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Research Article

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Spectroscopic approach of the association of heparin and its contaminant and related polysaccharides with polymers used in electrokinetic chromatography

S. Flor[1], V. Tripodi [1,2], M. Contin[1], C. Dobrecky[3] and S. Lucangioli[2,4]*

[1]Department of Analytical Chemistry and Physicochemistry, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina
[2]Consejo Nacional de Investigaciones Científicas y Tecnológicas, CONICET, Argentina
[3]Phoenix Laboratories, Argentina
[4]Department of Pharmaceutical Technology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

Heparin (Hep) is a linear sulfated polysaccharide used in clinical treatment of tromboembolic diseases. In 2008, contaminated batches of Hep appeared in the pharmaceutical market. The contaminant was identified as an over sulfated form of chondroitin sulfate (oversulfated chondroitin sulfate, OSCS). Different analytical methods have been developed to identify and quantify OSCS and other impurities expressed as dermatan sulfate from Hep. In this sense, we have developed a capillary electrophoretic method based on the use of a mixture of polymers such as polymeric β-cyclodextrin and poloxamine. As a result of this combination, not only the resolution of Hep from its impurities and contaminant was obtained but the sensitivity was extremely improved. The association of Hep and its related polysaccharides with the polymers was characterized by spectroscopic techniques as ultraviolet (UV) and infrared (IR). The results suggest that the interaction between polysaccharides and polymers would principally be through the sulfate groups of polysaccharides.

Keywords: heparin and related polysaccharides; polymeric □-cyclodextrin; spectroscopic techniques.

INTRODUCTION

Heparin (Hep) is a natural, linear, complex, polydisperse, and sulfated polysaccharide that belongs to the glycosaminoglycan family (GAGs). Chemically, Hep consisting of 1 → 4 linked pyranosyluronic acid and 2-amino-2-deoxycopyranose (with either N-sulfo or N-acetyl substitution) repeating units (Fig. 1) [1-2]. Hep has clinically used in the prevention and treatment of the thromboembolic diseases for many years [3].

Hep is a natural product extracted from animal tissues, most commonly from porcine intestine. As a result of an incomplete purification, other sulfated linear polysaccharides as dermatan sulfate (DS) and chondroitin sulfate (CS) are present in the manufactured product as impurities [4] (Fig. 2).

Hep is parenterally administered due to its degradation when it is orally taken and in some cases it has to be injected at high doses [5]. However, in 2008 appeared contaminated lots of injectable Hep in many of the pharmaceutical market. The adulterant was identified as a chemically modified chondrointin sulfate, oversulfated chondroitin sulfate (OSCS), which causes several effects and in some cases, death of the patients. [1-2]. OSCS is a semisynthetic polysaccharide containing four sulfonate groups per repeating units (Fig. 3) [6].

Government agencies, pharmaceutical laboratories and research institutes have developed different analytical methods to characterize and identify contaminants and impurities of Hep.

In this sense, we have presented a novel and very highly sensitive capillary electrophoretic chromatography (EKC) method by capillary electrophoresis, based on the use of polymers as pseudostationary phases. The association of these polymers like polymeric-β-cyclodextrin (Pβ-CD), allowed not only the resolution of Hep from its contaminant and impurities but also specially, the quantitation in injectable products [7].

The characterization of the interaction between Hep and related polysaccharides and polymeric β-cyclodextrin (Fig. 4) and poloxamine like tetronic tetronic® 1107 (Fig.5) can be studied by spectroscopic techniques like ultraviolet (UV) and even better infrared spectroscopy (IR) [8-11].

In terms of structural analysis, IR spectroscopy gives a complete "molecular finger print" of the studied sample. Different authors have reported IR spectra for Hep and related polysaccharides in solid-state and in aqueous solution, from different sources. Several peaks can be identified by spectral comparison, specially those associated with sulfate vibration in the molecule [12-13].

Therefore, the purpose of the present work was to investigate by spectroscopic techniques the interaction between Hep and its related polysaccharides and polymers used in electrokinetic chromatography.

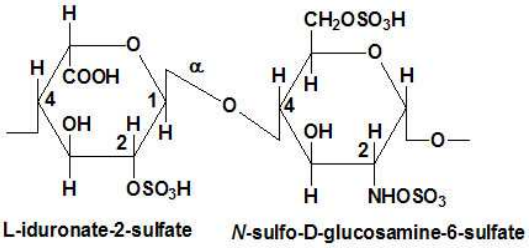

Figure 1. Chemical structure of Heparin

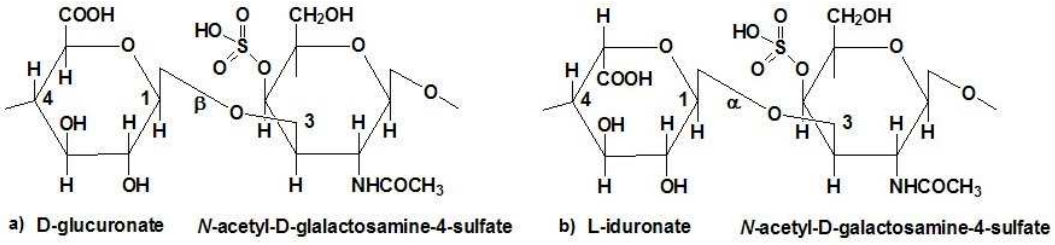

Figure 2. Chemical structures of a) chondroitin sulfate; b) dermatan sulfate.

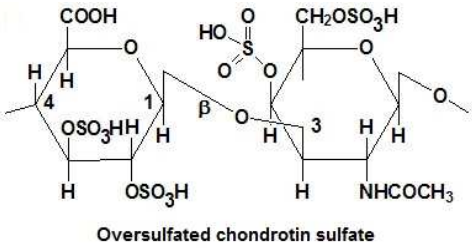

Figure 3. Chemical structure of oversulfated chondroitin sulfate

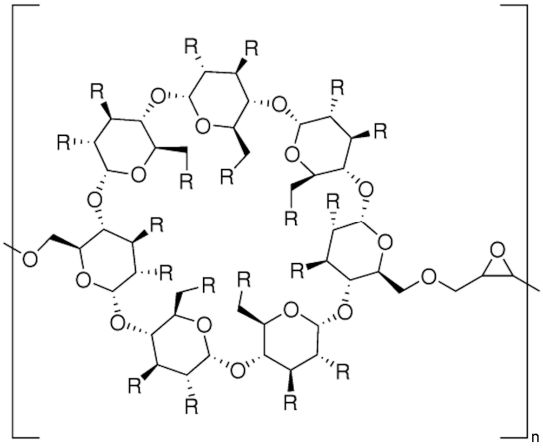

Figure 4. Chemical structure of polymeric-β-cyclodextrin (n: MW range from 2,000 to 4,130)
(from reference 7).

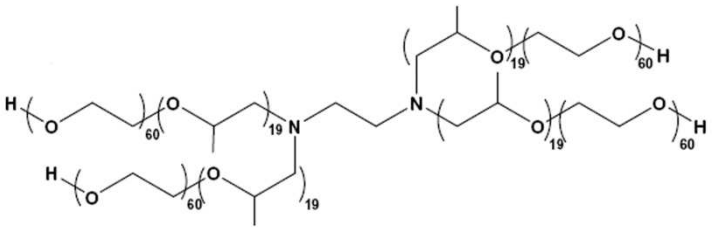

Figure 5. Structure of poloxamine tetronic® 1107.

EXPERIMENTAL SECTION

Materials and Chemicals

Heparin (Hep), dermatan-4-sulfate (DS) and oversulfated chondroitin (OSCS) were kindly supplied by Syntex S.A. Laboratories. Polimeric β-cyclodextrin (Pβ-CD) was purchase from Sigma Aldrich (St. Louis, USA). Tetronic 1107® was supplied by BASF Argentina. Tris (tris(hydroxymethyil)aminomethan) and potasium bromide were obtained from Merck (Darmstead, Germany). The water used in all the experiments was obtained from a Easy Pure™ RF equipment (Barnstead Dubuque, IA, USA)

Preparation of the samples

Sample solutions

For the spectral analysis seven solutions were prepared according to table 1. All solutions were prepared in Tris buffer 400 mM pH 3.5 and tetronic 0.4 w/v.

Tabla 1. Sample solutions of Hep and it related polysaccharides

Sample	*Concentration (µg/mL)*	*Pβ-CD (%w/v)*	*Tetronic (%w/v)*	**Abv**
Heparin	100	-	0.4	Hep
	100	0.5	0.4	Hep-PβCD
Dermatan-4-sulfate	10	-	0.4	DS
	10	0.5	0.4	DS-PβCD
Oversulfated Chondroitin	10	-	0.4	OSCS
	10	0.5	0.4	OSCS-PβCD
Blank	-	0.5	0.4	Pβ-CD

Freeze drying procedure

Five mililiters of each solution presented on table 1 were frozen at -70°C and stored for 72 hours. Then the solutions were lyophilizated on a freeze dryer, FIC L 05 (FIC, Scientific Instrumental, Argentina) under the following parameters: Freeze dryer shelf temperature -14 °C, condenser temperature -40 °C, pressure 0,03 mbar, time 24 hs.

ATR-IR spectroscopy

Prior to lyophilization, the seven solutions mentioned in table 1 were analyzed by ATR- IR spectroscopy. The measurements were carried out using HATR unit fitted to a FTIR Nicolet 380 spectrometer (universal diamond/zinc selenide). The number of scans was set to 32 and the resolution was 4 cm^{-1}. The spectrum was obtained in a range between 4000 to 650 cm^{-1} and the incidence angle was 45 degrees.

FTIR spectroscopy

For FTIR sample preparation the KBr pastille method was used. The liophilizated samples were pulverized to a fine powder, together with pure potassium bromide and pressed at 200 MPa to obtain a transparent pastille.

The number of scans was 32, resolution was 4 cm^{-1} and the spectral range analyzed was from 4000 to 650 cm^{-1}.

RESULTS AND DISCUSSION

In a previous paper [7], we have developed a mixed-polymeric electrokinetic chromatography (EKC) system by capillary eelctrophoresis for the simultaneous determination of Hep and contaminants like OSCS and impurities expressed as DS. The EKC system consisted of 0.5 % w/v polymeric β-cyclodextrin (Pβ-CD), 0.4 % w/v tetronic® 1107 and 400 mM tris-phosphate buffer at pH 3.5 (Fig. 6). This highly sensitive method showed low values of LOD, 0.07 µg/ml (OSCS) and 0.1 % w/w 0.1 µg/ml (DS), at a concentration level of heparin in samples as low as 0.1 mg/mL compared with HPLC or NMR methods, in which a concentration of 100 mg / ml of Hep in the sample is necessary [14].

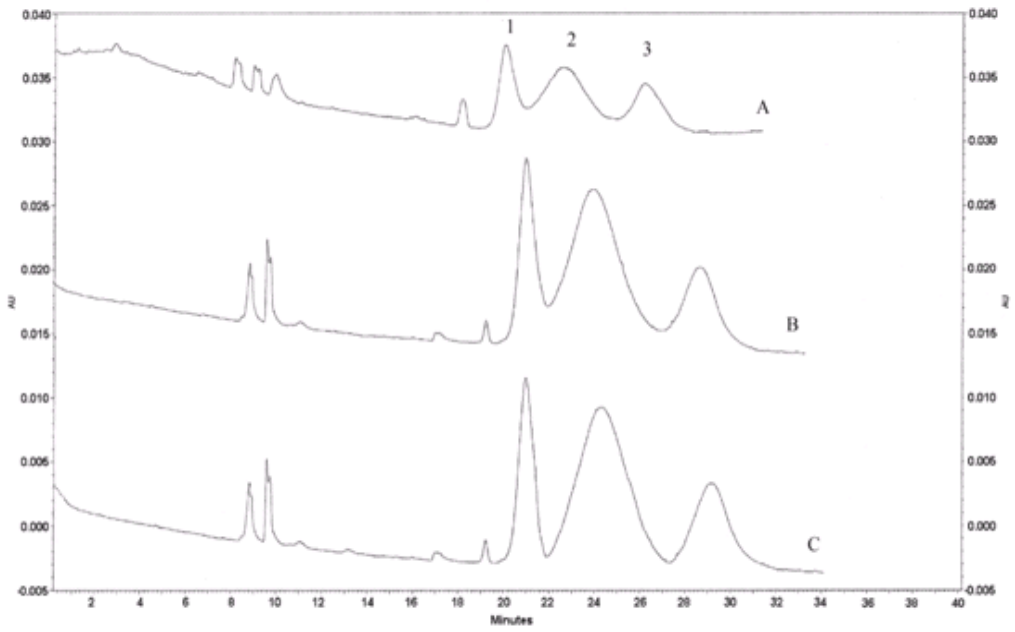

Figure 6. Electropherograms of Hep (2) (0.1 mg/mL), OSCS (1) (10 µg/mL) and DS (3) (10 µg/mL) (A) Electrolyte: 400 mM Tris-phosphate at pH 3.5, (B) Electrolyte with 0.5 % polymeric-β-cyclodextrin (C) Electrolyte with 0.5 % w/v polymeric-β-cyclodextrin and 0.4 % w/v Tetronic® 1107. From reference 7.

Analysis of UV-spectra

Figure 7 shows the UV-Vis spectra of Hep, Pβ-CD and their combination. In table 2, it is presented the UV-Absorbance values at 200 nm of each one in solution. These data show an interaction between both components, to obey the law of Lambert and Beer it is necessary that the total absorbance correspond to the sum of the individual absorbance values and the interaction between the components has to be ruled out.

Table 2. Absorbance values at 200nm

Solution	Abs
Hep	0.0431
P-β-CD	0.0741
Hep- P-β-CD	0.0508

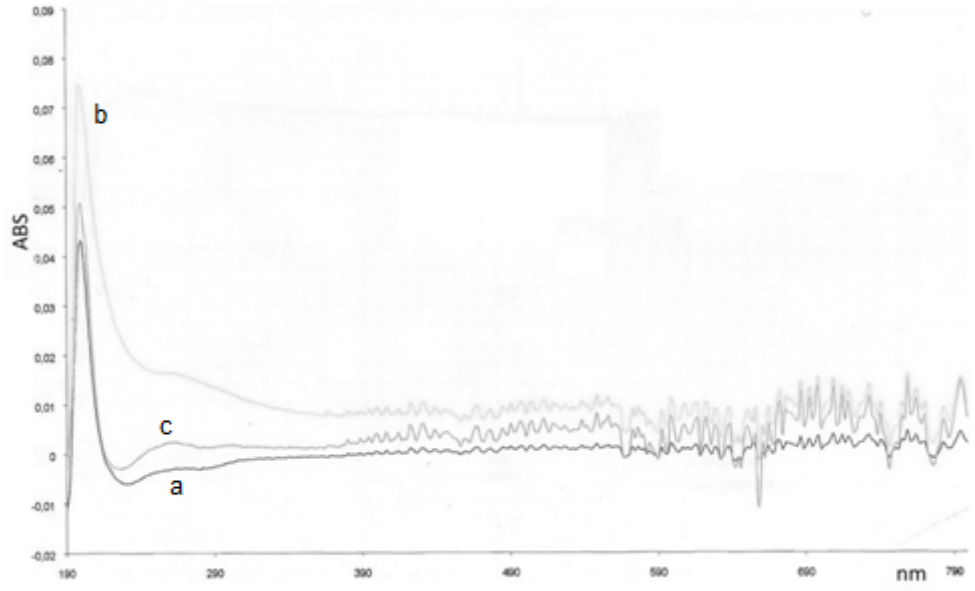

Figure 7. UV-Vis spectra of a) Hep (100 µg/ml), (b) Pβ-CD 0.5 % p/v and (c) HepP-β-CD (Experimental conditions in Table 1).

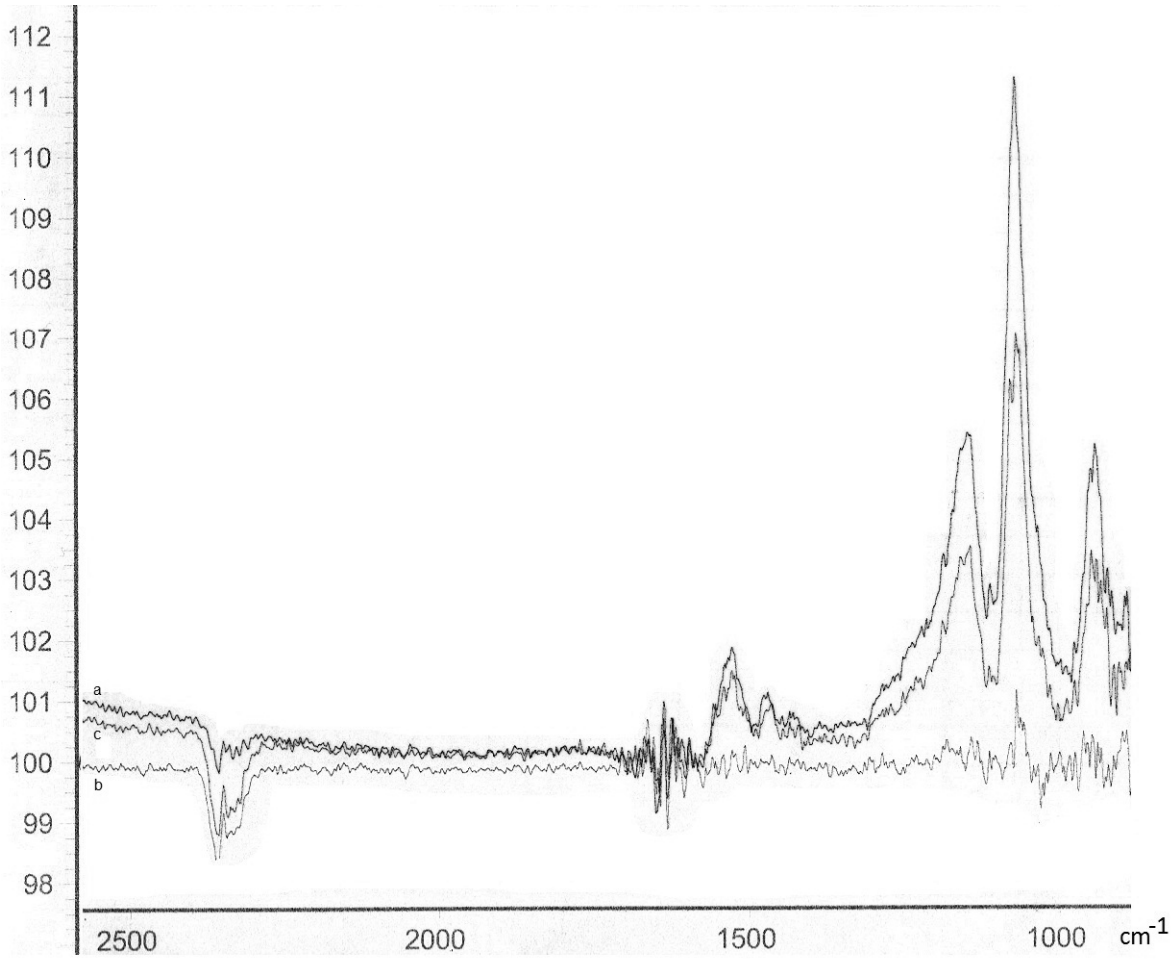

Figure 8. ATR-IR spectra a) Hep (100 µg/ml), (b) Pβ-CD 0.5 % p/v and (c) Hep-Pβ-CD (Experimental conditions in Table 1).

Analysis of IR-spectra for GAGs

ATR-IR spectra of Hep

ATR-IR spectrum of Hep solution (100 µg/mL), Pβ-CD as well as the ATR-IR spectrum of solution of Hep with Pβ-CD (0.5% w / v) using the ATR cell is presented in figure 8a, 8b and 8c, respectively. Both ATR-IR spectra do not present too much information, only a difference in bands at 1250 cm^{-1} and 1000 cm^{-1} related to molecule sulfonation. Instead, data for IR Spectra are presented in the next section for GAGs in solid-state by lyophilization and subsequent preparation of KBr.

Solid-states IR spectra for GAGs

The solid state IR spectra for GAGs like Hep and related polysaccharides are usually presented between 2000 cm^{-1} and 600 cm^{-1}, with characteristic peaks. These peaks are 1248 cm^{-1} and 1065 cm^{-1} related to molecule sulfonation. Also, it can be observed, peaks at 1410 cm^{-1} and 1620 cm^{-1} corresponding to symmetrical and asymmetrical stretching vibration of COO^- groups. In the same way, IR peaks are observed below 1015 $cm^{-1}$and they can be assigned to different sulfate group vibrations. The peak at 820 cm^{-1} and shoulder centered at about 1000 cm^{-1}correspond to vibrations of an equatorial sulfate group on C6 of galactosamine, while peaks at 730 cm^{-1} and 853 cm^{-1} are assigned to vibrations of an axial sulfate groups on C4 of the galactosamine residues (Fig. 1 to 3) [12].

Figure 9a shows the Hep spectrum with characteristic peaks in comparison with figure 9b corresponding to Hep with Pβ-CD. The last spectrum, presents differences in some bands like 1065 cm^{-1} and 1248 cm^{-1} and in less intensity at 700 cm^{-1}. These results suggest the possible interaction between Hep and Pβ-CD related to sulfonic groups. Moreover, the Pβ-CD spectrum is completely different from Hep and Hep- Pβ-CD (data not shown).

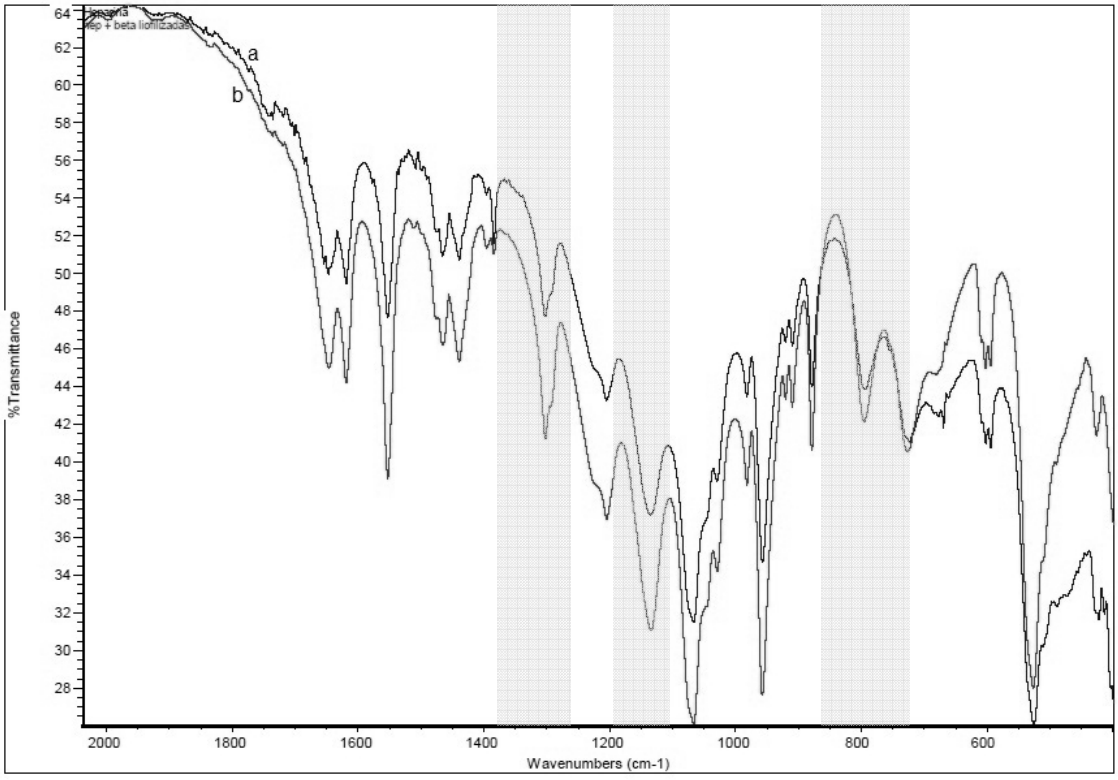

Figure 9. IR spectra for a) Hep and b) Hep- Pβ-CD. Gray zones indicate the peaks with differences between the spectra (Experimental conditions in Table 1).

In the case of DS (Fig. 10a), characteristic peaks are observed at 1065 cm^{-1} and 1248 cm^{-1}, as well as peaks at 1410 cm^{-1} and 1620 cm^{-1}, but in particular, the bands corresponding to lower wave numbers appearing at 730 cm^{-1} and 850 cm^{-1}, which correspond to OSO_3^- in axial and equatorial position. Figure 10b also shows the interaction between DS and Pβ-CD. In this spectrum, it can be observed, a higher modification in the peaks at 1248 cm^{-1} and 1000 cm^{-1}, respect to the spectrum of Hep- Pβ-CD shown in Fig. 9b.

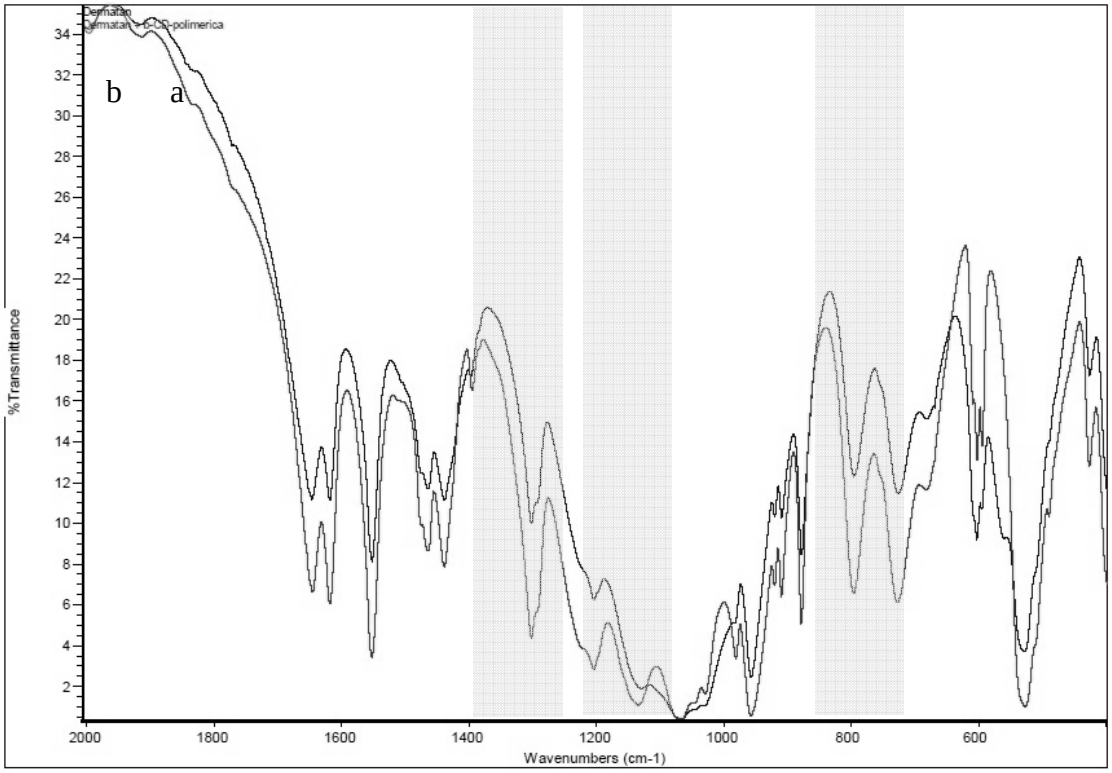

Figure 10. IR spectra for a) DS and b) DS- Pβ-CD. Gray zones indicate the peaks with differences between the spectra. (Experimental conditions in Table 1).

Similarly, in the spectra of OSCS and OSCS-Pβ-CD (Fig. 11a and 11b) it can be seen modifications in the peaks at 1066 cm^{-1}, 1248 cm^{-1} and 1000 cm^{-1} and specially at 730 cm^{-1}, corresponding to sulfonic group at C4 (Fig. 3). Note that this sulfonic group is present in DS and OSCS but absent in the structure of Hep.

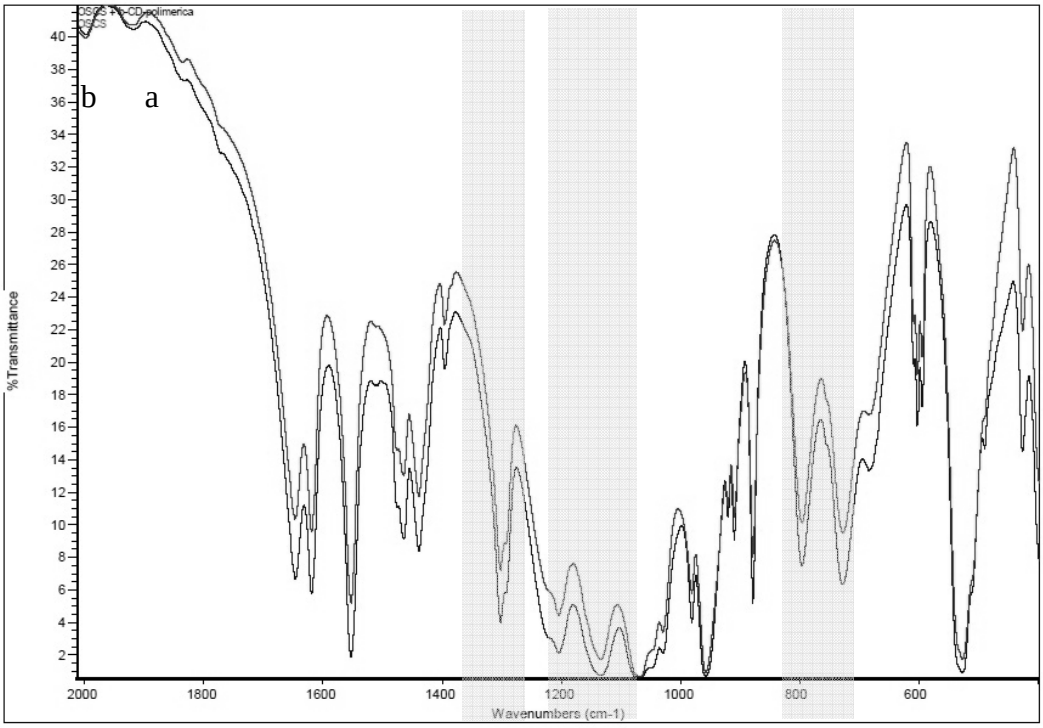

Figure 11. IR spectra for a) OSCS and b) OSCS- Pβ-CD. Gray zones indicate the peaks with differences between the spectra. (Experimental conditions in Table 1).

These results are closely in agreement to the difference in UV detector response shown for OSCS and DS in electrokinetic chromatography in comparison with Hep. The same UV detector response for Hep solution of 100 μg / ml is produced by 10 μg/ml of solution of DS and OSCS each one [7].

In regard to Tetronic, no differences were observed in the IR-spectra respect to individual and combined IR-spectrum (Tetronic® and polysaccharides) (Data not shown).

CONCLUSION

The interaction of polysaccharides like Hep, DS and OSCS with polymers such as Pβ-CD is evidenced by spectroscopic methods as IR, specially in solid state samples. This characterization allows understanding the role of the polymers like Pβ-CD in the analysis of Hep and its related polysaccharides. The use of polymers can increase the response of UV detector for the group of GAGs in electrokinetic chromatography methods by capillary electrophoresis. This evidence suggests that the interaction between polysaccharides and polymers would principally be through the sulfate groups of polysaccharides.

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