Accepted Manuscript

A multi-analytical approach to better assess the keratan sulfate contamination in animal origin chondroitin sulfate

Odile Francesca Restaino, Rosario Finamore, Paola Diana, Mariacarmela Marseglia, Mario Vitiello, Angela Casillo, Emiliano Bedini, Michelangelo Parrilli, Maria Michela Corsaro, Marco Trifuoggi, Mario De Rosa, Chiara Schiraldi

PII: S0003-2670(16)31445-3

DOI: 10.1016/j.aca.2016.12.005

Reference: ACA 234949

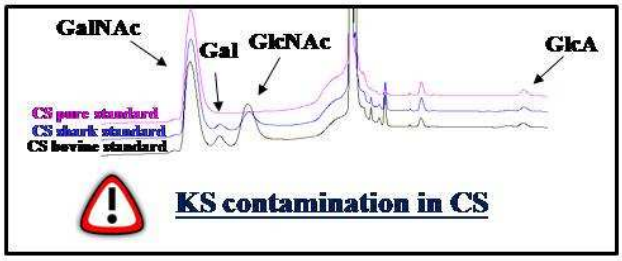
To appear in: *Analytica Chimica Acta*

Received Date: 30 July 2016

Revised Date: 22 November 2016

Accepted Date: 3 December 2016

Please cite this article as: O.F. Restaino, R. Finamore, P. Diana, M. Marseglia, M. Vitiello, A. Casillo, E. Bedini, M. Parrilli, M.M. Corsaro, M. Trifuoggi, M. De Rosa, C. Schiraldi, A multi-analytical approach to better assess the keratan sulfate contamination in animal origin chondroitin sulfate, *Analytica Chimica Acta* (2017), doi: 10.1016/j.aca.2016.12.005.



A multi-analytical approach to better assess the keratan sulfate contamination in animal origin chondroitin sulfate

Odile Francesca Restaino[1], Rosario Finamore[1], Paola Diana[1], Mariacarmela Marseglia[1], Mario Vitiello[1], Angela Casillo[2], Emiliano Bedini[2], Michelangelo Parrilli[3], Maria Michela Corsaro[2], Marco Trifuoggi[2], Mario De Rosa[1] and Chiara Schiraldi[1§].

[1]Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, Second University of Naples, Via De Crecchio 7, 80138, Naples, Italy.

[2]Department of Chemical Sciences, University of Naples "Federico II", Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Naples, Italy.

[3]Department of Biology, University of Naples "Federico II", Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Naples, Italy.

[§] Corresponding authors: Prof. C. Schiraldi; Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, Second University of Naples, Via De Crecchio 7, 80138, Naples, Italy. E-mail: chiara.schiraldi@unina2.it. Phone: +39815667679. Fax: +39815667546.

Dr. O.F. Restaino E-mail: odilefrancesca.restaino@unina2.it

R. Finamore E-mail: rosario.finamore@unina2.it

P. Diana E-mail: paola.diana@unina2.it

Dr. M. Marseglia E-mail: marimars84@hotmail.it

M. Vitiello E-mail: mariovitiello.ita@gmail.com

Dr. A. Casillo E-mail: angela.casillo@unina.it

Dr. E. Bedini E-mail: emiliano.bedini@unina.it

Prof. M.M. Corsaro E-mail: mariamichela.corsaro@unina.it

Prof. M. Parrilli E-mail: michelangelo.parrilli@unina.it

Dr. M. Trifuoggi E. mail: marco.trifuoggi@unina.it

Prof. M. De Rosa E-mail: mario.derosa@unina2.it

Prof. C. Schiraldi E-mail: chiara.schiraldi@unina2.it.

Abstract

Chondroitin sulfate is a glycosaminoglycan widely used as active principle of anti-osteoarthritis drugs and nutraceuticals, manufactured by extraction from animal cartilaginous tissues. During the manufacturing procedures, another glycosaminoglycan, the keratan sulfate, might be contemporarily withdrawn, thus eventually constituting a contaminant difficult to be determined because of its structural similarity. Considering the strict regulatory rules on the pureness of pharmaceutical grade chondrotin sulfate there is an urgent need and interest to determine the residual keratan sulfate with specific, sensitive and reliable methods. To pursue this aim, in this paper, for the first time, we set up a multi-analytical and preparative approach based on: i) a newly developed method by high performance anion-exchange chromatography with pulsed amperometric detection, ii) gas chromatography-mass spectrometry analyses, iii) size exclusion chromatography analyses coupled with triple detector array module and on iv) strong anion exchange chromatography separation. Varied KS percentages, in the range from 0.1 to 19.0% (w/w), were determined in seven pharmacopeia and commercial standards and nine commercial samples of different animal origin and manufacturers. Strong anion exchange chromatography profiles of the samples showed three or four different peaks. These peaks analysed by high performance anion-exchange with pulsed amperometric detection and size exclusion chromatography with triple detector array, ion chromatography and by mono- or two-dimensional nuclear magnetic resonance revealed an heterogeneous composition of both glycosaminoglycans in terms of sulfation grade and molecular weight. High molecular weight species (> 100 KDa) were also present in the samples that counted for chains still partially linked to a proteoglycan core.

Keywords: Chondroitin sulfate, keratan sulfate, GC-MS, HPAE-PAD, pharmacopeia methods, SEC-TDA.

1. Introduction

Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) present in the extracellular matrix of the animal cartilaginous tissues as a proteoglycan, in which the polysaccharide chain is covalently linked to a protein core. CS has a 4)-β-GlcA-(1→3)-β-GalNAc-(1→ disaccharide repeating unit (GlcA=glucuronic acid, GalNAc=*N*-acetyl-galactosamine) with a variable sulfation pattern 1-4]; within a single chain the type and the grade of sulfation could vary, and non-sulfated disaccharides (CS-0 type disaccharide) coexist with monosulfated (CS-A and CS-C types with one sulfate group at *O*-4 or *O*-6 position of the aminosugar, respectively) and disulfated units (the most common ones are CS-E displaying a 4,6-di-*O*-sulfated GalNAc, as well as CS-B and CS-D with sulfation at position *O*-2 of GlcA and at position *O*-4 or *O*-6 of the aminosugar, respectively) [5]. The relative percentages of these different sulfation patterns as well as the chain molecular weight vary according to the species, the organ and the tissues of origin. CS from bovine, pig and chicken cartilage has higher percentages of CS-A type disaccharides and lower molecular weights (14-26 KDa) compared to CS samples from marine organisms that shows higher percentage of CS-C and di-sulfated disaccharides and higher molecular weight values (30-70 KDa) [2, 4]. It has been widely demonstrated that CS is a symptomatic slow acting molecule and a structure/disease modifying anti-osteoarthritis drug (S/DMOAD) that shows a good anti-inflammatory pharmacological activity, comparable to the non-steroidal anti-inflammatory molecules. It has also a chondro-protective effect by inducing cellular growth at both bone and cartilage levels [6-9]. Beside the long tradition of using CS as the active principle of anti-osteoarthritis drugs, CS has been recently assessed as an active component in tissue engineering scaffolds too [10-13]. More diffused, especially in the USA, are the CS-based nutraceutical formulations and dietary supplements, even in veterinary field [4, 14]. CS has been traditionally formulated alone or in mixture with glucosamine for oral administration, but formulations with hyaluronic acid for intra-articular injections have been also developed in the last decades [9; 15-19]. Nowadays, CS is mainly obtained by extraction and purification from the animal cartilaginous tissues of shark fins, bovine and chicken trachea or pig muzzle for a total world market, as raw material, of about 600 metric tons per year and a price ranging from 65 to 150 $ per kg, according to the purity grade [4]. The extracted CS for pharmaceutical applications has to be highly pure in order to satisfy the legislation constraints and it has also to fit precise structural characteristics, in terms of disaccharide compositions, sulfation patterns and molecular weight distribution to obtain the desired biological and pharmaceutical action. Indeed, differences reported in the therapeutic efficiency of CS, during clinical trials, might be attributed either to the variations of the polysaccharide structure or to the presence of contaminants [20, 21]. Furthermore, a too high sulfation level in CS can induce a strong allergic response [22], as proved by the 574 serious adverse events, including 149 deaths in USA and Europe, that have been associated to the contamination of some heparin lots with per-*O*-sulfated chondroitin [23, 24]. To

solve the issue of the high heterogeneity of the CS structure, in the last years some semi-synthetic approaches have used chemical sulfation processes to obtain structural tailored-cut CS from microbial sourced unsulfated chondroitin [25-32]. Diverse analytical methods are reported in literature for the identification of the type and origin of CS and to determine its purity grade. The European Pharmacopeia testing monograph suggests methods based on infrared absorption spectrometry, specific optical rotation, intrinsic viscosity analyses and by visual or photometric titration with cetylpyridinium chloride (CPC) to identify and determine the concentration of CS in drug samples [33]; in order to establish the different animal origin of the samples the United States Pharmacopeia added to these methods also an identification based on the disaccharide composition and sulfation pattern, performed after enzymatic digestion of the polysaccharide chain [34]. It is recommended to determine the CS purity by agarose gel electrophoresis or by cellulose acetate membrane analyses [33, 34]. Testing monographs consider chloride and sulfate ions, proteins and heavy metals as possible impurities of CS [33, 34], but recent studies have demonstrated that keratan sulfate (KS) constitutes the main residual glycosaminoglycan contaminant of pharmaceutical grade and commercial CS products. KS is present in the animal articular cartilaginous tissues as well, and thus it might be contemporarily extracted with CS and then not completely removed in the purification procedures [21, 35, 36]. KS has a polysaccharide structure similar to CS, with a 4)-β-GlcNAc-(1→3)-β-Gal-(1→ disaccharide repeating unit (GlcNAc=*N*-acetyl-glucosamine; Gal=galactose), mainly sulfated at the *O*-6 position of both sugars. It generally shows a molecular weight distribution ranging from 10 to 40 KDa [35, 37]. Although the limit of total contaminants in CS pharmaceutical grade formulations should be lower than 5% (w/w), according to the legislation constraints of different countries [33, 36], so far, no specific methods have been reported yet in either European or United States pharmacopeia testing monographs to precisely determine the residual KS [33, 34]. In literature, instead, recent papers have tried to estimate the relative percentages of CS and KS by using different methods. Agarose gel electrophoresis analyses resulted not successful in separating the two GAGs because they co-migrated [36], differently from what was reported for the separation of chondroitin sulfate, dermatan sulfate and heparan sulfate [2]. High performance size exclusion liquid chromatography resulted very useful to distinguish between CS of different animal origins but not in detecting KS in CS samples [36]. Strong anion exchange high performance liquid chromatography (SAX-HPLC) was more successful in determining an averaged 16% KS contamination in shark origin CS batches and a lower than 1% contamination in bovine CS samples by integration of the obtained CS and KS peak areas [36]. In addition, in that paper, the peaks of the two GAGs, obtained by SAX-HPLC, were then further characterised by carbazole assays, by ^{1}H, ^{13}C and ^{1}H, ^{13}C heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy and by agarose gel electrophoresis after enzymatic digestion with chondroitin AC lyase or keratanase. Nonetheless it is worth to note that a third peak, present at low retention times in many chromatograms, remained unknown [36]. In an another paper, instead, quantification of KS in CS samples was performed

by immnunoblotting analyses that revealed only an approximate 1% of KS contamination, probably due to the lower sensitivity of the method [21; 38]. Considering 2% a sound quality parameter as a reasonable limit of KS contamination in CS samples, the necessity to develop highly sensitive and reliable methods to determine it are mandatory. In this study we aimed to set up a new multi-analytical approach to determine the KS contamination in CS samples. First a new high performance anion exchange chromatography method with pulsed amperometry detection (HPAE-PAD) was set up to detect KS in CS samples on the basis of the monosaccharide composition, after acid hydrolysis. Seven standards (both pharmacopeia and commercial ones) and nine commercial samples from three different suppliers of both terrestrial and marine animal origin CS were screened by using this new method. Results were compared to gas chromatography-mass spectrometry (GC-MS) analyses of the monosaccharide composition as acetylated methyl glycosides. Size exclusion chromatography with triple detector array (SEC/TDA) was used to determine sample molecular weights. Strong anion exchange (SAX) chromatography profiles of some CS samples revealed the presence of different peaks (from three to four) whose composition was determined by HPAE-PAD, GC-MS, SEC/TDA chromatography, ion chromatography and mono- or two-dimensional NMR analyses.

2. Material and Methods

2.1 Materials

N-acetyl-galactosamine, *N*-acetyl-glucosamine, galactose and glucuronic acid used as standards for HPAE-PAD and GC-MS analyses were from Sigma-Aldrich (USA), as well as the sodium hydroxide used for SAX chromatography, the sodium nitrate used for the SEC/TDA buffer and the sodium acetate used for both HPAE-PAD analyses and SAX chromatography, the acetyl chloride and the anhydrous methanol used to prepare hydrogen chloride in methanol, the pyridine and the acetic anhydride used for GC-MS analyses and the deuterium oxide (99.9%) used for the NMR spectroscopy. The magnesium oxide and the sodium carbonate used to prepare the Eschka mixture for the determination of sulfates were from Fluka, Sigma Aldrich (USA), the nitric acid solution (≥69% v/v TraceSELECT® water solution) and the sodium carbonate used as eluent for the ion chromatography, the K_2SO_4 used to prepare standard solutions for the instrument calibration was from Sigma Aldrich (USA), as well. Also the protease from *Aspergillus oryzae* and the α-amylase from *Bacillus licheniformis*, used in the enzymatic digestions, were from also the same supplier. The NaOH solution used to prepare the buffers in HPAE-PAD analyses and to neutralize the samples before the determination of sulfates was from J.T. Baker (The Netherlands). The hydrochloric acid used for the hydrolysis reactions was from Carlo Erba (Italy). Certified reference solutions for the ion chromatography quality control were from Romil LTD (UK).

European pharmacopeia CS standards of bovine and marine origins (code number Y0000280 and Y0000593) and US CS pharmacopeia standards of bovine origin (code number 1133570), the commercial analytical grade CS standards from bovine trachea (code number C9819), and from bovine cartilage (code number C6837), and the two from shark (code number C4384 and 27043-Fluka) were from Sigma-Aldrich (USA). Nine commercial samples of bovine, pig and shark CS from three different suppliers (named Supplier #1, #2, #3) were tested; six of them, commercially available, were either purchased or obtained as test/gift samples, the three pharmaceutically formulated ones of bovine CS (from Supplier #1), of CS supplemented with galactosamine (from Supplier #1) and of avian CS (Supplier #4) were purchased in a drug store. Since a KS standard was not available on the market, to the best of our knowledge, it was obtained by purification of contaminated CS samples by SAX chromatography as described below (paragraph 2.5).

2.2 Monosaccharide composition analyses by HPAE-PAD

The pharmacopeia and analytical grade commercial standards as well as the commercial and pharmaceutical grade samples, or their purified and desalted peaks, obtained after SAX chromatography, were hydrolyzed with 5 M HCl (2.4 $mg \cdot ml^{-1}$) for 6 hours at 100 °C and 600 rpm (Thermomixer comfort, Eppendorf, Germany), slightly modifying a procedure reported by the US Pharmacopeia [39] for the hydrolysis of heparin, or eventually at the same concentration with 1 M HCl for 18 hours at 100 °C and 600 rpm. The monosaccharide composition analyses for each sample was performed by an high pressure ion chromatography system (ICS3000, Thermo Fisher Scientific, Italy), equipped with a pulsed amperometric detector (reference electrode Ag-AgCl; measuring electrode Au), by using an anion exchange column (Carbopac PA1, Thermo Fisher Scientific, Italy), injecting 25 µl of the sample and eluting in gradient conditions in 41 minute run, at a flow rate of 1 $ml \cdot min^{-1}$ (0-12 min from 1 to 4 mM NaOH, 12-14 min 4 mM NaOH, 14-16 min from 4 to 100 mM NaOH, 16-30 min 100 mM NaOH + 20mM $NaCH_3COOH$, 30-39 min from 100 mM NaOH + 20mM $NaCH_3COOH$ to 1 mM NaOH; 39-41 min 1 mM NaOH). Quantitative analyses on the area of the four monosaccharide standards were performed before and after hydrolytic treatment with both 5 M HCl for 6 hours or 1 M HCl for 18 hours, injecting the samples at both 8.0 and 2.0 $ng \cdot \mu l^{-1}$ concentrations; the percentage recovery for the each monosaccharide was calculated according to the following formula: Recovery = %[Area after hydrolysis/Area before hydrolysis]. GalNAc and GlcNAc were used as references for CS and KS quantification; repeatability of the retention time was determined by injecting consecutively ten samples at 8.0 $ng \cdot \mu l^{-1}$, for each monosaccharide. Linearity of the method was determined in the concentration range from 8.0 to 1.0 $ng \cdot \mu l^{-1}$and two calibration curves were built by plotting the averaged area values of the GalNAc and GlcNAc standards, after the hydrolytic treatment, obtained from three replicates. Equivalent calibration curves, in the same concentration range, were built plotting the averaged

area of the two monosaccharides, obtained after hydrolysis of the CS and KS polysaccharides purified by SAX chromatography as reported in the following paragraphs. The lowest detection limit of the method (LOD) was determined on the basis of a signal-to-noise ratio of 3:1 and the lowest quantification limit (LOQ) was determined on the basis of a signal-to-noise ratio of 10:1. Precision was determined as run to run repeatability by injecting consecutively ten samples at 8.0 $ng \cdot \mu l^{-1}$, for each monosaccharide; and as day to day reproducibility by injecting consecutively ten samples at 8.0 $ng \cdot \mu l^{-1}$, for each monosaccharide, on three different days. The percentage of CS and KS contents in the standards and in the commercial samples were calculated according to the following formulas: CS =%[$CS(g \cdot L^{-1})/(CS(g \cdot L^{-1})+KS(g \cdot L^{-1}))$] and KS = %[$CS(g \cdot L^{-1})/(CS(g \cdot L^{-1})+ KS(g \cdot L^{-1}))$]; in these formulas the concentrations of CS and KS were obtained by multiplying the concentration of GalNAc and GlcNAc, obtained from the analyses, for two averaged molecular weight values of the disaccharide of CS and KS (503 and 467 Da, respectively). The total recovery of the sample, after hydrolysis, was calculated as the percentage ratio of the sum of the CS and KS concentrations, obtained from the analyses, on the theoretical concentration of the sample calculated after dissolution in a precise volume of a weighed amount of the sample (w/v), without the water content, according to the following formula: Recovery = %[$(CS(g \cdot L^{-1})+ KS(g \cdot L^{-1})/sample(g \cdot L^{-1})$].

2.3 Monosaccharide composition analyses by GC-MS.

The pharmacopeia and analytical grade commercial standards as well as the commercial and pharmaceutical grade samples, or their purified and desalted peaks were analyzed by GC-MS. Their constituent monosaccharides were analyzed as acetylated methyl glycosides. Briefly a methanolysis reaction was performed in 2 M HCl in MeOH (1 ml, 80 °C, 20 h) and the samples were dried and acetylated with Ac_2O and pyridine (25 µl, 100 °C, 30 min). The samples were analyzed by a gas-chromatograph (6850A, Agilent Technologies) equipped with a mass selective detector (5973N, Agilent Technologies) and a Zebron ZB-5 capillary column (30m x 0.25mm i.d., film thickness 0.25 µm, Phenomenex) using He as gas carrier (flow rate 1 $ml \cdot min^{-1}$), following a temperature program of 180 °C for 3 min and 180 °C→220 °C at 1 $°C \cdot min^{-1}$. The percentage of CS and KS contents in the samples was calculated by considering the area corresponding to the GalNAc and GlcNAc peaks, respectively, according to the following formulas: %CS= [Area GalNAc/(Area GalNAc+Area GlcNAc)]*100, %KS= [Area GlcNAc/(Area GalNAc+Area GlcNAc)]*100.

2.4 Analyses by SEC/TDA.

The molecular weight analyses of the CS samples, or of the purified and desalted peaks obtained as chromatographic fractions by SAX chromatography, were performed by a high performance size exclusion chromatographic system (Viscotek, Malvern, Italy), equipped with an integrated gel permeation chromatography system (GPCmax VE

2001,Viscotek, Malvern, Italy) and a triple detector array module (TDA302,Viscotek, Malvern, Italy) including a refractive index detector (RI), a four-bridge viscosimeter (VIS), and an laser detector (LS) made of a right-angle light scattering (RALS) detector and a low-angle light scattering (LALS) one. Two gel-permeation columns (TSK-GEL GMPWXL, 7.8 × 30.0 cm, Tosoh Bioscience, Italy) equipped with a guard column (TSK-GEL GMPWXL, 6.0 × 4.0 cm, Tosoh Bioscience, Italy), were set in series to perform the analyses. An OmniSEC software (Viscotek, Malvern, Italy) program was used for the acquisition and analysis of the data. Samples were analyzed in a concentration range from 0.3 to 4 $g \cdot L^{-1}$ to have a column load (injection volume x sample concentration x intrinsic viscosity) for each analysis of approximately 0.2 dl and, at the same time, to have also appreciable LALS and VIS signals when analyzing low-molecular weight fragments. Elution was performed in isocratic conditions with 0.1 M $NaNO_3$ at pH 7.0, at a flow rate of 0.6 $ml \cdot min^{-1}$, at 40 °C in 50 minute runs. The calibration of the instrument was performed by using a polyethylene oxide (PEO) standard (22 kDa PolyCAL, Viscotek, Malvern, Italy). The fragment molecular weight distribution, the molecular size distribution, the polydispersity, the hydrodynamic radius were calculated by the data of the sample concentration, the molecular weight and the intrinsic viscosity that the system simultaneously determined, according to formulas previously reported [40, 41]. The $dn \cdot dc^{-1}$ values used for the CS and KS determination were 0.147 $ml \cdot g^{-1}$ and 0.100 $ml \cdot g^{-1}$ [42, 43]. The percentage representative of each peak in the analyses was calculated as the ratio between the single peak area divided by the sum of areas of all the peaks in the chromatogram.

2.5 SAX chromatography

Both standard and commercial sample chromatographic profiles were determined by strong anion-exchange chromatography by using an ÄKTA Explorer 100 purifier system (GE Healthcare, Milan, Italy), connected to the software UNICORN (GE Healthcare, Milan, Italy). About 20 mg of each sample were dissolved in 2 ml of buffer A (20.0 mM sodium acetate, 0.5 M sodium chloride, pH 7.4) and loaded on a strong anion exchange column (HiPrep Q Sepharose 16/10 HP, 1.6 × 10.0 cm, GE Healthcare, Milan, Italy), previously equilibrated with the same buffer. The samples were eluted by applying a linear gradient of buffer B from 0 to 100% (20.0 mM sodium acetate, 3.0 M sodium chloride, pH 7.4) in 4 column volumes, at flow rate of 3 $ml \cdot min^{-1}$. The chromatographic profiles were registered detecting the signal at 215 and 280 nm and the areas of the different peaks were obtained. The representativity of each peak in a single chromatogram was then calculated as the percentage ratio of the peak area on the sum of the areas of the all peaks, determined during the run, according to the following formula: $\%peak = \%[Area_{peak}/(\Sigma\ Area_{peak})]$. In order to collect enough material to characterize every single peak, obtained in the different chromatograms, about 250 mg of each sample were dissolved in 5 ml of buffer A (20 mM sodium acetate, 0.5 M sodium chloride, pH 7.4), loaded on a preparative strong anion exchange column (HiPrep Q Sepharose 26/10

HP, 2.6 × 10.0 cm, GE Healthcare, Milan, Italy) and eluted as previously reported. For each run 2 ml fractions were collected, the fractions containing a single peak were pooled together and then loaded on a desalting column (HiPrep 26/10 Desalting, 2.6 × 10.0 cm, GE Healthcare, Milan, Italy), previously equilibrated with pure water. Elution was performed by using the same ÄKTA purifier system at a flow rate of 1 $ml \cdot min^{-1}$ in 1.5 column volumes; the chromatographic profiles were detected at 215 and 280 nm and the eluted peaks were collected and then freeze-dried (Christ Epsilon 2-6D, Martin Christ, Germany). After lyophilization the peak fractions were analyzed by HPAE-PAD, SEC/TDA and GC-MS. Enzymatic digestions were performed on the unretained peak (4 $mg \cdot ml^{-1}$) by using an α-amylase (100 $U \cdot ml^{-1}$) and on the peak 1 (3 $mg \cdot ml^{-1}$) by using a protease (120 $U \cdot ml^{-1}$), for 5 and 60 hours at room temperature, under stirring, .respectively.

2.6 NMR spectroscopy

^{1}H spectra were recorded using a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe at 298 K. The heteronuclear experiment [$^{1}H-^{13}C$ DEPT−HSQC (distortionless enhancement by polarization transfer−heteronuclear single quantum coherence)] was performed using standard pulse sequence available in the Bruker software. Chemical shifts were measured at 298 K in D_2O.

2.7 Sulfates analyses

For the determination of the sulfates 20.0 ± 0.1 mg of each sample were weighed and then calcined by grinding them with 2.5 g of Eschka mixture (magnesium oxide and sodium carbonate in a 2:1 ratio) followed by treatment in a muffle at 600 °C for 3 hours. Samples were then dissolved in a 1N HNO_3 solution, neutralized by addition of 1N NaOH and diluted to a 100 ml-final volume with ultrapure water (conductivity <0:06 $S \cdot cm^{-1}$) [44]. Sample solutions were analyzed by using an ion chromatographer (IC 883; Metrohm, Switzerland), equipped with a conductometric detector and by using a polyvinyl alcohol stationary phase column (ASupp7, 250 mm length, 4.0 mm diameter, 5 μm particle size), functionalized with quaternary ammonium groups, and a 3.6 mM sodium carbonate as mobile phase. The analyses of the samples were compared with the blank values (the reagents in the absence of the sample), in order to assess the presence of interfering species. Determination of sulfates in the so obtained aqueous solutions was made according to the method APHA Standard Methods for Water and Wastewater ed 22nd 2012 - 4110B [45].

3. Results

3.1 HPAE-PAD method development.

In order to evaluate the residual KS contamination in CS samples of animal origin, a new multi-analytical approach was designed (Figure 1). First, a new HPAE-PAD method for the contemporary determination of CS and KS on the basis of their monosaccharide composition was developed; the GalNAc, GlcNAc, Gal, GlcA monosaccharide standards resulted separated showing four distinctive retention times (GalNAc at 9.80 minutes, Gal at 11.6 minutes, GlcNAc at 12.78 minutes, GlcA at 28.4 minutes) (Figure 2A; Tables 1); (In the same analytical conditions also the glucose was completely be separated and detected at 13.4 ± 0.2 minutes, *data not shown*). But quantitative analyses of the peak areas, before and after hydrolysis (5 M HCl at 100 °C for 6 h, *see Material and Methods*), revealed that only the two amino sugar areas remained unchanged (recovery of 97.3 ± 2.9% and 94.2 ± 1.4%, respectively), while the Gal and GlcA ones resulted greatly reduced (recovery 55.6 ± 5.5%and 49.7± 2.0%, respectively). Using a different hydrolysis protocol (1 M HCl at 100 °C for 18 h, *See Material and Methods*) higher GlcA recovery values were obtained (79.5 ± 5.6%) but the Gal ones resulted still very low (61.2 ± 4.8%). On the basis of these results the two amino-sugars were considered as the two reference residues for CS and KS quantification and two calibration curves were built in the range from 8.0 to 1.0 ng·μL^{-1} (Figure 2B). The linearity, the limit of detection and quantification and the precision of the method were tested too and they resulted consistent (Table 1).

3.2 Monosaccharide composition analyses of CS standards and commercial samples by HPAE-PAD and GC-MS.

Following the aim to build two equivalent calibration curves of the GalNAc and GlcNAc monosaccharides, obtained from CS and KS standards after the hydrolysis, we realized that no KS standards were available on the market, while by analyzing the CS ones, we found that most of them resulted consistently contaminated by KS. As a matter of fact on seven standards of both bovine or fish origins, analyzed by HPAE-PAD, only one resulted completely pure. All the three pharmacopeia ones (both Eu. and US. Ph. ones) and three commercial ones revealed a KS contamination higher than the 2.0% w/w, with values variously assorted according to the animal source (Figure 3): terrestrial ones revealed a contamination ranging from a limit value of 2.4 to 7.8%, while the marine ones showed higher values, from 8.5 to 19.0% (Figure 3; Figure S1 in *Supplementary material*). These data were confirmed by GC-MS analyses, that revealed KS contamination in the samples ranging from 2.5 to 40.2% (Figure 3; Figure S1 in *Supplementary material*). HPAE-PAD and GC-MS analyses were also performed of nine commercial grade CS samples of different origins from three different suppliers (three of them were pharmaceutically formulated) (Figure 3). In six of nine samples the contamination resulted over the limit, with two cases of borderline values: in HPAE-PAD analyses KS contamination ranged from 1.1 to 5.3% (except in the case of CS formulated by addition of glucosamine in which the value of GlcNAc resulted higher because of the supplementation) (Figure 3); similarly in GC-MS analyses a contamination higher than the acceptable level resulted in five of nine samples, in two of them it was around 2%, while in the remaining ones a very low contamination was noted (Figure 3).

3.3 Analyses of CS standards and commercial samples by SEC/TDA.

SEC/TDA analyses, performed on both standards and commercial samples, showed different molecular weight values according to the animal origin of the CS: in the range from about 18.6 to 21.0 kDa for the bovine CS, from about 17.7 to 20.4 kDa for the pig CS and from 32.0 to 52.0 kDa for the shark CS (Figure 4A and B). But almost all the chromatograms also showed the presence of other species having molecular weight values higher than 100 kDa. The percentage of representativeness of these species ranged from 0.5 to 13.5%, thus supporting the idea that the samples were complex mixtures (Figure 4A and B). In order to better evaluate the complexity of the standards and of the commercial samples, chromatographic profile analyses were performed by SAX, modifying a previously reported method [46].

3.4 SAX chromatography of CS standards and commercial samples.

The chromatographic profiles of CS standards and commercial samples were evaluated by SAX chromatography and the representativity of each peak was evaluated on the basis of their areas. In almost all the chromatograms three or even four different peaks were visible, their representativity changed according to the animal source of origin (Figure 5A and B). A portion of the samples resulted unretained (UR) by the column (at 19.4 ± 0.2 ml of elution), with a representativity ranging from 15 to 18% (Figure 5A and B). Another small sharp peak (Peak 1) (at 76.6 ± 0.3 ml) was mainly present in the terrestrial origin CS samples with a representativity of around 13-14% but it was in lower percentages in some marine sourced ones with average values of 3% (Figure 5A and B). The main peak (Peak 2) (89.5 ± 2.5 ml) in all the chromatograms showed a representativity from 68 to 73% with higher values in the terrestrial samples than in those of marine origin, while only in the latter ones a final small broad peak (Peak 3) was found, counting for about the 11% of representativity (at 112 ± 0.9 ml) (Figure 5A and B).

3.5 Analyses of SAX chromatography peaks by HPAE-PAD, NMR and ion chromatography.

Analyses of the peaks of six samples, performed by HPAE-PAD and NMR revealed that the unretained portion was made of a mixture containing CS (in percentages from 57.9 to 69.0%) and KS (from 31.6 to 47.1%) in terrestrial samples, and mainly KS in marine ones (Table 2). This mixture had a very low grade of sulfation (0.07-0.08 sulfate group for each disaccharide residues), molecular weights ranging from about 20.9 to 41.4 kDa (Table 2) and it also resulted contaminated by a polymer mainly constituted by α-glucose units, as revealed by NMR spectroscopy (*Data not shown*). A glycogen structure was suggested also because this polymer was easily degraded by α-amylase (Figure 6 A and B). The first small sharp peak (Peak 1), mainly present in the terrestrial samples, resulted to be almost pure CS (KS contamination lower than 7%, except in one case) that had the same sulfation pattern of the following main CS peak (Peak 2, *See the following data*) but lower molecular

weights (differences in the range from 8 to 21%) and a 25-30% lower sulfation grade *(See the following data*) (Figures 6 A and C; Table 2). In addition this peak was the only one in the chromatograms that showed absorption at 280 nm and its SEC/TDA analysis revealed the existence of high molecular weight species too (Figures 6 A and C). Starting from the hypothesis that this peak contained GAGs still partially linked to a proteoglycan core, an enzymatic hydrolysis reaction with protease was performed on it; after digestions the high molecular weight peak disappeared and changes in the molecular weight distribution were noted (Figure 6 A and C). Peak 2, in both terrestrial and marine origin samples, revealed to be highly pure CS, with molecular weight values and sulfation patterns corresponding to the ones already reported in literature for the CS extracted from different species [2, 4] (Table 2, Figure S2 in *Supplementary material*). Analysis of the ^{1}H-NMR spectra suggested an A,C sulfation pattern, as indicated by the peaks at δ = 4.64 and 4.11 ppm, that can be respectively associated to *H*-4 of 4-*O*-sulfated-GalNAc (GalNAc4S) and *H*-6 of 6-*O*-sulfated-GalNAc (GalNAc6S). The very small peak at δ = 4.01 ppm indicated a very low amount of unsulfated disaccharide units within the CS-A,C polysaccharide chain (Figure S2 in *Supplementary material*) [26, 46]. The final peak (Peak 3) displayed by the marine sourced samples was highly pure KS, the molecular weight and sulfation pattern of which were in accordance with the one already reported in literature [35, 37] (Figure 7; Table 2). Indeed, the analysis of the ^{1}H-^{13}C DEPT-HSQC spectrum (Figure 7) indicated a very extensive sulfation of the *O*-6 position of both GlcNAc and Gal units along the polysaccharide chain, as clearly showed by the much higher intensity of signals related to 6-*O*-sulfated GlcNAc and Gal (GlcNAc6S and Gal6S) residues at $\delta_{H/C}$ 4.52, 4.59/68.2 and 4.40, 4.43/69.1 ppm respectively, in comparison with those related to unsulfated units at $\delta_{H/C}$ 4.04, 4.16/61.8 and 3.96/62.6 ppm [36]. (Estimation of the representativity of each peak of SAX chromatograms was determined more precisely by gravimetric measurements too, after desalting. This allowed us to verify that the sum of KS contamination for each peak corresponded to the total one estimated in the initial sample).

4. Discussion

A precise determination of the purity grade of chondroitin sulphate of animal origin and of the presence of KS as contaminant has become an important and critical necessity from both commercial and scientific point of view in order to assure an efficient biological and pharmacological action. Indeed, the controversial reports on the diverse efficacy and effects of CS-based drugs may be due to the diverse quality and purity grade of the API, that although checked by pharmacopeia assays and analyses, could not actually contain “pure CS” when they are released on the market. In fact, pharmacopeia testing monographs have reported methods for the identification, quantification and purity grade determination of chondroitin sulfate or for the determination of impurities like proteins, heavy metals, chloride and sulfate residues [33, 34] but they have not reported any

specific one to detect the keratan sulphate, that latest studies have demonstrated to be widely diffused as contaminant [36]. Recent papers have tried to determine KS by using agarose gel-electrophoresis, immunoblotting or SEC chromatography analyses, but these methods lack of reproducibility and sensitivity to permit a precise detection of KS at low [21]. SAX chromatography resulted the most successful in identifying and quantifying the KS in CS samples by integration of the area of the correspondent peaks [36], although in that paper an un-identified peak at low elution time was not included in the estimation. Thus further, more sensitive, specific and accurate methods are necessary, considering that the limit of contaminants in CS samples should be lower than 5%, but a sound quality for CS pharma grade should include a limit of KS lower than 2% [2, 36]. In this study a multi-analytical approach was designed for the first time: a new high performance anion exchange chromatography method was developed and set-up to quantify the KS in CS samples, it was able to completely resolve and separate all the four monosaccharides constituting the glycosaminoglycan mixture, as never reported before, by using a gradient profile and not a linear one that required extensive pre-run and post run column washes (at least 20 minutes as reported before [39]); it resulted reliable, precise, reproducible and more sensitive than others previously reported with a detection limit on $ng \cdot \mu l^{-1}$ scale. By using this method, the KS contamination was determined in seven CS standards and nine commercial samples, and the results were compared with the ones obtained by using a GC-MS method, the application of which for the determination of the KS contamination has never been reported, as far as we know. Both methods were able to detect the presence of KS with higher sensitivity than the method used before, and to finely determine it in a range from 0.1 to 19.0 percentages. The HPAE-PAD method, quantifying the CS and KS monosaccharides on the basis of calibration curves and not of their area ratio, seems to be more precise, allowing to distinguish with higher precision whether the contamination was under or over the allowed limit. Results demonstrated that almost 50% of the samples presented a KS content not only higher than the 2% but also higher than the 5% w/w. Significant contamination was present in the marine samples but differently from what reported before a detectable KS contamination was also found in terrestrial origin CS. Only one supplier (Supplier #3) produces CS of both terrestrial and marine origin with a KS content under the 2% w/w. Surprisingly, also some pharmacopeia and commercial CS standards resulted contaminated over the limit (except for the shark one from Fluka, that resulted not available anymore on the market in the meanwhile this paper was written). These data suggest an additional potential issue on the reliability of the material used as reference in the CS analyses for purity assessment, in addition to the necessity of developing sensitive analytical methods. For example, the European Pharmacopeia bovine CS standard was characterised on the basis of its chromatographic profile by high performance SEC chromatography [2] and no KS contamination was found, probably because the two peaks of CS and KS in SEC partially overlap [36], as also our SEC/TDA analyses suggested. Besides, SEC chromatograms hitherto reported showed also some partially unresolved peaks, following the CS one, that were not taken into consideration or characterised at all [2]. But also analyses of the disaccharide composition of the European

Pharmacopeia bovine CS standard after enzymatic digestion, were not sufficient to reveal the contamination [2]. SAX-HPLC analyses of the not digested mixture allowed to identify a KS contamination in the European Pharmacopeia shark CS standard profile, but not in the United States Pharmacopeia bovine one [36]. As a matter of fact in the marine origin standards a KS peak, following the CS one, was determined; this peak was not visible in the terrestrial origin CS samples [36]. The determination of the KS content was thus performed taking into consideration only the contribution of this peak whose representativity, calculated on the basis of the area integration, was similar to the percentage we have determined in this study by SAX cromatography [36]. But in both terrestrial and marine origin standards also an unretained peak was visible. This peak has never been characterised before, according to our knowledge, but it contained undetected KS that contributes to the contamination content [36] as we have demonstrated in this work by characterising the unretained peak by using HPAE-PAD, SEC/TDA and NMR. Thus, so far, by using only SAX-HPLC analyses the KS contamination was not revealed in the terrestrial animal origin samples while it was under-estimated in the marine ones. SAX chromatography could be employed as technique useful to have CS sample finger print profiles, but not to have a precise KS content determination. Only the absence of both the unretained and the KS peaks could assure the absence of a KS contamination, as, for example, we noted in the Fluka standard. In the framework of this experimental research work, by analysing the unretained peak, we have also determined, for the first time, the presence of GAG chains still bounded to a protein core in many terrestrial standards and samples. Their presence was probably due to an incomplete enzymatic digestion of the proteoglycan chains during the purification steps of the manufacturing process. The presence of high molecular species (>100 KDa), was clearly detected by SEC/TDA analyses, even in small percentages, already in the initial samples. Although so far molecular weight characterisation of CS from different sources has been traditionally performed by agarose gel electrophoresis or by high performance size-exclusion chromatography coupled with UV and RI detectors, it could also be interesting to employ the SEC/TDA for CS characterisation to quickly find the presence of not easily detectable high molecular weight species present at low concentrations. In this study it was also surprising to find out that there are no pure KS standards available on the market (from Japan to US) as well as it may be argued that more pure, reliable CS standards (> 99% purity) are necessary to obtain a precise characterization of the extractive CS. To overcome this issue, in this research work, we have produced our own CS and KS standards by SAX chromatography, obtaining gram scale quantities of high purity grade material, sufficient to be then used as standard for HPAE-PAD and GC-MS analyses.

5. Conclusion

In conclusion this research work has tried to develop sensitive and reliable analytical methods to better assess the purity grade of CS extracted from animal tissue sources and to precisely determine the eventual residual KS content. A new

developed HPAE-PAD method and a new application of a GC-MS one have been proposed for the contemporary identification and quantification of CS and of KS. Data revealed a contamination over the allowed limit in almost 50% of both terrestrial and marine origin standards and commercial samples. These results have underlined also the need to produce CS standards of improved purity and to manufacture a KS one that is not available on the market for commercialization. SEC/TDA analyses have allowed a deeper characterization in terms of detection of high molecular weight species, while SAX chromatography have provided specific finger print profiles useful as a first screen of the CS standards and samples. Overall, new analytical tools were assessed towards a better understanding of the composition of extractive GAGs used as active pharmaceutical ingredient or nutraceuticals on human and animals.

6. Acknowledgements

This work was supported by the Ministero dell'Università e della Ricerca Scientifica [PON01_1226 "Nutrafast"].

HPAE-PAD	Elution time			Linearity		Limit		Precision	
Analyte	**$t_{elution}$** (min)	**$t_{elution}$ SD** (min)	**CV** (%)	**Range** (ng/ml)	**Correlation factor** (R^2)	**LOD** (ng/ml)	**LOQ** (ng/ml)	**Repeatability CV** (%)	**Reproducibility CV** (%)
GalNAc	9.80	0.03	0.35	8.0-1.0	0.997	0.3	1.0	0.83	0.72
GlcNAc	12.78	0.01	0.09	8.0-1.0	0.997	0.3	1.0	0.88	0.68

Table 1: High performance anion exchange chromatography method parameters.

	Origin	**UR**	**Peak 1**	**Peak 2**	**Peak 3**	**UR**	**Peak 1**	**Peak 2**	**Peak 3**
		HPAE-PAD				**SEC/TDA (kDa)**			
Sigma-Aldrich	Bovine trachea	31.6% KS 69.0% CS	1.3% KS 98.7% CS	0.6% KS 99.4% CS	- -	23.4	19.4	22.9	-
Sigma-Aldrich	Bovine cartilage	40.7% KS 59.3% CS	5.4% KS 94.6% CS	1.5% KS 98.5% CS	- -	35.2	20.2	25.5	-
Sigma-Aldrich	Shark	94.2% KS 5.7% CS	1.9% KS 98.1% CS	0.5% KS 99.5 % CS	95.9% KS 4.1% CS	5.42	-	33.8	33.5
Supplier #1	Bovine oral	42.0% KS 58.0% CS	7.0% KS 93.0% CS	0% KS 100.0% CS	- -	41.4	15.5	20.6	-
Supplier #1	Pig oral	45.1% KS 54.9% CS	4.9% KS 95.1% CS	0% KS 100.0% CS	- -	25.2	17.0	18.5	-
Supplier #2	Bovine oral	22.4% KS 77.6% CS	14.2% KS 85.8% CS	1.8% KS 98.2% CS	- -	20.9	16.0	21.9	-

Table 2: HPAE-PAD and SEC/TDA analyses of peaks derived by SAX chromatography of some representative CS standards and commercial samples.

Figure 1. Outline of the multi-analytical approach for the KS determination in CS standards and commercial samples.

Figure 2. HPAE-PAD analyses: A)-chromatogram reporting GalNAc, Gal, GlcNAc and GlcA peaks (* indicates the increase of NaOH and sodium acetate concentration in the eluent); B)-comparison of the calibration curves of GalNAc and GlcNAc monosaccharide standards and the ones of the two monosaccharides obtained after hydrolysis of purified CS and KS standards.

Figure 3. KS percentages in CS standards and commercial samples analyzed by HPAE-PAD and GC-MS.

Figure 4. SEC/TDA analyses of CS standards and commercial samples: A) representative chromatograms of CS different profile types: 1) bovine cartilage CS standard from Sigma-Aldrich; 2) bovine sample CS from Supplier #2; 3) pig CS sample from Supplier #1; 4) shark CS standard from Sigma-Aldrich. The chromatographic profiles were detected as RI (red), RALS (green), LALS (black) and viscosimeter (blue) signals; the high molecular weight specie and the main peak were indicated by blue and black arrow, respectively. B) Table of molecular weight, polydispersity and intrinsic viscosity values of the main peak in SEC/TDA analyses of the CS standards and commercial samples.

Figure 5. A) SAX chromatography profiles of standards and commercial samples as detected at 215 nm (in black) and at 280 nm (in blue) with the unretained (UR), 1, 2 and 3 peaks indicated by the arrows: 1) bovine CS standard from Eu. Ph.; 2) bovine CS standard from US. Ph.; 3) bovine trachea CS standard from Sigma-Aldrich; 4) bovine cartilage CS standard from Sigma -Aldrich; 5) marine CS standard from Eu. Ph.; 6) shark CS standard from Sigma-Aldrich; 7) shark CS standard from Fluka; 8) avian CS (pharma formulation) sample from Supplier #4; 9) bovine CS oral sample from Supplier #1; 10) bovine injectable CS sample from Supplier #1; 11) bovine oral CS sample from Supplier #2; 12) pig sample CS from Supplier #1; 13) shark sample CS from Supplier #3; 14) bovine sample CS (pharma formulation with glucosamine) from Supplier #1; 15) bovine CS (pharma formulation) from Supplier #1. B) Relative area percentages of the four different peaks detected in SAX profiles of CS standards and commercial samples, according to the different animal origins.

Figure 6. A) Representative SAX profile of terrestrial CS; B) HPAE-PAD analyses of the unbound peak before and after α-amylase hydrolysis and of the glucose released in the digestion; C) SEC/TDA analyses of peak 1 before and after protease hydrolysis with indication of the high molecular weight specie (blue arrow) and the low one (black arrow).

Figure 7. ^{1}H-^{13}C DEPT-HSQC spectrum (zoom, 600 MHz, D_2O, 298K) of KS (peak 4) isolated from shark sample by SAX chromatography.

Figure S1. A) Representative chromatograms of HPAE-PAD analyses (* indicates the increase of NaOH and sodium acetate concentration in the eluent): contaminated bovine CS standard from Sigma-Aldrich (black line), contaminated shark CS standard from Sigma-Aldrich (blue line), not contaminated shark CS standard from Fluka (pink line), KS standard not available on the market as isolated by SAX chromatography (brown line); B) and C) Representative chromatograms of GC-MS contaminated bovine and shark CS standards (both from Eu. Ph.).

Figure S2. ^{1}H NMR spectra (600 MHz, D_2O, 298K) of CS (peak 2) isolated from: A) bovine and B) shark CS samples by SAX chromatography.

References

[1] Raman R., Sasisekharan V. and Sasisekharan R. *Chemistry & Biology*. (2005); 12, 267-277. doi: 10.1016/j. chembiol. 2004.11.020.

[2] Volpi N. *J. Pharm. Sci.* (2007); 96(12), 3168-80. doi: 10.1002/jps.20997.

[3] Bedini E. and Parrilli M. *Carbohydr. Res.* (2012); 356, 75-85. doi: 10.1016/j.carres.2012.02.010.

[4] Restaino O.F., De Rosa M., Cimini D., Schiraldi C. in: Pomin V.H., *Chondroitin Sulfate: Structure, Uses and Health Implications*, Nova Science Publishers, New York, (2013), 41-56.

[5] Shi Y., Meng Y., Li J., Chen J., Liu Y. and Bai X. *J. Chem. Technol. Biotechnol.* (2014); 89, 1445-1465. doi: 10.1002/jctb.4454.

[6] Morreale P., Manopulo R., Galati M., Boccanera L., Saponati G., Bocchi L. *J. Rheumatol.* (1996); 3, 1385-91.

[7] Ronca F., Palmieri L., Panicucci P. and Ronca G. *Osteoarthritis Cartilage*. 6, 14-21 (1998).

[8] Volpi N., *Carbohydr. Polym.*-55, 273-281 (2004). doi:10.1016/j.carbpol.2003.09.010.

[9] Michel B.A., Stucki G., Frey D., De Vathaire F., Vignon E., Bruehlmann P., Uebelhart D. *Arthritis Rheum.* (2005); 52, 779-86. doi: 10.1002/art.20867.

[10] Van Vlierberghe S., Dubruel P. and Schacht E., *Biomacromolecules* 12, 1387-1408 (2011). doi: 10.1021/bm200083n.

[11] Weyers A. and Linhardt R.J. *FEBS J.* (2013); 280(10) , 2511-2522. doi: 10.1111/febs.12187.

[12] Zhao L., Liu M., Wang J. and Zhai G., *Carbohydr. Polym.* (2015); 133, 391-399. doi:10.1016/j.carbpol.2015.07.063.

[13] Bedini E., Laezza A. and Iadonisi A. *Eur. J. Org. Chem.* (2016); 18, 3018-3042. doi: 10.1002/ejoc.201600108.

[14] Sakai S., Otake E., Toida T., Goda Y., *Chem. Pharm. Bull*. 55(2), 299-303 (2007). doi: 10.1248/cpb.55.299.

[15] Coaccioli, S., Allegra A., Pennacchi M., Mattioli C., Ponteggia F., Brunelli A., Patucchi E., Puxeddu A. *Int. J. Clin. Pharm. Res.* (1998); 18(1), 39-50.

[16] Rovetta G., *Drugs Exptl. Clin. Res.* VII, 53-71 (1991).

[17] Fioravanti A., Franci A., Anselmi F., Fattorini L, Marcolongo R. *Drugs Exptl. Clin. Res*. (1991); 7, 41-4.

[18] Leeb B.F., Schweizer H., Montan K., Smolen J.S. *J. Rheumatol*. (2000); 27(1), 205-211.

[19] McAlindon T.E., LaValley M.P., Gulin J.P., Felson D.T. *JAMA*. (2000); 283(11), 1469-1475.

[20] Martel-Pelletier J., Farran A., Montell E., Vergés J., Pelletier J.-P. *Molecules* (2015); 20(3), 4277-4289. doi:10.3390/molecules20034277.

[21] Da Cunha A. L., de Oliveira L. G., Maia L. F., Cappa de Oliveira L. F., Michelacci Y. M., de Aguiar J. A. K., *Carbohydr. Polym.* (2015); 134, 300-308. doi:10.1016/j.carbpol.2015.08.006.

[22] Greinacher A., Michels J., Schäfer M., Keifel V. and Mueller-Eckhardt C. *Br. J. Haematol.* (1992); 81, 252-254.

[23] Kishimoto T.K., Viswanathan K., Ganguly T., Elankumaran S., Smith S., Pelzer K., Lansing J.C., Sriranganathan N., Zhao G., Galcheva-Gargova Z., Al-Hakim A., Bailey G.S., Fraser B., Roy S., Rogers-Cotrone T., Buhse L.F., Whary M., Fox J., Nasr M., Dal Pan G.J., Shriver Z., Langer R.S., Venkataraman G., Austen K.F., Woodcock J. and Sasisekharan R. *New Engl. J. Med.* (2008); 358, 2457-2467. doi: 10.1056/NEJMoa0803200.

[24] Guerrini M., Beccati D., Shriver Z., Naggi A., Viswanathan K., Bisio A., Capila I., Lansing J.C., Guglieri S., Fraser B., Al-Hakim A., Gunay, N.S., Zhang Z., Robinson L., Buhse L.F., Nasr M., Woodcock J., Langer R., Venkataraman G., Linhardt R.J., Casu B., Torri G. and Sasisekharan R. *Nat. Biotechnol.* (2008); 26(6), 669-675. doi: 10.1038/nbt1407.

[25] Zoppetti G. and Oreste P. *U.S. Pat. Appl. Publ.* 6777398, (2004).

[26] Bedini E., De Castro C., De Rosa M., Di Nola A., Iadonisi A., Restaino O.F., Schiraldi C. and Parrilli M. *Angew. Chem. Int. Ed.* (2011); 50, 6160-6163. doi: 10.1002/anie.201101142.

[27] Bedini E., De Castro C., De Rosa M., Di Nola A., Restaino O.F., Schiraldi C. and Parrilli M. *Chem. Eur. J.* (2012); 18, 2123-2130 . doi: 10.1002/chem.201102458.

[28] Restaino O.F., Cimini D., De Rosa M., Catapano A., De Rosa M and Schiraldi C. *Microb. Cell Fact.* (2011);10:10. doi: 10.1186/1475-2859-10-10.

[29] Schiraldi C., Carcarino I.L., Alfano A., Restaino O.F., Panariello A. and Mario De Rosa. *Biotechnol. Journal.* (2011); 6(4):410-9. doi: 10.1002/biot.201000266.

[30] Bianchi D., Valetti M., Bazza P., Miraglia N. and Valoti E. *PCT Int. Appl.* WO2012/152872. (2012).

[31] Schiraldi C., Alfano A., Cimini D., De Rosa M., Panariello A., Restaino O.F., De Rosa M. *Biotechnol Prog*. (2012); 28(4):1012-8. doi: 10.1002/btpr.1566.

[32] Laezza A., De Castro C., Parrilli M. and Bedini E. *Carbohydr. Polym.* (2014); 112, 546-555. doi:10.1016/j.carbpol.2014.05.085.

[33] European Pharmacopeia 7.0. (2009); 01/2009: 2064.

[34] United States Pharmacopeia, (2015); USP 39-NF34.

[35] Funderburgh J. L. *Glycobiology*. (2000); 10(10), 951-58.

[36] Pomin V.H., Piquet A.A., Pereira M.S., Mourão P.A.S. *Carbohydr. Polym.* (2012); 90, 839-846. doi: 10.1016/j.carbpol.2012.06.009.

[37] Huckerby T.N. *Prog Nucl Magn Reson Spectros*. (2002); 40, 35-110. doi:S0079-6565(01)00040-1.

[38] Nakano T. and Ozimek L. *Carbohydr. Polym.*(2014); 99, 547-552. doi:10.1016/j.carbpol.2013.08.052.

[39] United States Pharmacopeia, (2015); USP 37-NF32.

[40] Theisen A., Johann C., Deacon M. P. and Harding S.E. *in Refractive Increment Data-Book for polymer and biomolecular scientists.*(1999). Nottingham University Press. UK.

[41] La Gatta A., De Rosa M., Marzaioli I., Busico T., Schiraldi C. *Anal. Biochem.* (2010); 404(1), 21-29. doi:10.1016/j.ab.2010.04.014.

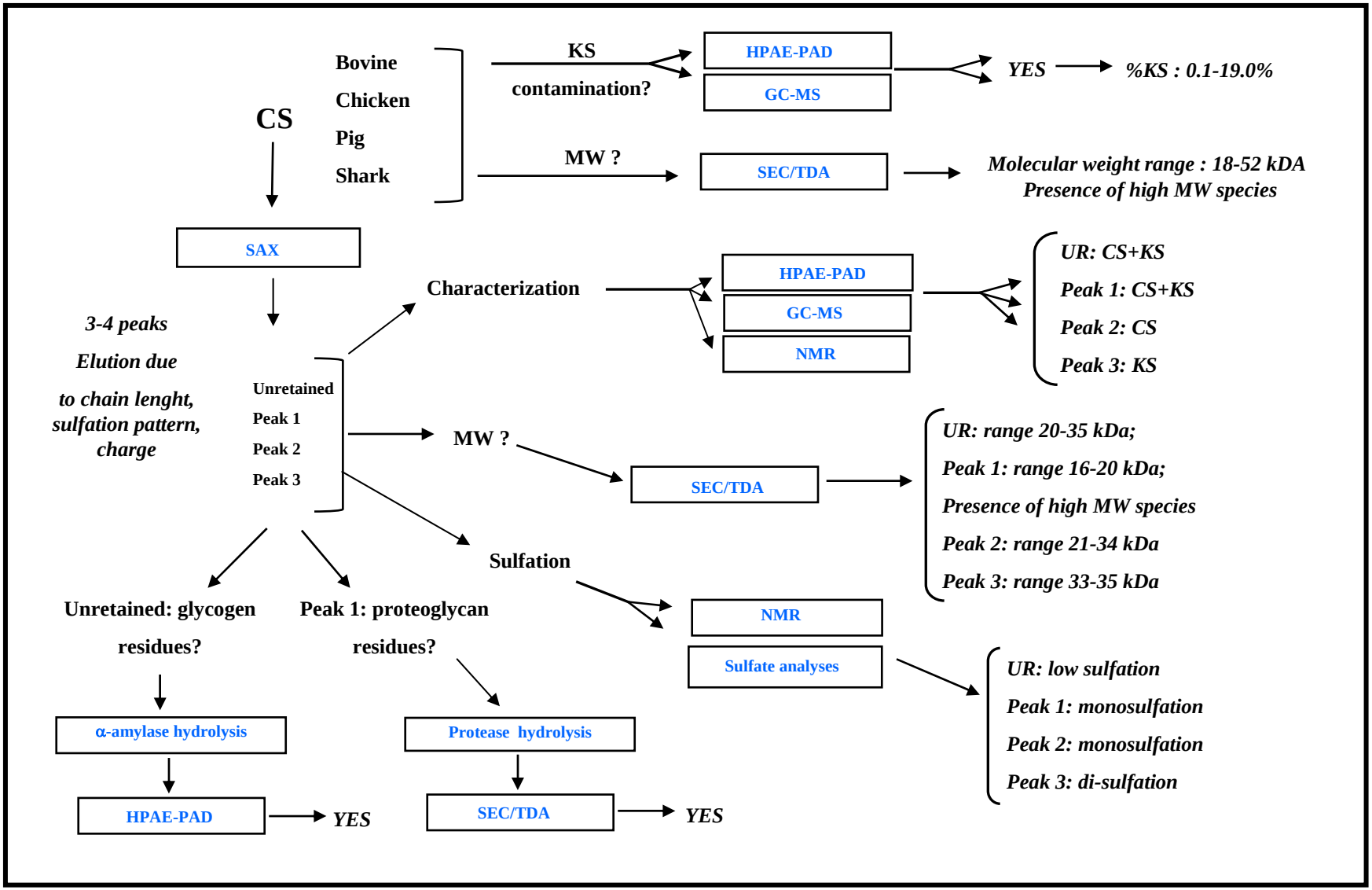
[42] Swann D.A., Garg H.A., Silver F.H: Larsson A. *J. Biol. Chem.* (1984); 259, 7693-7700.

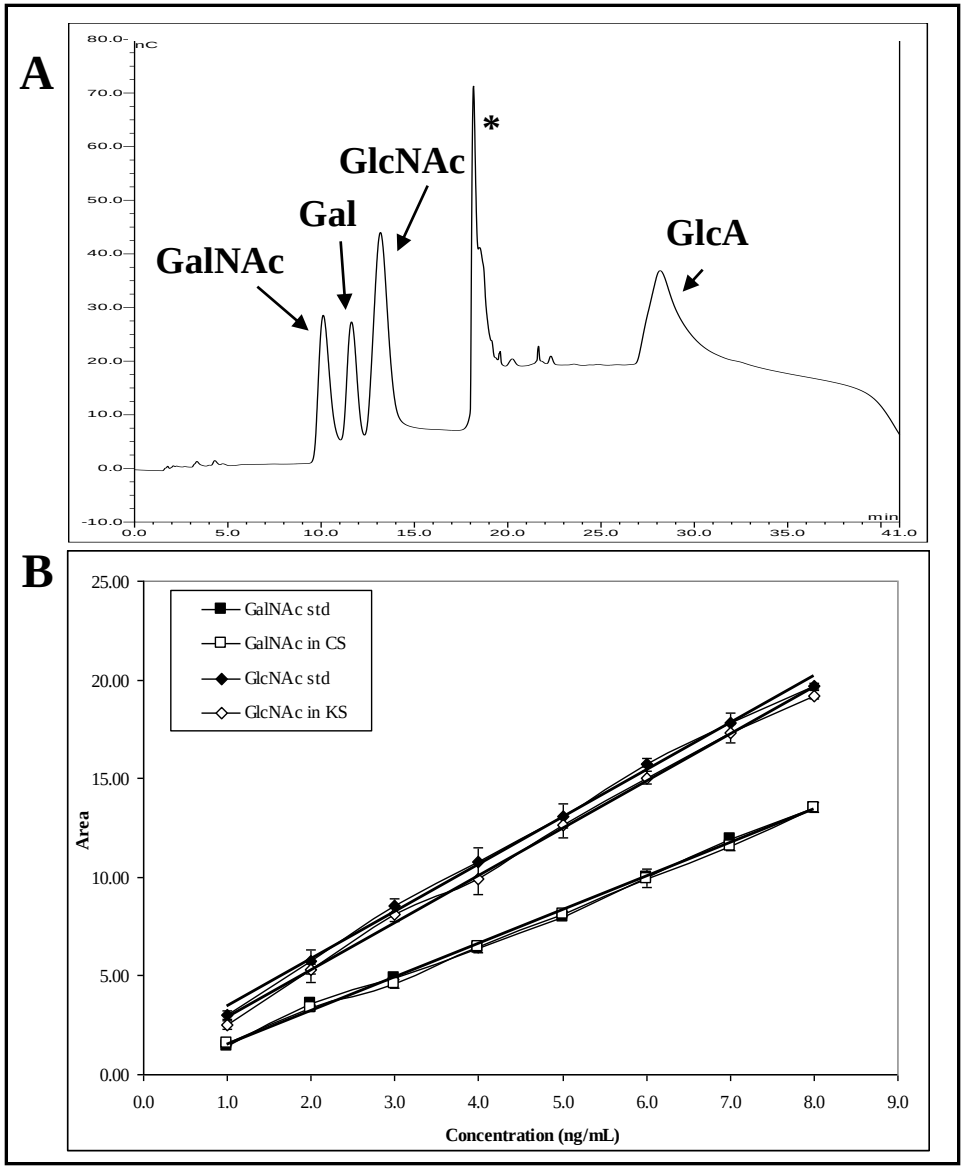
[43] Jumel K., Harding S.E., Sobol E., Omel'chenko A., Sviridov A., Jones N. *Carbohydr. Polym.* (2002); 48, 241-245. doi:10.1016/S0144-8617(01)00242-9.

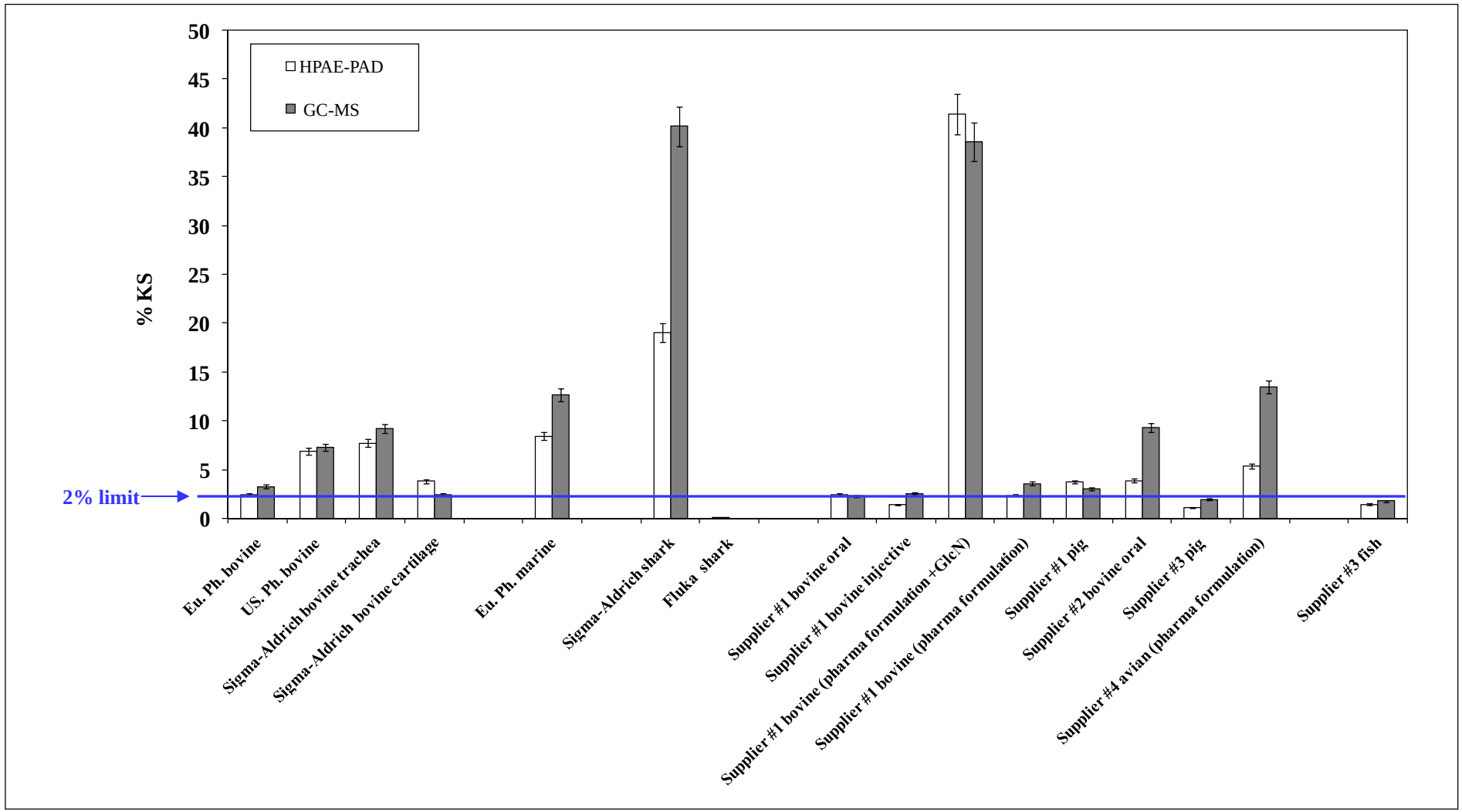
[44] Ubaldini I., Capizzi-Maitan F., Chimica e Industria. (1955) 37, 779-81.

[45] Rice E.W. Standard Methods for the Examination of Water and Wastewater 22nd Edition, APHA AWWA WEF Publishers, Washington, (2012).

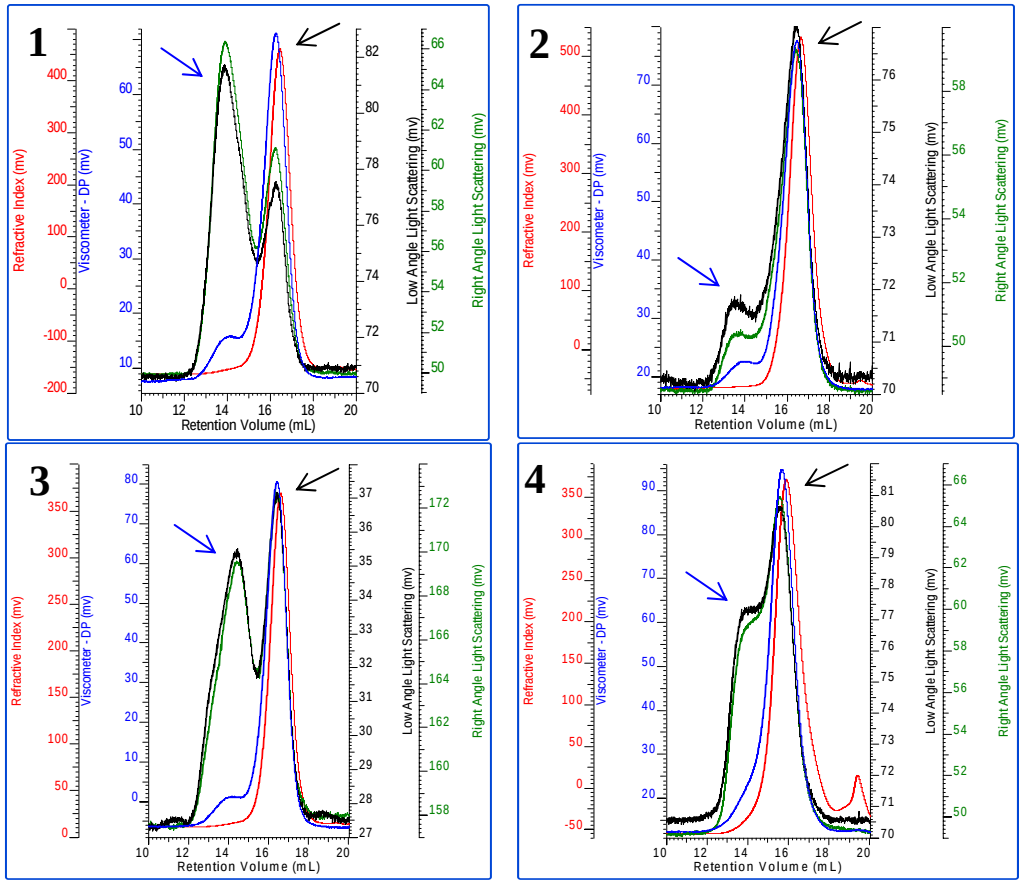
[46] Squillaci G., Finamore R., Diana P., Restaino O.F., Schiraldi C., Arbucci E., Ionata S., La Cara F., Morana A., *Appl Microbiol Biotechnol*. (2016); 100, 613-623. doi:10.1007/s00253-015-6991-5.


CS
Bovine
Chicken
Pig
Shark
KS
contamination?
HPAE-PAD
GC-MS
YES
%KS : 0.1-19.0%
MW ?
SEC/TDA
Molecular weight range : 18-52 kDA
Presence of high MW species
SAX
3-4 peaks
Elution due
to chain lenght,
sulfation pattern,
charge
Unretained
Peak 1
Peak 2
Peak 3
Characterization
HPAE-PAD
GC-MS
NMR
UR: CS+KS
Peak 1: CS+KS
Peak 2: CS
Peak 3: KS
MW ?
SEC/TDA
UR: range 20-35 kDa;
Peak 1: range 16-20 kDa;
Presence of high MW species
Peak 2: range 21-34 kDa
Peak 3: range 33-35 kDa
Sulfation
NMR
Sulfate analyses
UR: low sulfation
Peak 1: monosulfation
Peak 2: monosulfation
Peak 3: di-sulfation
Unretained: glycogen
residues?
α-amylase hydrolysis
HPAE-PAD
YES
Peak 1: proteoglycan
residues?
Protease hydrolysis
SEC/TDA
YES


A
nC
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
-10.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
41.0
min
GalNAc
Gal
GlcNAc
*
GlcA
B
25.00
20.00
15.00
10.00
5.00
0.00
Area
GalNAc std
GalNAc in CS
GlcNAc std
GlcNAc in KS
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Concentration (ng/mL)

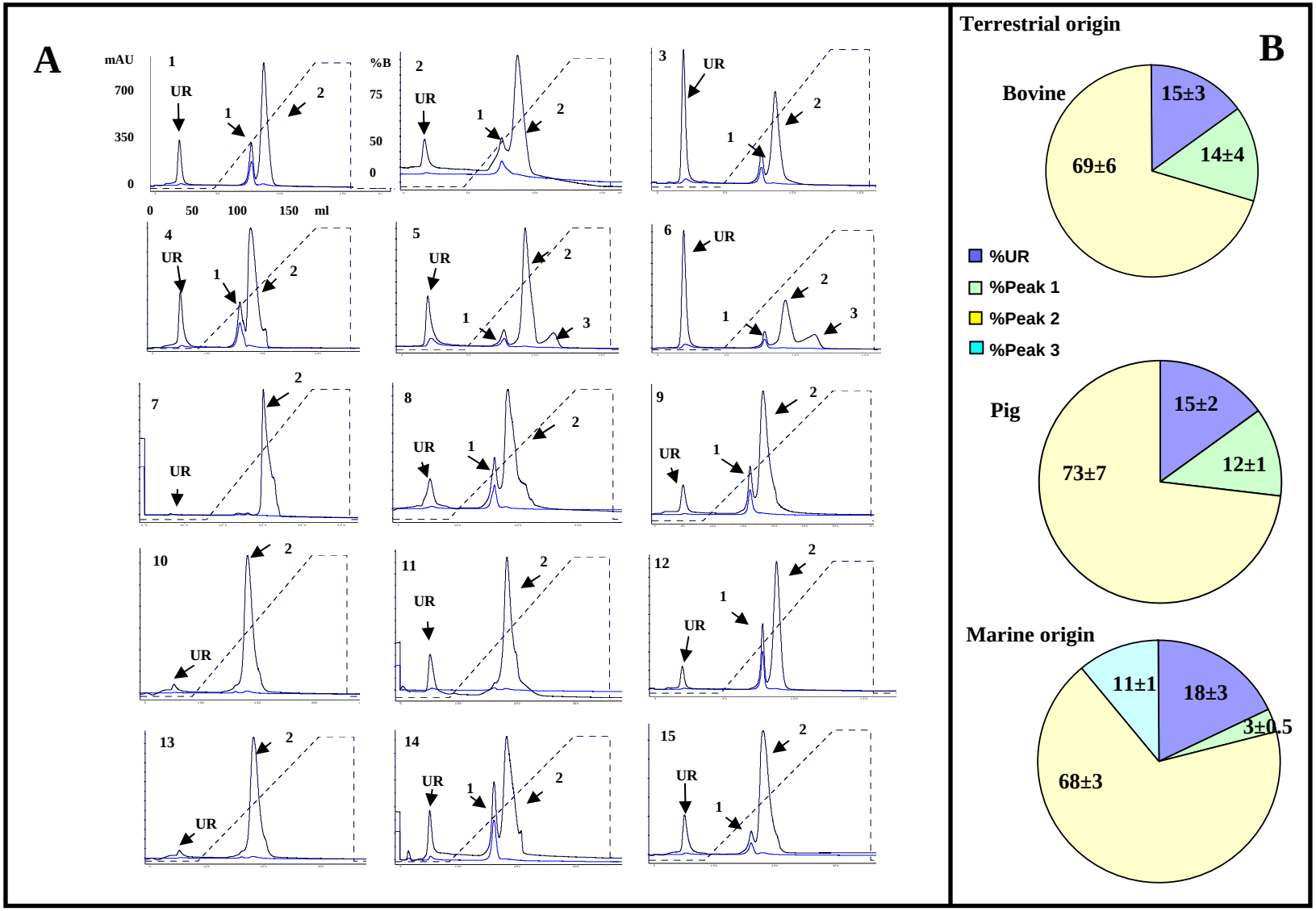

HPAE-PAD
GC-MS
% KS
50
45
40
35
30
25
20
15
10
5
0
2% limit
Eu. Ph. bovine
US. Ph. bovine
Sigma-Aldrich bovine trachea
Sigma-Aldrich bovine cartilage
Eu. Ph. marine
Sigma-Aldrich shark
Fluka shark
Supplier #1 bovine oral
Supplier #1 bovine injective
Supplier #1 bovine (pharma formulation+GlcN)
Supplier #1 bovine (pharma formulation)
Supplier #1 pig
Supplier #2 bovine oral
Supplier #3 pig
Supplier #4 avian (pharma formulation)
Supplier #3 fish

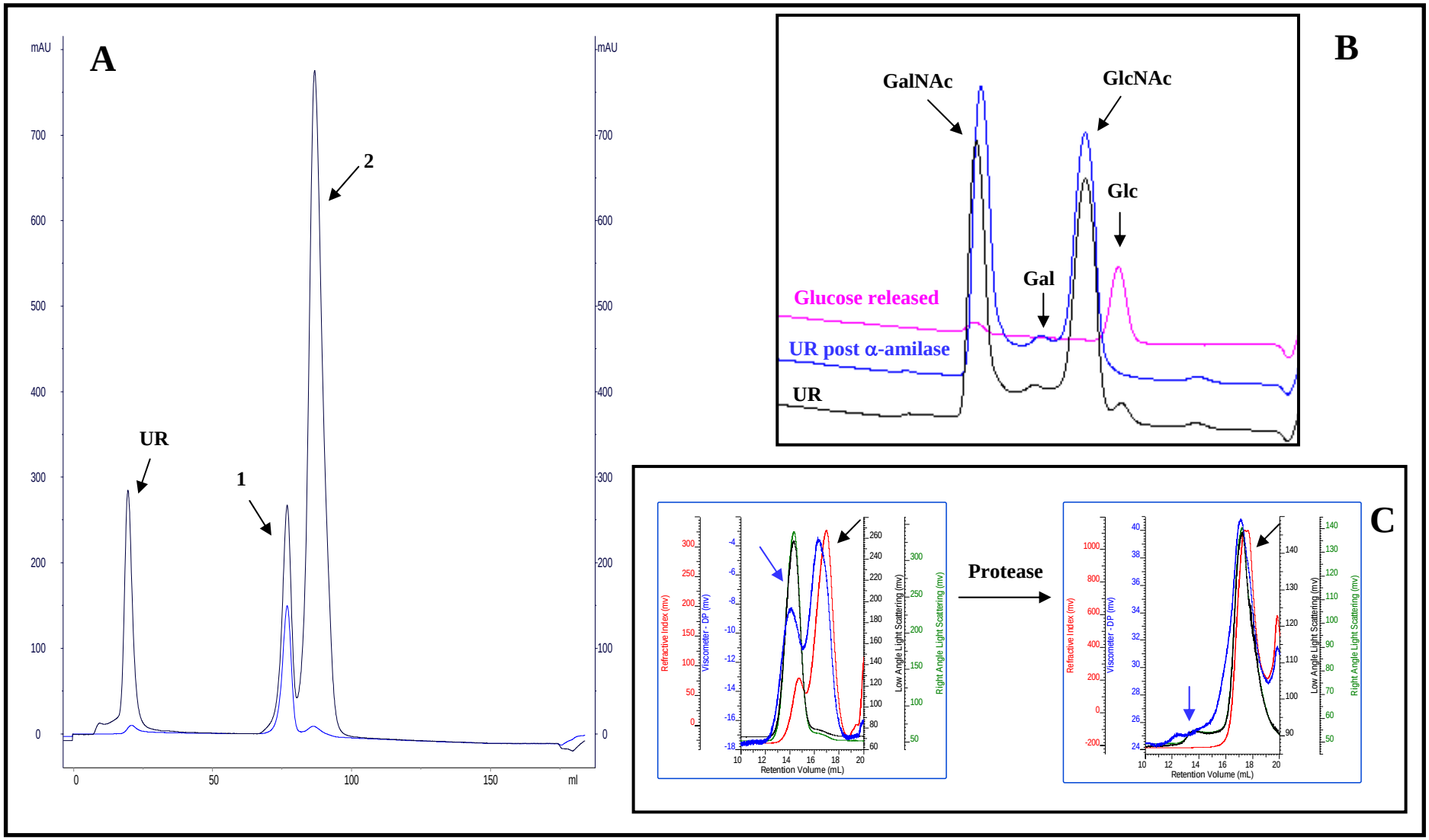
A

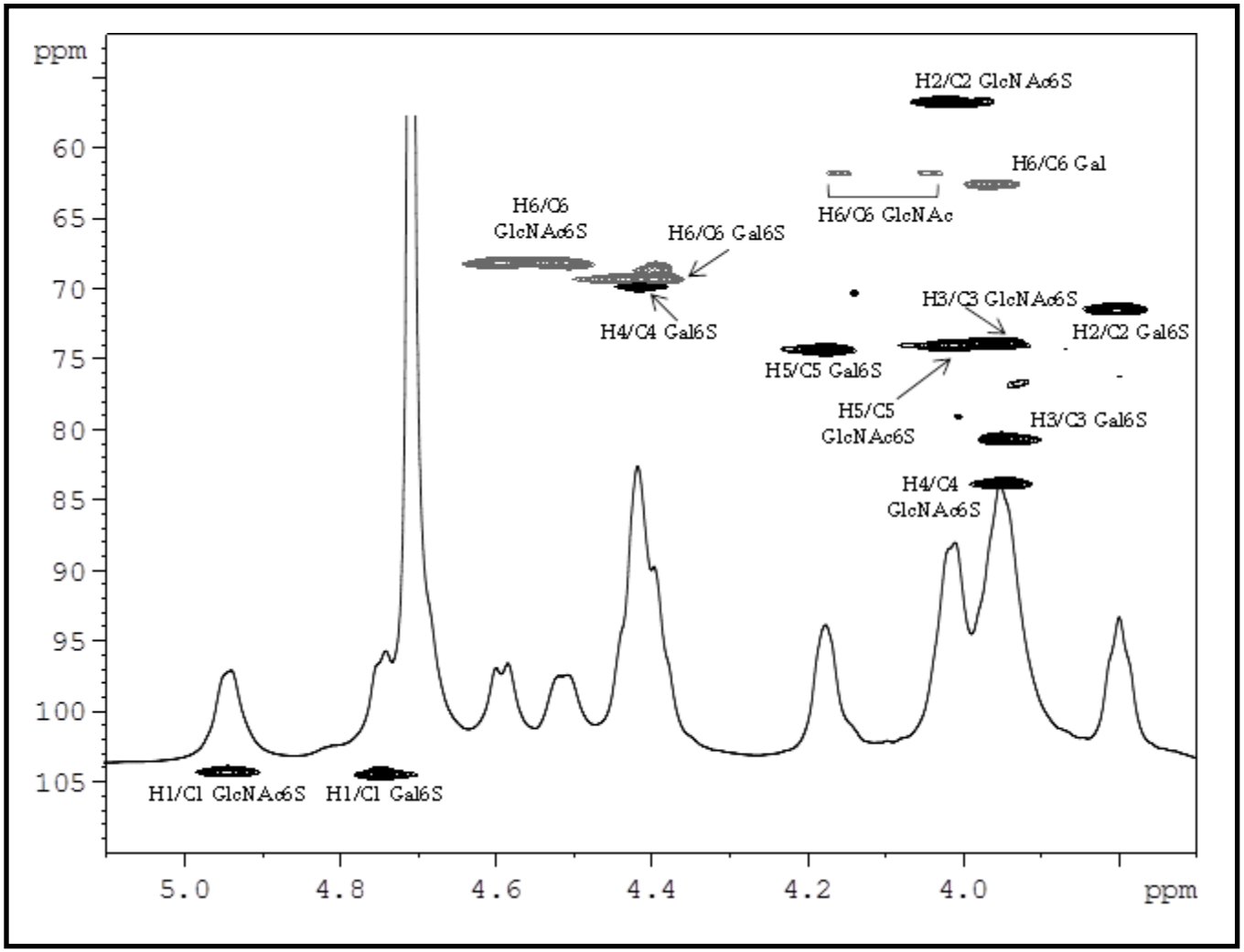


B

	Bovine CS	Porcine CS	Avian CS	Shark CS
Mw (kDa)	19.63 ± 0.7	18.1± 0.3	24.60 ± 0.5	32.0-52.0
Polydispersity	1.28 ± 0.2	1.2 ± 0.01	1.21± 0.02	1.18-1.60
Intrinsic Viscosity (m^3/kg)	0.05 ± 0.004	0.043± 0.001	0.061± 0.001	0.077-0.149


A
mAU
700
350
0
%B
75
50
0
0 50 100 150 ml
1
UR
1
2
2
UR
1
2
3
UR
2
1
4
UR
1
2
5
UR
2
1
3
6
UR
2
1
3
7
2
UR
8
UR
1
2
9
2
UR
1
10
2
UR
11
UR
2
12
2
1
UR
13
2
UR
14
UR
1
2
15
2
UR
1
B
Terrestrial origin
Bovine
15±3
14±4
69±6
%UR
%Peak 1
%Peak 2
%Peak 3
Pig
15±2
12±1
73±7
Marine origin
11±1
18±3
3±0.5
68±3


A
mAU
UR
1
2
ml
B
GalNAc
GlcNAc
Glc
Gal
Glucose released
UR post α-amilase
UR
C
Protease
Refractive Index (mv)
Viscometer - DP (mv)
Low Angle Light Scattering (mv)
Right Angle Light Scattering (mv)
Retention Volume (mL)


ppm
60
65
70
75
80
85
90
95
100
105
H2/C2 GlcNAc6S
H6/C6 Gal
H6/C6 GlcNAc
H6/C6
GlcNAc6S
H6/C6 Gal6S
H4/C4 Gal6S
H3/C3 GlcNAc6S
H2/C2 Gal6S
H5/C5 Gal6S
H5/C5
GlcNAc6S
H3/C3 Gal6S
H4/C4
GlcNAc6S
H1/C1 GlcNAc6S
H1/C1 Gal6S
5.0
4.8
4.6
4.4
4.2
4.0
ppm

Highlight 1: A multi-analytical approach was set up, for the first time, for the determination of the residual keratan sulfate contamination in chondoitin sulfate samples.

Highlight 2: A new HPAED method was developed, compared to a GC-MS one and used to screen standard and commercial samples.

Highlight 3: Keratan sulfate contamination was found in the range from 0.1 to 19%, in almost 50% of the samples tested it was over the 5% limit.

Highlight 4: First time detected keratan sulfate contamination in both bovine and marine origin standards.

Highlight 5:. SEC-TDA analyses, SAX chromatography and NMR studies allowed to finger print profile the chondrotin sulfate samples detecting the presence of low sulfated chains and proteoglycan bounded ones.

.