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European chondroitin sulfate and glucosamine food supplements: A systematic quality and quantity assessment compared to pharmaceuticals

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ABSTRACT

Chondroitin sulfate and glucosamine, commercialized as anti-osteoarthritis food supplements, do not undergo the strict quality controls of pharmaceuticals. In this paper a systematic multi-analytical approach was designed to analyse 25 food supplements from 8 European countries compared to 2 pharmaceuticals by using high performance anion-exchange chromatography with pulsed amperometric detection, size exclusion chromatography with triple detector array, capillary electrophoresis, mono and bi-dimensional NMR. Furthermore the biological activity was assessed on *in vitro* human synoviocyte and chondrocyte primary cell models. Most of the samples (over 19 out of 25) showed lower condroitin sulfate and glucosamine contents than the declared ones (up to -60.3%) while all of them showed a KS contamination (up to 47.1%). Mixed animal origin chondroitin sulfate and multiple molecular weight species were determined in more than 32% of the samples. Only 1 on 5 biologically screened samples had an effective action in *vitro* almost comparable to the pharmaceuticals.

1. Introduction

Osteoarthritis is a common, progressive, degenerative joint and musculoskeletal disease, that involves a mechanical overloading of the articular joints, loss of cartilage and synovial fluids, bone degradation, inflammation, reduction of mobility and quality of life of 40 million Europeans over 50 years (Chevalier & Conrozier, 2017; Conaghan, Kloppenburg, Schett, & Bijlsma, 2014). It also involves the activation of cell signaling and inflammation pathways, as the one mediated by NF-kB whose expression is correlated with the pro-inflammatory IL-6 and IL-8 induction (Goldring & Otero, 2011; Roman-Blas & Jimenez, 2006). Pharmacological therapy includes analgesics or non-steroidal anti-inflammatory drugs (Conaghan et al., 2014) or, in alternative to them, chondroitin sulfate (CS) and glucosamine (GlcN) as anti-osteoarthritis agents. CS is a glycosaminoglycan (GAG) that occurred in the extracellular matrix of the animal cartilaginous tissues. It is made of 4)−β-GlcA-(1→3)−β-GalNAc-(1→ disaccharide repeating units (GlcA = glucuronic acid, GalNAc = *N*-acetyl-galactosamine). CS has variable molecular weights (Mw), types and grades of sulfation according to the origin of the animal tissue or to the age of the animal (Collin et al., 2017). In the same chain non-sulfated disaccharides (di-0S CS) might coexist with monosulfated (di-4S CS or di-6S CS) and disulfated units (di-2,4S CS or di-2,6S CS or di-4,6S CS) (Shi et al., 2014). CS from terrestrial animal cartilages (*e.g.* bovine, pig, chicken) has lower Mw (14–26 KDa) and higher di-4S CS percentages than the CS from marine organisms (*e.g.* shark, skate, *etc.*) that has higher Mw (30–80 KDa) and more di-6S CS and di-sulfated units (Restaino, De Rosa, Cimini, & Schiraldi, 2013). GlcN is naturally produced in the human body as precursor of glycosylated lipids, proteins, glycosaminoglycans and proteoglycans. It could be obtained by extraction of chitin from crustacean shells followed by both alkaline or acid hydrolyses or by *de novo* chemical synthesis and it is commercialized as stabilized salt of glucosamine hydrochloride (from crab shell) or glucosamine sulfate (from shrimp shell) (Agiba, 2017). CS and GlcN are combined in pharmaceutical preparations (Ph) and in food supplements (FS) to be orally administered as anti-ostearthritis active principles even

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; IL, 6-Interleukin 6-; IL, 8-Interleukin 8; NF, kB-Nuclear factor kappa-light-chain-enhancer of activated B cells; PBS, Phosphate-buffered saline; $^{1}H-^{13}C$ DEPT−HSQC, distortionless enhancement by polarization transfer − heteronuclear single quantum coherence

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in long term therapy. They are nowadays recommended by the European League Against Rheumatism (EULAR) and by the European Society for Clinical and Economic aspects of Osteoporosis and Osteoarthritis and Musculoskeletal Disease (ESCEO), as symptomatic slow-acting molecules (SYSADOA) for the treatment of knee, hand or hip osteoarthritis, providing pain relief of the symptoms and increasing joint mobility (Bruyere et al., 2014; Zhang et al., 2005). Differently from the non-steroidal anti-inflammatory drugs, CS and GlcN alone or in combination have also a modifying structure/disease action (DMOADs-disease-modifying osteoarthritis drug), called chondro-protective effect, that may induce a correction of the degenerative disorder of the connective tissues by supporting new cellular growth at bone and cartilage levels and by inhibiting both the cytokines and the metalloprotease inflammation activity and the degradative enzymes (Michel et al., 2005). The daily CS and GlcN recommended dosages are between 800 to 1200 mg and between 1250 to 1500 mg respectively. As food supplements the CS and GlcN global market counts 1 billion dollar annual sales and it has a further 15% growth potentiality per year, due to newly formulated, registered and commercialized preparations in combinations with plant extracts like *Boswella serrata* or *Harpagofitum*, vitamins D or C, hyaluronic acid (HA), collagen, curcumina and methylsulfonylmethane (MSM) (Reddy, 2013). The biological activity of these food supplements could greatly vary according to 1) the CS and GlcN quantity and purity 2) the CS structural heterogeneity (*e.g.* different molecular weight, type and grade of sulfation pattern, mixed sourced origin), 3) the presence of contaminants or adulterants. Infact recent papers have demonstrated that the animal sourced CS could be contaminated by another glycosaminoglycan, the keratan sulfate (KS), that is present in the animal articular cartilaginous tissues as well and that could be contemporarily extracted with the CS (Restaino et al., 2017). Since the KS has a structure similar to the CS, having a 4)−β-GlcNAc-(1→3)−β-Gal-(1→ disaccharide repeating unit (GlcNAc = *N*-acetyl-glucosamine; Gal = galactose) and being di-sulfated, with a Mw ranging from 10 to 40 KDa, it could be difficult to selectively remove the KS in the CS purification process (Funderburgh, 2000; Pomin, Piquet, Pereira, & Mourão, 2012). So far only highly-purified pharmaceutical preparations have undoubtedly demonstrated clinical efficacy and have been recommended by ESCEO for a safe therapy at the initial state of osteoarthritis (Bruyere et al., 2018). Thus different therapeutic effects in clinical trials obtained by FS may be attributed to the above mentioned CS and GlcN structural variablity or possible precence of contaminants due to low standardized manufacturing processes (Agiba, 2017; Da Cunha et al., 2015; Martel-Pelletier, Farran, Montell, Vergés, & Pelletier, 2015). Since the CS and GlcN food supplements are not obliged by any national health regulators to satisfy the same mandatory quality controls of pharmaceuticals before being commercialised, the uncertainties concerning the FS biological activity are high (Chevalier & Conrozier, 2017). Thus there is a specific scientific interest to comparatively analyse CS pharmaceuticals and FS to provide clear structural data to support the FS biological activity (Chevalier & Conrozier, 2017). No official methods are reported in the European Pharmacopeia (Eu. Ph.) for FS analyses (European Pharmacopeia, 2009) while new, updated analytical strategies are needed (Bruyere et al., 2018; Chevalier & Conrozier, 2017). Besides the CS natural structure heterogeneity and the risk of KS contamination, the analytical challenges of characterizing FS is related to the presence of other contaminants coming from to the extractive procedures (*e.g.* organic solvents, surfactants and proteins) (Chevalier & Conrozier, 2017). In the worst case they could be intentionally adulterated by the addition of cheaper polysaccharides, like algal polymer carrageenan, in order to lower their cost (Agiba, 2017; Da Cunha et al., 2015; Martel-Pelletier et al., 2015; Volpi & Maccari, 2008, 2009). In literature the amount and the quality of CS in food supplements, but not of GlcN, have been determined by agarose-gel electrophoresis of the intact chain coupled with densitometric analyses. The CS origin was determined by analyzing the disaccharide composition by SAX-HPLC after chondroitinase digestion, while the CS molecular weight has been obtained by high performance size exclusion chromatography (HP-SEC) (Volpi & Maccari, 2008, 2009). To date the contemporary determination of CS and GlcN, and of the KS in the FS samples have not been reported. Since the quality of CS based product may affect the efficacy of clinical treatments, in this paper we present a multi-analytical systematic protocol to assess the quality of CS and GlcN in 25 European food supplements, and data were compared with 2 CS pharmaceuticals. The protocol includes a new application of a high performance anion exchange chromatography method with pulsed amperometric detection (HPAE-PAD) for CS, GlcN and KS content determination, mono or bi-dimensional NMR analyses and the determination of the FS insoluble part, the protein and metal content. It also included the CS molecular weight analysis by size exclusion chromatography with triple detector array (SEC-TDA), CS animal origin determination by high performance capillary electrophoresis (HPCE) after chondroitinase digestion. Biological activity assays were performed on two *in vitro* models based on human synoviocytes and chondrocytes cells from osteoarthritic joints.

2. Material and methods

2.1. Materials

Sodium nitrate, sodium acetate, Tris, sodium tetraborate, sodium dodecyl sulfate (SDS), sodium hydrogen phosphate were purchased from Sigma-Aldrich (USA), as well as *N*-acetyl-galactosamine, glucosamine, galactose and glucuronic acid and the ABC chondroitinase from *Proteus Vulgaris* used in the enzymatic reaction. The NaOH solution was from *J*.T. Baker (The Netherlands). The hydrochloric acid was from Carlo Erba (Italy). The medium and enzymes used in the biological experiments were from Gibco, Invitrogen (USA), unless differently specified. The six commercially available CS standards were purchased from Sigma-Aldrich (USA). The di-0S and di-4S CS disaccharide standards, used in HPCE analyses, were from Sigma-Aldrich (USA), the di-6S and di-2S CS disaccharide standards were from Santa Cruz Biotechnology (Texas, USA), the di-2,6S, di-2,4S, di-4,6S CS disaccharide standards were purchased from Iduron (UK). Purified bovine, pig, avian and shark CS samples, as well as purified HA and KS to be used as reference material in disaccharide composition analyses were obtained by SAX chromatography according to a previously described method (Restaino et al., 2017). The 25 commercially available food supplements, containing both CS and GlcN, in the forms of capsules or tablets or powder, and the 2 pharmaceuticals (Ph), containing only CS, in the forms of capsules, were from 8 different European countries and 10 different companies (indicated as Company #1, #2, #3) and they were either purchased or obtained as test/gift samples. Their compositions, as described in the labels, are reported in Table 1.

2.2. Insoluble solids and protein content determination

In order to evaluate the insoluble fraction and the protein content in the FS and Ph, 1 capsule or 1 tablet or 1 g of powder of each sample was dissolved in 20 ml of milliQ water in triplicate. Solutions were stirred for 18 h at 300 rpm and then let over night to sediment. The samples were centrifuged at 6500 rpm for 30 min (Avanti J20-XP, Beckman Coulter, USA) and the supernatants were separated from the insoluble fractions that were dried at 40 °C for 24 h in an oven (Binder GmbH, Germany) and then weighed. The insoluble solid weights were calculated as the ratio of the insoluble fraction on the total weight, in percentage. The supernatants were filtered on 0.22 μm filters (Millipore, France) and the protein content was determinated with a colourimetric method (Bradford, 1976) by using the Kit Protein assay Biorad (Bio-Rad Laboratories Inc, USA) and the bovine serum albumin (BSA) as standard (Bio-Rad Laboratories Inc, USA).

Table 1
Composition of the 25 FS and the 2 Ph as reported on their labels.

European Country	Company	Food supplements	CS (mg/unit)	GlcN (mg/unit)	HA (mg/unit)	Type II Collagen	Vegetable extracts	MSM	Vitamins	Others (mg/unit)
France	Company #1	FS1	150	500	–	x	–	x	Vit C	–
		FS2	50	375	–	–	*Harpagofitum*	x	–	–
		FS3	300	375	–	–	*Harpagofitum*	–	–	–
		FS4	444	526	7	–	*Harpagofitum*;Curcumine	x	–	
Slovakia	Company #2	FS5	200	500	20	x	–	x	Vit C	–
		FS6	50	750	–	x	Curcumine; *Boswella Serrata*; *Salix alba*	–	Vit C	Mn = 0.3
		FS7	200	500	18	x	–	x	Vit C	–
Spain	Company #3	FS8	300	375	–	–	*Harpagofitum*	–	–	–
		FS9	1200	1500	–	x	–	–	Vit C	Mn = 2 ; Cu = 1.1
Switzerland	Company #2	FS10	365	416	–	–	–	–	–	–
		FS11	166	250	–	–	–	–	Vit C	Cr = 0.02, Mn = 2, Cu = 0.5
		FS12	166	410	–	–	–	–	Vit C	Mn, Cu = contents not declared
Hungary	Company #2	FS13	200	500	–	–	–	x	–	–
		FS14	200	600	–	–	–	–	Vit C ; Vit D	–
		FS15	200	500	–	–	–	x	Vit C	–
Austria	Company #4	FS16	250	300	–	–	Rosehip	x	Vit C; Vit E; Vit D3	Mn = 1 ; Se = 0.05
		FS17	250	400	–	x	–	–	–	–
Greece	Company #5	FS18	400	500	–	–	Curcumine	–	Vit C	M n = 2
		FS19	110	665	–	–	–	–	–	–
		FS20	110	750	–	–	–	–	–	–
Italy	Company #6	FS21	400	500	–	x	–	–	–	L-carnitine = 200
	Company #7	FS22	400	415	–	–	–	–	–	–
		FS23	400	500	–	–	Curcumine	–	–	–
	Company #8	FS24	440	500	–	x	–	–	Vit C	Bromeline = 100
	Company #9	FS25	400	290	–	–	*Boswella Serrrata*	–	Vit C ; Vit D	M n = 1

European Country	Company	Pharmaceuticals	CS (mg/unit)
France	Company #10	Ph1	400
Switzerland	Company #2	Ph2	400

2.3. Analyses of metals

The heavy metal content in terms of mg/kg in the 25 FS and the 2 Ph were performed according to the UNI EN ISO 17294-2:2016 method; samples were subjected to oxidative digestion with 10 ml of HNO_3 + 2 ml of H_2O_2 + 2 ml of HCl at high temperature and pressure assisted by microwave (Mars-CEM). The mineralized samples were analysed by inductively coupled plasma mass spectrometry (Aurora M90 Bruker) and Be, Al, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Cd, Sb, Ba, Hg, Pb metals were determined. The quality of the data was ensured by the use of certificate reference material (SRM 3280, NIST). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the method of blank variability for each investigated metal (Pagano, Thomas, Di Nunzio, & Trifuoggi, 2019).

2.4. Structural characterization by NMR spectroscopy

^1H spectra were recorded using a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe at 298 K. The heteronuclear experiments ($^1H-^{13}C$ DEPT–HSQC) were performed using standard pulse sequence available in the Bruker software. Chemical shifts were measured at 298 K in D_2O.

2.5. Monosaccharide composition analyses by HPAE-PAD

The CS and GlcN content determination and the eventual KS contamination in FS and Ph were performed on the basis of the monosaccharide composition by HPAE-PAD analyses (ICS3000, Thermo Fisher Scientific, Italy). In particular aliquots of the supernatants (2 mL) that contained soluble components (*i.e.* CS, GlcN and KS), obtained as described in 2.2 paragraph, were ultra-filtered on 3 KDa filter devices (Sartorius Stedim, Germany) at 6000 rpm and at 4 °C (Avanti J20-XP, Beckman Coulter, USA). The permeate was collected and the retentate was diafiltered with two volumes of milliQ water. The permeate contained only the GlcN as free monosaccharide while the retentate contained heteropolysaccharides. The supernatants and the retentates were hydrolyzed according to a previously reported method (Restaino et al., 2017; Squillaci et al., 2015) in order to determine the CS content and the KS contamination on the basis of GalNAc and GlcN concentrations, respectively. The permeate samples were analyzed without hydrolysis for the determination of the GlcN content. Analyses were performed slightly modifying the method previously reported (Restaino et al., 2017). Specifically quantification was obtained on the basis of three calibration curves namely hydrolyzed GalNAc and GlcN standards and not hydrolyzed GlcN standard in a concentration range from 8.0 to 1.0 $ng{\cdot}\mu L^{-1}$. The percentage differences of the CS and GlcN contents, compared to the declared ones, were calculated according to the following formulas %CS = [declared CS (mg)- analysed CS (mg)/ declared CS (mg)]·100; %GlcN = [declared GlcN (mg)- analysed GlcN (mg)/ declared GlcN (mg)]·100. The percentage of KS contamination was calculated according to the following formula: $\%KS = [CS(g{\cdot}L^{-1})/(CS(g{\cdot}L^{-1}) + KS(g{\cdot}L^{-1}))]{\cdot}100$. In the three samples containing HA the hypothetical contribution of the GlcN coming from the hydrolysis of this polysaccharide was subtracted from the KS value.

2.6. Disaccharide composition analyses by HPCE after enzymatic digestion

The 25 FS, the 2 Ph, the 6 commercially available CS standards and the CS, KS, and HA samples, purified in our lab by SAX chromatography, were dissolved, in duplicate, in milliQ water in order to have a theoretical polysaccharide concentration of 10 $mg\,ml^{-1}$. Then they were centrifuged at 13,000 rpm and at 4 °C for 10 min (Avanti J20-XP, Beckman Coulter, USA) to remove the insoluble solid parts and the supernatants were enzymatically digested with chondroitinase ABC,

according to a previously described method (Volpi, 2004). Capillary electrophoresis analyses of the digested samples were performed according to a method previously reported (Volpi, 2004) by employing a HPCE instrument (P/ACE MDQ, Absciex, USA), equipped with a deuterium lamp and a photo diode array detector, with an un-coated fused-silica capillary (50 μm I.D., 70 cm of total length, 60 cm of effective length, Absciex, USA). The disaccharide peaks of the digested samples were identified by comparing their migration times with the CS disaccharide standard ones. The percentage of each disaccharide on the total was determined according to the following formula %dis-CS = [Peak Area dis-CS/ (Σ Peak Area all dis-CS)] ·100; the ratio of dis-4S/dis-6S CS was calculated according to the following formula dis-4 s/dis-6S CS = [Peak Area dis-4S/Peak Area dis-6S].

2.7. CS molecular weight analyses by SEC-TDA

The CS molecular weight analyses of the 25 FS and 2 Ph were performed according to a method previously described (Restaino et al., 2017) by using a high performance size exclusion chromatographic system (Viscotek, Malvern, UK), equipped with a triple detector array module including a refractive index (RI), a four-bridge viscosimeter (VIS) and two laser right-angle light scattering (RALS) and low-angle light scattering (LALS) detectors. An OmniSEC program was used for data acquisition and analyses. The molecular weight and size distribution, the polydispersity (Mw/Mn), the hydrodynamic radius of the major peak of RI signals were calculated by using the $dn \cdot dc^{-1}$ values of 0.147 $mL \cdot g^{-1}$ for CS and of 0.100 $mL \cdot g^{-1}$ for KS (Restaino et al., 2017), by using the following equations: $RI = K_1 \cdot dn/dc \cdot C$; $VIS = K_2 \cdot [\eta] \cdot C$; $LALS = K_3 \cdot M_w \cdot (dn/dc)^2 \cdot C$; where [η] is the intrinsic viscosity (dl/g), C is the mass concentration (mg/ml), dn/dc is the refractive index increment (ml/g) and K_1, K_2, and K_3 are instrumental constants. The representative of each peak in percentage was calculated as the percentage ratio between the single peak area divided by the sum of areas of all the peaks in each chromatogram.

2.8. Food supplement biological activity

2.8.1. Cell models, cell growth and proliferation studies using time lapse video microscopy

Primary cell isolation was obtained with a slight modification of the published protocol (Stellavato, De Novellis, Reale, De Rosa, & Schiraldi, 2016). Specifically, synoviocyte and chondrocytes obtained as previuosly described (Stellavato et al., 2019, under revision) were treated with diluted FS and Ph preparation (Sampler FS1, FS3, FS10, FS11, FS25, Ph1, Ph2) to obtain a concentration of 1.5 mg mL-1. Cell viability and proliferation were evaluated through time lapse videomicroscopy (TLVM) up to 96 h (Stellavato, Corsuto et al., 2016).

2.8.2. Western blotting analyses for NF-kB

Western blotting analyses were performed only on the best perfoming samples in cell viability tests (Ph1, Ph2 FS 25) in order to analyze expression of NF-kB as a biomarker of inflammation in chondrocytes. 2 μg of intracellular proteins were electrophoretically resolved on 10% SDS-PAGE (Stellavato, Tirino et al., 2016) and the separated proteins were transferred to nitrocellulose membrane (GE, Amersham, UK) and blocked with 5% non-fat milk in Tris-buffered saline and 0.05% Tween-20 (TBST) (Lonza, Italy). To detect NF-kB, a primary antibody (Santacruz Biotechnology, USA) was used at 1:500 dilutions and incubated for 2 h at room temperature. Immunoreactive bands were detected by chemioluminescence using corresponding horseradish peroxidase-conjugated secondary antibody (Santacruz Biotechnology, USA), diluted 1:10,000 for 1 h at room temperature and reacted with an enhanced chemiluminescence detection system (ECL) (Millipore, France). Normalization was performed compared to the signal obtained with an anti-Actin housekeeping protein (1:500 dilution) (Santacruz Biotechnology, USA), using the Gel Doc 2000 UV System (Bio-Rad Laboratories, USA) according to the manufacturer's protocols.

2.8.3. Quantification of IL-6 production using ELISA assay

IL-6 protein was assayed in synoviocytes treated with FS or Ph samples by using an ELISA Kit following the manufacturer's instructions (Boster antibody and ELISA experts, Tema Ricerca Italy). Briefly, supernatants were transferred in duplicate to microtitre plates and a specific biotinylated detection polyclonal antibody was added subsequently, followed by washes with PBS buffer. Avidin-Biotin-Peroxidase complex (Boster antibody and ELISA experts, Tema Ricerca, Italy) was added and unbound conjugates were washed away with PBS buffer. Horseradish peroxidase substrate (Boster antibody and ELISA experts, Tema Ricerca, Italy) was used to visualize the enzymatic reaction producing a blue colour product that changed into yellow after adding an acidic stop solution. The optical density was measured by a microplate reader (Bio-Rad Laboratories, USA) at 450 nm and concentrations ($pg \cdot mL^{-1}$) were extrapolated using a calibration curve provided by the kit.

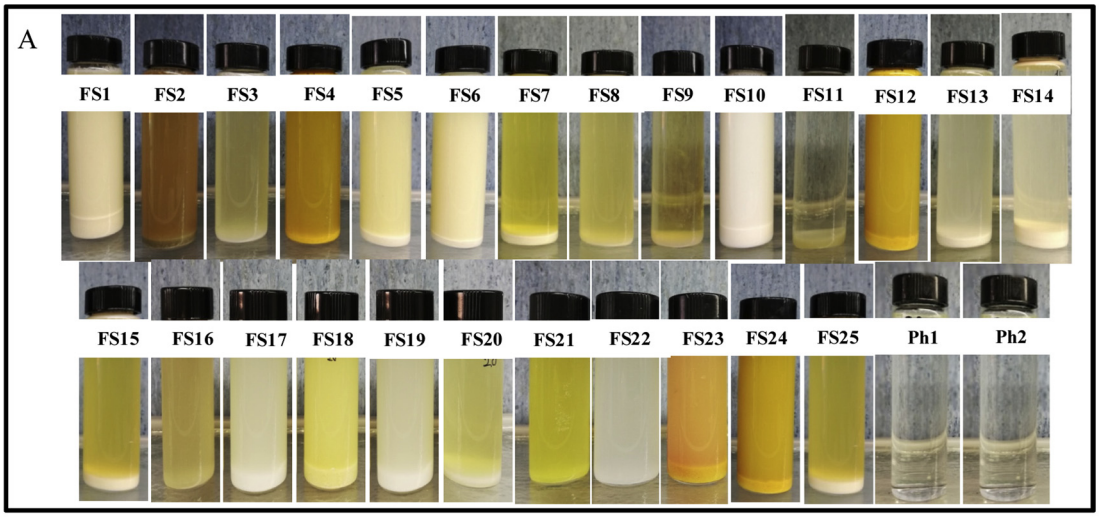
3. Results

3.1. Insoluble solid determination, protein content and metal analyses

The 25 FS and the 2 Ph samples were dissolved in milliQ water, as described above, and let over night to sediment. Pictures of the sedimented FS samples showed that the supernatants presented different colours, from trasparent white or yellow to opaque white, or pink-orange to dark brown (Fig. 1 A). These differences were clearly due to the various components and diverse vegetable extracts present in the FS, while, in comparison, the Ph samples were completely transparent (Fig. 1 A) The FS showed also diverse amounts of precipitated insoluble solids as sedimented material on the bottom of the vials that were in the range from 0.5 to 37.4% of the total weight (Fig. 1 B). 19 samples had values over the 10% (w/w) and 13 sample over the 20% (w/w). The protein content was declared only on the label of 7 FS (FS1, FS5, FS7, FS9, FS16, FS21, FS24) on 25 (Table 1), but our analyses demonstrated that it was lower than the declared one up to 42.5%. In the other 18 FS a protein content, in the range from 0.08 to 5.2 mg/dose, was found, although it was not declared on the labels (data not shown). All the FS showed a heavy metal (Pb, Cd, Hg, As) content lower than the legislation constraints (European pharmacopeia, CE N.1881/2006, CE N.629/2008). The concentrations of the other metals (Be, Al, V, Cr, Mn, Co, Ni, Cu, Zn, Se, Sb, Ba) were under the method detection limit. Manganese, copper, zinc, chrome and selenium were present in seven samples (FS6, FS9, FS11, FS12, FS16, FS18, FS25), as declared on the labels (Table 1), but they resulted to have concentrations from 38.0% to 92.0% lower than the claimed ones. The Ph samples presented metal contents below the detection limits (data not shown).

3.2. Structural characterization by NMR spectroscopy

The FS were analysed, as samples in their entirety, by ^1H and DEPT-HSQC 2D NMR and compared with the spectra of the Ph (Fig. 2). As expected, signals of the GlcN presence were found in the FS samples, differently from the pharmaceutical grade products that contained only CS. The analyses allowed to determine the Glc/CS molar ratios by relative integration of CS CH_3CO signal at δ 1.98 ppm *vs.* α- + β-GlcN signals at δ 5.44 and 4.92 ppm, respectively, in ^1H-NMR spectrum. Some FS samples showed a ^1H-NMR spectrum with the CS CH_3CO signal overlapped by the contaminant peaks; in this case a relative integration of CS GalNAc H-1 signal at $\delta_{H/C}$ 4.52/101.8 ppm *vs.* α- + β-GlcN signals δ 5.44/90.2 and 4.92/94.0 ppm, respectively, in DEPT-HSQC 2D-NMR spectrum, was performed. The GlcN/CS molar ratios varied from 2 to 24. The 4S/6S CS ratios might be also measured by DEPT-HSQC 2D NMR, by relative integration of 4-sulfated GalNAc *O*-4 CH signal at $\delta_{H/C}$



B	FS1	FS2	FS3	FS4	FS5	FS6	FS7	FS8	FS9	FS10	FS11	FS12	FS13	FS14
% Insoluble solids (w/w)	37.0	30.0	4.0	16.0	21.3	27.0	30.5	4.6	0.5	37.0	19.6	32.0	26.6	37.4
	FS15	**FS16**	**FS17**	**FS18**	**FS19**	**FS20**	**FS21**	**FS22**	**FS23**	**FS24**	**FS25**	**Ph1**	**Ph2**	
	30.0	11.4	33.8	21.2	15.4	9.7	37.0	30.0	4.0	16.0	21.3	-	-	

Fig. 1. Pictures of the 25 FS and 2 Ph solutions in milliQ water (A): an insoluble portion is visible as undissolved, precipitated material. The table reports the percentages of insoluble solids for each sample (B).

4.72/77.1 ppm *vs.* 6-sulfated GalNAc *O*-6 CH_2 signal at $\delta_{H/C}$ 4.20/67.7 ppm (Bedini et al., 2011). The ratio varied from 0.05 to 1.78 in the samples. In some cases the content of CS and GlcN was so low that it was difficult to determine it as well as the 4S/6S CS ratio. Also the signals of an eventual KS contamination were difficult to be precisely determined. In many samples traces of aliphatic compounds were found. It could be hypothesized that they are due to the presence of oils and/or grease as contaminants of the and CS and GlcN purification processes.

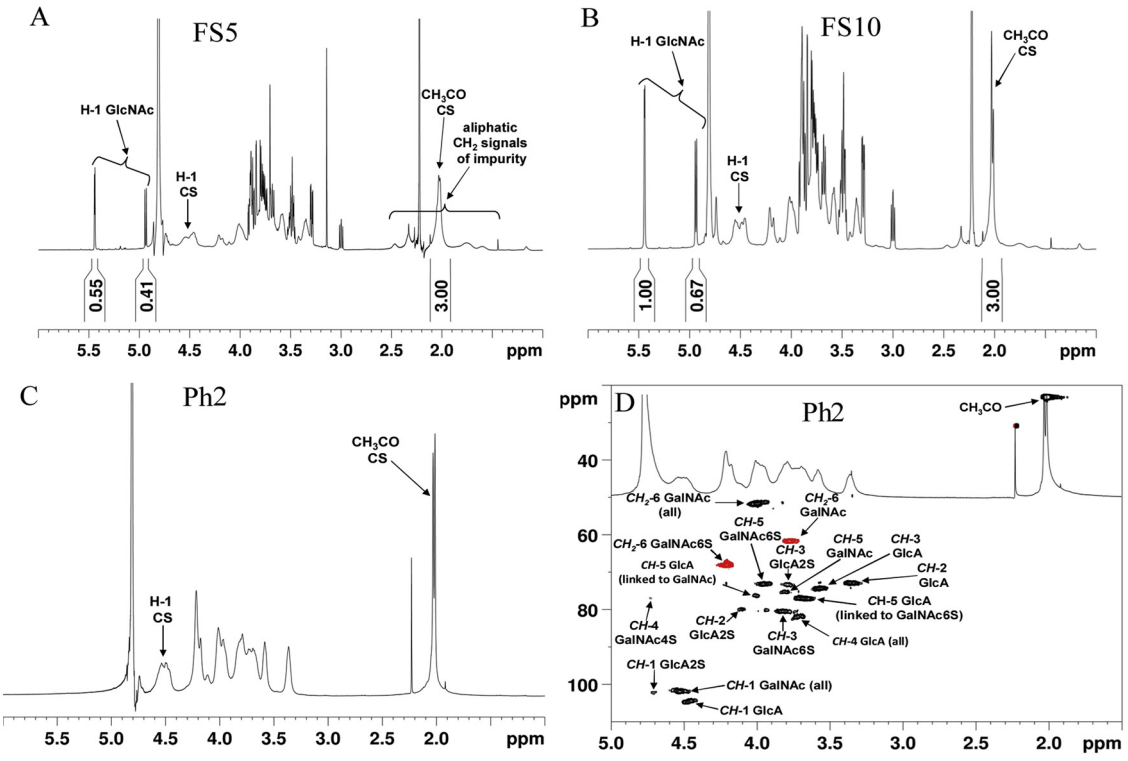


Fig. 2. ^{1}H NMR spectra (600 MHz, D_2O, 298 K) of FS5 (A), FS10 (B) and Ph2 (C). ^{1}H-^{13}C DEPT-HSQC spectrum (zoom, 600 MHz, D_2O, 298 K) of Ph2 (D).

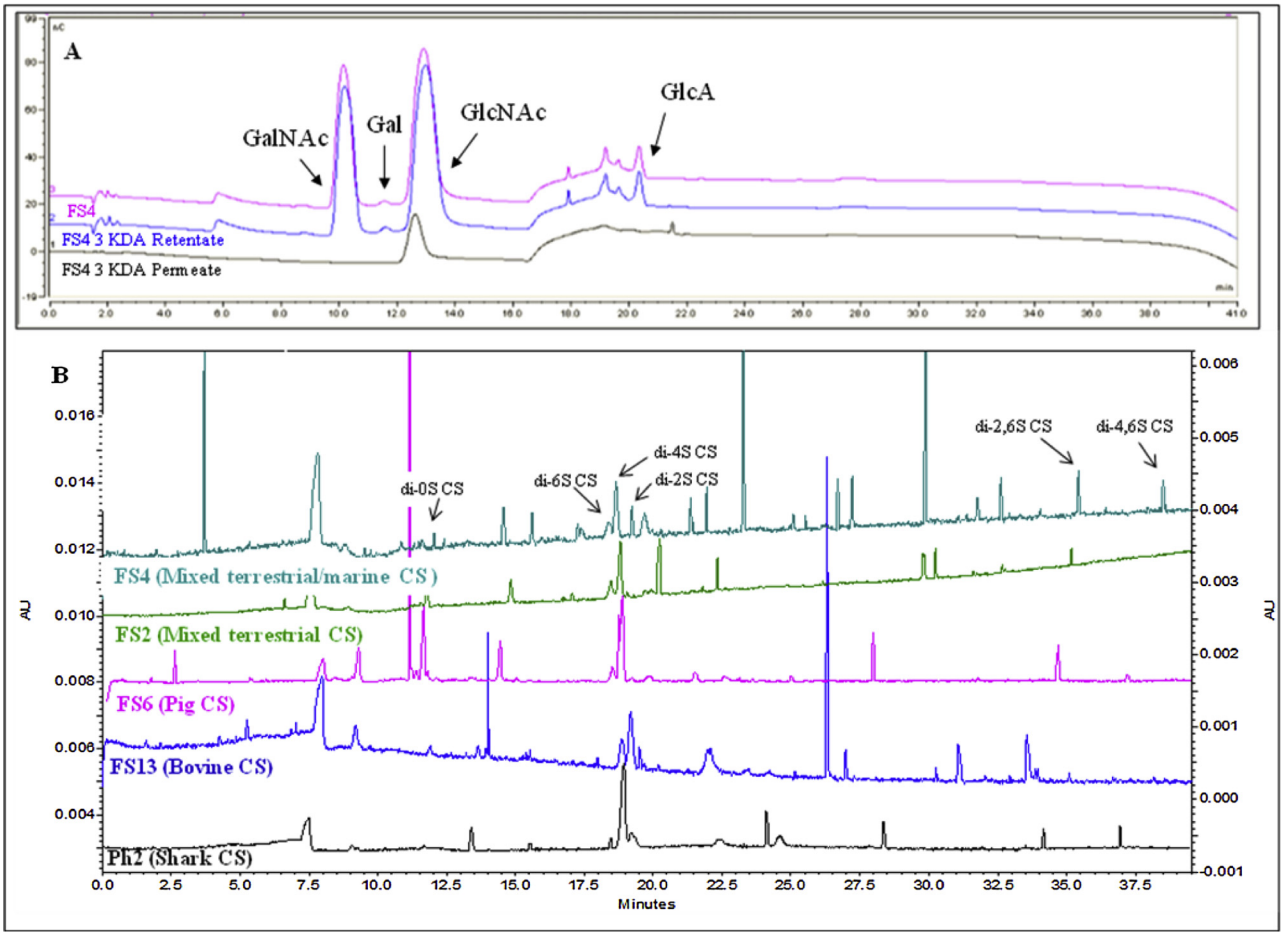


Fig. 3. Representative HPAE-PAD chromatogram of a FS (FS4) containing CS, glucosamine and the KS contaminant (pink line), overlaid with the chromatograms of its retentate after 3 KDa membrane ultrafiltration and acid hydrolysis (blue line) and of its not-hydrolysed permeate (black line) after 3 KDa membrane ultrafiltration (A). Representative HPCE analyses of the disaccharide composition of FS and Ph, after digestion with chondroitinase: Ph2 (Shark CS-black line), FS13 (Bovine CS-blue line), FS6 (Pig CS-pink line), FS2 (Mixed terrestrial CS-light green line) and FS4 (Mixed terrestrial/marine CS-green line) (B).

3.3. Monosaccharide composition analyses by HPAE-PAD

The CS and GlcN contents and the eventual keratan contamination of the 25 FS and 2 Ph were determined by HPAE-PAD. Initially the samples in their entirety were analyzed after acid hydrolysis. All of them showed the peaks of GalNAc and GlcA, representative of the CS and the peak of the GlcN. However the presence of a Gal peak indicated a KS contamination. That meant that in the samples in their entirety, the GlcN peak was the sum of both the free GlcN monosaccharide content and the GlcN derived from the hydrolysis of the KS polysaccharide (Fig. 3 A). The samples were ultra-filtered and the permeates were analyzed to quantify the free GlcN content that was higher than the declared ones in 6 out of 25 samples, in a range from 3.8 to 15.0%, and lower in 19 out of 25 samples, in a range from -10.0 to -60.3% (Fig. 3A, Fig. 4 A). As expected no free GlcN was found in the Ph. The retentate samples were hydrolyzed and then analyzed to determine the CS and KS contents (Fig. 3 A). In 24 FS samples on 25 the CS content was lower than the declared one in a range from -1.1 to -45.0% (Fig. 4 B). Only in one case was it higher. In all the FS a KS contamination was found ranging from 10.0% to 47.1% (Fig. 4 C). It has to be pointed out that in FS4, FS5, FS7, the HA was present in percentages of 1.5, 9.0 and 10.0% of the CS content, according to the label. HA produces GlcN and GlcA monosaccharides, when hydrolyzed. This was taken into consideration for the final calculation of KS contamination. The CS content was the same in the samples in their entirety and in the retentates, while the sum of the GlcN concentrations in the permeate and in the retentates was identical to the GlcN concentration determined in the samples in their entirety. The CS contents in the Ph samples were consistent to the declared ones (difference between -1.5 and -2.0%) and very low KS contaminations were found (Fig. 4 B and C).

3.4. Disaccharide composition analyses by HPCE after enzymatic digestion

To determine the CS animal origin the FS and Ph samples were enzymatically digested and their disaccharides were analyzed by HPCE in terms of un-sulfated (di-0S CS), mono-sulfated (di-2S CS; di-4S CS; di-6S CS) and di-sulfated disaccharides (di-2,4S CS; di-2,6S CS; di-4,6S CS), and the di-4S/6S CS ratios were calculated. The disaccharide profiles were compared with literature data (Lauder, Huckerby, & Nieduszynski, 2000; Maccari, Galeotti, & Volpi, 2015; Volpi, 2004) and with the disaccharide profiles of the CS Eu. Ph. and commercial standards of the purified bovine, pig, avian and shark CS samples experimentally obtained in our lab. Small disaccharide percentage variations were found in standards having the same CS animal origin: for example the bovine Eu. Ph. CS standard had 6.39% of di-2S CS that was not present in the commercial bovine cartilage or trachea CS standards, while these last two ones showed wide 4S/6S CS ratio differences (2.28 *vs* 1.72) (Table 2). Compared to Eu. Ph. marine CS standard, commercial shark CS standards showed also small percentages of di-0S CS (2.10 and 3.73%) and a high presence of di-2S CS (13.4%). Two additional profiles of pig and avian CS disaccharides were obtained by analyzing the purified CS samples (Table 2). In all the standards and purified CS samples no di-sulfated disaccharides were found. Because HA was present in three FS and all the FS contained also KS, purified HA and KS

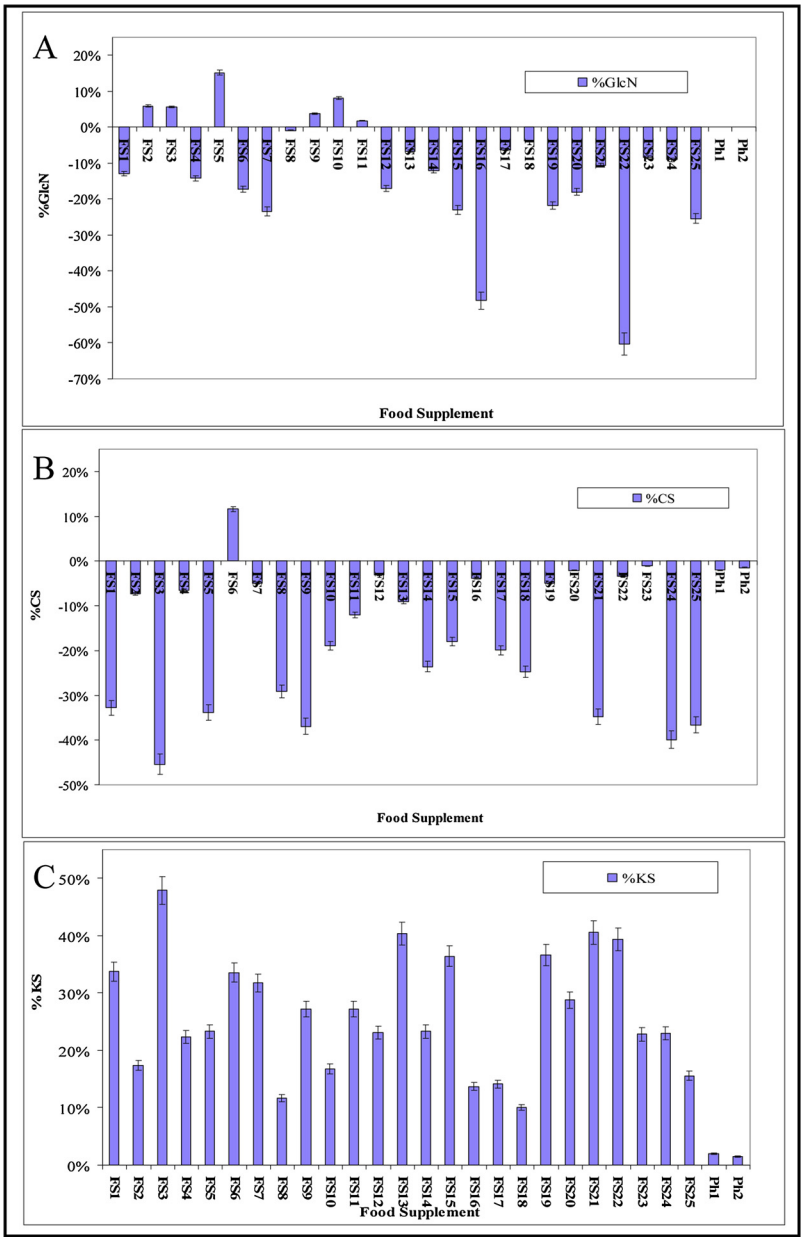


Fig. 4. HPAE-PAD analysis of the monosaccharide composition of the 25 FS and of the 2 Ph after acid hydrolysis: differences in percentage of GlcN content (A) and of CS content (B) compared to the declared values and percentage of the KS contamination (C).

polysaccharides were also enzymatically digested: as expected, the HA profile presented only di-0S disaccharides while the KS was not digested by the ABC chondroitinase (data not shown). 12 FS showed terrestrial disaccharide patterns, confirmed also by the di-4S/6S CS ratios that ranged from 1.16 to 1.63 and from 4.26 to 5.73 for bovine and pig CS respectively (Fig. 3 B, Table 3). 13 FS, instead, showed mixed bovine and pig compositions, although two of them (FS3 and FS8) might also be of avian origin (Fig. 3 B, Table 3). 4 FS presented mixed terrestrial and marine CS origin because of the presence of 2-sulfated and di-sulfated disaccharides. Although small percentages of 2-sulfated disaccharides (lower than 9.0%) were found also in two pig CS (FS6 and FS12) and in almost all the mixed terrestrial CS, the di-sulfated disaccharides clearly indicated a marine origin. Besides, in FS of mixed origin the dis-4S/6S CS ratios had intermediate values between the ratios of pure marine and terrestrial CS, ranging from 2.03 to 3.92 in mixed terrestrial samples and from 2.44 to 4.21 in mixed terrestrial and marine CS samples respectively. In FS where the HA was present, the value of the un-sulfated disaccharides (that included the di-0S CS and the diastereoisomer di-0S HA) was not higher than 9.0%, in agreement with the declared HA contents (Table 3). A pig-like and a fish-like CS disaccharide profile were found in the 2 Ph samples, with di-4S/6S CS ratios of 5.32 for pig CS and 0.58 for shark CS (Table 3).

3.5. CS molecular weight analyses by SEC-TDA

SEC-TDA analyses were performed to determine the CS Mw and its polydisperdity in all the FS and Ph samples (Fig. 5, Table 3). Diverse Mw values were noted in samples having the same animal origin while 8 FS showed more than one Mw specie. The bovine CS Mw ranged from small chains of 8.45 KDa up to 42.90 KDa (FS25 and FS23, Fig. 5, Table 3). The pig CS FS ranged from 11.28 KDa (FS17) to 44.20 KDa (FS6). Too high a Mw value for a pig CS sample was found in FS12 (78.0 KDa), while in FS9 a complex chromatographic profile was hampering the Mw determination (Table 3). In a mixed terrestrial (FS2) and terrestrial/marine CS (FS5) two different Mw peaks were found whose values clearly confirmed the identification of a mixed bovine and pig or

Table 2
CS disaccharide profiles of different animal origin: data reported in literature (A) and data experimentally obtained in our lab after enzymatic digestion of Eu. Ph. and commercial CS standards and purified CS samples (B).

A)

Literature data	Volpi, 2004				Maccari et al., 2015		Lauder et al., 2000
% CS dis	Bovine CS	Pig CS	Chicken CS	Shark CS	Tuna CS	Salmon CS	Whale CS
dis -0S CS	7.10	2.90	9.00	0.50	1.60	7.70	–
dis -6S CS	35.60	16.30	20.60	41.90	28.30	37.30	29.50
dis -4S CS	57.30	80.80	70.40	30.70	63.20	51.20	54.00
dis -2S CS	–	–	–	–	traces	traces	–
disulfated	–	–	–	21.00	6.90	3.80	0.30
dis 4S/6S CS	1.61	4.96	3.42	0.73	2.23	1.37	1.83

B)

Experimental data	Eu. Ph.	Sigma-Aldrich	Sigma-Aldrich	Eu. Ph.	Fluka	Sigma-Aldrich	CS purified by SAX chromatography			
% CS dis	Bovine CS	Bovine cartilage CS	Bovine trachea CS	Marine CS	Shark CS	Shark CS	Bovine CS	Pig CS	Avian CS	Shark CS
dis -0S CS	5.66	5.160	4.86	–	1.79	3.73	4.02	3.83	6.65	4.01
dis -6S CS	33.38	28.93	34.96	60.57	62.35	56.21	40.83	14.93	22.58	63.78
dis -4S CS	54.57	65.91	60.19	39.43	20.99	27.13	55.15	81.24	70.77	32.21
dis -2S CS	6.39	–	–	–	–	13.44	–	–	–	–
dis -2,6 CS	–	–	–	–	4.58	–	–	–	–	–
dis -4,6 CS	–	–	–	–	6.22	–	–	–	–	–
dis -2,4 CS	–	–	–	–	4.07	–	–	–	–	–
dis 4S/6S CS	1.63	2.28	1.72	0.65	0.3	0.50	1.35	5.44	3.1	0.5

Table 3
HPCE determined disaccharide composition after enzymatic digestion of 25 FS and the 2 Ph, corresponding animal origin, SEC-TDA determination of molecular weight (Mw) and polydispersity (Mw/Mn).

Food supplements	% dis-0S CS	% dis-6S CS	% dis-4S CS	% dis-2S CS	% dis-2,6 CS	% dis-4,6 CS	% dis-2,4 CS	dis-4S/6S CS	CS origin	CS Mw (KDa)	CS Mw/Mn
FS1	2.64	13.27	55.89	3.45	16.01	8.8	n.d.	4.21	Mixed Terrestrial/ Marine	27.72	1.18
FS2	2.1	27.61	63.04	7.25	n.d.	n.d.	n.d.	2.28	Mixed Terrestrial	16.94 / 29.73	1.02/1.31
FS3	4.01	17.96	70.4	7.63	n.d.	n.d.	n.d.	3.92	Mixed Terrestrial or Avian	33.00	1.35
FS4	4.24	15.72	39.89	16.07	13.69	10.39	n.d.	2.54	Mixed Terrestrial/ Marine	37.30	1.28
FS5	4.69	23.48	61	4.92	0.77	3.45	1.68	2.59	Mixed Terrestrial/ Marine	24.50/49.50	1.12/1.01
FS6	3.87	16.07	73.01	7.05	n.d.	n.d.	n.d.	4.54	Pig	44.22/49.39	1.07/1.01
FS7	8.14	22.5	65.00	4.37	n.d.	n.d.	n.d.	2.88	Mixed Terrestrial	34.34	1.24
FS8	4.35	21.52	74.12	n.d.	n.d.	n.d.	n.d.	3.44	Mixed Terrestrial or Avian	29.57	1.47
FS9	5.36	14.06	80.58	n.d.	n.d.	n.d.	n.d.	5.73	Pig	n.d.	n.d.
FS10	0.96	29.72	60.37	8.95	n.d.	n.d.	n.d.	2.03	Mixed Terrestrial	25.38	1.25
FS11	3.27	26.26	70.47	n.d.	n.d.	n.d.	n.d.	2.59	Mixed terrestrial	20.29	1.52
FS12	5.59	16.61	70.78	7.02	n.d.	n.d.	n.d.	4.26	Pig	78.04	2.28
FS13	4.81	43.92	51.27	n.d.	n.d.	n.d.	n.d.	1.16	Bovine	33.62	1.18
FS14	1.34	19.33	47.27	4.08	22.09	2.66	3.23	2.44	Mixed Terrestrial/ Marine	27.57	1.45
FS15	11.89	19.89	63.68	4.44	n.d.	n.d.	n.d.	3.18	Mixed Terrestrial	228.00/348.00	1.31/1.60
FS16	16.14	13.3	70.56	n.d.	n.d.	n.d.	n.d.	5.3	Pig	34.36	1.20
FS17	2.03	17.86	78.61	n.d.	n.d.	n.d.	n.d.	4.4	Pig	14.71/11.28	1.04/1.00
FS18	3.06	25.17	66.85	4.92	n.d.	n.d.	n.d.	2.66	Mixed Terrestrial	13.27/6.64	1.22/1.02
FS19	1.54	29.2	69.26	n.d.	n.d.	n.d.	n.d.	2.37	Mixed Terrestrial	45.38/23.86/ 5.97	1.08/1.04/ 1.48
FS20	2.89	35.35	61.76	n.d.	n.d.	n.d.	n.d.	1.74	Bovine	19.71	1.14
FS21	2.25	35.74	51.48	0	10.53	n.d.	n.d.	1.44	Bovine	33.70	1.05
FS22	5.13	38.16	56.71	0	n.d.	n.d.	n.d.	1.48	Bovine	17.90	1.16
FS23	4.87	36.09	59.04	0	n.d.	n.d.	n.d.	1.63	Bovine	42.90	1.01
FS24	3.64	38.33	57.92	0	n.d.	n.d.	n.d.	1.51	Bovine	29.11/5.77	1.31/1.13
FS25	5.4	43.71	50.9	0	n.d.	n.d.	n.d.	1.16	Bovine	8.45	n.d.
Pharmaceuticals											
Ph1	2.12	15.49	82.38	n.d.	n.d.	n.d.	n.d.	5.32	Pig	19.00	1.22
Ph2	3.45	44.91	26.27	n.d.	n.d.	n.d.	n.d.	0.58	Shark	36.22	1.23

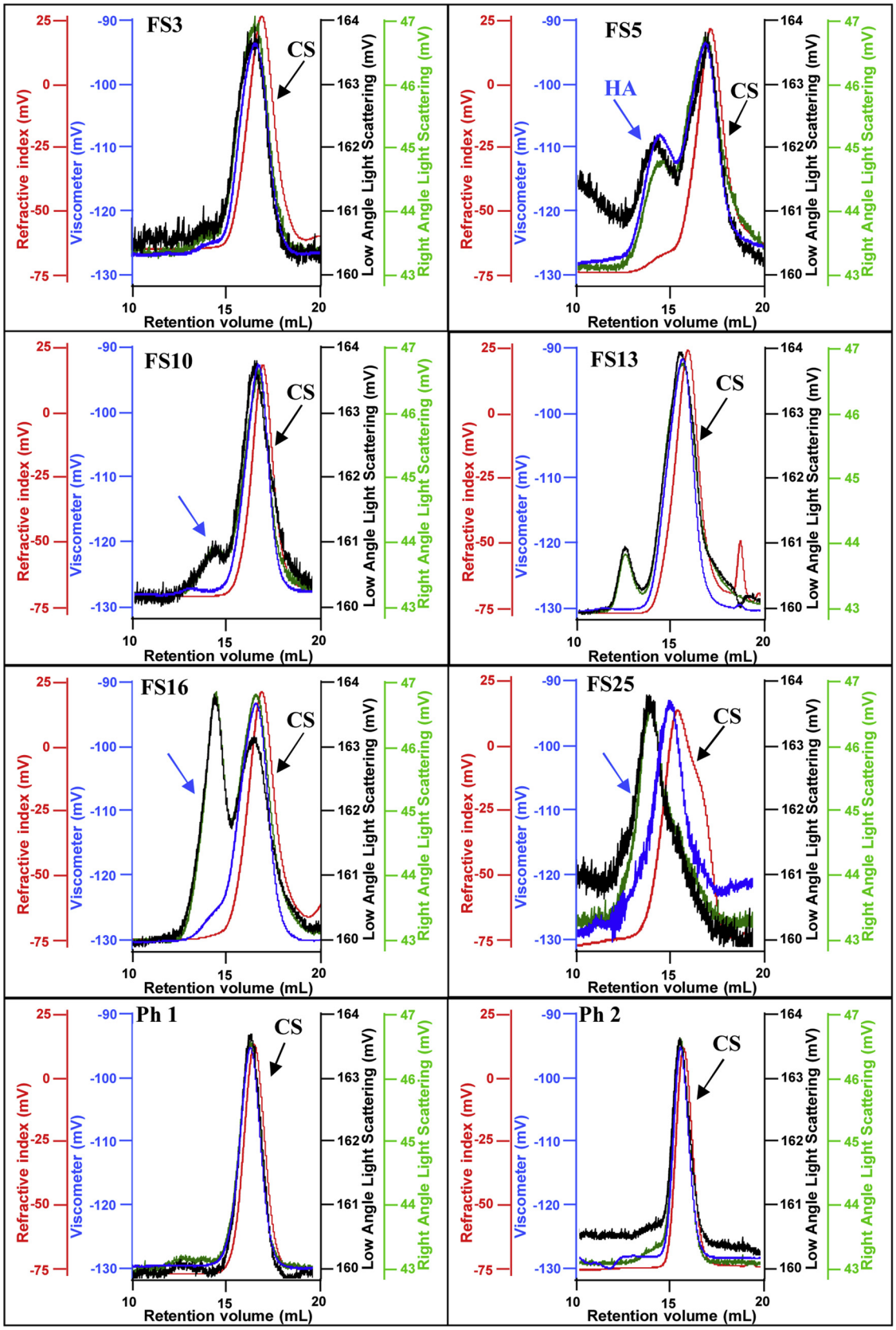


Fig. 5. Representative SEC-TDA analyses of FS and Ph, as detected by the refractive index, (red line), by the viscosimeter (blue line), by the LALS (black line) and the RALS (green line) detectors: FS3 (France, Company #1), FS5 (Slovakia, Company #2), FS10 (Switzerland, Company #2), FS13 (Hungary, Company #3), FS16 (Austria, Company #4), FS25 (Italy, Company #9), Ph1 (France, Company #10), Ph2 (Switzerland, Company #2). The CS peaks are indicated by the black arrows, while the high molecular weight species, such as a hyaluronic acid peak, are indicated by the blue arrows.

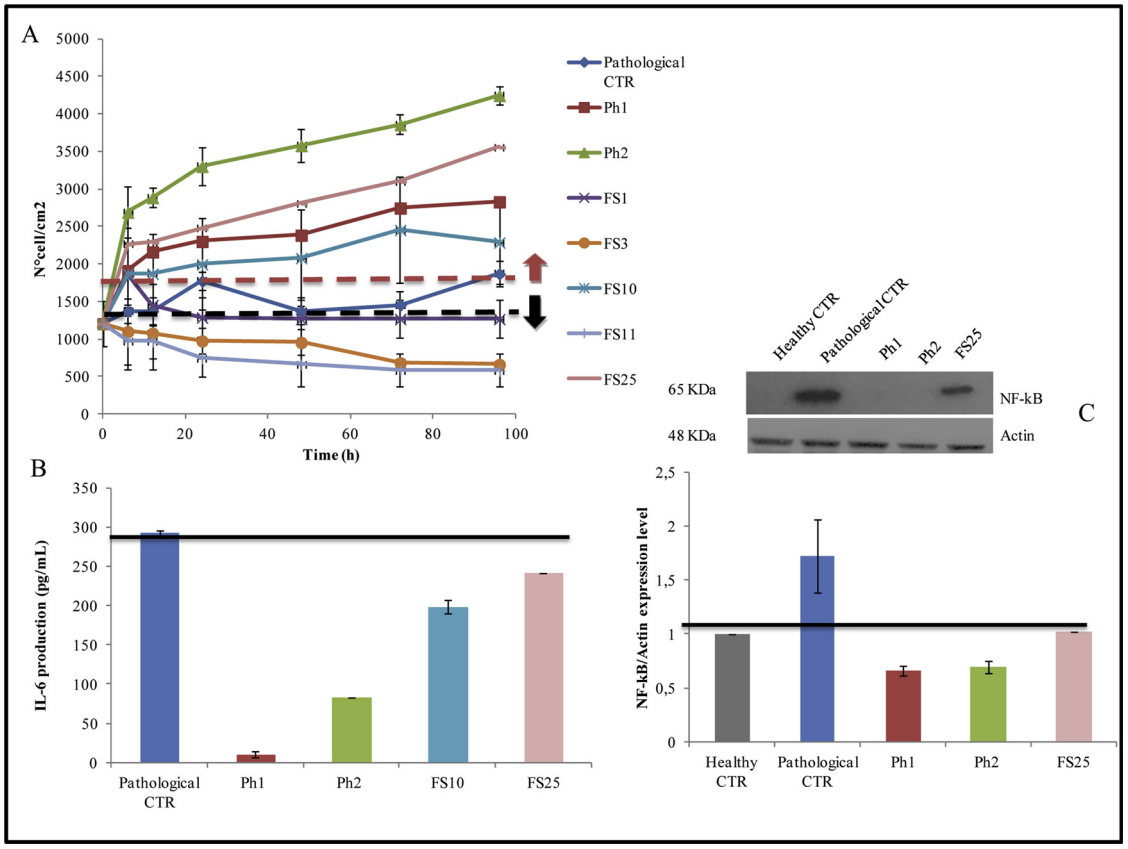


Fig. 6. Effect of FS (FS1, FS3, FS10, FS11, FS25) and Ph (Ph1 and Ph2) on the synoviocyte growth and proliferation evaluated by TLVM (A), on IL-6 quantification assayed by ELISA (B), and on the NF-kB protein expression assayed on articular chondrocytes (C).

a mixed pig and fish CS (Fig. 5, Table 3). The other mixed terrestrial samples showed single CS peaks ranging from 13.27 to 43.58 KDa while the mixed terrestrial and marine CS Mw, instead, ranged from 27.72 to 37.30 KDa (Table 3). In the FS the CS polydispersity varied from 1.01 to 1.60, while the representativity of the CS varied from 9.1% to 99.9% (data not shown). Some chromatograms also showed the HA presence or of plant extract molecules of Mw > 100 KDa. Representativity of high molecular weight species ranged from 0.1 to 5.1% (Fig. 5). The Ph of pig and fish CS origin had Mw of 19.00 and 36.22 KDa, respectively, with a low polydispersity, a representativity of 98.0–99.0 and a clean and consistent chromatographic profile. (Table 3).

3.6. Food supplement biological activity

The ability of 5 FS (FS1, FS3, FS10, FS11, FS25) to sustain the viability and proliferation of human synoviocytes, as well as the anti-inflammatory activity of their CS content were tested and compared with the two Ph samples. Specifically, the proliferation rate and the secreted IL-6 were analyzed on human synoviocytes (Fig. 6 A and B); while osteoarthritic chondrocytes were used to confirm the biological data and to evaluate the modulation of NF-kB transcription factor as a key trigger marker of inflammatory cascade (Fig. 6 C). Concerning cell growth and proliferation, Ph1 and Ph2, FS10 and FS25 positively affected the synoviocyte growth compared to the pathological control and increased the cell number (curves above the red dotted line in Fig. 6 A). However, differences between the FS10 and FS25 and Ph1 and Ph2 were noted: in particular Ph2 supported cell proliferation better than the other preparations with a 1.5 fold increase compared to the pathological control. FS1, FS3 and FS11, instead, reduced the cell number of about 50.0% compared to the pathological control (curves below the black dotted line in Fig. 6 A). In particular, the cell number was significantly reduced within the first 12 h of incubation in the case of FS3 and FS11 (Fig. 6 A). Ph1 and Ph2, FS10 and FS25 proved to sustain growth demonstrating also to reduce the IL-6 expression of 14.0, 4.0, 1.47 and 1.2 folds, respectively, compared to the positive control (pathological), thus proving their anti-inflammatory activity (Fig. 6B). In particular Ph1, Ph2 and FS10 reduced the IL-6, in comparison to the positive control (pathological). Since the FS25 sample was the best food supplement in terms of improvement of cell growth and proliferation, showing a trend similar to Ph samples, we evaluated its efficacy also on the cellular model of osteoarthritic human chondrocytes. Ph1 and Ph2 as well as FS25 were all able to counteract the NF-kB activation on articular chondrocytes, compared to the pathological control, showing lower NF-kB expression levels (Fig. 6 C).

4. Discussion

The issue of low purity, of the inferior quality ingredients, of the contaminated or adulterated composition of CS and GlcN is significant in the food supplement market due also to minor regulatory contraints (Burns, Walker, & Mussell, 2018; Chevalier & Conrozier, 2017). In absence of any mandatory analytical controls, as required for pharmaceutical compounds by Eu. Ph., each manufacturer could establish its own analytical procedures for the determination of identity, content and purity of active ingredients in the FS (Burns et al., 2018). This problem was recently approached by the Association of Official Analytical Chemists International (AOAC) that suggested the use of structurally sensitive multi-analytical techniques such as HPLC, MS or NMR for the determination of CS in FS, eventually after specific enzymatic hydrolysis (AOAC, 2014). However no protocols were reported for the contemporary determination of GlcN or of the residual KS. Instead a deep structural analyses of the FS might be necessary in order to support evidence of safety, beyond their biological and pharmacological activity. In fact, low CS and GlcN contents, structural heterogeneity of

the CS material as well as the presence of contaminants could make differences in therapeutic effectiveness (Agiba, 2017; Martel-Pelletier et al., 2015). To fill the gap of the absence of any specific official indications, in this study a systematic multi-analytical approach was designed to assess the CS and GlcN quality and quantity of 25 European FS, compared to 2 Ph. A new application of a high performance anion exchange chromatography method was used to determine both the CS and GlcN content and to quantify the KS contamination. In many of the analyzed FS the CS and GlcN contents were lower than the declared ones, with more than half of them having a 10.0% lower titre. In a previous paper only the CS content of 10 FS was evaluated and characterized but not the GlcN one. CS resulted lower compared to the declared values, however the techniques used for the analyses, namely agarose gel electrophoresis and separation of the CS disaccharides by SAX HPLC, gave consistently different results in the range from 10.7% to 61.0% in 8 out of 10 screened samples (Volpi & Maccari, 2008, 2009). So far the difference of CS and GlcN contents, compared to the declared values, were contemporarily determined only in pharmaceuticals but by using less sensitive methods such as colorimetric assays (differences were in the range from zero to -7.0%) (Santos et al., 2017). For the first time our HPAE-PAD method allowed a precise determination of both CS and GlcN contents even in complex matrices (as FS) and it resulted more reliable than ^{1}H NMR that has a too high threshold sensitivity. For the first time HPAE-PAD also allowed to determine the residual KS contamination in FS. It was present in all the 25 samples, with values higher than 10.0% in most of them. These values were much higher than the ones found in the Ph samples analyzed in this and in a previously reported paper (Restaino et al., 2017). So far the KS was determined only in pharmaceutical grade products by using less sensitive techniques like SAX-HPLC analysis of the intact polysaccharides or by immunoblotting (Pomin et al., 2012; Nakano & Ozimek, 2014). This KS contamination, and the one revealed by the ^{1}H NMR regarding aliphatic compounds, highlighted the problem of low quality raw materials and/or of unreliable CS extraction and purification processes. Although the heavy metals and proteins were under the regulatory limits, the plant extracts lowered the FS solubility compared to the Ph, as only 5 samples had an insoluble fraction value below the 5.0%. Differently from what has been reported before, the CS animal origin was determined by analyzing all the possible mono and disulfated disaccharides (Lauder et al., 2000; Maccari et al., 2015; Volpi, 2004). The high presence of 2-sulfated disaccharide (about 14.0–20.0%) and disulfated disaccharides was considered indicative of a marine origin CS, although low percentages of dis-2S CS were also found in terrestrial CS (around 6.0–7.0%), as reported before. Attributing the CS animal origin, on the basis of the disaccharide composition, was not straightforward considering that even standards of the same animal origin showed different profiles. Furthermore, the attribution is more complex in case of CS mixture of different animal sources. In previous papers only mixed terrestrial origin CS was found (Volpi & Maccari, 2008, 2009), while our analyses revealed FS containing also mixed terrestrial and marine sourced CS. Our data indicated that there is an attitude in FS manufacturing in mixing starting materials, thus obtaining low quality products of unclear origins that may arise medical doctors', pharmacists' and finally consumers' concerns. SEC-TDA analyses of the CS Mw helped, in many cases, to confirm the animal origin, but they also revealed the enormous CS heterogeneity also possibly related to a partial digestion of the proteoglycan core bounded to the CS chain (Restaino et al., 2017). As a matter of fact in some analyses the Mw values of bovine and pig CS resulted higher than the data reported in literature (Restaino et al., 2013). In many cases both high (40–50 KDa) and low (8–10 KDa) Mw chains were present and this again may account for low standardized FS manufacturing processes. However, in some samples, the presence of plant extracts hampered a sound evaluation of CS Mw. This instrumental technique, based on a triple detector, proved able to give contemporary information on different Mw species more than the simple agarose gel electrophoresis analyses used in previous papers (Volpi & Maccari, 2008, 2009). Besides the powerful analytical tool presented here, more and more interest has arisen in developing a realistic *in vitro* model to predict *in vivo* effects. For instance functional FS containing several anti-inflammatory or anti-arthritic molecules (*e.g.* HA, collagen peptides, *Boswellia serrata*, devil's claw or curcumin) were tested on synovial cells, evaluating the NF-kB activation and IL-8 production (Yamagishi, Someya, Imai, Nagao, & Nagaoka, 2017). Tests performed *in vitro* showed that CS treatment had an anti-inflammatory effect and induced an anabolic response (e.g NF-kB traslocation, SOD-2 activation) and also the activation of hyaluronic acid biosynthesis in synoviocytes culture (Hochberg, Chevalier, Henrotin, Hunter, & Uebelhart, 2013). Kilborne and collaborators proposed a model based on synovial cell cultures exposed to LPS treatment, to study the protective and anti-inflammatory effects of HA alone or in combination with CS and GlcN (Kilborne, Hussein, & Bertone, 2017). In previous papers, the therapeutic efficacy of pharmaceutical grade CS, orally administered, was also demonstrated *in vivo* by the reduction of the osteoarthritis symptoms (Volpi & Maccari, 2009). The biological activity evaluation of 5 FS was performed in the present research work by using two cellular models derived from osteoarthritic joints, following protocols already reported (Stellavato et al., 2019). Specifically, a similar procedure was used to compare extractive CS to biotechnological chondroitin in their anti-inflammatory action (Stellavato, Tirino et al., 2016). The NF-kB activity is implicated in the pathogenesis of several inflammatory diseases, including osteoarthritis, and its activation plays a crucial role in the development and progression of the pathology. In the experiments reported in this paper the NF-kB is activated in pathological chondrocytes isolated from osteoarthritic patients and reduced during the treatment with pharmaceuticals. On the contrary 4 of 5 FS tested did not show efficacy. Our data demonstrated that only a FS sample of bovine CS origin, which had both high and low Mw CS chains, and that also contained *Boswella serrata* extract, had a positive effect on the two cellular models, thus being able to both induce growth and lower the activation of NF-kB. Although only 5 FS were screened for their biological activity, the data obtained clearly indicated that FS quality may limit their efficacy. Differently, the Ph proved sound biological activity in all the assays performed.

5. Conclusion

In conclusion, in this research work, a systematic multi-analytical approach was employed for the characterization of 25 European FS. A new application of a HPAE-PAD method was used to precisely determine the GlcN and the CS contents and the residual KS contamination. The results indicated that the analysed FS do not have a composition corresponding to the label descriptions, showing lower CS and GlcN titers. Compared to the pharmaceutical grade products, they contained low quality CS, highly contaminated by keratan sulphate, in some cases of multiple animal origins and of dis-homogeneous molecular weights. The presence of high insoluble solids and solvent residues contribute to confirm that less controlled manufacturing procedures or poor raw materials were used in the FS preparation compared to the pharmaceuticals. Data from the biological assays demonstrated that only one of the screened FS presented a sound biological activity on both the proposed models of chondrocytes and synoviocites, thus suggesting that further bio assays are required to confirm the correlation between the analytical quali-quantitative results and the FS biological activity. Overall, in this paper a systematic assessment of the structural composition of the European chondroitin sulfate and glucosamine food supplements was performed towards a better understanding of their effective biological activity in order to be more conscious in analyzing and interpreting clinical outcomes.

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