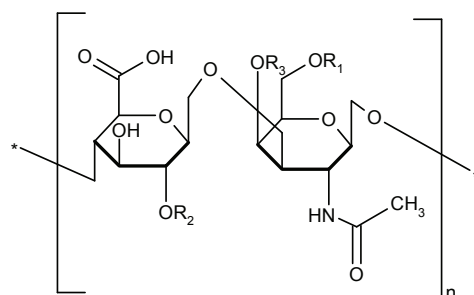


0037 - Chondroitin Sulfate by HPLC

Common Names: Chondroitinsulfuric acid

Molecular Weight: Varies, depending upon source and method of extraction/purification, up to 50,000.

Chemical Formula: Chondroitin sulfate is a polymeric sulfated glycosaminoglycan.



R₁, R₂, and R₃ can be either H or SO₃⁻
Chondroitin sulfate

Solubility: Very water soluble, insoluble in organic solvents.

Other Physical/Chemical Data: No significant UV absorption above 210 nm; very hygroscopic.

Uses: Used in dietary supplements to help relieve symptoms of osteoarthritis. It is often found in dietary supplements in conjunction with glucosamine.

Modes of Action:

Chondroitin sulfate supplements may relieve symptoms of osteoarthritis by increasing levels of chondroitin sulfate in articular cartilage and may inhibit enzymes that are responsible for cartilage deterioration. Chondroitin sulfate may also increase bioavailability of glucosamine.

Methods of Analysis

Chondroitin sulfate has been difficult to analyze in dietary supplements for several reasons. It is a polymer with a wide molecular weight range and has virtually no chromophore, and the sulfate groups make it extremely hydrophilic. In addition, the structure of chondroitin sulfate is similar to other glycosaminoglycan polymers, such as keratan sulfate, heparin, and heparin sulfate, which may be present as impurities or adulterants. Four general techniques have been used to characterize and quantify chondroitin sulfate in dietary supplements: carbazole reaction, cetyl pyridinium chloride (CPC) titration, size-exclusion chromatography, and enzymatic hydrolysis followed by HPLC.

Carbazole Reaction:

In the carbazole titration method, chondroitin sulfate is treated with sulfuric acid and heat to hydrolyze the chondroitin sulfate to hexuronic acid and hexosamine. Carbazole is then added and reacts with the hexuronic acid to form a colored product that can be measured by colorimetry. Although relatively easy to use and rugged, the method is nonspecific; any other compounds containing hexuronic acid moieties, such as glycosaminoglycans, will react with the carbazole to give a positive response.

Standard and sample preparation:

Add about 0.5 mL of standard or sample solution containing about 0.05 to 0.5 mg/mL of chondroitin sulfate to a test tube containing about 5.5 mL of sulfuric acid–water (6:1) that is immersed in an ice bath. Allow the solution to warm to room temperature, and then place the test tube in a water bath at 60°C for 90 seconds. Cool to room temperature, add 200 µL of a 0.1% solution of carbazole, and shake vigorously. After 1 hour, measure the absorption of the solution at 527 nm.

CPC Titration:

Cetyl pyridinium chloride (CPC) is a positively charged polymer that will form an ion-pair with chondroitin sulfate. This ion-pair is insoluble in water and will cause turbidity. By titrating a solution of chondroitin sulfate and measuring the resulting turbidity with a phototrode, the amount of chondroitin sulfate can be determined. The technique² is reproducible, but it has several drawbacks. Like the carbazole reaction technique, it is subject to interferences from other glycosaminoglycans, as well as other large, anionic molecules, such as surfactants and some proteins. Very low molecular weight chondroitin sulfate may not yield a positive response. Lastly, the CPC titrant requires special safety precautions and disposal procedures.

Standard and Sample Preparation: Prepare both samples and chondroitin sulfate standard in a dilute phosphate buffer (pH 7.2) at a concentration of 1 mg/mL. Transfer 5 mL of standard or sample to a titration vessel with about 30 mL of water. Titrate the solution with a 1-mg/mL solution of cetylpyridinium chloride in water. The end point can be determined visually, or, if using an automatic titrator with a phototrode detector in transmittance mode, the end point can be determined automatically.

Size-exclusion Chromatography:

Because chondroitin sulfate is a polymeric species, size-exclusion chromatography (SEC) can be used to separate it from small molecules often found in dietary supplements. Its lack of a chromophore, however, necessitates use of either short-wavelength UV detection (<210 nm) or refractive index detection. In addition, SEC cannot distinguish between chondroitin sulfate and other polymers of similar size; therefore, its selectivity is not very good.

Way et al.³ presented a method utilizing SEC for the assay of chondroitin sulfate in dietary supplements. They noted, however, that the presence of other glycosaminoglycans in the samples could cause overestimation of the chondroitin sulfate content.

Standard and Sample Preparation: Dissolve standards and samples in water. Prepare linearity standard concentrations ranging from 0.20 to 0.80 mg/mL. Prepare samples so that the final chondroitin sulfate concentration is about 0.40 mg/mL

Chromatography:

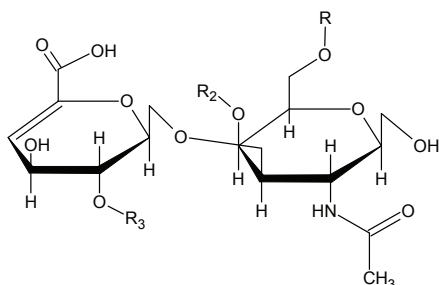
Column: Phenomenex BioSep-SEC-S2000.
 Mobile phase: 0.1 M sodium phosphate, pH 7.0.
 Flow rate: 0.8 mL/minute
 Injection: 50 μ L
 Detection wavelength: UV at 207 nm

Validation data:

Linearity: Correlation coefficient >0.999.
 Accuracy: 98.7% average recovery of spiked samples.
 Precision: 2.48% RSD for 10 samples.
 Selectivity: It was demonstrated that ingredients magnesium stearate, manganese ascorbate, and glucosamine hydrochloride did not interfere.
 Ruggedness: 104.8% of first analysis
 Robustness: Not specified
 LOD/LOQ: Not specified

Enzymatic Hydrolysis with HPLC:

Chondroitin sulfate can be hydrolyzed to disaccharide units by the enzyme chondroitinase ABC.⁴ The resulting disaccharide units are unsaturated, with a UV absorbance maximum of 232 nm:



	R	R ₂	R ₃
Di-0S	H	H	H
Di-4S	H	SO ₃	H
DI-6S	SO ₃	H	H

Enzymatic hydrolysis using chondroitinase ABC followed by HPLC offers several advantages compared to other techniques for characterizing chondroitin sulfate. Chondroitinase ABC will only hydrolyze chondroitin sulfate, allowing the method to distinguish between chondroitin sulfate and related glycosaminoglycans. In addition, the ratios of the resulting disaccharide units can be used to determine the source of the chondroitin sulfate (e.g., bovine, porcine, avian, or shark).^{5,6} The disaccharides can be separated by ion-exchange, ion-pair, or normal-phase HPLC, with detection at 232 nm, allowing for easy quantitation. Ion-exchange chromatography using amino columns has been most widely used for the separation of the disaccharides. The major disadvantage to the technique is the cost and availability of the chondroitinase ABC enzyme. Typical ion-exchange chromatographic conditions for the separation of the disaccharides are given.

Chromatography:

Column: Phenomenex Phenosphere NH₂, 4.6 × 150 mm.

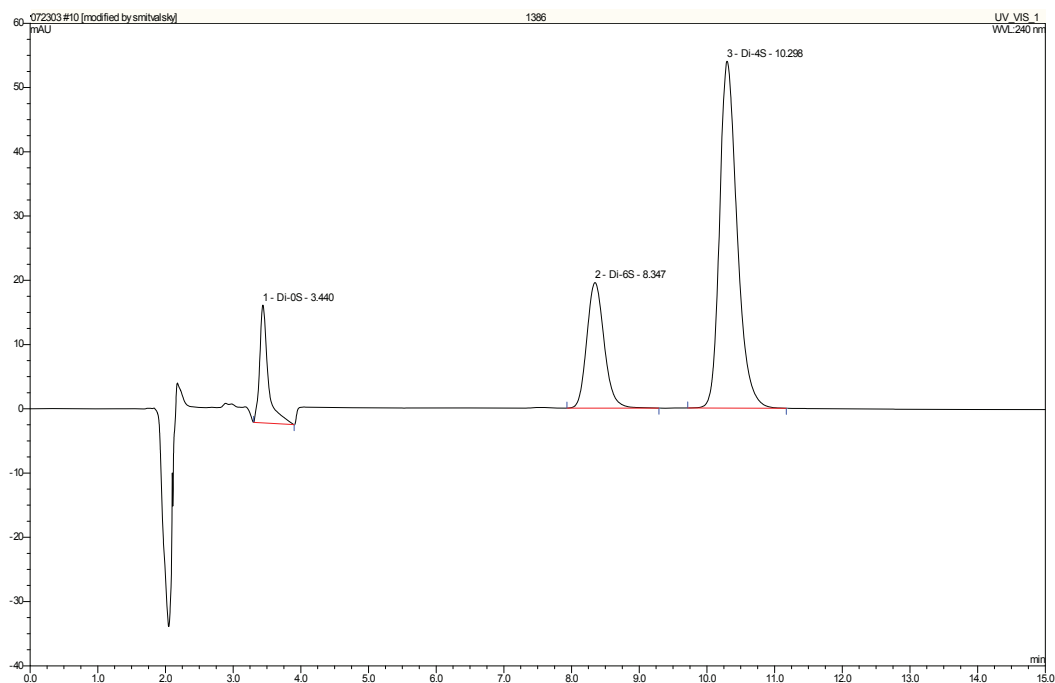
Mobile phase: Acetonitrile–0.08 M sodium acetate buffer (pH 5.0) (30:70).

Flow rate: 1.5 mL/minute

Injection volume: 30 µL

Detection wavelength: 240 nm

Run time: 20 minutes

Typical HPLC chromatogram using enzymatic hydrolysis**References:**

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Way WK, Gibson KG, Breite A. *J Liq Chromatogr Relat Technol.* 2000;23(18):2851–60.
3. Ototani N, Sato N, Yosizawa Z. High performance liquid chromatography of unsaturated disaccharides produced from chondroitin sulfate by chondroitinase. *J Biochem.* 1979;85:1383–5.
4. Karamanos NK, Syrokou A, Vanky P, et al. Determination of 24 variously sulfated galactosaminoglycan- and hyaluronan-derived disaccharides by high-performance liquid chromatography. *Anal Biochem.* 1994;221:189–99.
5. Volpi N. Hyaluronic acid and chondroitin sulfate unsaturated disaccharides analysis by high-performance liquid chromatography and fluorometric determination with dansylhydrazine. *Anal Biochem.* 2000;277:19–24.