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Short communication

Improved impurity fingerprinting of heparin by high resolution ^{1}H NMR spectroscopy

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ABSTRACT

For improving the identification of potential heparin impurities such as oversulfated chondroitin sulfate (OSCS) the standard 2D ^{1}H-^{1}H NMR NOESY was applied. Taking advantage of spin diffusion and adjusting the experimental parameters accordingly additional contaminant-specific signals of the corresponding sugar ring protons can easily be detected. These are usually hidden by the more intense heparin signals. Compared to the current 1D ^{1}H procedure proposed for screening commercial unfractionated heparin samples and focusing on the contaminants acetyl signals more informative and unique fingerprints may be obtained. Correspondingly measured ^{1}H fingerprints of a few potential impurities are given and their identification in two contaminated commercial heparin samples is demonstrated. The proposed 2D NOESY method is not intended to replace the current 1D method for detecting and quantifying heparin impurities but may be regarded as a valuable supplement for an improved and more reliable identification of these contaminants.

1. Introduction

Unfractionated heparin is an anticoagulant used to prevent or treat thromboembolic disorders. It is also common in dialysis and cardiac surgery. Recently, hundreds of cases of anaphylactoid-type adverse effects, some fatal, were reported to U.S. and European Health Authorities [1,2]. For example, in January 2008 a cluster of adverse effects was observed in a Missourian pediatric hospital among patients undergoing hemodialysis. Symptoms were facial edema, tachycardia, hypotension, urticaria, and nausea [1]. In March 2008, the U.S. Food and Drug Administration (FDA) declared a "heparin-like" contaminant to be responsible for these adverse effects [1]. This compound was identified as oversulfated chondroitin sulfate (OSCS) [3] activating the contact and complement systems [4].

NMR spectroscopy has been recommended by the FDA as one of the analytical techniques – together with capillary electrophoresis (CE) – for rapidly and effectively screening for the presence of a specific impurity highlighted as a safety concern. This technique has already been proposed in the past for detecting variations in chemical composition of heparin [5–7] and heparin-derived oligosaccharides [8,9] as well as for checking impurities from other glycosaminoglycans, such as dermatan sulfate (chondroitin sulfate B, CSB) [2,10], chondroitin-4-, and chondroitin-6-sulfate [5,6]. Due to its high sensitivity to even minor structural variations one-dimensional ^{1}H NMR spectroscopy has proven to be most promising and suitable for assessing routine methods for analyzing complex mixtures. With improved detection limit, this simple technique has become even more popular, when performed on spectrometers equipped with high field magnets and cryogenically cooled probeheads [11] and when combined with microcoil technology [12–15]. Fast sample preparation and straightforward spectra evaluation taking advantage of the high potential of ^{1}H NMR spectra as unique fingerprints make the method most popular for quality and purity control by means of quantitative NMR (qNMR) [16]. ^{1}H NMR was consequently proposed for the quantitative determination of OSCS in unfractionated heparin and an optimized ^{1}H NMR method with increased detection sensitivity especially for OSCS and dermatan sulfate was presented [2]. This method concentrates on the *N*-acetyl region and takes advantage of multivariate statistics and quantification. Very recently it has been shown [17] that diffusion-ordered spectroscopy (DOSY-NMR) [18] can be applied for screening the lots of heparins. Concentrating on the ^{1}H signals of the *N*-acetyl peaks impurity profiles of these samples based on different translational diffusion coefficients D_t of the components may be obtained.

However, the great potential of NMR is the arsenal of sophisticated 1D and 2D NMR techniques [19] exploited for unraveling molecular structures. This has been demonstrated with the characterization of the solution conformations of heparin [20] and very

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recently with the detailed structural analysis of the contaminant of commercial heparin finally identified as oversulfated chondroitin sulfate [3].

Although highly valuable for structure elucidation most of these sophisticated techniques, especially heteronuclear experiments relying on the natural abundance of rare isotopes, e.g. ^{13}C or ^{15}N, are rather time-consuming. Furthermore and with mixtures of compounds the analysis of the corresponding spectra is normally not straightforward. Therefore, these methods are usually too insensitive and spectra analysis is too complicated for checking the quality of drug samples such as heparin on a routine level.

In the present study, we describe the application of a method based on the 2D ^1H-^1H NOESY experiment [21–24] which yields more informative fingerprints for the contaminants of heparin samples. The method is not intended to replace the current procedure for their quantitative detection. It should be regarded as a valuable experimental supplement applied to reliably prove the identity of these contaminants in case additional verification is needed.

2. Experimental

2.1. Materials and sample preparation

The commercial heparin samples were provided by Bichsel Laboratories, Interlaken, Switzerland. These were produced by Changzhou Bio-Pharma Qianhong Co. Ltd., Changzhou, China, and imported to Europe by Welding and Helm AG, Hamburg, Germany. Chondroitin sulfate A sodium salt (CSA, from bovine trachea) and chondroitin sulfate B (CSB, from porcine intestinal mucosa were provided by Sigma–Aldrich, Buchs, Switzerland.

According to the FDA specifications 20 mg of pure and of two contaminated commercial heparin samples were dissolved in 0.6 ml of D_2O (99.9%) spiked with 0.1% (w/v) of trimethylsilylpropionic acid (Na-salt, TSPA). Corresponding solutions with 5–9 mg of OSCS, CSA and CSB were prepared for NMR analysis. Samples with mixture of these solutions and the solution of pure heparin at the ratio of 20/80% (v/v), respectively, served as reference samples.

2.2. NMR experiments and NMR data analysis

The NMR experiments were performed at room temperature (298 K) on a BRUKER AV II-500 spectrometer operating at a ^1H resonance frequency of 500.13 MHz. The instrument was equipped with a 5-mm dual probe (BBI) for inverse detection with a z-gradient coil. The TOPSPIN 2.0 BRUKER Software was used for data acquisition and subsequent data processing. The 1D ^1H-spectra (using the standard BRUKER *zg* pulse program) were recorded according to the FDA specifications: 32 transients; spectral width of 8012 Hz; data size of 32 K points; acquisition time of 2.05 s; relaxation delay of 20 s; measuring time 12 min. The free induction decays (FID) were exponentially weighted with a line broadening factor of 0.5 Hz line broadening prior to Fourier transformation.

The gradient enhanced 2D NOESY experiment using the standard BRUKER noesygpph pulse program, slightly modified for selective presaturation of the strong water signal was performed with the following parameters: spectral widths in both dimensions 3000 Hz; time domain data sizes in the two time dimensions t2 and t1 of 1024 and 256 points (increments) respectively; 2 transients per increment; NOE build-up time of 0.6 s; relaxation delay of 0.5 s; total measuring time 14 min. Water presaturation was performed during the relaxation delay using a power of 0.031 mW. Postprocessing of the 2D time domain signal was performed with frequency domain data sizes in f2 and f1 of 1024 and 512 points respectively and exponential window functions with a line broadening factor of 5 Hz in both dimensions prior to 2D Fourier transformation. The spectra were manually phased and (polynomial) base line corrected in f1.

3. Results and discussion

The 2D ^1H-^1H NOESY experiment [21–25] proposed for obtaining superior NMR fingerprints of acetylated impurities in heparin samples was originally developed and has widely been used for the detection of intra- or intermolecular spatial proximities among protons within the investigated or between different molecules. The physical basis of the experiment is the mutual and direct (through space) dipole–dipole (DD) coupling among nuclei giving rise to

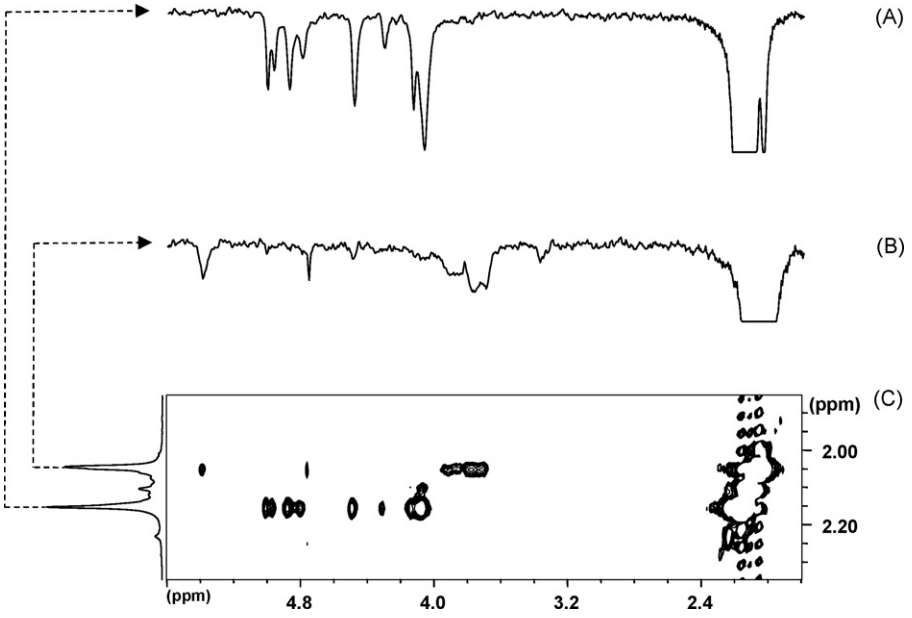

Fig. 1. Results of the 2D NOESY experiment applied to a sample of heparin contaminated with OSCS. (C) Expansion of the 2D NOESY spectrum with the intense contours of the acetyl signals (right) and the weaker contours obtained by spin diffusion of the corresponding sugar protons (left) of heparin and OSCS; corresponding 1D rows extracted from the 2D spectrum at the acetyl resonances of (B) heparin and (A) OSCS.

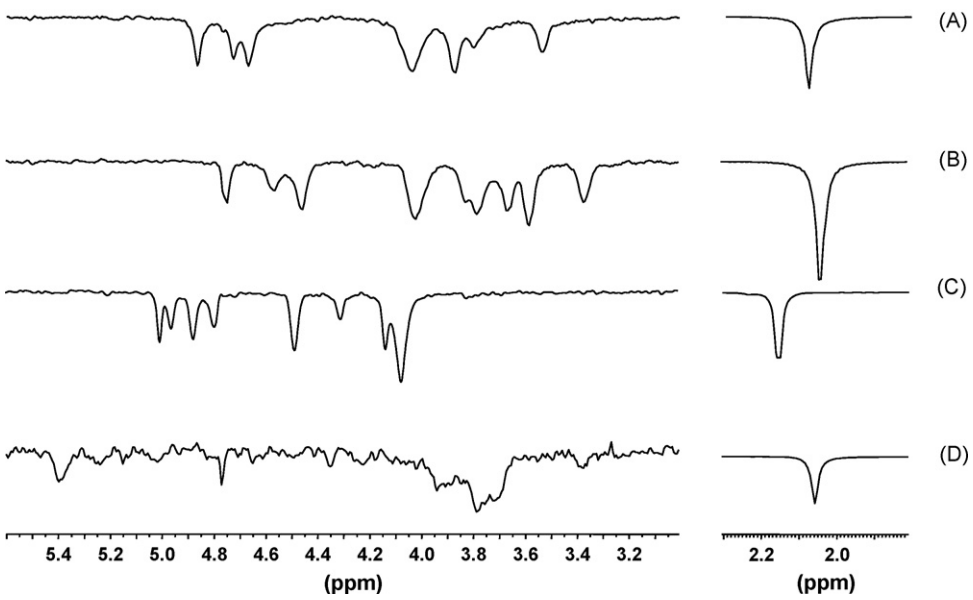

Fig. 2. NOESY reference rows of pure (D) heparin and three potential acetylated contaminants: (C) OSCS; (B) CSA and (A) CSB. The expansions showing the acetyl signals (right) are scaled down by a factor of 32 compared to the corresponding regions of the sugar protons (left).

small, but meaningful changes of signal intensities, the so-called Nuclear Overhauser Effect (NOE). These intensity changes may be transformed qualitatively or quantitatively into internuclear distances and hence structure information and makes NMR most popular for the determination of the 3D structure of biomolecules.

However, and especially when applied to large molecules experimental parameters, primarily the so-called mixing time, governing the build-up of transient NOEs [26,27], have to be adjusted carefully. With the equilibrium polarization of a selected proton disturbed initially NOEs are built up almost exclusively in the very first part of the mixing period for the spatially closest protons, leaving the intensities of more remote protons unperturbed. With increasing mixing times however and most pronounced for larger molecules a second process, the so-called spin diffusion [28,29] becomes more and more dominant. NOE intensity changes built up for the nearest protons at the beginning of the mixing period propagate snowballing to the next spheres of nearby protons and finally, with a mixing time long enough, are distributed more or less uniformly across the whole network of dipolar coupled nuclei of a molecule.

Spin diffusion usually avoided carefully in structural studies has been exploited in the present study to enlarge the ^{1}H-fingerprints of heparin impurities. Thereby the acetyl protons of these impurities serve as the main source for spin diffusion. With mixing times long enough intensity changes originating from and induced by the

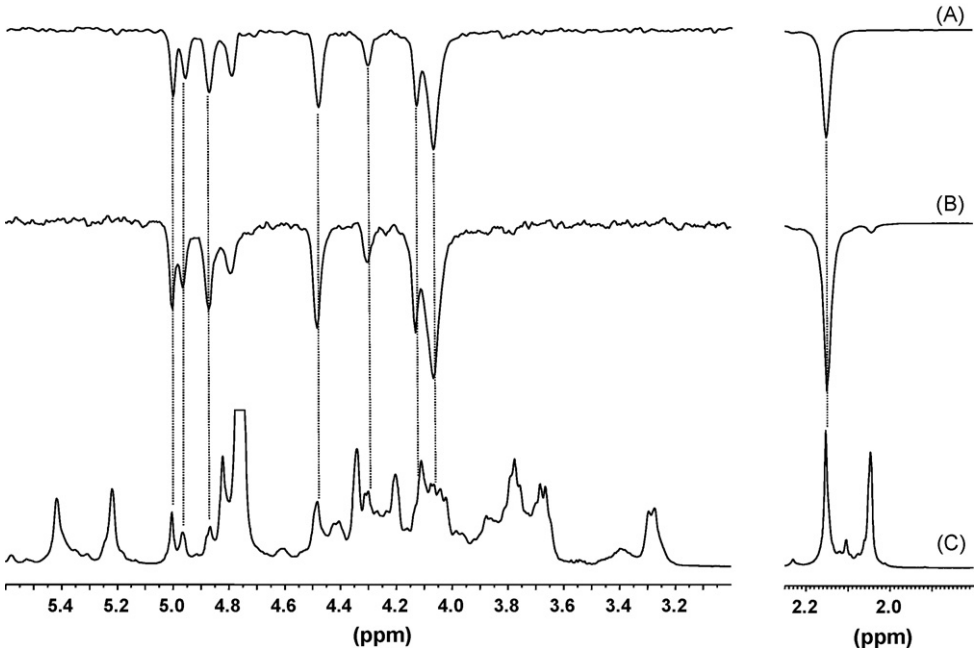

Fig. 3. Application of the proposed 2D NOESY experiment to a heparin sample contaminated with OSCS. (C) Expansions of the 1D ^{1}H spectrum (obtained in 12 min) of contaminated heparin with the acetyl signals (right) and the sugar ring signals (left); (B) 1D row extracted from the 2D NOESY spectrum (obtained in 14 min) at the acetyl resonance at 2.15 ppm; (A) NOESY reference row of OSCS. The contaminant is unambiguously identified as OSCS. The expansions showing the acetyl signals in (A) and (B) are scaled down by a factor of 32 compared to the corresponding regions of the sugar protons.

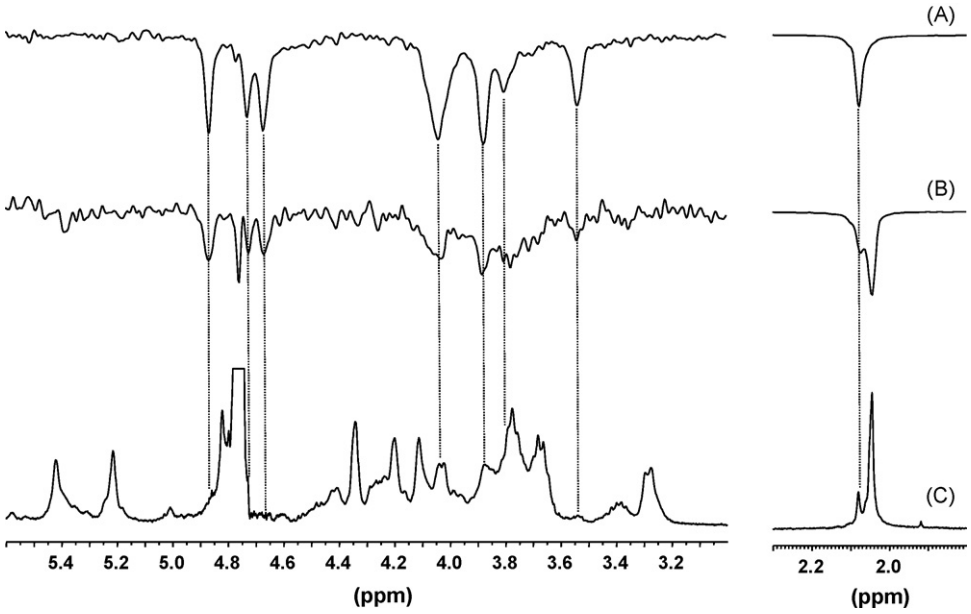

Fig. 4. Application of the proposed 2D NOESY experiment to a heparin sample contaminated with CSB. (C) Expansions of the 1D ^{1}H spectrum (obtained in 12 min) of contaminated heparin with the acetyl signals (right) and the sugar ring signals (left); (B) 1D row extracted from the 2D NOESY spectrum (obtained in 14 min) at the acetyl resonance at 2.09 ppm; (A) NOESY reference row of CB. Although and due to partial overlap with the heparin acetyl signal in (B) some weak heparin peaks are visible as well, the contaminant is unambiguously identified as CSB according to a few unique resonances. The expansions showing the acetyl signals in (A) and (B) are scaled down by a factor of 32 compared to the corresponding regions of the sugar protons.

strong signal of the acetyl group(s) may be detected for most if not all sugar ring protons in the corresponding impurities. Since the acetylation degree of these impurities is usually high (1 acetyl group in every disaccharide repeat unit) compared to heparin (1 acetyl group in every seventh or eighth disaccharide repeat unit) the NOESY fingerprints are rather sensitive and adequate for detecting and identifying impurities even for weakly contaminated heparin samples.

In a first step the experimental parameters for the acquisition and processing of ^{1}H-^{1}H NOESY data for heparin samples had been optimized. Parameters for data acquisition and data processing have been chosen to get highest sensitivity with the NOESY experiment within a given measuring time, e.g. comparable to the time used for the current ^{1}H NMR experiment recommended by the FDA.

For corresponding sample characterization rows across the acetyl signals in the NOESY spectrum – instead of depicting 2D contour plots – turned out to be most informative and directly comparable with the corresponding 1D ^{1}H spectra (Fig. 1).

In a second step and to provide reference data ^{1}H-^{1}H-NOESY spectra were acquired of pure heparin, the potential contaminants

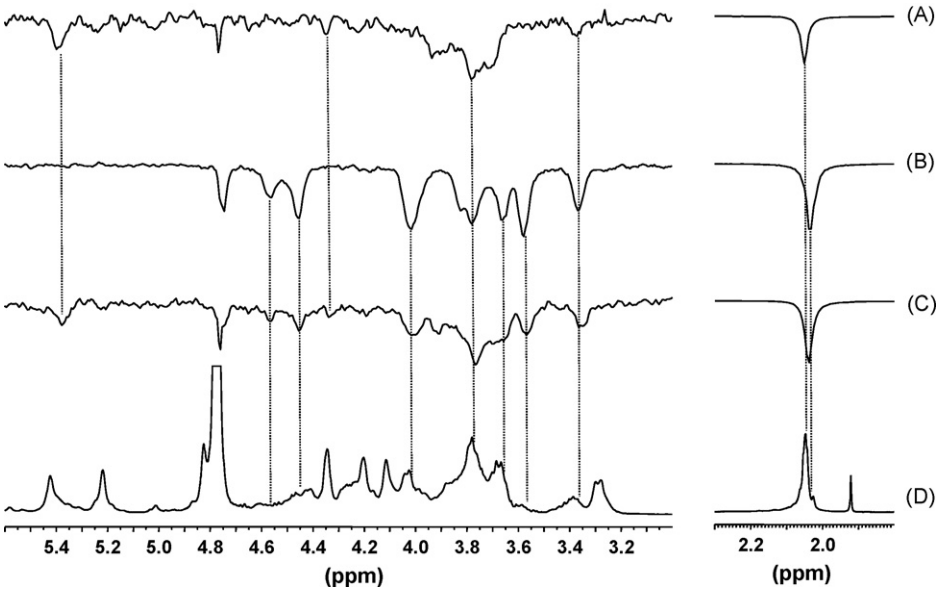

Fig. 5. Application of the proposed 2D NOESY experiment to a heparin sample contaminated with a small amount of CSA. (D) Expansions of the 1D ^{1}H spectrum (obtained in 12 min) of contaminated heparin with the acetyl signals (right) and the sugar ring signals (left); (C) 1D row extracted from the 2D NOESY spectrum (obtained in 14 min) at the acetyl resonance at 2.05 ppm; (B) NOESY reference row of CSA. (A) NOESY reference row of pure heparin. Although the two acetyl signals of the contaminant and heparin overlap giving rise to weak heparin peaks in (C) additional and CA unique signals are recognized in the same spectrum. This allows the contaminant to be identified. In (D) an additional acetyl signal of a trace contaminant is visible. The expansions showing the acetyl signals in (A) and (B) are scaled down by a factor of 32 compared to the corresponding regions of the sugar protons.

OSCS, CSA, CSB, and heparin samples spiked with these contaminants. Fig. 2 shows the corresponding rows measured for the pure samples. Unique fingerprints are obtained allowing individual contaminants to be recognized and identified reliably.

Comparing the two NOESY profiles obtained for these potential contaminants when dissolved in pure D_2O and when dissolved in heparin solutions small but nevertheless distinct and non-uniform shifts for the fingerprint signals of OSCS are detected. Such mixture induced shifts, not observed with CSA and CSB, must be attributed to specific intermolecular interactions between heparin and OSCS. Consequently and instead of using solutions of the pure contaminants, NOESY spectra of heparin samples spiked with the selected contaminants should be used as reference data for controlling the quality of commercial heparin.

The search for and the identification of contaminants is demonstrated for two heparin samples in Figs. 3 and 4. In the NOESY spectra of the corresponding samples the row across the acetyl signal of one of the contaminants had been extracted. This row is depicted together with the 1D ^{1}H spectrum (bottom) and the NOESY reference spectrum of the identified contaminant (top). With the unique fingerprints of the corresponding contaminants OSCS in the first sample (Fig. 3) and CSB in the second (Fig. 4) and with the additional contaminant specific peaks a higher reliability for identification is obviously achieved compared to the current procedure relying on a single acetyl signal. On the other hand and comparing the results of the two methods for equal measuring times there is no doubt that the proposed NOESY technique is less sensitive. Consequently and depending on the contaminant concentration longer measuring times have to be taken into account.

A further aspect in the context of the two procedures is demonstrated in Fig. 5. The two 1D ^{1}H spectra of a pure heparin sample and a commercial heparin sample contaminated with CSA reveal on a first glance no distinct differences. Besides a weak sharp acetyl signal at 1.92 ppm of a trace impurity there is only one acetyl signal visible and only minor differences may be recognized in the region of the ring protons. In the NOESY row across the single acetyl signal however – besides the weak heparin responses – a few additional but unique signals can be recognized which fit to the NOESY reference spectrum of CSA. Therefore, contaminants with the same acetyl chemical shift and with their ring proton signals overlapped by the intense heparin signals may easily be overlooked with the current 1D ^{1}H method but may be detected and identified with the proposed 2D ^{1}H-^{1}H NOESY experiment.

4. Conclusion

A standard 2D ^{1}H-^{1}H NMR method (NOESY) was optimized and applied for obtaining improved and easily to interpret fingerprints of potential acetylated contaminants of heparin such as OSCS. Exploiting the effect of spin diffusion the fingerprint of these contaminants is extended to the signals of the corresponding sugar ring protons.

Compared to the current 1D ^{1}H procedure focused on the acetyl signal region the NOESY method yields additional contaminant specific ^{1}H signals and hence more unique fingerprints even in case of overlapping acetyl signals. These highly informative ^{1}H fingerprints may easily be extracted and allow the corresponding contaminants to be characterized and identified more reliably. However, the method is less sensitive compared to the standard 1D ^{1}H procedure and consequently longer measuring times have to be taken into account with low concentrated contaminants. Therefore, the proposed method will not replace the current procedure for detection and quantification of contaminants but should be considered as a valuable supplement for their reliable identification.

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