Analysis of Chondroitin in Supplements

Statutory Analysis Government Chemist Programme Ad Hoc Project 3

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Contents

1. Summary

This report covers the assessment of four selected, published, analytical methods that are recommended for the analysis of chondroitin in commercial supplements. The aim of this work was to find a suitable procedure that could be routinely used by Public Analysts (PA) as well as a referee method that could be used by LGC to appraise samples for which there might be disputed results.

The general findings of this project agree with previous studies carried out by LGC and a Public Analyst Laboratory that in most cases the content of chondroitin found by analysis is lower than the labelled value claimed by manufacturers. However, the work also highlights some industry wide problems that impinge upon accurate or reproducible analysis of chondroitin in commercial formulations. Firstly there is no consensus on which analytical method is the most appropriate for the analysis of chondroitin in the material. The work also highlights that for accurate analyses it is crucial to choose a reference material that is produced from the same animal source and has similar properties to the chondroitin used in each formulation.

It is suggested that there needs to be a broader based approach to this problem involving input from the supplement manufacturers as well as the reference houses that produce the chondroitin standard materials. The Association of Official Analytical Chemists has championed this approach in the USA. The AOAC are about to complete the single laboratory validation (SLV) of a new analytical procedure for chondroitin in supplements. It should be possible for LGC to share knowledge and to participate in the final multi-laboratory collaborative trial that will be used to prove their new analytical method.

2. Introduction

2.1 Background

Chondroitin is an essential component of connective tissues that plays an important role in the elasticity and function of articular cartilage. It has been used for the treatment of chronic diseases such as degenerative arthritis, cirrhosis and chronic photo damage. Supplements may also be formulated to contain other components such as glucosamine, vitamins, methyl sulfonyl methane (MSM) and inorganic salts, which the body can use either to synthesise chondroitin or to aid its inclusion into the cellular structure of cartilage and connective tissue.

In early 2005, LGC were notified by a PA that they had analysed 15 samples of chondroitin sulfate and found them all to be deficient of the declared amounts. The PA informed us that the Local Authority was intending to submit the majority if not all 15 referee samples to LGC for analysis under the Government Chemist function.

LGC carried out assay analyses on a set of commercial supplements that had been analysed by the Public Analyst. The results on tablet samples agreed well with the data supplied by the PA Laboratory. For liquid based samples the agreement was poor. LGC used the PA laboratory method throughout their analysis. It is therefore important for LGC to have validated methods for the determination of chondroitin sulfate.

2.1.1 Chondroitn Properties

Chondroitin is a polymeric sugar – an aminoglycan sulfate. The molecular weight of the material available from animal extracts normally falls in the range 5000 to 100 000 daltons. The molecular

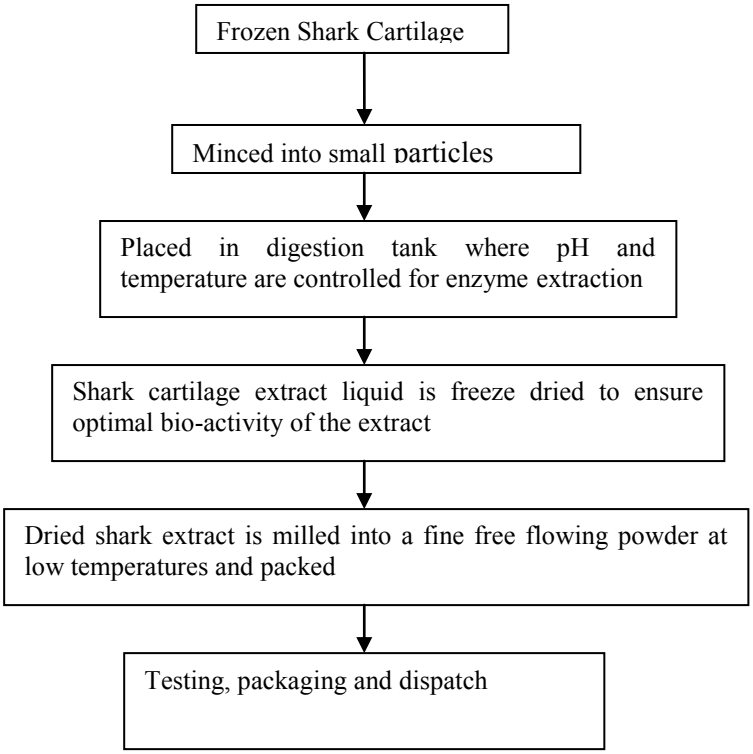
weight of the base material commonly used in supplements is at the lower end of the range, around 15000-20000 Daltons.

The three main types of chondroitin, A, B and C are characterised by the number and position of sulfate groups in the repeated disaccharide units of the polysaccharide chain. Types A and B are found in bovine and porcine land animals, whereas chondroitin C is available from fish and shark species. It is stressed that although, for example, chondroitin C reportedly contains mainly the 6S disaccharide, the material is by no means isomerically pure. All sources and species of land and sea animals contain, to a greater or lesser degree, amounts of the other isomers as well.

2.1.2 Preparation of Chondroitin Raw Materials and Standards

Chondroitin has not been synthesised so the raw materials for supplement formulation and reference standards are only available through the extraction of animal products.

The chondroitin in animal tissue is bound to proteins in the form of a proteoglycan. The initial processing of the tissue is done to free the polymeric material from the protein. The digestion conditions may also be harnessed to reduce the average molecular weight of the product. The following flow chart illustrates how shark material for chondroitin C is processed and purified before it is supplied to the supplement manufacturers.



The detail and comments attached to this process suggests a final product of consistent physical properties and bioactivity, rather than a chondroitin standard material of known chemical purity.

Worldwide, cartilage is available from many species of shark for use in supplement formulations. Raw materials include the skull, backbone and fin of the shark. In the UK and Europe the most important source for chondroitin C is dogfish or rock salmon (*Squalus Acanthias*).

Limited information is available on the approach the manufacturers use for preparing reference standard materials of chondroitin. More attention is probably given to finding a consistent source

material, on the age, species and part of the animal sourced and on improved extraction and cleanup procedure to ensure a purer product.

3. Methods Studied

There are two main approaches to the analysis of chondroitin in supplement samples

- The intact polymer is extracted into water and is separated and analysed by virtue of its unique properties of high molecular weight and negative charge. This approach is used in the dye forming colorimetric methods, the cetylpyrridium titration, the Gel Permeation Size Exclusion (GPC-SEC) and the Exclusion HPLC procedures. Calibration of these methods is based on the use of commercial polymeric chondroitin A and C reference standard materials.
- Alternatively the polymer is enzyme hydrolysed to its base disaccharide units, which then are separated and analysed as low molecular weight, negatively charged molecules. Calibration of these methods can again be accomplished by using commercially available chondroitin A and C. These materials are subjected to the same enzyme treatment as the samples. Where disaccharides are available these can be used for quantitative analyses. However, most of these materials are sold without any indication of purity. Ion pair, strong ion exchange and amine column chromatographic methods form the basis of the end analysis for these procedures.

3.1 Samples and Standards Used in The Methods

- Table 1 in the Annex lists the samples used in the initial work carried out by LGC. In the coding of samples CSP refers to commercial supplement product
- Table 2 in the Annex lists the samples that were analysed by the four analytical methods in 2006.
- Section 6.3 lists the standard materials used the analysis of chondroitin supplements.

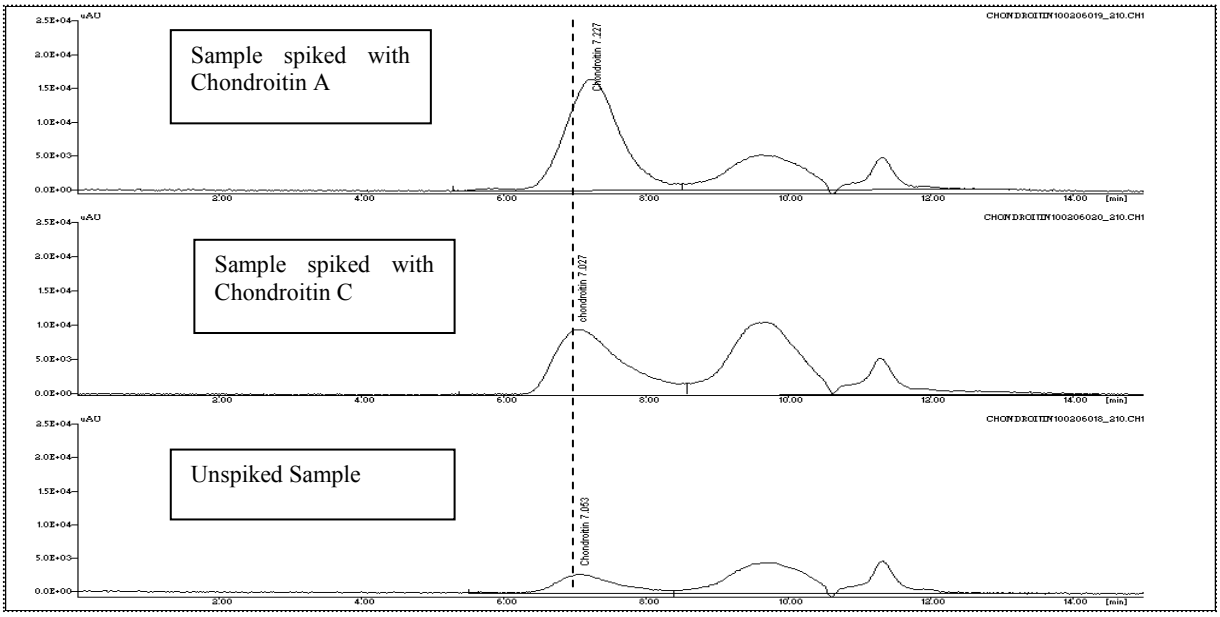
3.2 Gel Permeation Size Exclusion Chromatography

The method of Choi[1] et al was tested against the set of samples containing chondroitin

The principle of this approach is that chondroitin is the highest molecular weight (MW) water soluble component in each supplement and it will be excluded from a low MW gel column as a moderately sharp peak with the shortest retention time. The other components, due to their smaller size, are able to permeate the pores of the gel particles and so take longer times to reach the detector. In the classic GPC method the molecular weight of components is inversely proportional to their retention times of their peaks.

It is clear from the chromatograms of a spiked and unspiked sample in Figure 1 that the chondroitin peak (closest to the hashed line) is very broad and not totally separated from other matrix components. The sample was declared to contain shark chondroitin. This is supported by the information in the middle chromatogram where the shape (slightly tailing) and retention time of the chondroitin peak is retained after spiking with chondroitin C. A chromatogram of the chondroitin C spike alone was found to contain more than one peak. This is not consistent with the purity information supplied by the manufacturer. When the same concentration of chondroitin A is used for spiking the sample, the resultant chromatogram contains a more intense and more symmetrical chondroitin peak. This peak is not centred at the same retention time as in the unspiked trace, indicating that there is a difference in molecular weight between the chondroitin A and the shark chondroitin. Results obtained for spiked samples indicate excellent recovery for both chondroitin A and chondroitin C.

Figure 1: Chromatograms of Spiked and Unspiked sample

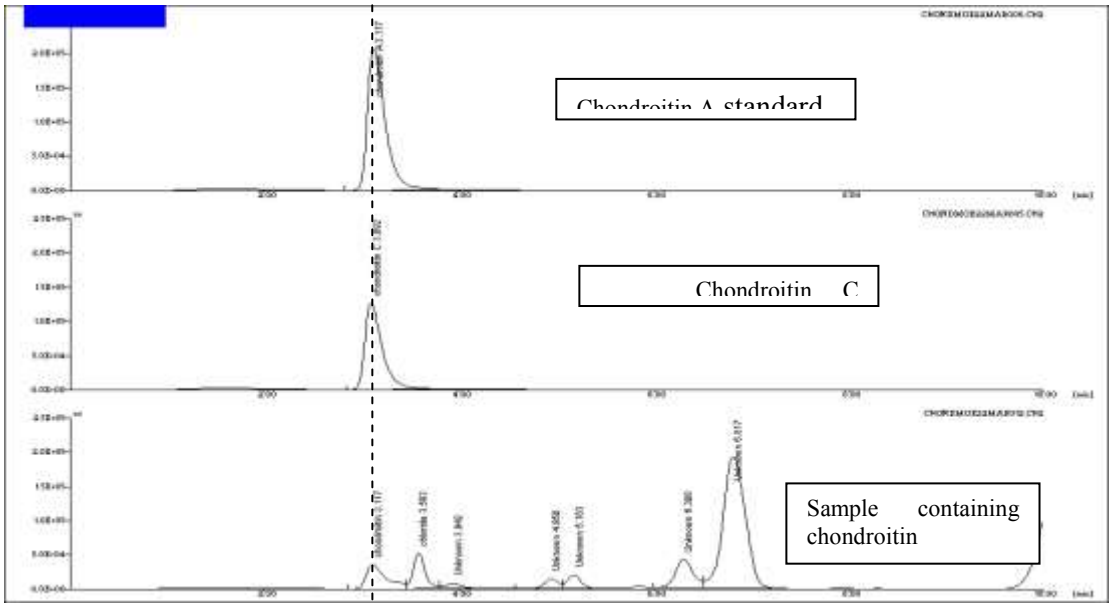


All these findings, together with the difficulty we experienced in repeating some of the analyses on the samples and standards, suggest that using GPSEC as a quantitative method for the determination of chondroitin in supplements is not wholly satisfactory.

3.3 Exclusion HPLC

This method by Tyler[2] is proposed for the analysis of raw materials and tablets. In this procedure a C18 column is conditioned with a surface negative charge. Chondroitin because of its large molecular size and negative charge is excluded from the column as a peak eluting close to the injection point, before the solvent peaks. Smaller sized ions and neutral molecules elute with longer retention times compared to chondroitin.

Figure 2: Exclusion HPLC Chromatograms of Chondroitin Samples and Standards



The traces in Figure 2 show the chondroitin peak as a well shaped peak at the shortest retention time in each chromatogram. Chondroitin A and C elute at the same position. Chondroitin A, at the same concentration as chondroitin C, gives a larger response on the UV detector. The chondroitin peak in the sample is well resolved from the nearest component in the chromatogram. According to the literature the adjacent peak in the chromatogram is due to chloride ions. Because the concentration of chloride in the product can often be calculated from other declarations on each supplement, it could serve as a form of internal standard in second-checking the chondroitin assay results.

There are disadvantages in adopting this system of analysis. Because the chondroitin analyte is excluded by the column its position and retention time are not governed by the normal chromatographic parameters e.g. mobile phase composition, column type and column temperature. Further, the exclusion mode is difficult to set up and is obtained only after conditioning the column for several hours with mobile phase prior to use. Results for the determination of chondroitin content in samples are shown in Table 3.

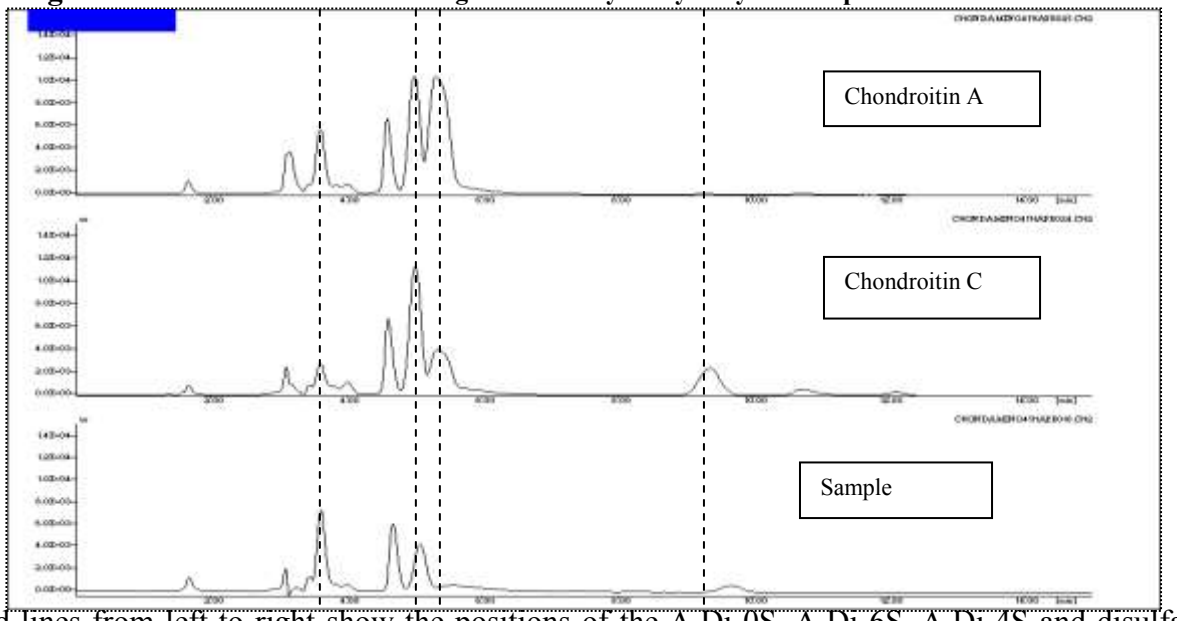
3.4 Enzyme Hydrolysis and Amine Column HPLC

Chondroitin is converted to its base disaccharides by enzymes such as Chondroitinase ABC. There are 7 possible disaccharides that can be produced from the hydrolysis of the chondroitin types. Each disaccharide is characterised by the number and positions of sulfated groups in the sugar molecule. However, because the Δ Di-4S and Δ Di-6S (sulfated in the 4 or 6 ring position respectively) mono-sulfated disaccharides are known to be the most abundant of the isomers present, their peak areas alone are normally used in any quantitative calculations.

To remove potential interferences from materials such as gelatine and other proteins the samples were separated from their capsules before the enzyme hydrolysis stage was reached.

When the methodology offered by the Public Analyst[5] was applied to disaccharide standards run on a new amine column, it was found that each analyte peak was split into two or sometimes three components and that retention times were much longer than predicted. To overcome these problems the mobile phase was replaced by that recommended by Baker et al[3].

Figure 3 –Amine Column Chromatograms of Enzyme hydrolysed Samples and Standards



Dotted lines from left to right show the positions of the Δ Di-0S, Δ Di-6S, Δ Di-4S and disulfate disaccharides respectively.

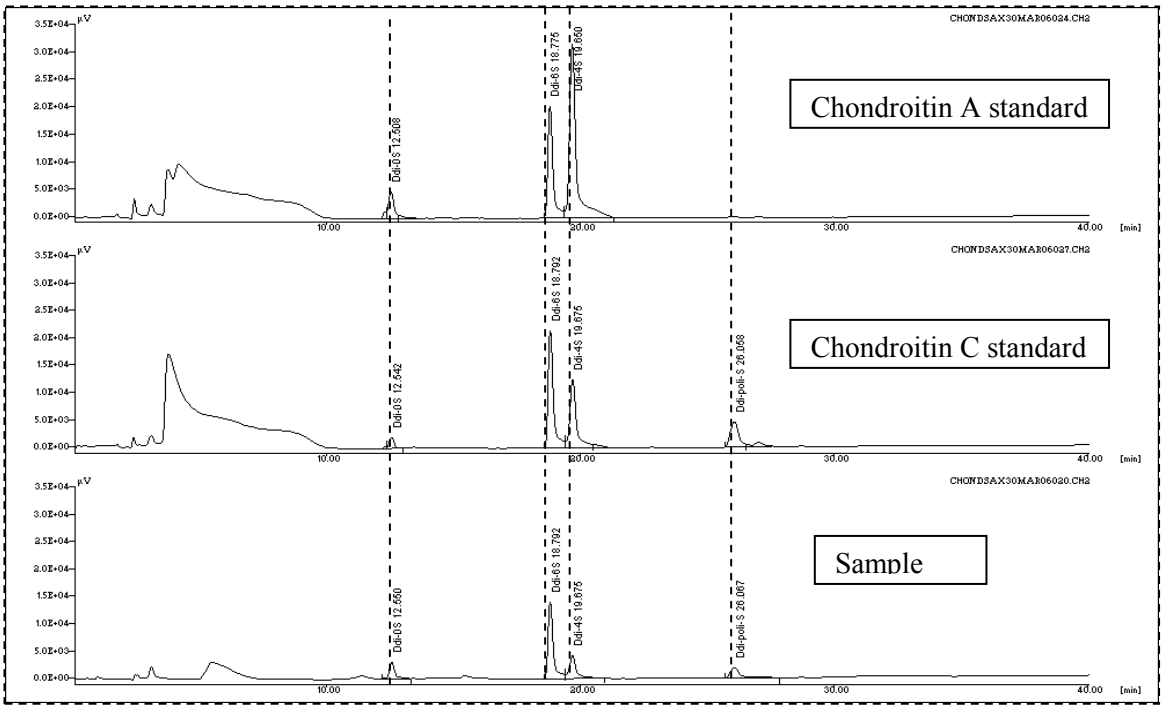
The chromatograms show that for chondroitin A the Δ Di-4S isomer is more abundant than the Δ Di-6S isomer. In contrast, for the sample containing shark chondroitin or for the chondroitin C standard, the Δ Di-6S isomer predominates with also a large peak for the di-sulfated disaccharide, Δ Di-2,6 Di. The chromatograms all show a significant peak at the position of the Δ Di-0S disaccharide. This peak occurs close to the solvent and is more likely to originate from an interfering compound rather than the disaccharide. It can also be noted that the retention times of the main components shorten throughout the analysis run indicating that the performance of the amine column is slowly deteriorating. The peak shape of the ΔDi-4S isomer is fairly broad, consequently the separation from the adjacent Δ Di-6S peak is still not as good as reported by Baker et al. Results for the samples using this method are shown in Table 4.

3.5 Enzyme Hydrolysis and Strong Ion Exchange HPLC

The sample treatment and extraction is exactly the same as for the amine column method. Extracts prepared for the amine column method were run on the SAX HPLC.

This method[4] differs from the amine column procedure in that anion analytes are eluted from the SAX columns using a gradient of chloride ions. Because the interaction of the anionic chondroitin is stronger with the SAX column than with the amine column retention times for the disaccharides are much longer.

Figure 4: SAX HPLC Chromatograms of Sample and Standards



The dotted lines show from left to right the positions of the Δ Di-0S, Δ Di-6S, ΔDi-4S and Δ Di-2,6 Di disaccharides respectively.

The chromatograms show better separation and excellent definition for the disaccharide peaks compared to the amine column method. The Δ Di-0S peak is small compared to the other isomers.

The chondroitin content found in samples by using this method is shown in Table 5.

4. Results

The following tables show the results of analysis. Brand names have not been included as it was not the primary aim of this work to survey the market and no systematic attempt was made to do so.

Table 3: Sample Content by the Exclusion HPLC Method

Code	Analysis1+ Content (milligrams)	Analysis1 # Content (milligrams)	Analysis 2 Content (milligrams)	Declared amount (milligrams)
CSP1	175	193	154	**300**
CSP2	604	734	619	**400**
CSP3	282	328	265	**400**
CSP4	317	401	330	**400**
CSP5	-----	-----	-----	**100**
CSP6	152	421	358	**200**
CSP7	136	226	187	**200**
CSP8	122	202	165	**200**

+ based on chondroitin C calibration at the beginning of analysis sequence 1

based on chondroitin C calibration at the end of the analysis sequence 1

Table 4: Sample Content by the Amine Column Method

Code	Analysis 1 Content= (milligrams)	Analysis 1 Content * (milligrams)	Declared amount (milligrams)
CSP1	189	168	**300**
CSP2	611	500	**400**
CSP3	260	235	**400**
CSP4	441	419	**400**
CSP5	----	-----	**100**
CSP6	341	278	**200**
CSP7	186	181	**200**
CSP8	165	161	**200**

= calculated against chondroitin C standards - based on disaccharides Δ Di-4S and Δ Di-6S

* calculated against chondroitin C standards - based on Δ Di-4S, Δ Di-6S and Δ Di-2,6diS

Table 5: Disaccharide Ratios-Amine Column Method

Code	Ratio Δ Di-4S/ΔDi-6S	Ratio Δ Di-4S/Δ Di-2,6 DiS
CSP1	1.3	5.8
CSP2	0.9	∞
CSP3	1.4	4.6
CSP4	0.2	0.7
CSP5	6.1	∞
CSP6	1.9	∞
CSP7	0.5	1.3
CSP8	0.1	0.2
Chondroitin C Standard#	0.5	1.1
Chondroitin A Standard	1.7	∞
Chondroitin B Standard	19.5	∞

#average of five chondroitin C standards

Table 6: Sample Results by the SAX HPLC Method

Code	Analysis 1 Content (milligrams)	Analysis 2 Content (milligrams)	Declared amount (milligrams)
CSP1	----	129	**300**
CSP2	649	568	**400**
CSP3	240	221	**400**
CSP4	411	381	**400**
CSP5	----	-----	**100**
CSP6	321	270	**200**
CSP7	174	182	**200**
CSP8	237	193	**200**

5. Conclusions

Agreement between the chondroitin content of the samples found by the three methods is quite reasonable, considering that different analytes are being measured in two of the three cases. The GPSEC method was deemed to be unsuitable for quantitative analysis.

In contrast the analytical results are generally lower than the declared or label content of the samples. The data in the tables was calculated by comparison to a set chondroitin C standards. Because the chondroitin A standards, at the same concentration, always gave a larger response than their chondroitin C equivalents, results up to 30% lower could be expected with this alternative calibrant. It is possible that the CSP2 & CSP6 supplements, from the same supplier, were formulated on the basis of chondroitin A. If this was true, the calculated concentrations for chondroitin in the samples would be much more in line with the content declared by the manufacturer.

Only the CSP7 product had similar disaccharide ratios to the chondroitin C standard used even though four of the products were reported to contain chondroitin from a fish or shark source. For this material the LGC results are close to the label value of 200 mg/capsule.

The general conclusions are that for accurate analyses, irrespective of the method being used, the source of the chondroitin must be known and a suitable standard material similar in molecular weight, purity and disaccharide ratio to this must be available. Because the disaccharide ratio is known to be dependent on the source species, the age and condition of the animal and that the molecular weight is affected by the extraction procedure, the chance of matching reference standard and sample chondroitin for a routine assay analysis is low.

As mentioned earlier, methods involving enzyme hydrolysis can be calibrated against disaccharide standards. However, there is little agreement on which or how the disaccharides standards should be used in quantitative calculations.

Without this information or the appropriate standards it is not possible to validate the analytical methods or propose them to Public Analysts for use on the determination of chondroitin in supplements.

It is suggested that there needs to be a broader based approach to this problem involving input from the supplement manufacturers as well as the references houses that produce the chondroitin standard materials. The Association of Official Analytical Chemists has championed this approach in the USA. The AOAC are about to complete the single laboratory validation (SLV) of a new analytical procedure for chondroitin in supplements. It should be possible for LGC to share knowledge and to participate in the final multi-laboratory collaborative trial that will be used to prove their new analytical method.

6. Appendix

6.1 Samples of supplements Containing Chondroitin

The samples from the previous study were available for analysis. Out of these the following were used for the early GPSEC qualitative tests only.

Table 1: Old Samples Analysed Qualitatively By GPSEC

Supplement Code	Form	Claimed Content of Chondroitin sulfate (milligram)	Source of Chondroitin
CSP A	Dry Capsules	400	shark
CSP B	Tablets	400	fish
CSP C	Tablets	400	unknown
CSP D	Wet Capsules	250	unknown
CSP E	Tablets	400	unknown
CSP F	Wet Capsules	200	unknown
CSP G	Dry Capsules	0	not applicable

For the quantitative tests and analysis, fresh chondroitin supplements were purchased.

These materials are described in Table 2

6.2 Samples used in Method Evaluation

Fresh samples similar to the materials analysed previously were purchased at local supermarkets and health stores for the evaluation of the analytical methods.

Table2: New Samples Used In Quantitative Analyses

	Form	Declared Content per tablet or capsule Chondroitin sulfate (milligram)	Indicated source of Chondroitin	Average weight of tablet or content of capsule* (grams)
CSP 3	Tablets	400	shark	1.31
CSP 1	Tablets	300	shark	0.9
CSP 4	Dry Capsules	400	shark	0.97
CSP 2	Tablets	400	unknown	1.32
CSP 6	Wet Capsules	200	unknown	2.41
CSP 9	Dry Capsules	0	not applicable	0.87
CSP 5	Tablets	100	unknown	4.2
CSP 7	Wet Capsules	200	fish	1.0
CSP 8	Wet Capsules	200	unknown	1.14

* = average weight of capsule minus average weight of empty capsule

6.3 Chondroitin Standards used for quantitation

Chondroitin sulfate A Sigma Cat. No. C 9819 – Chondroitin sulfate A from bovine trachea. Approximately 70 %, the balance is Chondroitin sulfate C.

Chondroitin sulfate C, Sigma cat. no. C 4384 – Chondroitin C from shark cartilage. Approximately 90% with the balance Chondroitin sulfate B

Chondroitin sulfate A, CPC standard, Sigma cat. no. C 6737 - Chondroitin sulfate from bovine cartilage (for cetylpyridinium chloride titration) min 99% by CPC titration.

Chondroitin Δdi-4S disaccharide sodium salt, Sigma cat. no. C4045 – no stated purity.

Chondroitin Δdi-6S disaccharide sodium salt, Sigma cat. no. C 5945 – no stated purity.

Chondroitin Δdi-0S disaccharide sodium salt, Sigma cat. no. C 3920 –no stated purity.

7. References

1) A Size-exclusion Method for the Determination of Sodium Chondroitin Sulfate in Pharmaceutical Preparations. Choi et al J Pharm Biomed Anal. 2003 Apr 10;31(6):1229-36.

2) Determination of Chondroitin Sulfate in Raw Materials by Liquid Chromatography. Tyler et al J-AOAC-Int May Jun 2002; 85(3): 567-571

3) An Unsulphated Region of Rat Chondrosarcoma Chondroitin Sulfate Chain and its Binding to Monoclonal Antibody 3B3. Baker et al Biochem. J. (1991) 273, 237-239.

4) Quantitative Analysis of Chondroitin Sulfate in raw Materials, Ophthalmic Solutions, Soft capsules and Liquid Preparations Sim et al Journal of Chromatography B, 818 (2005) 133-139.

5) Chondroitin Method – Private communication with Somerset PA Laboratory

6) Chemical Selection Working Group report on chondroitin use in supplements. ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/chondroitin.pdf