STAKEHOLDER PANEL ON DIETARY SUPPLEMENTS

Working Group on Chondroitin Sulfate
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Gaithersburg, MD USA

• Uses
  – Joint health
  – May help treat symptoms of osteoarthritis*
  – Products may contain CS alone or in combination with glucosamine, MSM, and/or SAM-e

*This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure or prevent any disease.
• Chondroitin Sulfate Chemistry
  – negatively charged polymeric glycosaminoglycan (GAG)
  – alternating uronic acid and N-acetylhexosamine residues connected by β1-3 hexuronidic and β1-4-N-acetylhexosaminidic bonds

  \[
  \begin{align*}
  \text{R1, R2, R3} &= \text{SO}_3^- \text{ or H}
  \end{align*}
  \]

• Chondroitin Sulfate Chemistry
  – Structurally similar to other GAGs including:
    • Dermatan sulfate, heparin, heparan sulfate, keratan sulfate
  – Similar chemical properties to other anionic polymers
    • Sodium alginate, carrageenan
  – MW range of 40 – 500 kD
    • Special hydrolysis procedures can create “low MW” CS (4 – 10 kD)
• Chondroitin Sulfate Sources
  – Bovine trachea, porcine rib cartilage, shark & avian cartilage.
  – Amount of sulfation and sulfation positions can vary between source species.
    • Chondroitin sulfate “A” sulfated at 4- position
    • Chondroitin sulfate “C” sulfated at 6- position
    • Chondroitin sulfates “D” and “E” are disulfated
    • “Chondroitin sulfate B” now recognized as dermatan sulfate and not actually chondroitin sulfate

• Chondroitin Sulfate Products
  – One of the most popular supplement ingredients
  – Used in supplements to support healthy joints
  – Also used in veterinary products for joint health
  – May be present as single ingredient or in combination with ingredients such as glucosamine, MSM and/or SAM-e.
**Chondroitin Sulfate Products**

- Popularity of ingredient, limited sources, and challenges of analytical testing make CS a prime candidate for economic adulteration.
- Manufacturers often perform inadequate testing of CS raw materials to ensure identity, purity, and/or assay.
- Adulterants so far found masquerading as CS:
  - Carrageenan
  - Alginates
  - Dermatan Sulfate
  - Proteins
  - Sodium Hexametaphosphate

**Industry Needs for Chondroitin Sulfate**

- Tests for Identity, Purity, Assay
  - Methods to verify the identity of the bulk material is chondroitin sulfate
  - Methods to demonstrate that material does not contain economic adulterants
  - Methods to quantify the amount of chondroitin sulfate in the material (possibly in the presence of economic adulterants)
• Identity Methods for Chondroitin Sulfate
  – FTIR
    • polymeric nature of CS yields broad bands
    • similarity to other GAGs
    • Limited utility
  – Optical Rotation (Specific Rotation)
    • CS is optically active and has characteristic specific rotation
    • [α]=−20.0° to −30.0° (c=30 mg/mL)
    • Not necessarily unique to CS

• Identity Methods for CS
  – Enzymatic HPLC
    • CS is selectively digested by chondroitinase AC enzyme into unsaturated disaccharides.
    • Unsaturated disaccharides can be quantified by HPLC.
    • Related GAGs do not interfere with test.
    • Problems of availability for disaccharide standards (esp. Δdi-6S) and enzyme.
Enzymatic-HPLC Analysis of Bovine CS

1 = Δdi-0S
2 = Δdi-4S
3 = Δdi-6S

- Other potential identification techniques
  - Electrophoresis
    - Paper electrophoresis migration factor
  - NMR
    - Ability to distinguish oversulfated CS from heparin
• Identity Standard Method Performance Requirements
  – Must be able to positively identify material as CS when material contains at least 90% CS with 95% confidence.
  – Must be able to distinguish CS from related GAGs, proteins, alginates, carrageenan, and polyphosphates.
  – May be combination of two or more techniques.

• Purity Methods
  – Ability to identify/quantify impurities in CS raw materials.
  – Unintentional Adulterants
    • Heavy Metals
    • Microbiological
  – Intentional Adulterants
    • Related GAGs
    • Alginates
    • Carrageenan
    • Proteins
    • Polyphosphates
• Purity Methods
  – Paper Electrophoresis
    • USP37
      – Uses running buffer of barium acetate, pH 5.0
      – Cellulose acetate membrane
      – Running time of 2 hours
      – Stained with toluidine blue solution

• Purity of CS by Paper Electrophoresis

Lane 1 = CSRS
Lane 2 = 2% CSRS
Lane 3 = ASD
Lane 4 = Polyphosphate

Lane 1 = CS w/ 0.1% ASD
Lane 2 = CS w/ 0.3% ASD
Lane 3 = CS w/ 0.5% ASD
Lane 4 = CS w/ 0.8% ASD
• Purity by Paper Electrophoresis
  - Need to demonstrate selectivity to:
    • Carrageenan
    • Dermatan Sulfate and other GAGs
    • Proteins
  - Advantages
    • Can run multiple samples in parallel
    • Sensitive (can see at least some adulterants down to 0.1%)
    • Relatively inexpensive equipment
  - Disadvantages
    • Paper electrophoresis not a common technique
    • Some specialized expertise/training may be needed

• Other Purity Techniques
  - Folin-Ciocalteu colorimetric technique for total protein
    • Reacts with any phenolic compound
  - NMR?
• Purity Standard Method Performance Requirements
  – Must be able to detect known adulterants in CS down to a level 2%
    • Determine POD
  – Must be able to detect related GAGs, proteins, alginates, carrageenan, and polyphosphates.
  – May be combination of two or more techniques.

• Assay Methods for CS
  – Quantify amount of CS in the presence of other ingredients and potential adulterants
  – Most common techniques:
    • Cetylpyridinium Chloride (CPC) Titration
    • Enzymatic HPLC
  – Other Techniques:
    • Carbazole Reaction
    • Size Exclusion Chromatography
• **Enzymatic HPLC Method**
  - Single Laboratory Validation (SLV) published in J AOAC 90(3) 2007, 659-669.
    - HorRat values ranged from 0.79 – 2.25
    - Recoveries 100.8% - 105.8%
    - Specificity against carrageenan, dermatan sulfate, glucosamine demonstrated
    - Lack of inhibition from minerals such as zinc, calcium, magnesium, and manganese demonstrated
    - Method applicable to raw materials and finished products

• **Enzymatic HPLC Method**
  - Collaborative Study conducted in 2008
  - 13 Laboratories returned data on nine test materials
  - Method had higher variability than desired, especially for raw materials
  - Method variability however was comparable to similar methods, e.g. Fructans in Foods
• Enzymatic HPLC Method

- Modifications to method may improve performance
  - Increasing retention of Δdi-OS away from void peak (several labs did not achieve resolution)
    - USP method uses anion exchange chromatography
  - Performing reaction in 2- mL or 5- mL conical bottomed reaction vials to eliminate transfer step
  - For raw materials, dry sample in oven prior to weighing, and place desiccant in balance chamber

Table 3. Statistical Analysis of Blind Replicates – Repeatability and Reproducibility

<table>
<thead>
<tr>
<th>Material</th>
<th>Average mg/g S</th>
<th>RSD %</th>
<th>S_0 RSD %</th>
<th>HORRAT Ratio</th>
<th>Outlier Labs</th>
<th>Number of Labs Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>726</td>
<td>21.6</td>
<td>2.97</td>
<td>106</td>
<td>14.6</td>
<td>6.94</td>
</tr>
<tr>
<td>B</td>
<td>566</td>
<td>149</td>
<td>24.6</td>
<td>175</td>
<td>30.9</td>
<td>14.2</td>
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<tr>
<td>C</td>
<td>51.6</td>
<td>6.88</td>
<td>13.3</td>
<td>27.0</td>
<td>52.3</td>
<td>16.8</td>
</tr>
<tr>
<td>D</td>
<td>379</td>
<td>16.3</td>
<td>4.27</td>
<td>34.7</td>
<td>9.10</td>
<td>3.94</td>
</tr>
<tr>
<td>E</td>
<td>303</td>
<td>14.9</td>
<td>4.93</td>
<td>20.8</td>
<td>6.89</td>
<td>2.88</td>
</tr>
<tr>
<td>F</td>
<td>140</td>
<td>4.66</td>
<td>3.34</td>
<td>13.0</td>
<td>9.33</td>
<td>3.47</td>
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<td>G</td>
<td>15.3</td>
<td>1.04</td>
<td>6.84</td>
<td>2.53</td>
<td>16.7</td>
<td>4.44</td>
</tr>
<tr>
<td>H</td>
<td>36.6</td>
<td>5.33</td>
<td>14.6</td>
<td>5.33</td>
<td>14.6</td>
<td>4.44</td>
</tr>
</tbody>
</table>

A = Chondroitin sulfate raw material from bovine trachea
B = Chondroitin sulfate raw material from shark cartilage
C = Negative control low spike
D = Negative control high spike
E = Chondroitin sulfate/glucosamine HCl capsules
F = Chondroitin sulfate/glucosamine HCl tablets
G = Chewable tablets
H = Chondroitin sulfate/glucosamine HCl/methylsulfonylmethane tablets
• Enzymatic HPLC Method Drawbacks
  – Lack of availability of reference standards (Δdi-6S) has been out of stock at least ~6 months
    • Using USP Chondroitin Sulfate RS may address issues of availability and purity
  – Limited supply of enzyme
    • IBEX is currently only supplier
  – Expensive to run
  – Technically Challenging

• CPC Titration
  – CPC cation forms ion pair with large CS anion
  – Ion pair has poor solubility and causes turbidity
  – Amount of turbidity is proportional to amount of CS in solution and can be measure with optical sensor (phototrode)
  – Poor selectivity
    • Will react with any large polyanion
    • Can not be used as stand-alone method; must be used in conjunction with appropriate identity and purity tests.
• Other Quantitative Techniques
  – Carbazole Method
    • Hydrolyzes CS down to the individual sugar units (glucuronic acid and N-acetylglactosamine) with concentrated sulphuric acid
    • Glucuronic acid residues are then reacted with carbazole in presence of borate to form red complex
    • Will yield a positive result with any GAG, and even some salts such as NaCl, and hexose sugars
    • Can not be used as stand-alone method; must be used in conjunction with appropriate identity and purity tests.

• Other Quantitative Techniques
  – Size Exclusion Chromatography
    • Separation based on molecular weight
    • CS can be reacted with dimethylene blue to yield colored compound that can be detected with UV detector
    • Dimethylene blue reacts with any polyanionic polymer
    • Calibration difficult due to difference in MW distribution between samples/standards
    • Can not be used as stand-alone method
• Assay Standard Method Performance Requirements
  – Must “accurately quantify chondroitin sulfate A and chondroitin sulfate C in dietary supplements and in-process materials, as a neat product and in the presence of potential interferences including heparin, heparin sulfate, keratan sulfate, dermatan sulfate (i.e., chondroitin sulfate B), hyaluronic acid, carrageenin, glucosamine, MSM, and minerals such as calcium and manganese.”
  • From original ERP report

• Assay Standard Method Performance Requirements
  – Repeatability HorRat of 0.3 – 1.3
    • Is this reasonable?
  – Reproducibility HorRat of 0.5 – 2.0
    • Is this reasonable?
  – Recoveries: Follows AOAC recommended guidelines based on concentration
QUESTIONS/DISCUSSION