STAKEHOLDER CONSENSUS

SMPRs Approved for High-Priority Dietary Supplements: Chondroitin Sulfate

The following article on chondroitin sulfate is the first in a series on dietary supplements for which AOAC INTERNATIONAL has established voluntary consensus standards, under contract with the National Institutes of Health-Office of Dietary Supplements (NIH/ODS). In future ILM issues, installments will focus on anthocyanins, PDE5 inhibitors, and other ingredients chosen as priority dietary supplements by AOAC's Advisory Panel on Dietary Supplements, based on industry feedback. Information will be included on the ingredient's use, biochemistry, analytical issues, and current methodologies, together with a summary of method performance requirements.

Chondroitin sulfate is an important structural component of cartilage, and has become a widely used dietary supplement for treatment of osteoarthritis. However, supplies of raw material are limited, giving some suppliers incentive to use inferior materials. The industry has developed a number of methods to evaluate the quality and amount of chondroitin in dietary supplement ingredients and finished products.

Chondroitin sulfate is a sulfated glycosaminoglycan (GAG) composed of a chain of alternating hexose sugars (Figure 1). It is usually found attached to proteins as part of a complex known as proteoglycan. A chondroitin chain can have over 100 individual sugars, each of which can be sulfated in variable positions and quantities.

Chondroitin sulfate sodium consists mostly of the sodium salt of the sulfate ester of N-acetylgalactosamine-2-acetamido-2-deoxy-d-galactopyranose, usually abbreviated as (GalNAc), and d-glucuronic acid copolymer. These hexose sugars are alternately linked -1,4 and -1,3 in the polymer. Both of the hexose sugars can be sulfated at different positions. The amount and position of sulfation varies based on the species, age of the animals, and anatomic location of the source cartilage. Chondroitin sulfate "A" is sulfated at the 4- position. Chondroitin sulfate "C" is sulfated at the 6- position. Chondroitin sulfate "D" and "E" are di-sulfated. What used to be designated as chondroitin sulfate "B" is now recognized as dermatan sulfate and is not actually a chondroitin sulfate.

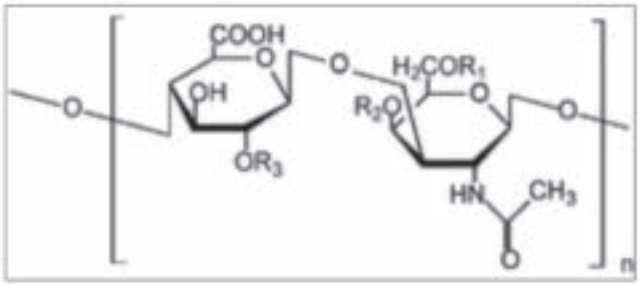

Figure 1. Chemical structure of one unit in a chondroitin sulfate chain. Chondroitin-4-sulfate: R1 = H; R2 = SO_3H; R3 = H. Chondroitin-6-sulfate: R1 = SO_3H; R2, R3 = H. Chondroitin-6-sulfate: R1 = SO_3H; R2, R3 = H. Chondroitin sulfate has also a linkage region to consisting of GlcAβ-1-3Galβ-1-3Galβ-1-4Xylβ-1-O-Ser, and a capping trisulfated monosaccharide. Commercial chondroitin sulfate has a varying content of nonsulfated disaccharides and it may contain some degree of decarboxylation depending on the isolation and purification treatment. Sulfation position depends on the species from which it is derived, age of the animals and anatomic location of the cartilage.

Supplies of raw material are limited, giving some suppliers incentive to use inferior materials. The industry has developed a number of methods to evaluate the quality and amount of chondroitin in dietary supplement ingredients and finished products.

Use

A number of studies suggest that chondroitin sulfate may be an effective treatment for osteoarthritis, a type of arthritis characterized by the breakdown and eventual loss of cartilage, either due to injury or to normal wear and tear. It commonly occurs as people age. In some studies, chondroitin sulfate supplements have decreased the pain associated with osteoarthritis. In the past, some researchers thought chondroitin sulfate may actually slow progression of the disease, unlike other current medical treatments for osteoarthritis. Chondroitin sulfate is often combined in dietary supplements with glucosamine as a treatment for osteoarthritis.

Biochemistry

GAGs exhibit a high

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Table 1. SMPR for quantitative determination of total chondroitin sulfate salts in dietary ingredients and dietary supplements

Type of study	Parameter	Acceptance criteria	
Single-laboratory validation	Limit of quantitation, % (w/w)	1	
	Analytical range, % (w/w)	1-10	>10-100
	Repeatability (RSD_r), %	≤3	≤2
	Recovery, %	92-105	98-102
Multi-laboratory validation	Reproducibility (RSD_R), %	≤6	≤4

degree of heterogeneity with regards to molecular mass, disaccharide construction, and sulfation due to the fact that GAG synthesis, unlike proteins or nucleic acids, is not template driven, but rather is modulated by processing enzymes. It is this heterogeneity that makes the analytical evaluation of chondroitin sulfate such a challenge.

GAGs are synthesized in the Golgi apparatus within the cell, where protein cores made in the rough endoplasmic reticulum are post-translationally modified with O-linked glycosylations by glycosyltransferases to form the proteoglycans found in cartilage.

Sources and Processing

Chondroitin sulfate can be harvested from bovine trachea, porcine rib cartilage, and shark and avian cartilage. The raw material must be collected following strict hygiene conditions, and frozen immediately after collection. The extraction process must be carefully controlled to preserve the molecular integrity of the product and ensure that there is no protein, polysaccharide, or bacteriological contamination. GAGs can be denatured through disulfation, disamination, or depolymerization of the polysaccharide chain.

Analytical Issues

Dietary supplements with chondroitin sulfates are some of the most popular supplements on the market. This popularity, combined with limited sources and the challenges of analytical testing, make these supplements a prime candidate for economic adulteration. A variety of economic adulterants have been found such as carrageenan, alginates, dermatan sulfate, proteins, and sodium hexametaphosphate.

Adebowale et al. reported in 2000 that of 32 chondroitin supplements they analyzed, only five were labeled correctly, and more than half contained less than 40% of the labeled amount, according to their analytical methodology (1).

> It is this heterogeneity that makes the analytical evaluation of chondroitin sulfate such a challenge.

Current Methodologies for Identification

- **Carbazole–Colorimteric**
  This method was originally developed by Dische and Borenfreund in the early 1950s and is based on the principle of strong acid hydrolysis to break the components of disaccharides into their monosaccharides (2). Glucuronic acid is the major monosaccharide product when chondroitin sulfate is hydrolyzed and is measured by a color reaction. The method is easy to use, low cost, and relatively rugged. However, it is not specific. Other mucopolysaccharides containing glucuronic acid, such as heparin or free glucuronic acid itself, will give a similar response as chondroitin sulfate.

- **CPC Titration**
  This method is based on formation of turbidity when cetyl pyridinium chloride (CPC) reacts with organic anions such as sulfate or carboxylate ions under slightly basic condition. The CPC method is not specific for chondroitin sulfate. Other mucopolysaccharides will react to the reagent in the same way as chondroitin sulfate. Even carboxylic acid groups on proteins will react the same way as chondroitin sulfate. Turbidity can be measured by either auto- or manual-titrator. This method must be combined with an array of other techniques to obtain reliable confirmation of the purity and identity of chondroitin sulfate.

- **Cellulose Acetate Membrane Electrophoresis**
  Cellulose acetate membrane electrophoresis (CAME) is one of the USP methods developed for the detection of impurities in CS dietary ingredients and supplements. This method combines the binding ability of CPC to organic ions (previously discussed) with the separation ability of electrophoresis. Toluidine blue is used as a stain after electrophoresis to visually reveal any impurities that have reacted with the CPC. When CAME and CPC titration are used in combination, adulterants can be visualized and estimated, and a true value for CS can be assigned. CAME is an inexpensive procedure with a low initial setup cost. The apparatus has a small footprint, requiring about 1 M of bench

space. The operating cost is also low, and each membrane can accommodate between four and 10 samples. The electrophoretic separation requires about 2 hours following about 45 minutes of sample preparation (3).

- **Enzymatic High-Performance Liquid Chromatography (eHPLC)**
  Samples are selectively digested into unsaturated disaccharides using chondroitinase AC enzyme. The resulting disaccharides are then measured by HPLC with a UV detector at 240 nm. The method is specific (virtually free of interference) because of the selective reaction of enzyme chondroitinase AC. The technique uses a standard HPLC with UV detector, and is rugged, robust, and accurate.

The extraction process must be carefully controlled to preserve the molecular integrity of the product and ensure that there is no protein, polysaccharide, or bacteriological contamination.

- **High-Performance Liquid Chromatography**
  Undigested (no enzyme treatment) high-performance liquid chromatography methods have been widely used by the industry. HPLC methods typically separate analytes based on a number of factors, including polarity, size, and pH. However, chondroitin actually elutes before the solvent front because there is no interaction between the chondroitin and the stationary phase(4). Therefore, these methods are nonspecific, demonstrating neither separation nor specific UV absorption.

- **Fourier Transformation Infrared (FTIR)**
  FTIR spectroscopy using the KBr pellet technique has been used for determination of chondroitin sulfate from different sources of cartilage (5).

- **Optical Rotation (Specific Rotation)**
  Chondroitin sulfate is optically active and has a characteristic specific rotation. Chondroitin sulfate has a strong, negative, optically active band near 210 mμ, arising from the carboxylate and N-acetyl groups. The method is not very specific to chondroitin sulfate but could be used in tandem with other methods.

Table 2. SMPR for screening method for selected adulterants in dietary ingredients and supplements containing chondroitin sulfate

Type of study	Parameter	Parameter requirements	Target test concentration, % (w/w)	Minimum acceptable results
Single-laboratory validation	Matrix studies	Minimum of 33 replicates representing ideally all target compounds in Annex I and all matrix types listed in Annex II, spiked at or below the designated low level target test concentration [annexes available at www.eoma.aoac.org]	≤5	90% POD[a] of the pooled data for all target compounds and matrices
		High concentration; minimum of five replicates per matrix type spiked at the designated high level target test concentration	ca 20	100% correct analyses are expected per matrix type[b]
		Zero concentration; minimum of five replicates per matrix type that have tested negative with a second method and have not been spiked	0	
Multi-laboratory study	LPOD	Use Appendix N: *ISPAM Guidelines for Validation of Qualitative Binary Chemistry Methods* [www.eoma.aoac.org]	≤5	≥0.85 LPOD[c]
			ca 20	≥0.95 LPOD[c]
			0	≤0.05 LPOD[c]

[a] Probability of detection with 95% confidence interval.

[b] 100% correct analyses are expected. Some aberrations may be acceptable if the aberrations are investigated, and acceptable explanations can be determined and communicated to method users.

[c] LPOD = Laboratory probability of detection. LPOD is not required for First Action *Official Methods of Analysis* approval.

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- **Size-Exclusion Chromatography (SEC)**
  SEC, also known as gel filtration chromatography (GFC), is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight. It is usually applied to large molecules or macromolecular complexes such as proteins, and polymers like chondroitin. A typical setup for chondroitin measurement uses a 7.5 x 300 mm column packed with 5 µ polyvinyl alcohol and a refractive index detector.

Two SMPRs have been approved for chondroitin sulfate: one for measurement of total chondroitin sulfate; and another for detection of selected adulterants.

Other Potential Methodologies for Identification

- **Nuclear Magnetic Resonance (NMR) Spectroscopy**
  NMR spectroscopy has been used for characterization of oligosaccharides from the chondroitin sulfates (6). NMR spectroscopy appears to suffer from low sensitivity, especially toward polymeric components giving less marked resonances (7). However, McEwen et al. report the accuracy of quantification as being rather good using ^{1}H-NMR (8). Bigler et al. reported better fingerprinting by high-resolution ^{1}H NMR spectroscopy using the two-dimensional Nuclear Overhauser effect spectroscopy (2D NOESY) method. Two-dimensional NMR spectra provide more information about a molecule than one-dimensional NMR spectra and are especially useful in determining complicated molecular structure (9).

Summary of Method Performance Requirements

Two SMPRs have been approved for chondroitin sulfate: one for measurement of total chondroitin sulfate; and another for detection of selected adulterants. The minimum performance requirements for each SMPR are provided in Tables 1 and 2. The SMPRs were prepared by the AOAC Chondroitin Working Group, led by **Jana Hildreth,** director of New Technology and Scientific Affairs, Synutra Pure.

See the related story in the September/October 2014 issue of ILM regarding the approval of the SMPRs by the AOAC Stakeholder Panel on Dietary Supplements. ■

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