Identification of the Origin of Chondroitin Sulfate in "Health Foods"

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Twelve "health foods" products containing chondroitin sulfate (CS) were purchased from the Japanese market and the origin of the CS was investigated by conducting disaccharide compositional analysis after enzymatic depolymerization and by ^{1}H-NMR spectroscopy. Nine of the 12 products had labels indicating that the origin of the CS was shark cartilage. However, two of them were found to contain mammalian CS. Next, we compared the ratio of the sulfate group to the galactosamine residue after the acid hydrolysis of CS. The results suggest that all of the CS from sharks had a ratio of more than 1.0, while the CS from mammals had a ratio of less than 1.0. Since this comparative analysis does not require expensive purified enzyme, it would be an economical way to identify the origin of CS in "health foods." Being able to determine the origin of the ingredients in natural products is very important for ensuring their quality, safety, and efficacy. Therefore, we think that regulatory requirements for accurately indicating the origin of "health foods" and effective enforcement of these requirements are needed.

Key words chondroitin sulfate; chemical compositional analysis; health food origin; high performance liquid chromatography; NMR

Chondroitin sulfate (CS) is composed of a repeating disaccharide unit of the structure [-4)GlcA(β1-3)GalNAc(β1-]$_n$, where GlcA is glucuronic acid and GalNAc is *N*-acetylgalactosamine. As shown in Fig. 1, ordinary CS contains monosulfated disaccharide units -4)GlcA(β1-3)GalNAc4S(β1- and -4)GlcA(β1-3)GalNAc6S(β1-, which have been designated as the A and C units, respectively. Apart from these disaccharide units, over-sulfated CS contains disulfated disaccharide units such as D, E, and K. However, a disaccharide unit containing iduronate instead of glucuronate (B unit) is known as dermatan sulfate (DS), which is a stereoisoform of CS, differing at C-5 of the hexuronic acid moieties. The numbers and positions of the *O*-sulfo groups vary among CS samples obtained from different sources.

CS is widely distributed in animal tissues and possibly plays an important role in different types of metabolic reactions as well as being a protective agent of joints, the internal wall of blood vessels, skin, bones, *etc*.[2,3)] In cartilage, glycosaminoglycans have a protective function. CS in particular stabilizes fibrous and cellular elements of the connective tissue and at the same time lubricates and protects the membranes in joints.[4—7)] Because of this, CS has become increasingly popular as an ingredient in "health foods", and has been unlawfully advertised as effective in the treatment of symptoms related to arthritis and cartilage repair, though several proprietary drugs approved in Japan contain CS as an effective constituent.

The origin of the CS in drugs is completely regulated by their letters of approval to ensure their safety and efficacy in Japan. However, no such regulations are in place for foods sold as "health foods", and it is thus unclear what kind of animal is used for the origin of the CS contained in these foods. In the course of our continuous studies to identify the origin of natural commercial products,[8,9)] we purchased "health

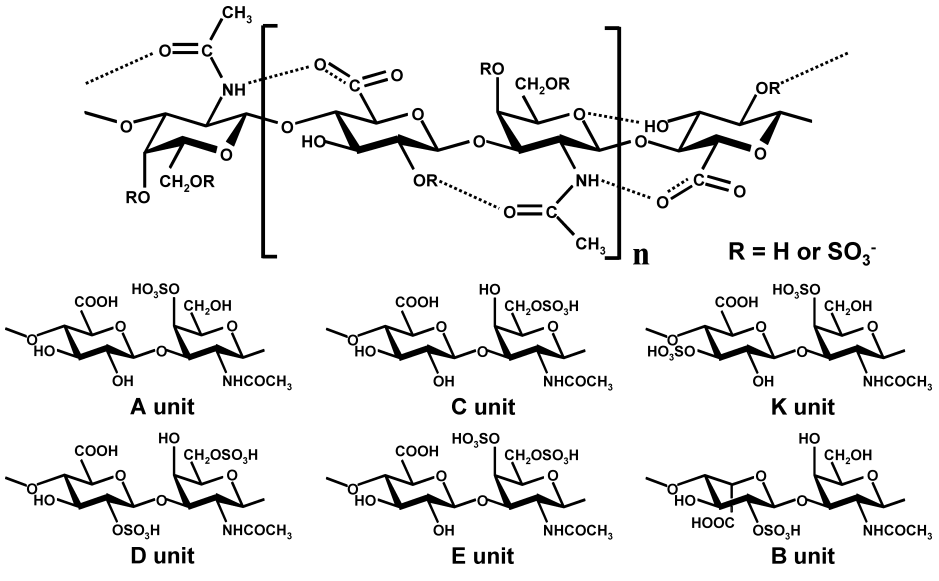

Fig. 1. Chemical Structure of CS

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foods" from the Japanese market that contained CS, and investigated their CS origin by known chemical analyses after enzymatic depolymerization and by ^{1}H-NMR spectroscopy. The results suggest that two of the twelve samples stated a false origin for the CS on their labels. In addition, we describe the comparison of the ratio of the sulfate group to GalN (galactosamine) in CS as a novel method for the determination of the origin of CS. Finally, we discuss the quality control of "health foods" and their regulatory requirements.

Experimental

Materials and Reagents CS standards from bovine tracheal cartilage and shark fin cartilage were obtained from Shin-Nippon Yakugyo Co. (Tokyo, Japan) and Seikagaku Kogyo Co. (Tokyo, Japan), respectively. Twelve "health food" products were purchased through the internet from the Japanese market. Of these, sample **A** had a picture of a shark on its package, but there was no label describing the origin of the CS in the product. The labels of samples **B**, **C**, **D**, **E**, **F**, **G**, **K** and **L** listed the CS as originating from shark fins. The labels on samples **H** and **I** listed the CS as having a bovine origin, while the label on sample **J** listed a porcine origin. Samples **A**, **F** and **H** were capsules and the other samples were tablets.

The unsaturated disaccharide standards, including 2-acetamido-2-deoxy-3-*O*-(β-D-*xylo*-hex-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-4-*O*-sulfo-3-*O*-(β-D-*xylo*-hex-4-enepyranosyluronic acid)-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-6-*O*-sulfo-3-*O*-(β-D-*xylo*-hex-4-enepyranosyluronic acid)-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-*xylo*-hex-4-enepyranosyluronic acid)-D-galactose (ΔDi-UA2S), 2-acetamido-2-deoxy-4-*O*-sulfo-3-*O*-(2-*O*-sulfo-β-D-*xylo*-hex-4-enepyranosyluronic acid)-D-galactose (ΔDi-diS_B), 2-acetamido-2-deoxy-6-*O*-sulfo-3-*O*-(2-*O*-sulfo-β-D-*xylo*-hex-4-enepyranosyluronic acid)-D-galactose (ΔDi-diS_D), 2-acetamido-2-deoxy-4,6-di-*O*-sulfo-3-*O*-(β-D-*xylo*-hex-4-enepyranosyluronic acid)-D-galactose (ΔDi-diS_E) and 2-acetamido-2-deoxy-4,6-di-*O*-sulfo-3-*O*-(2-*O*-sulfo-β-D-*xylo*-hex-4-enepyranosyluronic acid)-D-galactose (ΔDi-triS), and chondroitin ABC (EC 4.2.2.4.) lyase were also obtained from Seikagaku Kogyo. Acetonitrile was purchased from Merck (Darmstadt, Germany) and the other reagents were of the analytical grade available.

Purification of CS The tablet samples were smashed into powder using a mill. For the capsule samples, the capsule was removed and the contents were used for extraction. The purification procedure of CS was conducted according to the method reported by Emura and Mukuda[10] with minor modifications. Two hundred milligrams of each capsule or tablet was then treated with 0.5 M NaOH containing 0.3 M sodium borohydride (10 ml) at 4 °C for 12 h to release the CS chains from the core proteins by the β-elimination reaction. The mixture was then neutralized with 1.0 M hydrochloric acid, and impurities were removed by ethanol precipitation. Next, isolated CS chains were precipitated by the addition of cetylpyridinium chloride (CPC, final concentration at 0.1%) containing 0.03 M NaCl at 4 °C for 3 h. The CS-CPC complex was collected by centrifugation at 2300×***g*** for 15 min. The precipitate was washed twice with 0.1% CPC. The CS chains were extracted from the CS-CPC complex by the addition of 2.5 M NaCl, and the mixture was centrifuged at 2300×***g*** for 15 min. The CS chains were precipitated from the supernatant by the addition of 11 vol. of 85% ethanol at 4 °C for 16 h and were collected by centrifugation at 2300×***g*** for 15 min. The CS chains were then isolated through dialysis against distilled water at room temperature for 16 h followed by lyophilization to afford partially purified CS.

Enzymatic Depolymerization of CS The CS was depolymerized by a solution containing 20 μg of each partially purified sample in 10 μl of 50 mM sodium phosphate buffer at pH 7.6 for chondroitin ABC lyase treatment.[11] Chondroitin ABC lyase catalyzes the eliminative cleavage of *N*-acetylhexosaminide linkages in chondroitin sulfate, dermatan sulfate, chondroitin, and hyaluronic acid, yielding mainly disaccharides with Δ^4-hexuronate at the non-reducing ends.[12] Chondroitin ABC (1 mU) lyase in 10 μl of the same buffer was added to each sample solution, and the reaction mixtures were incubated in polyethylene vials at 37 °C in a water bath under gentle shaking. After 12 h of incubation, the enzymatic reaction was stopped by dipping the tubes in boiling water for 5 min, and then the tubes were analyzed by the post-column HPLC method.

HPLC Analysis of Disaccharide Composition The determination of unsaturated disaccharides prepared from CS was performed on the lyase-digested samples using HPLC. Unsaturated disaccharides were monitored by the fluorescence detection using 2-cyanoacetamide as a post-column labeling reagent.[13,14] The post-column HPLC system was constructed with two PU-980 intelligent HPLC pumps (Jasco, Tokyo, Japan), a double-plunger pump for the fluorogenic reagents (SPU-2.5W; Shimamura Instrument, Tokyo, Japan), a sample injector with a 20 μl loop (Model 7725i; Reodyne, CA, U.S.A.), a fluorescence spectrophotometer (FP-1520S; Jasco), a column thermocontroller (Mini-80; Saitama, Japan), a chromatointegrator (D-2500; Hitachi, Tokyo, Japan), and a dry reaction bath (DB-3; Shimamura Instrument). A DOCOSIL column (150 mm×4.6 mm i.d.; Senshu Kagaku, Tokyo, Japan) was used at 60 °C. The HPLC conditions were as follows: eluent A, 0.8 mM tetrabutylammonium hydrogen sulfate in 8% acetonitrile; eluent B, 0.8 mM tetrabutylammonium hydrogen sulfate and 0.2 M sodium chloride in 8% acetonitrile; gradient, 0—10 min (1—4% B), 10—11 min (4—15% B), 11—20 min (15% fixated B), 20—22 min (15—60% B), 22—29 min (60% fixated B), 29—33 min (60—77% B), and then equilibrated with 1% B for 20 min at a flow rate of 1.1 ml/min. To the effluent, aqueous 0.5% (w/v) 2-cyanoacetamide and 0.25 M sodium hydroxide at the same flow rate of 0.25 ml/min were delivered using a double-plunger pump. The mixture passed through a reaction coil (10 m×0.5 mm i.d.) set in a dry reaction bath thermostated at 110 °C and then through a cooling coil (3 m×0.25 mm i.d.) dipped in a cold water bath. The effluent was monitored fluorometrically (Ex. 346 nm, Em. 410 nm). A 10 μl portion of the standard and sample solutions were loaded onto the HPLC. A typical chromatogram of the unsaturated disaccharide standards is shown in Fig. 2.

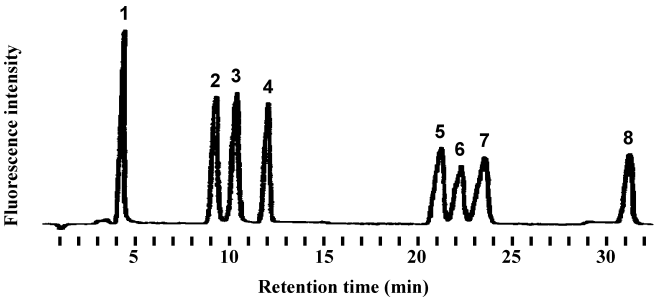

Fig. 2. Typical Chromatogram of the Unsaturated Disaccharides from CS

Peaks: 1, ΔDi-0S; 2, ΔDi-4S; 3, ΔDi-6S; 4, ΔDi-UA2S (as internal standard); 5, ΔDi-diS_E; 6, ΔDi-diS_B; 7, ΔDi-diS_D; 8, ΔDi-triS.

^{1}H-NMR Spectroscopy ^{1}H-NMR spectroscopy was performed using the conditions described previously by Toida *et al.*[15—17] Three milligrams of each standard and each purified CS sample were treated repeatedly with 0.5 ml of ^2H_2O, followed by desiccation over P_2O_5 *in vacuo* to exchange the labile proton with deuteron. Then the thoroughly dried standards and samples were redissolved in 0.5 ml of ^2H_2O and transferred to the NMR tube (5.0 mm o.d.×25 cm, PP-528; Willmad Glass Co., Buena, NJ, U.S.A.). All spectra were obtained by a JNM-ECP600 spectrometer (JEOL, Tokyo, Japan). The operation conditions for the one-dimensional (1D) spectra were as follows: frequency, 600 MHz; sweep width, 8 kHz; flip angle, 90 (10.8 μs); sampling points, 32×10^3; accumulation, 500 times; temperature, 333 K.

Chemical Compositional Analysis of CS The chemical compositional analysis of CS was followed as described previously.[18,19] In order to prepare the samples for chemical composition, the partially purified CS was further refined by exhaustive dialysis (MWCO 3500) against distilled water, lyophilization, and drying for 2 d in a desiccator over P_2O_5. Determination of the sulfate groups was performed by anion-exchange HPLC (see below) after acid hydrolysis of the sample in 2.5 M trifluoroacetic acid (TFA) at 100 °C for 6 h using conductivity detection. Hexosamines were analyzed by the post-column HPLC method after acid hydrolysis. The sulfation degree of CS was expressed by the molar ratio of sulfate ions to GalN molecules.

Ion Chromatography for Determination of Sulfation Ions in CS The determination of sulfate groups was performed by anion chromatography using suppressed conductivity detection after acid hydrolysis of the samples in TFA under the identical conditions described above. The HPLC system was constructed with a L-6000 Pump system (Hitachi), a sample injector with a 20 μl loop (Model 7725i; Reodyne), a conductivity detector (CM-8020; Tosoh, Tokyo, Japan), and a chromatointegrator (D-2500; Hitachi). A TSKgel IC-Anion-PW column (50 mm×4.6 mm i.d.; Tosoh) which induced the quaternary ammonium salt substitution to polymer was used at a flow rate of 0.8 ml/min. Sulfate ions were eluted by the use of 0.1 M carbonate (pH 9.5) as the eluting agent. A 10 μl portion of the standard and sample so-

lutions were loaded onto the HPLC.

Ion Exchange Chromatography for Determination of GalN in CS The determination of hexosamine was carried out with ion exchange chromatography using the post-column HPLC method after acid hydrolysis of the samples in TFA under the identical conditions described above. The post-column HPLC system was constructed with a PU-980 intelligent HPLC pump (Jasco), a double-plunger pump for the fluorogenic reagents (SPU-2.5W; Shimamura Instrument), a sample injector with a 20 μl loop (Model 7725i; Reodyne), a fluorescence spectrophotometer (FP-1520S; Jasco), a column thermocontroller (Mini-80; Taitech), a chromatointegrator (D-2500; Hitachi), and a dry reaction bath (DB-3; Shimamura Instrument). A TSK gel SCX column (150 mm×4.6 mm i.d.; Tosoh; 70 °C) was used at a flow rate of 0.6 ml/min. Hexosamines were eluted by the use of 0.35 M Borate–NaOH buffer (pH 7.6) as the eluting agent. To the effluent, aqueous 1% 2-cyanoacetamide solution and 1.0 M sodium hydroxide at the same flow rate of 0.25 ml/min were added by using a double-plunger pump. The mixture was passed through a reaction coil (10 m×0.5 mm i.d.), set in a dry reaction bath thermostated at 120 °C, and then through a cooling coil (3 m×0.25 mm i.d.). The effluent was monitored fluorometrically (Ex. 331 nm, Em. 383 nm). A 10 μl portion of the standard and sample solutions were loaded onto the HPLC. These conditions were used to test for the presence of other hexosamines, such as glucosamine and mannosamine.

Results and Discussions

Purification of "Health Food" Samples We purchased twelve "health foods" containing CS as a main constituent through the Internet from the Japanese market. Normally, natural non purified CS materials contain contaminated polysaccharides such as chitin, chitosan, and hyaluronic acid. We therefore purified the materials according to the purification method described in the experimental section before composition analysis.

Disaccharide Compositional Analysis In a review of CS, Volpi has suggested that the variety of sulfate substitution of CS depends on its origin.[20] As far as we know, it has been observed without exception that CS from mammals contains GalNAc with a higher percentage of sulfation at the C4-position than that at the C6-position, while CS from shark contains GalNAc with a lower percentage of sulfation at the C4-position than that at the C6-position.[20—24] More specifically, Volpi has reviewed that the ratios of ΔDi-4S to ΔDi-6S from land animals are between 1.72 and 4.82, whereas that from shark cartilage is 0.76 as the highest value.[20,21] Also, Sim *et al.* have reported that the ratio of CS from land animals in pharmaceuticals is distributed from 1.35 to 4.22, while that from shark was 0.26.[22] Nadkarni *et al.* have reported that the ratio of shark cartilage is 0.17.[23] Compositional analyses of the CS standard reagent materials originating from bovine and shark by our group also support these previous results.[25] We therefore thought it possible to discriminate between shark and mammals by comparing the ratio obtained by the compositional analysis of unsaturated disaccharides after enzymatic depolymerization of CS.

We enzymatically depolymerized partially purified CS and analyzed the disaccharides to clarify the origin of "health foods". Table 1 shows the results of the disaccharide composition of CS from "health food" samples and CS standard samples by using the post-column HPLC method. The standard sample from bovine and shark indicated the ratios of ΔDi-4S to ΔDi-6S as 1.23 and 0.35, respectively, both of which are consistent with literature values. In the same manner, the CS from samples **A**, **C**, **H**, **I**, and **J** showed ratios between 1.08 to 3.11, while the CS from samples **B**, **D**, **E**, **F**, **G**, **K**, and **L** showed ratios between 0.42 to 0.81. Since the highest reported ratio of ΔDi-4S to ΔDi-6S from shark is 0.76,[20—25] these observations strongly suggest that the CS from samples **A**, **C**, **H**, **I**, and **J** were not derived from sharks but from mammals, or that they at least contained CS from a mammal. Among the samples revealed as being of mammalian origin, or at least containing something of mammalian origin, samples **A** and **C** had labels suggesting shark origin. These results imply that these samples were falsely advertised as being of shark origin.

It is known that DS is normally prepared from porcine and bovine intestinal mucosa or porcine skin, and ΔDi-diS_B is the main disaccharide unit in DS.[20,26,27] In this study, ΔDi-diS_B was not detected from all of the samples as shown in Table 1. We therefore estimated that all of the samples tested did not contain DS.

"Health food" products of CS do not contain DNA residue, and we were therefore not able to utilize DNA authentication. However, chemical compositional analysis enabled us to comparatively reveal the origin. This is the first report to show the origin of the CS in "health foods" using disaccharide compositional analysis.

NMR Spectroscopy In order to confirm the results of the disaccharide compositional analysis, we directly measured ^{1}H-NMR of the CS standards and purified CS from

Table 1. Disaccharide Components of CS from "Health Foods"

Sample	Unsaturated disaccharide (%)							ΔDi-4S/ ΔDi-6S
	ΔDi-0S	ΔDi-4S	ΔDi-6S	ΔDi-diS_E	ΔDi-diS_B	ΔDi-diS_D	ΔDi-triS	
A	8.4	47.5	44.1	N.D.	N.D.	N.D.	N.D.	1.08
B	5.8	27.9	59.1	0.2	N.D.	9.7	N.D.	0.47
C	5.1	67.3	26.6	0.3	N.D.	0.6	N.D.	2.53
D	1.9	34.6	51.9	1.5	N.D.	10.2	N.D.	0.67
E	4.2	25.7	61.6	0.9	N.D.	7.7	N.D.	0.42
F	1.7	37.8	46.8	2.0	N.D.	11.8	N.D.	0.81
G	1.2	28.5	54.1	2.8	N.D.	13.4	N.D.	0.53
H	3.3	66.5	28.7	1.0	N.D.	0.6	N.D.	2.32
I	4.0	51.4	44.3	0.4	N.D.	N.D.	N.D.	1.16
J	7.5	70.0	22.5	N.D.	N.D.	N.D.	N.D.	3.11
K	4.0	27.3	62.6	1.4	N.D.	4.7	N.D.	0.44
L	9.3	28.6	56.1	N.D.	N.D.	6.0	N.D.	0.51
CS from bovine	10.2	49.2	40.0	0.2	N.D.	0.4	N.D.	1.23
CS from shark	6.2	21.7	62.0	1.7	N.D.	8.4	N.D.	0.35

N.D., not detected.

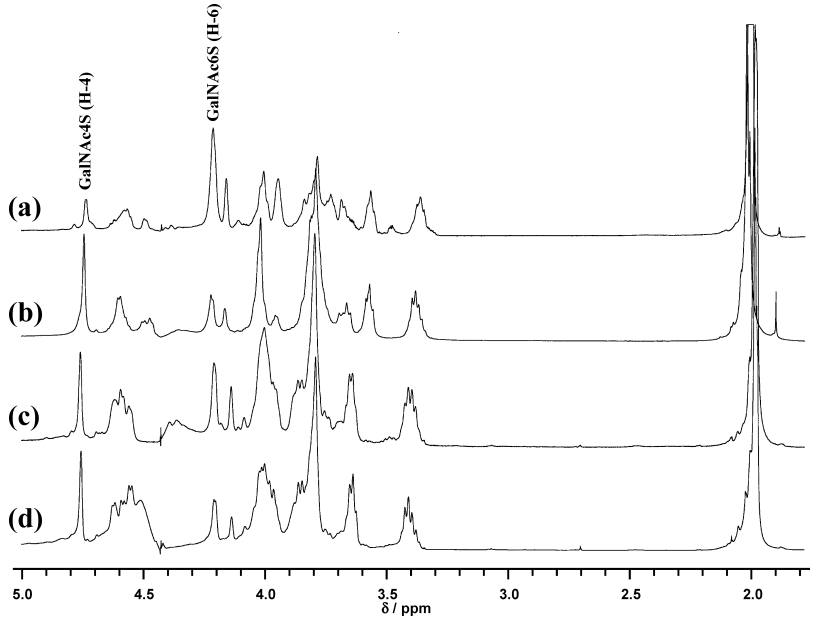

Fig. 3. [1]H-NMR Spectra of CS Standards and CS from "Health Foods"

(a) CS from shark fin, (b) CS from bovine tracheal cartilage, (c) CS from sample **A**, (d) CS from sample **C**. The letters in the spectra refer to the corresponding residues in the structures. Assignments were determined using 2D NMR.

samples **A** and **C**. It could be clearly observed that the CS standard from bovine tracheal cartilage, which is depicted in panel (b) of Fig. 3, shows a characteristic signal at 4.7 ppm, which has been assigned as H4 of the *O*-sulfonate substituted on the C4-position of GalNAc, whereas the CS standard from shark fins, depicted in panel (a) of Fig. 3, shows a characteristic signal at 4.2 ppm, which has been assigned as H6 of the *O*-sulfonate substituted on the C6-position of GalNAc.[23,25] Paying attention to these structure reporter signals, the [1]H-NMR spectra of samples **A** and **C** closely resembled the spectrum of the CS standard from bovines, as shown in panels (c) and (d) of Fig. 3, respectively. Consequently, it was confirmed that the CS from samples **A** and **C** originated from mammals or at least contained some mammalian CS.

Chemical Compositional Analysis of CS It is known that the CS from sharks contains more disulfated disaccharide units than that from mammals.[20,21,25] The results of the disaccharide compositional analysis in this study also supports this finding. We therefore thought it possible to discriminate between shark and mammals by comparing the molar ratio of the sulfate ions to the GalN molecule after the acid hydrolysis of CS. The results are shown in Table 2. Samples **A**, **C**, **H**, **I**, and **J** were found to have originated from mammals showing a ratio lower than 1.0. In contrast, samples **B**, **D**, **E**, **F**, **G**, **K**, and **L** were found to have originated from shark showing a ratio higher than 1.0. The difference in the two groups could be regarded as significant by the Student's *t*-test (probability values less than 0.01). These findings suggest that discrimination is possible by comparing the molar ratio, although more experimental data are needed to clarify the borderline ratio. The method comparing the molar ratio of sulfate ions to the GalN residue after the acid hydrolysis of CS does not consume chondroitinase ABC. Since purified chondroitinase ABC is very expensive, the above method would be useful for performing an economical identification of the CS origin in "health foods".

Table 2. Contents of Galactosamine and Sulfate Ion in CS from "Health Foods"

Sample	GalN (μg/10 μg)	Sulfate ion (μg/10 μg)	Sulfate ion/ GalN
A	0.73	0.58	0.80
B	1.48	1.64	1.11
C	1.97	1.70	0.86
D	1.74	1.92	1.10
E	0.80	0.81	1.01
F	1.53	2.30	1.50
G	1.64	2.56	1.56
H	2.06	1.71	0.83
I	2.35	1.47	0.63
J	1.90	1.75	0.92
K	1.76	1.89	1.07
L	0.67	0.85	1.27
CS from bovine	1.78	1.20	0.67
CS from shark	1.88	2.10	1.12

Quality Control of "Health Foods" Shark fins are used in Chinese cuisine, and the price is higher than bovine or porcine cartilage. Therefore, because of economic factors, cartilage from mammals may be used as the origin of CS instead of shark fins. There are some regulations in place ensuring that material from mammals used for drugs are prepared from healthy mammals because of the risk of bovine spongiform encephalopathy, foot-and-mouth disease, influenza spread in birds, and other animal diseases. However, there are no definite regulations on the origin of the ingredients in so-called "health foods." The origin of the ingredients in natural products is the most important factor ensuring quality, and thus safety and efficacy. In fact, the Japanese Pharmacopoeia states that the origin of crude drugs is the standard for judging propriety. However, the labeling on products sold as "natural" foods or health supplements is not always accurate or truthful. Recently, we revealed by chemi-

cal and DNA analyses that the ingredients used to make alkanet color were not identical to those listed in the official description.[9] In the United States, Jiang *et al.* have reported that the origin of three of the 11 food supplement products of black cohosh (*Actaea racemosa*) was an Asian *Actaea* species other than *A. racemosa*.[28] Consumers cannot estimate the origin of natural products by external appearance if these products are sold as tablets or in capsule form. Therefore, we think that regulatory requirements should be implemented on the manufacturers of "health foods," and that effective enforcement of these requirements is needed so that the ingredients in "health foods" are correctly listed.

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