

Determination of Chondroitin Sulfate in Raw Materials and Dietary Supplements by HPLC-UV

After Enzymatic Hydrolysis

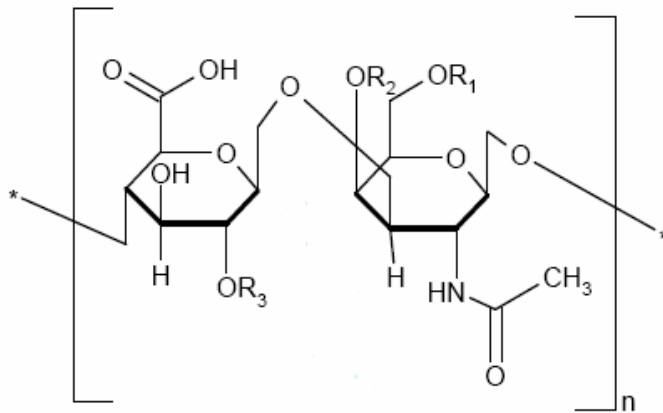


Alternate Methods

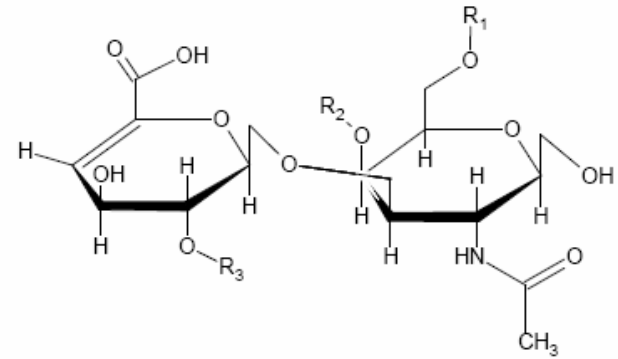
- **CPC Titration**
 - Commonly used to determine raw material purity.
 - Non-specific: will give positive results for any anionic polymeric material, such as carrageenan, proteins, surfactants, etc.
- **Carbazole Reaction**
 - Colorimetric method.
 - Not specific for chondroitin sulfate; will react with any glycosaminoglycan.
- **Size Exclusion Chromatography**
 - Based on molecular weight.
 - Any polymer with similar molecular weight to chondroitin sulfate will interfere.
- **HPLC without enzymatic hydrolysis**
 - Chondroitin sulfate is unretained and has poor UV absorbance
 - Virtually any unretained material will interfere with analysis

Principle of Enzymatic Hydrolysis

- Enzymes known as chondroitinase enzymes will hydrolyze chondroitin polymers creating unsaturated disaccharides.
- Chondroitinase ABC will hydrolyze chondroitin sulfate and dermatan sulfate (sometimes referred to as chondroitin sulfate B).
- Chondroitinase AC will only hydrolyze chondroitin sulfate.
- Chondroitin sulfate content is sum of total disaccharides created.



Chondroitin Sulfate



Δ -Disaccharide

Disaccharide	R1	R2	R2
Δ di-0S	H	H	H
Δ di-4S	H	SO ₃ ⁻	H
Δ di-6S	SO ₃ ⁻	H	H
Δ di-di(2,6)S	SO ₃ ⁻	H	SO ₃ ⁻
Δ di-di(4,6)S	SO ₃ ⁻	SO ₃ ⁻	H
Δ di-tri(2,4,6)S	SO ₃ ⁻	SO ₃ ⁻	SO ₃ ⁻

Advantages to Enzymatic Hydrolysis-HPLC

- Highly selective.
 - Possible contaminants/adulterants do not affect assay results
 - Carrageenan
 - Other GAGs
 - Proteins
- Can be used with finished products.
- No special waste disposal issues.

Disadvantages to Enzymatic Hydrolysis-HPLC

- Standards can be expensive, of limited availability, and uncertain purity.
- Enzyme solution has limited stability in solution.
- Longer analysis time than CPC Titration.

Current Proposed AOAC Chondroitin Sulfate Method

- Method was selected by an AOAC Expert Review Panel
- Method was developed by David Ji (Analytical Laboratories in Anaheim) and Joseph Zhou (NOW Foods).
- David Ji, Joseph Zhou, & Mark Roman (Tampa Bay Analytical Research) served as co-study coordinators for SLV.
 - Recommended modifications to method to incorporate di- and tri- sulfated disaccharides.

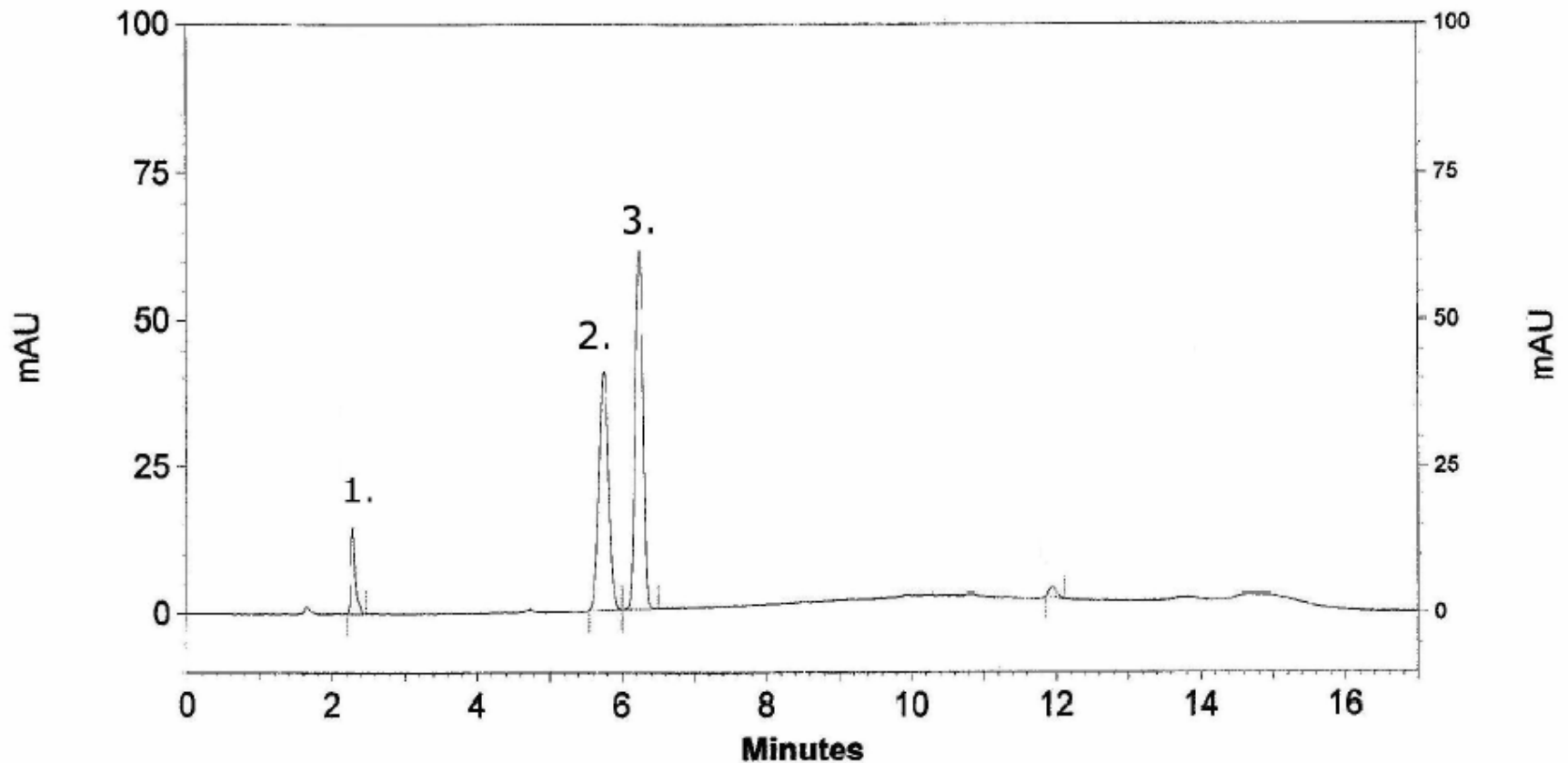
Methodology

- Samples are dissolved in dissolved in water with sonication.
- 20 μL of sample solution are mixed with 20 μL TRIS buffer and 30 μL of enzyme solution.
- Mixture is warmed at 37° C for 3 hours, dilute to 1 mL and injected on HPLC system.

Methodology – Chromatographic Conditions

- Column: Phenomenex Synergi Polar-RP, 4.6 x 150 mm
- Mobile Phase: A – Tetrabutylammonium Bisulfate in Water
B – TBAB in 33:67 Water:ACN
- Gradient: 20%B – 65% B over 7 minutes, hold 5 minutes
- Flow Rate: 1.1 mL/min
- Injection Volume: 30 μ L
- Detection: 240 nm

Methodology - Standard Chromatogram



Single Laboratory Validation

- Accuracy
 - Spike/Recovery studies on FPDF negative control (3 levels x 3 replicates x 3 days).
 - Spike/Recovery studies on heparin negative control (3 levels x 3 replicates x 3 days).
- Repeatability
 - 4 replicates x 3 days on 5 different materials
 - 4 replicates x 1 day on additional 4 materials

Single Laboratory Validation

- Linearity/Range – confirmed over approximately 2 orders of magnitude concentration range.
- Selectivity – Possible interferences from related GAGs, vitamins, minerals, MSM, glucosamine investigated.
- Youden Ruggedness Study

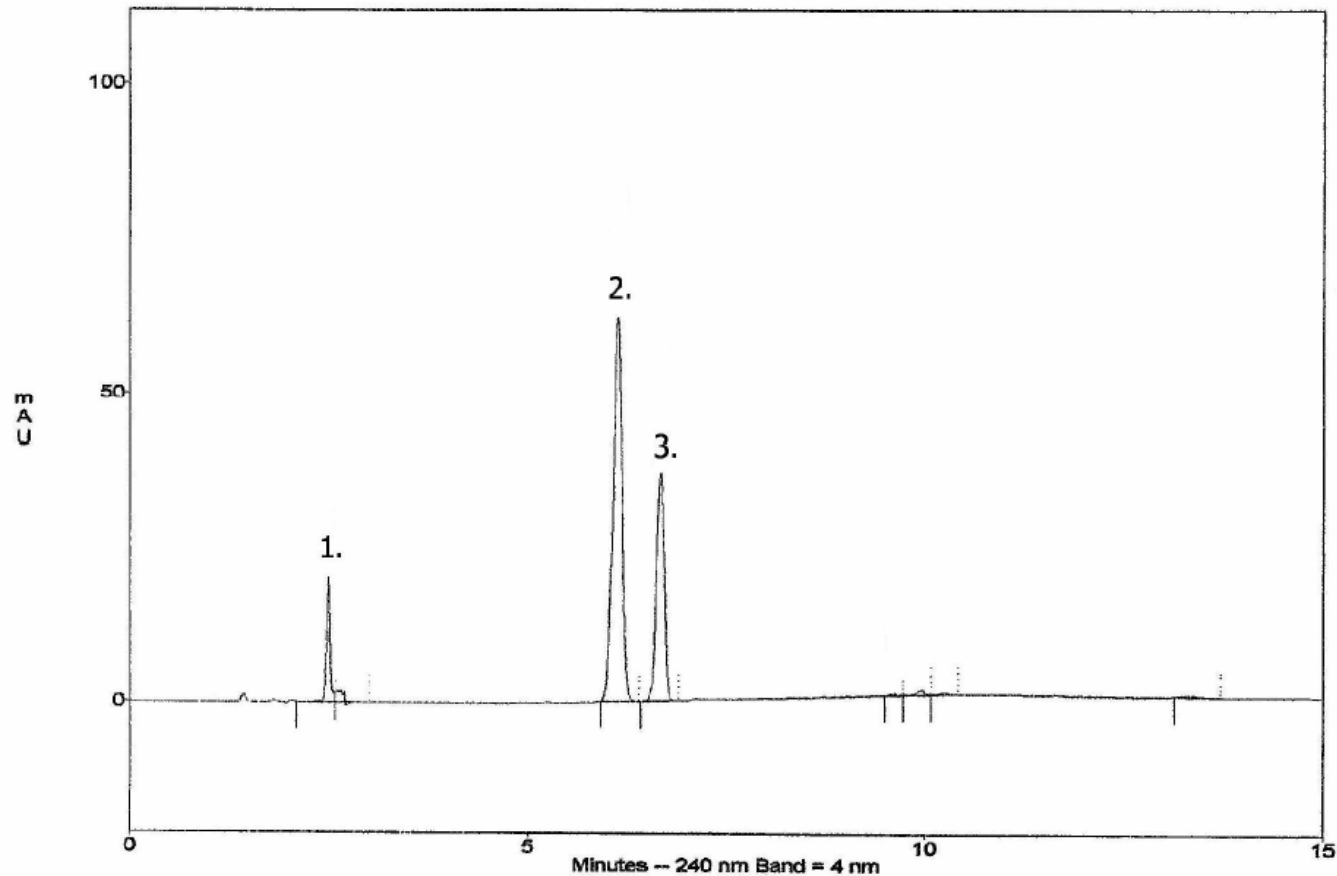
Results of SLV - Repeatability

Material	Result (mg/g)	SD	% RSD
Raw Material	923.6	14.8	1.6
Hard-Shell Capsules	740.4	34.9	4.7
Chewables	21.70	1.00	4.6
CS + Glucosamine Tablets	202.6	9.2	4.5
CS + Glucosamine Softgels	210.9	9.4	4.5

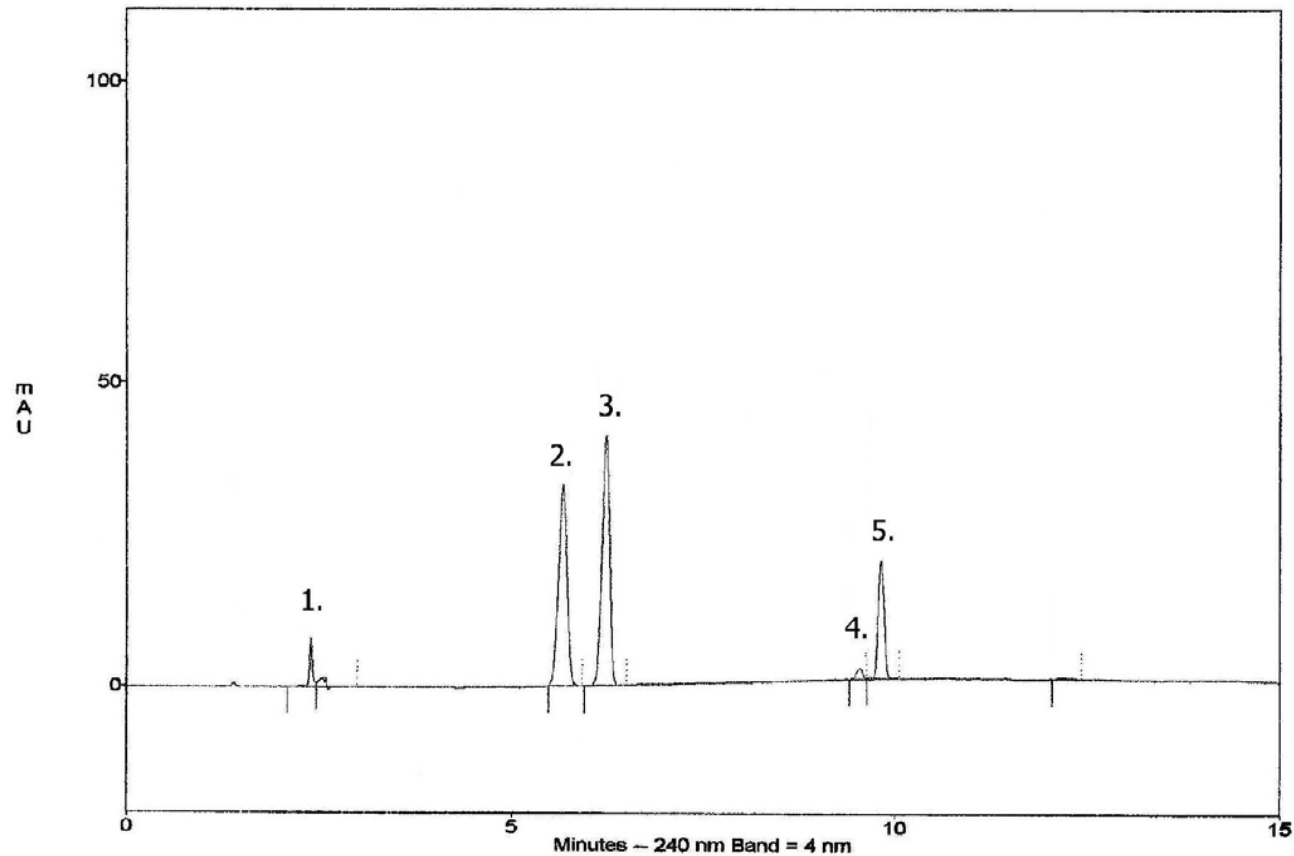
Results of SLV - Accuracy

	Level 1 % Recovery	Level 2 % Recovery	Level 3 % Recovery
FPDF Negative Control	105.6	105.4	105.8
Heparin Negative Control	101.6	101.1	100.8

Chromatographic Profiles - Bovine



Chromatographic Profile - Shark



Current Method Status

- SLV Manuscript has been accepted for publication in J AOAC.
- Revised AOAC Collaborative study protocol has been submitted to AOAC.
- 17 Laboratories have agreed to participate in collaborative study.

Current Method Status

- Reference Standard Solutions are being prepared by Cerilliant.
 - Powdered standards are being sourced from Dextra and Sigma (50 mg each).
 - Cerilliant will first characterize powdered standards for purity determination.
 - Standard Solutions will be prepared at concentrations specified in method.

Timeline

- Sample sent out to collaborative laboratories by mid-April.
- Results received back from laboratories within 30 days (mid- May).
- Draft collaborative study manuscript submitted to J AOAC by mid- June.
- Final collaborative study manuscript submitted by end of July.
- AOAC First Action Approval by end of August.