NMR Spectroscopy
Identity and purity test of heparin

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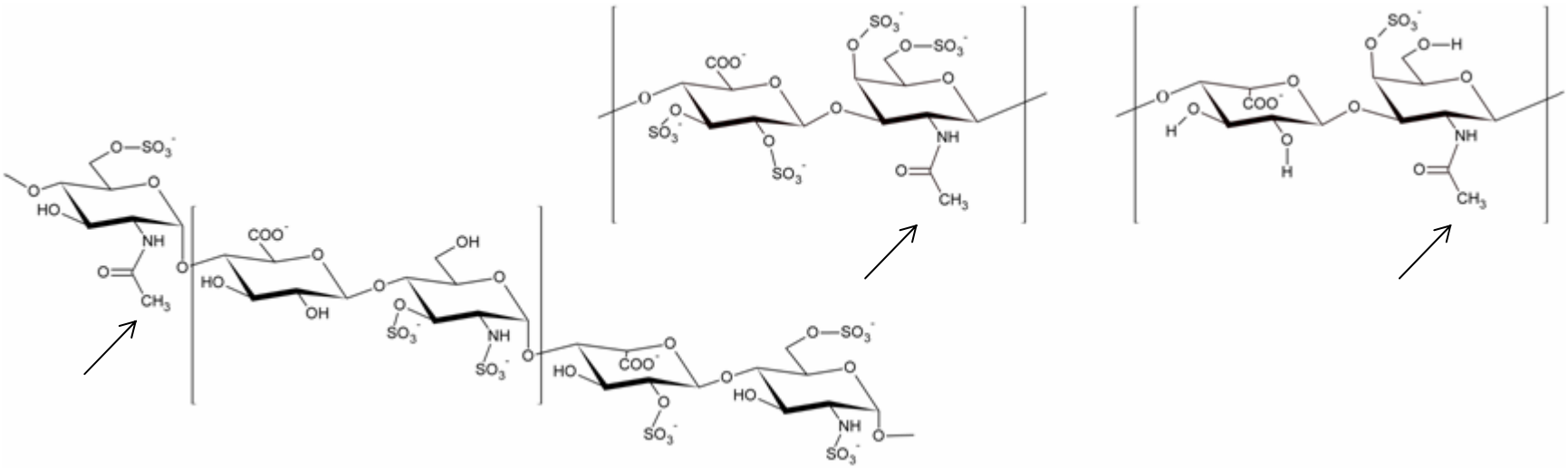
Oversulphated chondroitin sulphate (OSCS)

Dermatan sulphate (DS)



Heparin

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Identification of heparin test samples

- ^{1}H-NMR
- 2D NMR

Purity test:

1. Detection and quantification of OSCS and DS
    - ^{1}H-NMR

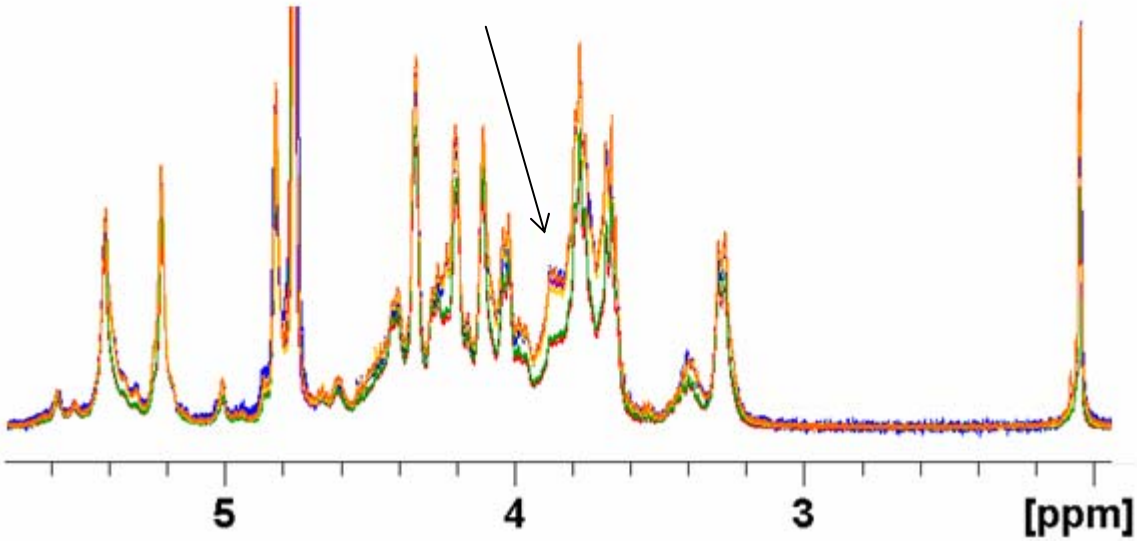
2. New and unknown impurities / contaminants
    - ^{1}H-NMR
    - 2D NMR

Identification of heparin test samples

- ^{1}H-NMR
- 2D NMR

Identification by ^{1}H-NMR

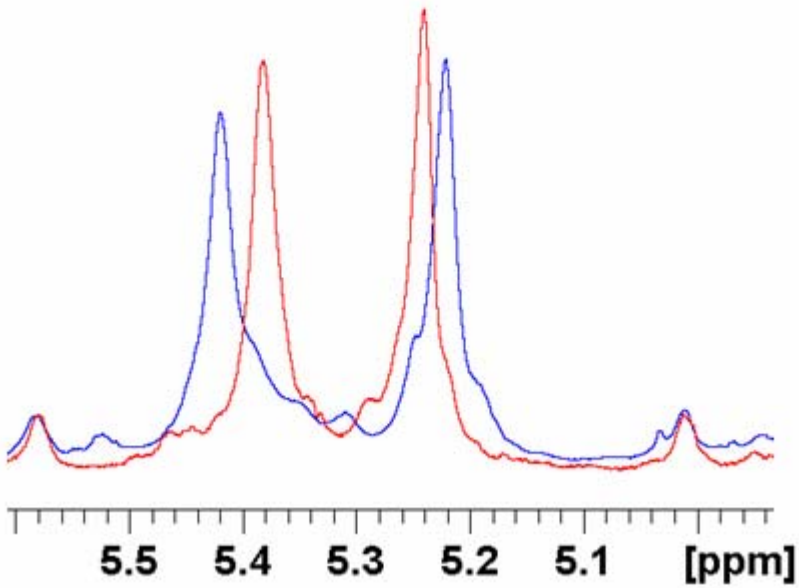
Over 350 heparin sodium NMR spectra have been compared. All spectra showed similar signal patterns



Seven superimposed ^{1}H-NMR spectra of heparin. Some variations in the intensity of a few signal regions have been observed. The more prominent one is the region between 3.81 and 3.89 ppm

Identification by ^{1}H-NMR

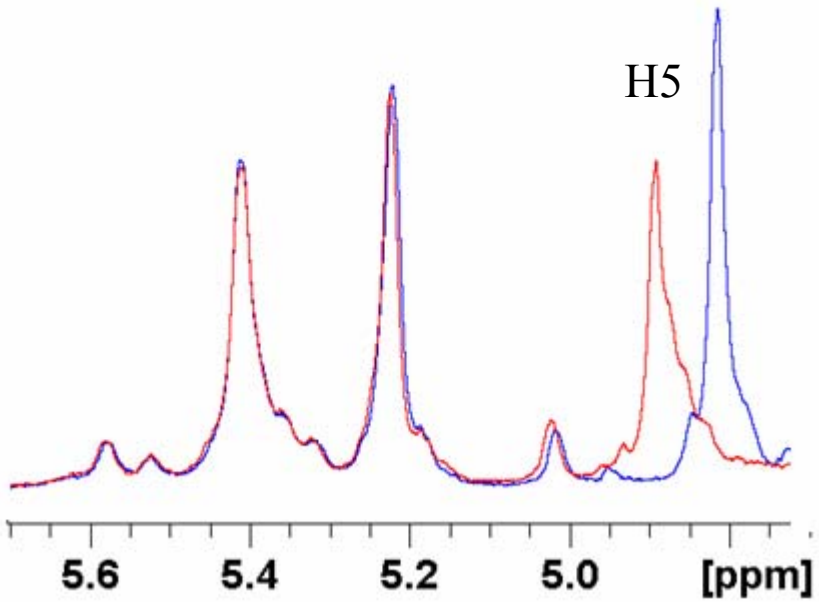
Three outliers were found when comparing the 350 NMR spectra. The effect of salt. The more salt, the closer the two signals shown in the NMR spectra come together

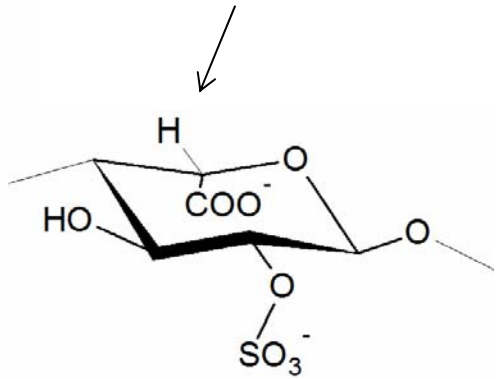


The effect of salt (red trace) on two major signals of the ^{1}H-NMR spectrum of heparin

Identification by ^{1}H-NMR

Three outliers were found when comparing the 350 NMR spectra. The effect of acidic pH. The lower the pH, the more the H5 signal moves to the left



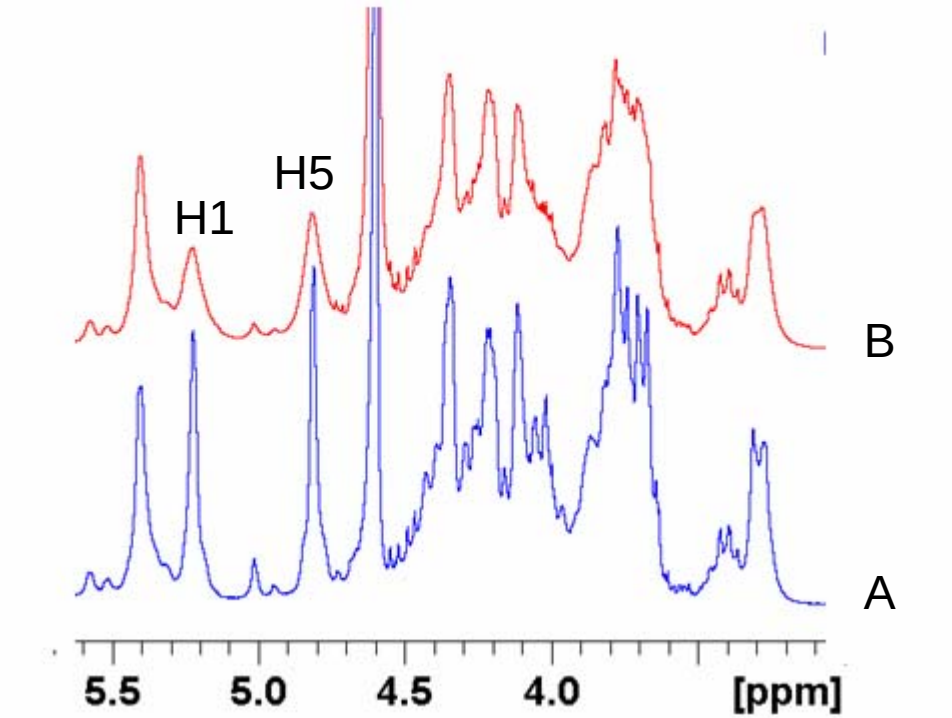


The effect of lowering the pH (red trace) on the H5 proton signal of the heparin uronic acid moiety

Identification by ^{1}H-NMR

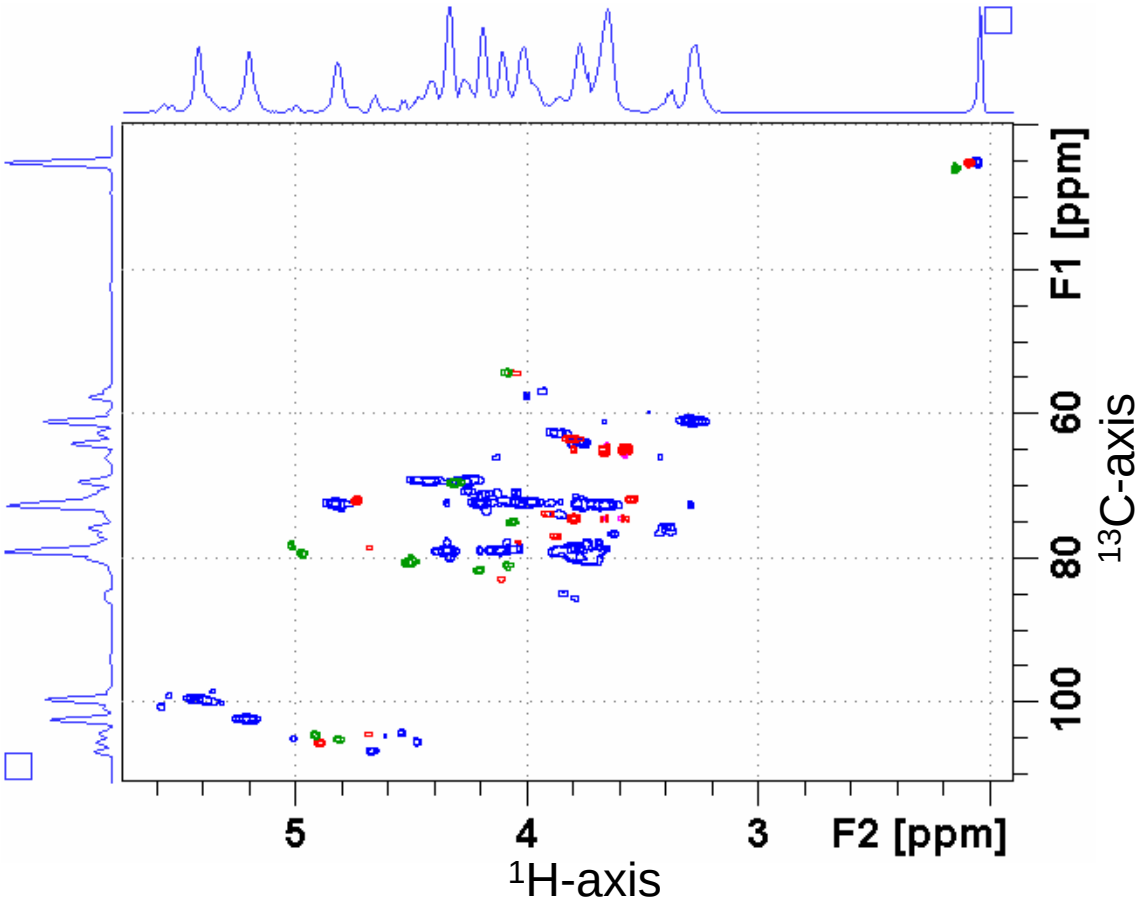
Three outliers were found when comparing the 350 NMR spectra.

<u>The effect of metal ions</u>



The effect of some transition metal ions (trace amounts) on the ^{1}H-NMR spectrum of heparin (spectrum B); and after the addition of EDTA (spectrum A)

Identification by 2D NMR



Superimposed 2D NMR HSQC experiments of heparin (blue), DS (red) and OSCS (green). The three polysaccharides have scarcely any signal whose position coincides

Identification by 2D NMR

A proposed list of proton-carbon correlated signals for the identification of heparin

	Chemical shifts (δ)	
Signal	$^{1}H \pm 0.10$ ppm	$^{13}C \pm 1.5$ ppm
1	5.42	99.3
2	4.34	78.8
3	4.20	72.1
4	3.68	72.4
5	3.28	60.7
6	2.04	24.7

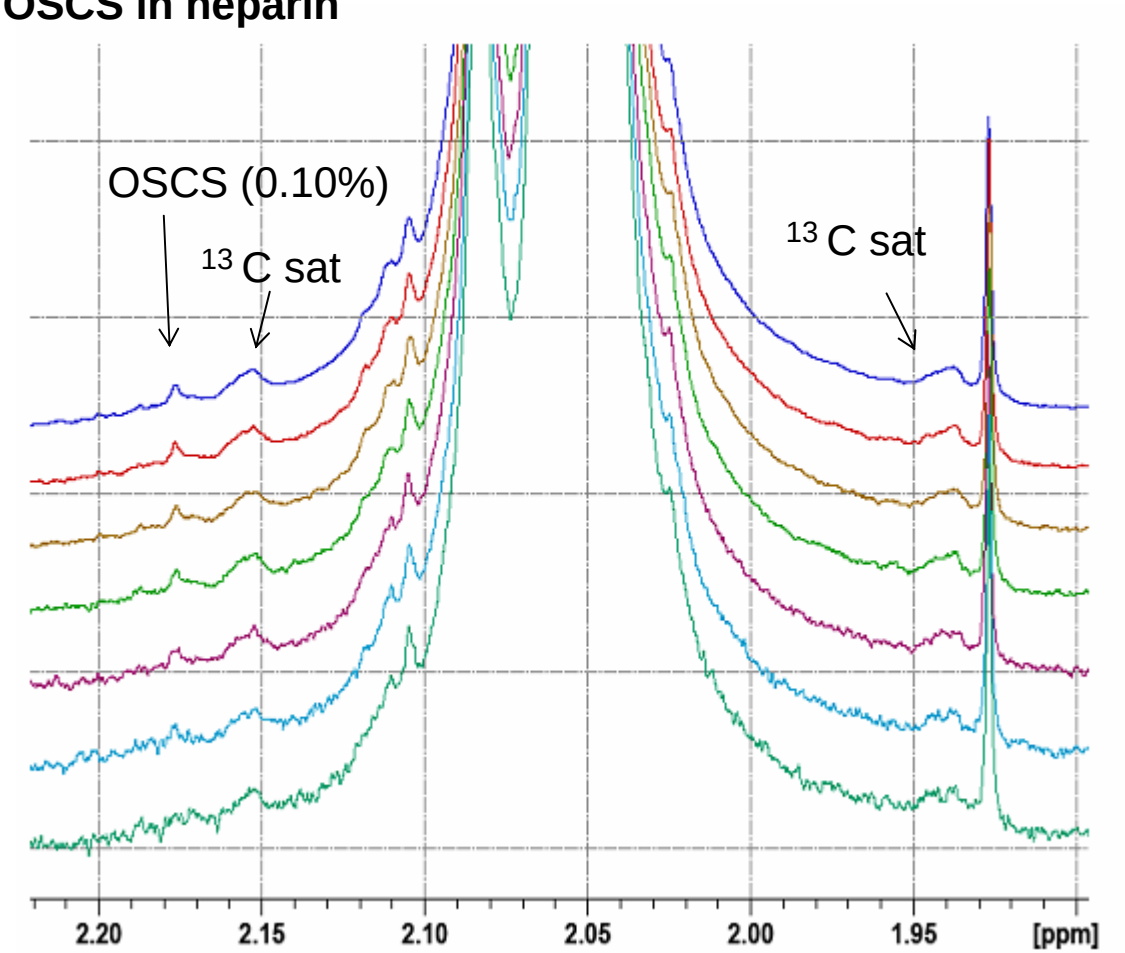
Purity test of known impurities / contaminants.
Detection and quantification of OSCS and DS

- ^{1}H-NMR

LoD of OSCS in heparin

S/N of the heparin methyl signal

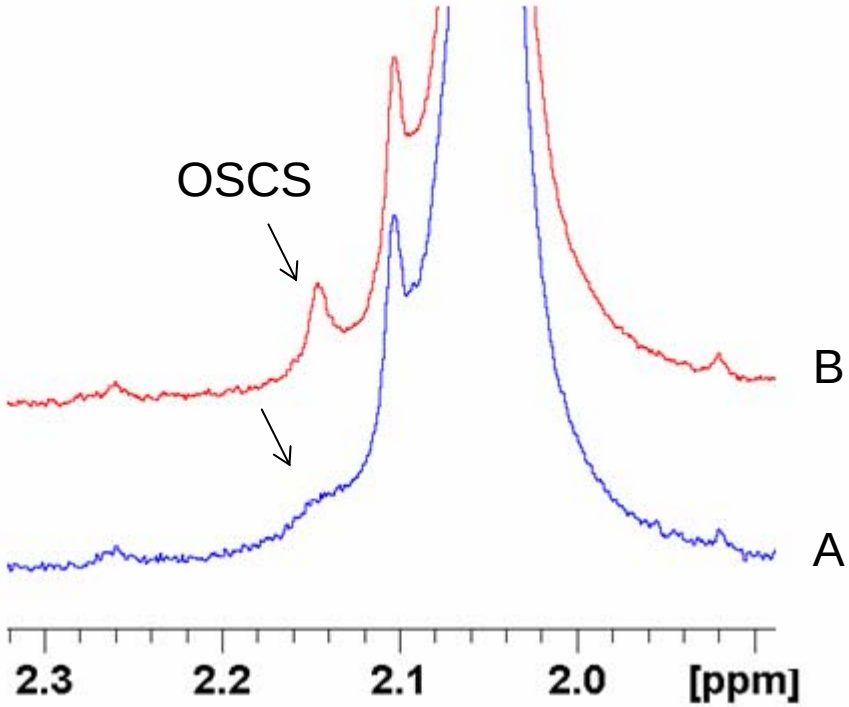
5346
3264
2721
1840
1376
922
677



By increasing the number of transtients (scans) the LoD is improved.
The OSCS signal has been shifted to the left by addition of Ca^{2+} ions

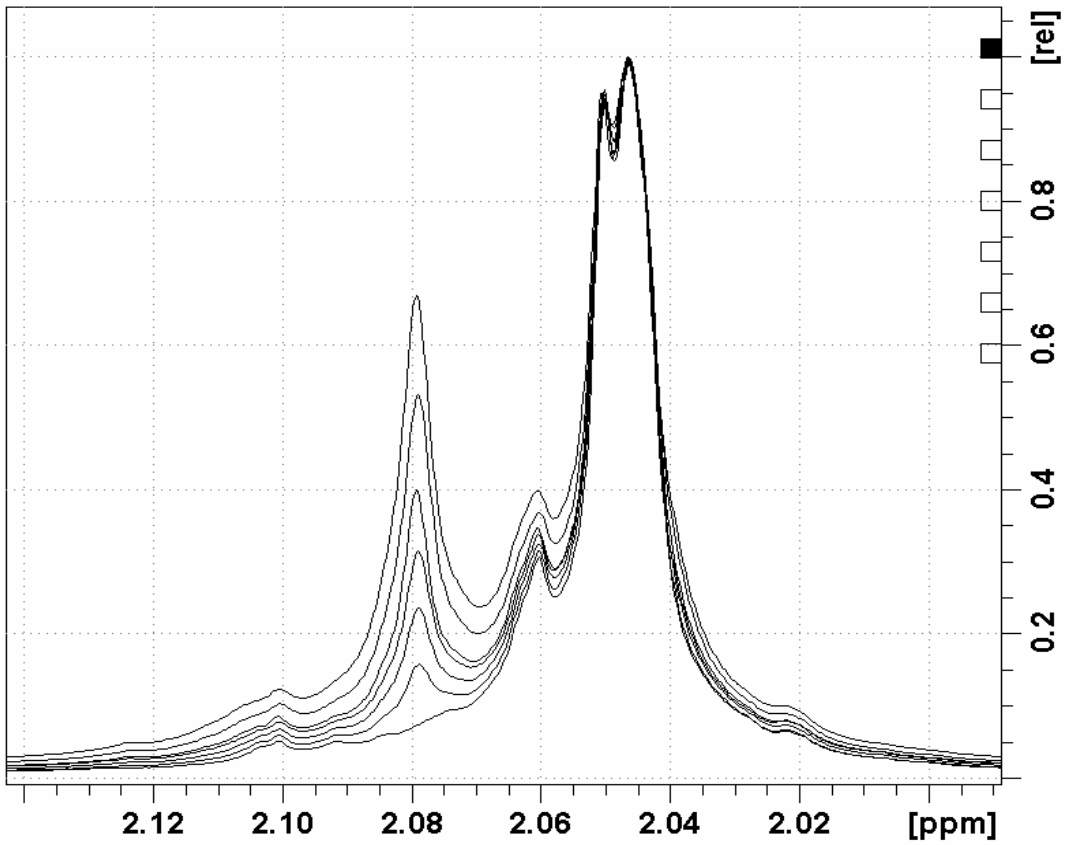
Detection of OSCS by ^{1}H-NMR

The effect of metal ions on the OSCS signal



The effect of some transition metal ions (trace amounts) on the OSCS methyl NMR signal (spectrum A); and after the addition of EDTA (spectrum B)

Detection of DS by ^{1}H-NMR



Dermatan sulphate: 1, 2, 3, 4, 6 and 8% in heparin

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Summary:

DS at 2.08 ± 0.01 ppm
OSCS at 2.15 ± 0.02 ppm (0.01 ppm). LoD = 0.1% at S/N of 2000:1. The S/N refers to the heparin methyl signal.

The OSCS signal can be shifted to 2.18 ± 0.01 ppm by adding Ca^{2+} to the solution. This can be used for OSCS identification instead of spiking, or when the NMR spectrum is obtained with a 600 MHz NMR instrument (otherwise the OSCS signal will coincide with the ^{13}C satellite of the heparin methyl group)

There is a unidentified signal at 2.10 ppm that is often present in pure heparin (for instance the EDQM heparin standard)

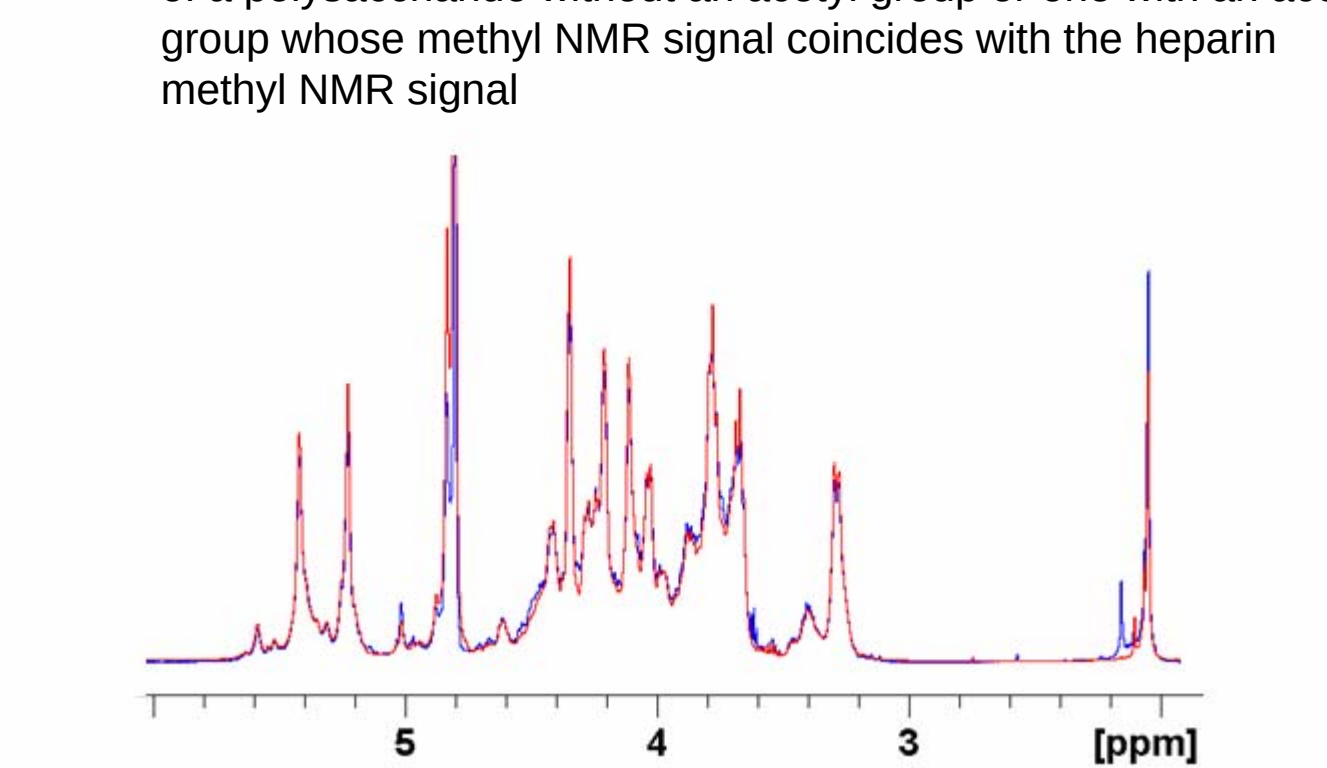
For quantification of OSCS and DS see publication in Pharmeuropa Bio, 2008-1

Purity test of new and unknown impurities / contaminants

- ^{1}H-NMR
- 2D NMR

Purity test by ^{1}H-NMR

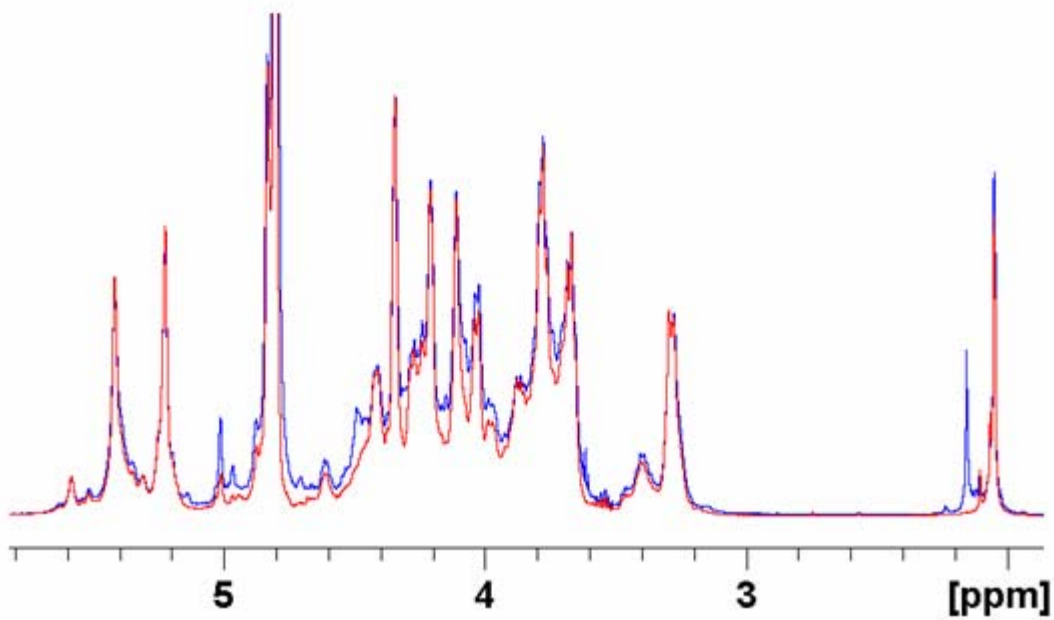
At levels below 5-7%, it would be difficult to detect the presence of a polysaccharide without an acetyl group or one with an acetyl group whose methyl NMR signal coincides with the heparin methyl NMR signal



^{1}H-NMR spectra of heparin free from OSCS (red trace) and of heparin spiked with 4% OSCS (blue trace)

Purity test by ^{1}H-NMR

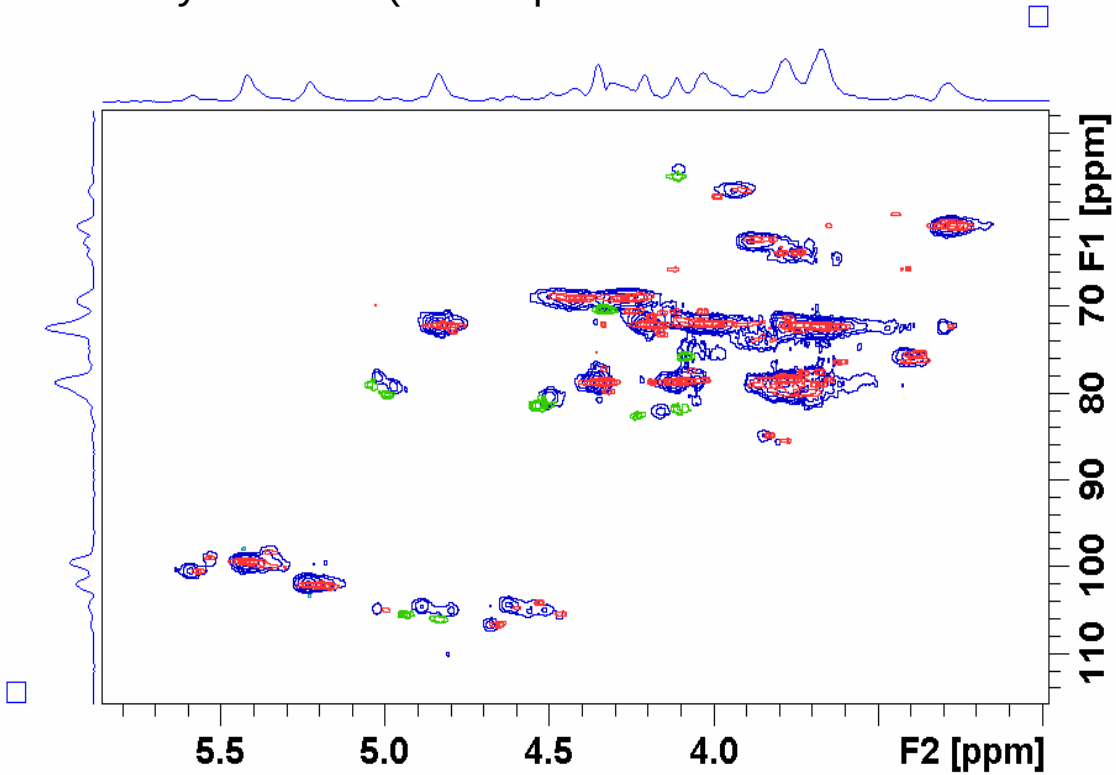
At levels above 8-10%, it would be possible to detect the presence of a polysaccharide without a methyl signal or with a methyl signal hidden under the heparin one



^{1}H-NMR spectra of heparin free from OSCS (red trace) and of heparin spiked with 9% OSCS (blue trace)

Purity test by 2D NMR

The presence of a polysaccharide without a methyl signal can be detected at levels of 1-2% by 2D NMR (with experiment times of more than two hours).



2D NMR spectra (HSQC) of heparin free from OSCS (red trace) and of heparin with 4% OSCS (blue trace). The NMR spectrum of OSCS is plotted in green and shifted a bit down to the left for comparison

Summary:

^{1}H-NMR will detect new and unknown impurities at levels of 0.5-2% if signals are well resolved or levels of 7-10% if signals are hidden under the heparin signals (an example would be a de-acetylated OSCS)

2D NMR. Detection of unknown impurities can reach levels as low as 1-2%

Conclusions

For identification of heparin test samples:
• 2D NMR, HSQC or HMQC (less than 15-20 minutes experiment time)

For detection and quantification of DS and OSCS:
• ^{1}H-NMR (a ^{1}H-NMR spectrum is normally obtained before a 2D NMR experiment is carried out)

For detection of new and unknown impurities / contaminants:
• 2D NMR, HSQC or HMQC (more than two hours experiment time)

Publications:

McEwen I, Rundlöf T, Ek M, Hakkarainen B, Carlin G, Arvidsson T. *Effect of Ca $^{2+}$ on the 1H-NMR chemical shift of the methyl signal of oversulphated chondroitin sulphate, a contaminant in heparin. J Pharm Biomed Anal. 49 (2009) 816–819*

McEwen I, Mulloy B, Hellwig E, Kozerski L, Beyer T, Holzgrabe U, Wanko R, Spieser JM, Rodomonte A. *Determination of Oversulphated Chondroitin Sulphate and Dermatan Sulphate in unfractionated heparin by 1H-NMR -Collaborative study for quantification and analytical determination of LoD. Pharmeuropa Bio. 2008 Dec; 2008(1): 31-9*

McEwen I. *Broadening of the ^{1}H-NMR signals in the spectra of heparin and OSCS by some transition metal ions. The use of EDTA to sharpen the signals. J Pharm Biomed Anal (submitted)*