *Turbo cornutus*, was investigated by use of GLC. Mass spectrometry and IR spectrometry.

Meterials and Methods

Animals Abalones, *Haliotis discus hannai* Ino, were collected in early January, 1975, at Onagawa Bay, Miyagi prefecture, Japan. *Mactra sulcataria* were obtained at Sunjae-ri, Oku-gun, Jeolrabug-do, Korea, on February 15, 1975. *Spisula sachalinensis* and *Turbo cornutus* were purchased on Kita-Sendai Fish Market, Sendai, Japan, on July 6, 1974.

Isolation of sterols Lipids were extracted according to the method of Bligh *et al.*[19]. The chloroform layer was evaporated on a rotary evaporator and the residue dried at 70℃ to constant weight for determination of the total lipids. The lipids were saponified with a solution of 10% KOH in 90% ethanol for 2 hours under an atmosphere of nitrogen. Upon cooling, the solution was diluted with distilled water, the unsaponifiable matters were extracted with anhydrous ether, and the ether phase was washed several times with distilled water. The sterol fraction was collected from the non-saponifiables by a solution of digitonine-ethanol, or colum chromatography. In column chromatography[20], Silica Gel(Mallinckrodt, 60—100mesh) was used as an adsorbent, and the elution was carried out with a mixture of petroleum ether (p.e.) and ether. The eluates were checked on TLC(Wakogel B-5, Wako Pure Chemical Industries, Ltd., Osaka, Japan) by using p.e. : ether : acetic acid(80 : 20 : 1) as a developing solvent. The sterol fraction was repeatedly rechromatographed, if contaminated with impurities.

The pure sterol fraction so obtained was acetylated[21] with a solution of anhydrous pyridine and anhydrous acetic acid(1 : 1) in a refrigerator overnight.

GLC apparatus used in this study was a dual column Hitachi Model GC-2C with a flame ionization detector. The coiled stainless (2*mm*×3*m*, i.d.) column packed with 1.5% OV-17 on Chromosorb[21] W was used. The column operating temperature was 250℃. The identification of sterols was achieved by comparing the relative retention times(relative to cholesterol) to those of authentic sterols and, in some cases, confirmed by IR and Mass spectrum.

Spectra analysis Infrared absorption spectra were carried out on a KBr disc, Mass spectra were measured on the Hitachi-6M instrument (Chamber voltage, 70 ev.)

Preparative argentation TLC[8] Silica Gel HF254(30*g*) was blended with a solution of silver nitrate(4.5*g*) in water(60*ml*). After shaking approximately 30 seconds, the mixture was poured onto TLC plates(4, 20×20*cm*) to 250μ. After air-drying in a dark place, the plates were stored in an tightly light-proof oven(35℃) until used. Developments were achieved in the solvent system, n-hexane: benzene 5:2 or 5:3 in an unlined tank. After two developmets (to 18*cm*), the plates were air-dried and sprayed with 0.1% rhodamine 6G-acetone solution, and then the separated bands appeared when viewed in longwave(365μ) ultraviolet radiation.

Results

The sterols content of *Mactra sulcataris*, *Spisula sachalinensis*, *Haliotis discus hannai* Ino, and *Turbo cornutus* examined are given in Table 1. The contents of unsaponifiables and sterols in the pelecypoda were less than those in the gastropoda, *Haliotis discus hannai* Ino, and *Turbo cornutus*.

Table 1. Sterol content of marine mollusks

Mollusks	lipide (% of fresh meat)	unsaponifiables*	sterol*
Mactra sulcataria	3.2**	13.0	12.0
Spisula sachalinensis	1.3	14.2	11.8
Haliotis discus hannai Ino	1.8	21.9	16.2
Turbo cornutus	1.5	18.2	15.3

*% of total lipids **% of dry matter

Table 2. Sterol composition

Rrt	Sterol	A	B	C	D* (%)
0.62	22-trans-24-norcholesta-5, 22-dien-3β-ol	3.0	3.9		
0.74	unknown		trace		
0.90	22-dehydrocholesterol	6.7	10.2	trace	trace
1.00	cholesterol	39.6	48.6	98.0	97.5
1.12	brassicasterol	14.1	13.8	trace	trace
1.18	desmosterol			trace	trace
1.34	24-methylenecholesterol	19.4	11.5	trace	
1.39	stigmasterol	2.4			
1.59	β-sitosterol	10.5	11.9		
1.74	fucosterl	4.3		trace	

*A, *Mactra sulcataria* B, *Spisula sachalinensis*
C, *Haliotis discus hannai* Ino D, *Turbo cornutus*

1. GLC of the sterols

In GIC on 1.5% OV-17, the sterol mixture isolated from *Mactra sulcataria*, were found to be composed of eight components, as shown in Fig. 1. The peaks 1, 2, 3, 4, 5, 6, 7 and 8 were identified in the relative retention times(to cholesterol) with the authentic 22-trans-24-norcholesta-5, 22-dien-3β-ol, 22-dehydrocholesterol, cholesterol, brassicasterol, 24-methylenecholesterol, stigmasterol, β-sitosterol and fucosterol. And the sterol composition of *Spisula sachalinensis* is very similar to that of *Mactra sulcataria*, except stigmasterol and fucosterol. Table 2 presents the sterols composition of the two pelecypoda and two gastropoda.

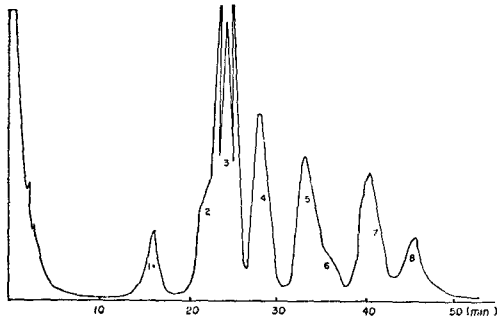


Fig. 1. Gas-liquid chromatogram of the sterol acetates from *M. sulcataria*
1: 22-trans-24-norcholesta-5, 22-dien-3β-ol
2: 22-dehydrocholesterol
3: Cholesterol
4: Brassicasterol
5: 24-methylenecholesterol
6: Stigmasterol
7: β-sitosterol
8: Fucosterol

On the other hand, the other sterols such as 22-dehydrocholesterol, brassicasterol, desmosterol and fucosterol were detected in addition to cholesterol as minor components in *Haliots discus hannai* Ino, as shown in Fig. 2, and *Turbo cornutus* also contained cholesterol, 22-dehydrocholesterol, brassicasterol and desmosterol. As presented in Table 2, the cholesterol content of the two gastropoda amounted to 98.0% and 95.5%, respectively.

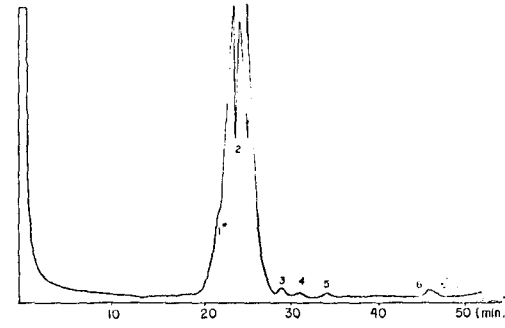


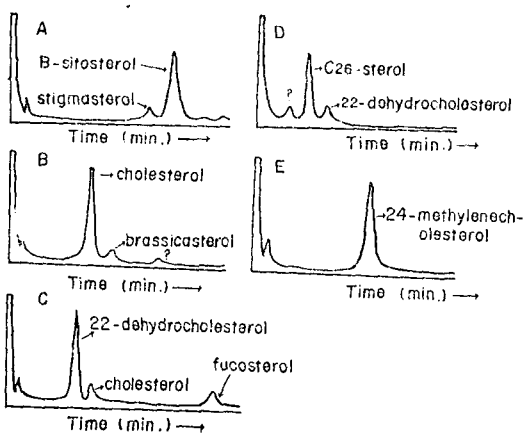
Fig. 2. Gas-liquid chromatogram of the sterol acetates from abalone, *Haliotis discus hannai* Ino
1: 22-dehydrocholestrol
2: cholesterol
3: brassicasterol
4: dermosterol
5: 24-methylenecholesterol
6. fucosterol

2. TLC of the sterols

The *Mactra sulcataria* sterol acetates mixture (Fig. 3-a) were separated into distinct bands which fluoresced in long wave violet light. Each individual band, except band E(pure 24-methylenecholesterol acetate) was shown to be an "enriched area" of one or more major components, each slightly contaminated with the sterol acetates from the neighboring bands. By selecting only the center portion of the band or, probably, rerunning the band separatively, the contamination from the adjoining band could be decreased. The constituents of each band, as determined by GLC, are shown in Fig. 3-b. All of the bands showed bright fluorescence which absorbed the UV-light.

3. Mass and IR spectra of the sterol acetates from abalone

Identification of brassicasterol. The mass spectrum of the peak 3 in Fig. 2 shows no molecular ion peak corresponding to brassicasterol acetate, but the peaks of mass spectrum(Fig. 4-a) are interpreted as follows: $380^{22)}$ ($M-CH_3CO-OH$), 365[$M^+-(CH_3COOH+CH_3)$], 337[$M^+-(CH_3COOH+43)$], 255[$M^+-(CH_3COOH+R$, R side



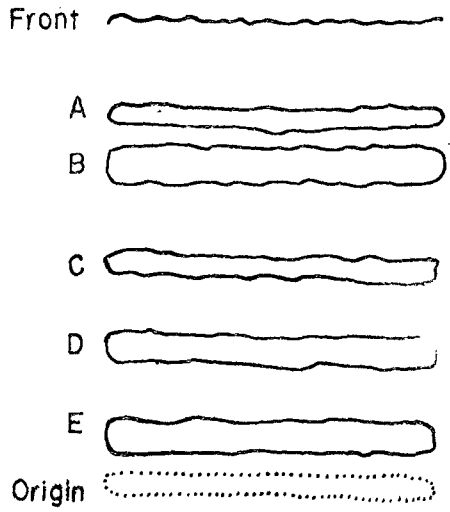


Fig. 3-a. TLC of the sterol acetates from *M. sulcataria*
plate: Kieselgel HF 254 impregnated with 15% silver nitrate
solvent: n-Hexane: Benzene(5:3)

b. GLC recordings of the *M. sulcataria* sterol acetate bands

chain)], 253[M^+-(CH_3COOH+2H)]. The fact[23] that the intensity of ion peak 255 is stronger than that of 253, suggests the presence of a double bond at the site of C22-C23. As shown in Fig. 5-a, the absorption peaks at 970cm^{-1} and 960cm^{-1} on[24] the IR spectrum show the presence of a trans double bond in the side chain.

4. Identification of desmosterol

The mass spectrum of the peak 4 on GLC(Fig. 2) gave prominent peaks at m/e 366, 351, 281, 255 and 253 as shown in Fig. 4-b. Though the molecular ion peak doesn't appear, the highest peak is seen at m/e 366 corresponding to that for the loss of acetic acid from the molecular ion(M^+). The other peaks are interpreted as follows: 366(M-CH_3COOH), 351[M^+-(CH_3COOH+CH_3)], 283[M^+-(CH_3COOH+63), 281[M^+-(CH_3COOH+85), 255[M^+-(CH_3COOH+R)], 253 [M^+-(CH_3COOH+R+2H)]. As shown on IR spectrum, the absorption peaks at 1640cm^{-1} and 840cm^{-1} suggests[25] that the presence of threes-ubstituted ethylene radical, and the absorption near 840cm^{-1} and 820cm^{-1} seems[25] to be characteristic of $\Delta^{5,24}$-sterol acetate(Fig. 5-b).

Identfication of 24-methylenecholesterol. In case of the peak 5 on GLC, the mass spectrum gave prominent peaks at m/e 380, 365, 296, 255 and 253. The peak at m/e 440 corresponding to the molecular ion peak of the sterol acetate is not observed, as shown in Fig. 4-c. However, a relatively high peak is seen at m/e 380 corresponding to one for the loss of acetic acid from the molecular ion(M^+). The other peaks are interpreted as follos: m/e 365[M^+-(CH_3COOH+CH_3)], 296[M^+-(CH_3COOH+CH_3+C_6H_{12}

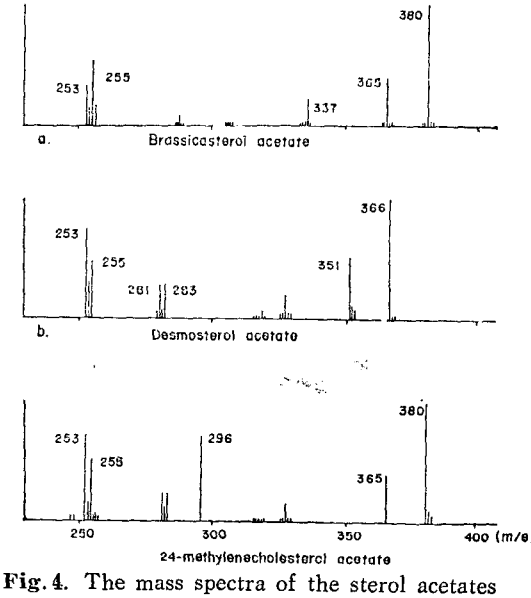


Fig. 4. The mass spectra of the sterol acetates isolated from abalone, *Haliotis discus hannai* Ino.

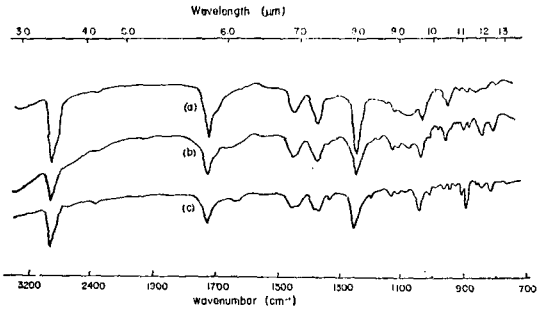


Fig. 5. IR spectra of sterol acetates from abalone, *Haliotis discus hannai* Ino.
(a) brassicasterol acetate
(b) desmosterol acetate
(c) 24-methylenecholesterol acetate

)], 255[M^+-CH_3COOH+R)], 253[M^+-(CH_3COO-H+R+2H)]. The ion peak 296 is characteristic in all the sterols included the side chains with a double bond at C24-C28. On the IR spectrum, as shown in Fig. 5-c, the absorption peaks at 885cm^{-1} and 1640cm^{-1},[24] suggests the presence of vinylidene (CH_2=C$\langle^{R}_{R}$) in the side chain of the sterol acetate.

Discussion

Vroman and Cohen[27] separated the acetates of cholesterol and desmosterol on Silica Gel H impregnated with silver nitrate, developing with n-hexane: benzens 5:3. Idler *et al.*[28] completely separated the sterol acetates mixture from scallop on Silica Gel-HF 254 and 366 impregnated silver nitrate. According to them, in order to separate a slightly less polar mixture, n-hexane-benzene 5:2 solvent with two developments gave more distinct band made for further separation. The more polar bands with low Rf values could be rerun in n-hexane-benzene 5:4 to obtain greater separations of pairs of compounds.

In this study, the most successful preparative separation of the sterol mixture had been obtained on 10% $AgNO_3$-HF356 TLC. Though each band separated on TLC with one development was proved to be contaminated with other sterols except the band E by 1.5% OV-17 GLC, we obtained pure sterol by rechromatographing each band if necessary.

Species of both pelecypoda and gastropoda contain varied and complex mixture of sterols. Cholesterol is the predominent sterol of both classes with the gastropoda containing a higher average percentage. Both of the pelecypoda examined, *Spisula sachalinensis* and *Mactra sulcataria*, have very complex sterol composition similar to other sterols of pelecypoda. In this study, stigmasterol and fucosterol were not detected in *S. sachalinensis*.

C26-sterol, 22-trans-24-norcholesta-5, 22-dien-3β-ol, and fucosterol were isolated from *Placopecten magellanicus*[28], and *Topes philippinarum* Deshayes[22], repectively. According to Itoh[29], after injection of 1-C-acetate into *P. magellanicus*, little radioactivity was detected in the fractions of C26-sterol and C29-sterol. Joh[30] reported the presence of C26-sterol in diatom, *Chaetoceros decipiens* Cleve, which was important feed of pelecypoda. It is still a question whether these unsual sterols are exogenous or endogenous.

Bergmann[1] states: "Mollusk sterols and, in particular, bivalves sterols are among the richest natural sources of $\Delta^{5,7}$-sterol, or provitamin D". Yasuda[9] reported the presence of $\Delta^{5,7}$-sterol in the pelecypoda and gastropoda he examined. But little amount of this sterol is found in the pelecypoda and gastropoda we examined. According to Idler *el al.*[5], there were no $\Delta^{5,7}$-sterols in 6 species of pelecypoda, *Solemya*(Say), *Mytilus edulis* Linnaeus, *Crassostrea virginica*, *Artica islanca* L., *Spisula solidissima*, *Mya arenaria* and *Placopecten magellanicus* In a series of early studies, elucidation of sterols structure was obscure, in most cases, mainly because efficient techniques for separating and identifying sterols had not been developed.

Early studies of gastropoda sterols indicated cholesterol as the principal of all species with the exception of *Buccinum undatum*, which contained principally 7-dehydrocholesterol[10]. In most

species only one or two sterols were identified. Tsujimoto *et al.*[11] found cholesterol and small amounts of the pelecypoda sterol "conchasterol" in ear and top shells, and Kind *et al.*[31] found the sterol of oyster drill, *Urosalpink cinereus*, consisted of 90% cholesterol and 10% $\Delta^{5,7}$-sterol.

According to Idler *et al.*[5], ten species of gastropoda contained complex sterol mixtures similar to those of pelecypoda, although generally containing higher levels of cholesterol. Of particular interest were the sterols of periwinkle, *Littorina littorea*, of which desmosterol percentage amounted to 29.5% of the total sterols. And they reported that Pteropod, *Spiratella helicina*, also had interesting sterol constituents; cholesterol 45.6%, β-sitosterol 12.0%, desmosterol 9.8% and 24-methylenecholesterol 8.1%.

But the abalone, *Haliotis discus hannai* Ino, archaeogastropoda, consists of cholesterol 98.0 % and small amounts of 22-dehydrocholesterol, brassicasterol, desmosterol and fucosterol. Teshima *et al.*[32] also reported the sterols of abalone, *Haliotis gurneri*, were composed of cholesterol 93% with small amounts of 22-methylenecholesterlol. The sterols of *Turbo cornutus*, archaeogastropoda, are composed of cholesterol 97.1% and small amounts of 22-dehydrocholesterol, brassicasterol and desmosterol, has a resemblance to those of abalone, *Haliotis discus hannai* Ino. Toyama *et al.*[12] also indicated cholesterol as a main sterol in *Turbo cornutus*.

The distinct difference of sterol composition is present between the Pelecypoda and the Gastropoda we studied. It will be of interest to investigate further whether the sterols of Pelecypoda and Gastropoda are exogenous or endogenous.

All Archaeogastropoda are herbivorous Gastropoda, and they can synthesize their sterols from acetate or mevalonate. Joh[28] also observed that abalone could synthesize their sterols, at least cholesterol and its precursor, desmosterol from acetate, and that fucosterol, 24-methylenecholesterol and brassicasterol are converted into cholesterol in abalone in vivo with isotope tracer techniques.

Summary

The sterol compositions of the Pelecypoda, *M. sulcataria*, *S. sachalinensis*, and the Gastropoda, *H. discus hannai* Ino, *T. cornutus* were investigated. The results areas follows:

1. The contents of the unsaponifiables and sterols of the Pelecypoda, *M. sulcataria*, *S. sachalinensis*, and the Gastropoda, *H. discus hannai* Ino, *T. cornutus*, are 12.0%, 11.8% and 16.2%, 15.3%, respectively.
2. The complex sterols from the Pelecypoda and Gastropoda are well separated on Silica Gel HF 254 TLC impregnated with 15% silver nitrate.
3. The prominent sterols of the Pelecypoda, *M. sulcataria* and *S. sachalinensis*, are 22-trans-24-norcholesta-5, 22-dien-3β-ol 3.0%, 3.9%, 22-dehydrocholesterol 6.7%, 10.2%, cholesterol 39.0%, 48.6%, brassicasterol 14.1%, 13.8 %, 24-methylenecholesterol 19.4%, 11.5%, stigmasterol 2.4%, 0%, β-sitosterol 10.5%, 11.9%, and fucosterol 4.3%, 0%.
4. Abalone, *H. discus hannai* Ino, and *T. cornutus* contain cholesterol 98.0%, 97.5% as main component with small amounts of 22-dehydrocholesterol, brassicasterol and desmosterol. In *H. discus hannai* Ino, 24-methylenecholesterol and fucosterol are also found.

Acknowledgements

The authors are much indebted to Professor M. Hata and T. Nomura, Tohoku University, Sendai, Japan, for their kind advice. They wish to express their thanks to Dr. M. Hata, Tohoku University, for his continued interest and guidance during the present study.

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