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Improving reliability of chemometric models for authentication of species origin of heparin by switching from 1D to 2D NMR experiments

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ABSTRACT

Nuclear magnetic resonance (NMR) spectroscopy is regarded as one of the most powerful and versatile analytical approaches to assure the quality of heparin preparations. In particular, it was recently demonstrated that by using ^{1}H NMR coupled with chemometrics heparin and low molecular weight heparin (LMWH) samples derived from three major animal species (porcine, ovine and bovine) can be differentiated [Y.B. Monakhova et al. J. Pharm. Anal. 149 (2018) 114–119].

In this study, significant improvement of existing chemometric models was achieved by switching to 2D NMR experiments (heteronuclear multiple-quantum correlation (HMQC) and diffusion-ordered spectroscopy (DOSY)). Two representative data sets (sixty-nine heparin and twenty-two LMWH) belonged to different batches and distributed by different commercial companies were investigated. A trend for animal species differentiation was observed in the principal component analysis (PCA) score plot built based on the DOSY data. A superior model was constructed using HMQC experiments, where individual heparin (LMWH) clusters as well as their blends were clearly differentiated. The predictive power of different classification methods as well as unsupervised techniques (independent components analysis, ICA) clearly proved applicability of the model for routine heparin and LMWH analysis.

The switch from 1D to 2D NMR techniques provides a wealth of additional information, which is beneficial for multivariate modeling of NMR spectroscopic data for heparin preparations.

1. Introduction

Heparin is a heterogeneous polymer, which belongs to the glycosaminoglycan (GAG) family [1]. Along with low molecular weight heparins (LMWHs), which are produced by depolymerization of heparin starting material, it is a widely used anticoagulant in medical and surgical indications [1].

Among instrumental methods, ^{1}H NMR spectroscopy is recognized as one of the most powerful tools used for the structural elucidation of heparin as well as for quantitative assessment of contaminant levels and qualitative features such as animal origin or producer [2,3]. However, for large carbohydrate polymer mixtures like heparin, spectral overlap in 1D NMR impedes excellent data evaluation even using chemometric techniques.

This problem could be overcome to some extent by using multidimensional NMR experiments. Indeed, 2D NMR spectra, which have two frequency dimensions, under certain circumstances encode a larger amount of information then 1D data. Previously, a number of 2D NMR methods have been used to characterize the composition and structure of heparin active pharmaceutical ingredients (APIs) [4–6]. It should be mentioned that Heteronuclear Single Quantum Coherence (HSQC) and a similar approach Heteronuclear Multiple-Quantum Correlation (HMQC), which both reflect direct ^{13}C–^{1}H coupling, are the most frequently applied 2D NMR experiments for heparin surveillance [7–10]. In the case of contaminated heparin found on the market in 2007 and 2008, HSQC was crucial for the identification of the contaminants [7]. This type of spectroscopy has been also utilized to determine variously substituted monosaccharide components obtained by

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chemical modifications of the *Escherichia coli* polysaccharide, which is structurally related to heparin [8]. Moreover, HSQC NMR revealed differences in the composition of four LMWH biosimilar brands produced by different manufacturers [9].

Quantitative HSQC was also applied to the study of heparin and LMWH [8,10]. For example, several commercially available LMWHs (enoxaparin, dalteparin, and tinzaparin) were investigated by HSQC focusing on the quantification of the reducing and non-reducing residuals [9]. To further develop and validate this approach, the HSQC technique was applied to a larger set of samples obtained from multiple sources [10]. The data showed that the HSQC assay was robust to small variations in signal to noise and relaxation effects [10].

Another type of 2D NMR spectroscopy, diffusion-ordered spectroscopy (DOSY), was also applied for the screening of heparin samples [11–13]. DOSY combines information, which is specific to ^{1}H NMR chemical shifts and is related to molecular weight distribution for a given species [11,12]. Therefore, this type of 2D NMR enabled the investigation of unfractionated and depolymerized heparins for the presence of contamination (e.g., oversulfated chondroitin sulfate) and process related impurities (e.g., dermatan sulfate) as well as their polydisperse degradation products [12]. It was demonstrated that DOSY NMR could be used to follow the course of the enzymatic reaction non-invasively and at the desired time point [11]. Recently, the DOSY technique was employed for the calculation of average molecular weight of heparin products as a complementary measurement to standard 1D NMR quality control [13].

Thus, the use of 2D NMR experiments permits a detailed structural and quantitative analysis of the monosaccharide features of heparins and LMWHs with an acceptable error [9–13]. Therefore, the switch from 1D to 2D NMR could also provide a wealth of additional information for multivariate modeling. Despite the fact that several attempts were made to employ chemometric approaches to ^{1}H NMR data of heparin [14,15], none of the existing applications of 2D NMR techniques utilized multivariate analysis to provide quality control of heparin regarding its animal origin. Therefore, in this study HMQC and DOSY NMR methods combined with chemometrics were evaluated for their ability to discriminate the animal origin of heparin samples. The method was validated on a representative dataset of authentic heparin and LMWH samples from various origin (porcine, bovine, ovine).

2. Materials and methods

2.1. Samples and chemicals

A total of fifty-seven heparin (30 bovine, 9 ovine, and 18 porcine) and twenty-two LMWH (10 ovine and 12 porcine) samples were investigated. All investigated samples were commercial active pharmaceutical ingredients (APIs) distributed by different commercial companies (all samples belonged to different batches), which encompasses at least eight various producers. This is the prerequisite to prove the heterogeneity of our reference library regarding samples structure (e.g., sulfation and acetylation profiles as well as the linkage region content). Deuterated water of 99.8% purity containing 0.1% trimethylsilyl propanoic acid (TSP) as internal standard was purchased from Euriso-top (Saarbrücken, Germany).

2.2. Sample preparation

For sample preparation, 100 mg of a heparin (LMWH) sample was mixed with 0.7 mL D_2O. A series of eighteen blends with a total weight of 100 mg was prepared from selected heparin and LMWH samples of different origin. The blends were prepared from the heparin (LMWH) batches not included in the reference database. The amount of ovine or bovine species in the mixtures with porcine material was 5%, 10%, 15%, 20%, 50% and 75% (w/w).

2.3. NMR measurements and processing

NMR measurements were performed on Bruker Avance III 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) with BBO cryo probe equipped with Bruker Automatic Sample Changer (B-ACS 120) at 297 K ^{1}H NMR spectra were recorded with standard pulse program (zg30 in Bruker language) using 16 scans (NS) and 2 prior dummy scans (DS). The data of 132k points (TD) were acquired with a spectral width (SW) of 24.0155 ppm, a receiver gain (RG) of 72, an acquisition time (AQ) of 4.5438 s.

2D HMQC spectra were recorded with the following parameters: NS = 64, DS = 16, TD = 2048, RG = 2050, AQ = 0.3047 (F2) and 0.003852 (F1), SW = 5.60 ppm (F2) and 110 ppm (F1). The transmitter offset was set to 4.5 for ^{1}H and 65 ppm for ^{13}C.

Several regions (rectangles) in HMQC data were tested to choose the optimal one for the multivariate statistics for heparin data set: 5.8–1.8 & 110–15 ppm; 5.8–4.9 & 110–90 ppm; 5.8–4.3 & 110–90 ppm; and 4.8–3.0 & 85–50 ppm. Bucketing with 0.01 ppm (^{1}H NMR axis) and 0.1 ppm (^{13}C NMR axis) width as well as unfolding were successively applied to the data in order to align the spectral data. To normalize the intensities in different samples, buckets were scaled to total intensity.

2D DOSY (diffusion ordered spectroscopy) experiments were performed using standard DOSY pulse sequence with longitudinal eddy current delay (LED) with bipolar gradient pulse pair and 2 spoil gradients. The length of the gradient pulse (δ) was set to 1400 μs and diffusion time (Δ) was set to 0.05 s. 2 scans provided enough sensitivity for heparin measurements.

For the processing of DOSY spectra the following diffusion fit function was used:

$f(x) = I_0 + e^{\left(-y^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right) D\right)}$, where D is the diffusion coefficient, g is the gradient strength and y is the gyromagnetic ratio. I_0 and I represent the maximum and observed signal intensity. The 2D plots show diffusion coefficient values D in [m^2/s].

The DOSY spectra were normalized to TSP signal at −9.3 m^2/s and 0.0 ppm. The free induction decay to an exponential multiplication associated with line broadening was set to 1 Hz in the ^{1}H direction. The data points from spectral regions (5.10–5.70 ppm, and 1.80–4.50 ppm for heparin) were then pre-processed by bucketing with 0.01 ppm width. Each resultant matrix was unfolded to an array (1 × 1500), so that traditional chemometric methods could be applied.

The data were recorded automatically under the control of ICON-NMR (Bruker Biospin, Rheinstetten, Germany). All NMR spectra were manually phased and baseline-corrected using Topspin 3.2 (Bruker Biospin, Rheinstetten, Germany). Dynamic Center v. 2.2 (Bruker Biospin, Rheinstetten, Germany) was used for the treatment of DOSY raw data.

2.4. Chemometric modelling and validation

Matlab 2015a (The Math Works, Natick, MA, USA) and SAISIR package for MATLAB was used for statistical calculations [16].

Principal component analysis (PCA), factor discriminant analysis (FDA), partial least squares – discriminant analysis (PLS-DA), and linear discriminant analysis (LDA) were utilized as chemometric approaches.

Validation of the discriminant analysis (DA) models was performed using leave-out-one cross validation (LOOCV) as well as independent test set. The calibration set for the heparin model con-

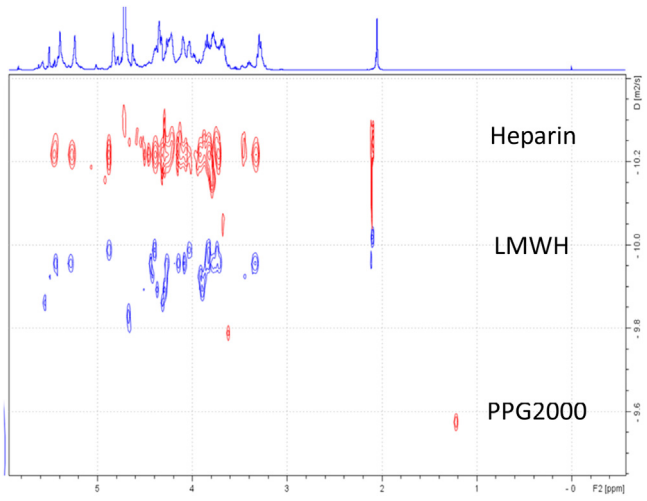

Fig 1. An overlay of the typical DOSY profiles of heparin, LMWH and polypropylene glycol standard with the average MW of 2 kDa.

sisted of 50 samples (7 ovine, 22 bovine, 13 porcine, 4 ovine-porcine and 4 bovine–porcine mixtures). Data analysis methods (LDA, FDA, and PLS- DA) were evaluated for predicting class membership of heparin samples from the test set consisted of 19 objects (2 ovine, 8 bovine, 5 porcine, 2 ovine-porcine and 2 bovine–porcine mixtures).

An unsupervised technique, independent components analysis (ICA), was also utilized in this study [17]. In the present paper, the Mutual Information Least Dependent Component Analysis (MILCA) ICA algorithm was applied [18]. To determine the number of significant independent components (ICs) recently proposed random modification of the ICA-by-Blocks method was used [19].

3. Results and discussion

3.1. Authentication of heparin animal origin using 2D DOSY NMR experiment

Differences in molecular weight distribution were observed among heparins produced from porcine, ovine, and bovine species [13,20]. For example, based on the size-exclusion chromatographic measurements it was found that porcine heparin had the highest molecular weight (MW) around 14.5 kDa among three major species, while ovine and bovine heparin have lower values [13,20]. LMWHs have an average MW between 4000 and 5000, and MW values of ovine LMWH samples were higher than those of porcine [14].

In this regard, DOSY NMR, which resolves the spectra of the individual components based on their size (molecular weight), could contain additional useful information for the characterization of heparin animal origin in comparison with 1D NMR profiles [11,12,21,22]. The combination of DOSY measurements and trilinear data analysis was shown to be powerful for the investigation of the reaction kinetics of sugar hydrolysis and lovastatin fermentation [21,22].

Fig. 1 shows the DOSY NMR spectra of representative heparin and LMWH samples in comparison with polypropylene glycol (PPG) reference standard with a defined molecular weight of 2 kDa. It is evident that the larger polymer heparin diffuses slower than the smaller LMWH and the PPG standard. However, if diffusion profiles of different heparin species are visually compared, the unambiguous characterization of heparin origin is not possible due to similar values of diffusion coefficients for heparins derived from different animal species.

Therefore, exploratory multivariate analysis using PCA was applied to a set of DOSY NMR data (Fig. 2). A tendency for species differentiation was observed in the 3D scatter plot constructed from the score values for the first three principal components (PCs). However, significant cluster overlap was found between the ovine and porcine groups, which would prevent reliable classification.

There are several plausible explanations for this observation. First, despite the possibility to align the 2D spectra along ^{1}H axis by bucketing, positions of the peak maxima are not stable along Y-axis of diffusion coefficient values (Fig. 1). Additional standardization using an appropriate polymeric internal standard with a defined molecular weight is required [14]. Second, each particular heparin sample showed considerable distribution in molecular weight values, which results in broad indecipherable 2D DOSY signals, which are difficult to be modelled.

Therefore, the use of diffusion coefficients along with the ^{1}H NMR profile of heparin is not advantageous for determining its animal origin. The correlation of DOSY NMR profiles with actual molecular weight values of heparins using multivariate regression techniques was more reliable [13].

3.2. HMQC spectroscopy for the characterization of animal origin of heparin and LMWH

Among ^{1}D NMR techniques, ^{13}C NMR spectroscopy has been also proved to be useful for the characterization of heparin and LMWH samples regarding their sulfation and disaccharide patterns as well as average molecular weight values [23,24]. Therefore, heteronuclear multiple-quantum correlation spectroscopy (HMQC), which is selective to direct C—H coupling, was selected as the second 2D NMR technique to improve the quality of multivariate modelling of NMR profiles of heparin samples.

Fig. 3 shows an overlay of HMQC spectra of representative ovine, porcine and bovine heparin samples. Heparins from different animal origin yielded quite similar HMQC patterns. Three major spectral regions were recognized: anomeric, sugar ring signals and the signal of *N*-acetyl group (Fig. 3) [8,10]. For the multivariate analysis the data within the rectangle limited by 5.8–4.9 ppm along ^{1}H and 110–90 ppm along ^{13}C axes, respectively, produced the best results regarding heparin animal origin. Similar to ^{1}H NMR spectra, no undisturbed peaks characteristic for each group of heparin samples were found, however, the integral values of the signals in this region varied significantly.

This area of anomeric signals was also highly specific for the characterization of heparin animal origin using 1D ^{1}H NMR data [15].

PCA was first carried out to evaluate the suitability of HMQC data for heparin origin differentiation. The projections of heparin samples as well as two types of heparin blends (bovine–porcine and ovine-porcine) on the first three PCs, which together explained almost 68% of data dispersion, were shown in Fig. 4. All five clusters were clearly differentiated, and the model was superior in comparison with that constructed based on ^{1}H NMR data, where slight overlap led to uncertainty in sample assignment between porcine and ovine groups [15,25]. It should be mentioned that the ovine-porcine and bovine-porcine blends were prepared from the external heparin batches not included in the reference library. The groups M1 and M2 were clearly distinguished from the clusters of independent heparin samples. Moreover, there was a clear trajectory of the increasing content of porcine material in blends on the score plot (Fig. 4).

As the heterogeneous group of heparin batches regarding structural variability was used to construct the PCA model, it can be concluded that the interspecies variability (ovine, bovine and porcine) is bigger than the structural differences within each origin group.

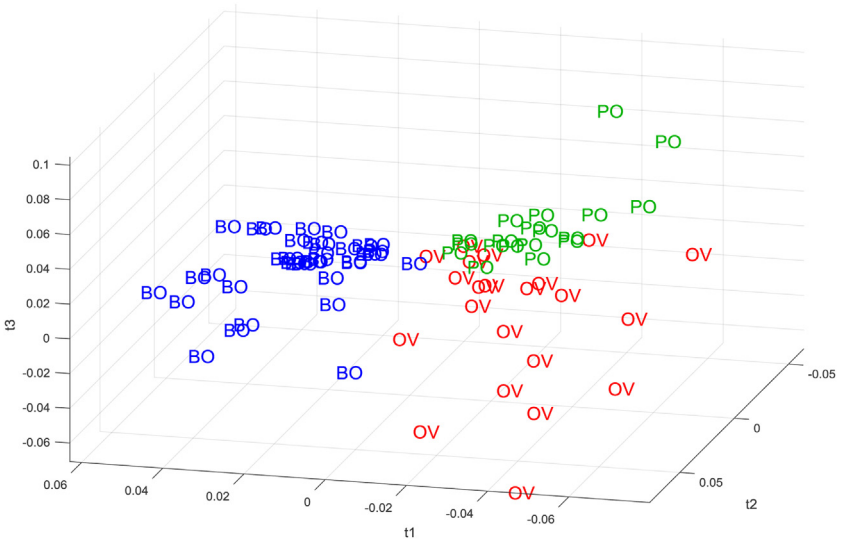

Fig. 2. 3D scatter plot of the FDA scores values for the authentication of heparin animal origin: BO – bovine, OV – ovine, PO – porcine.

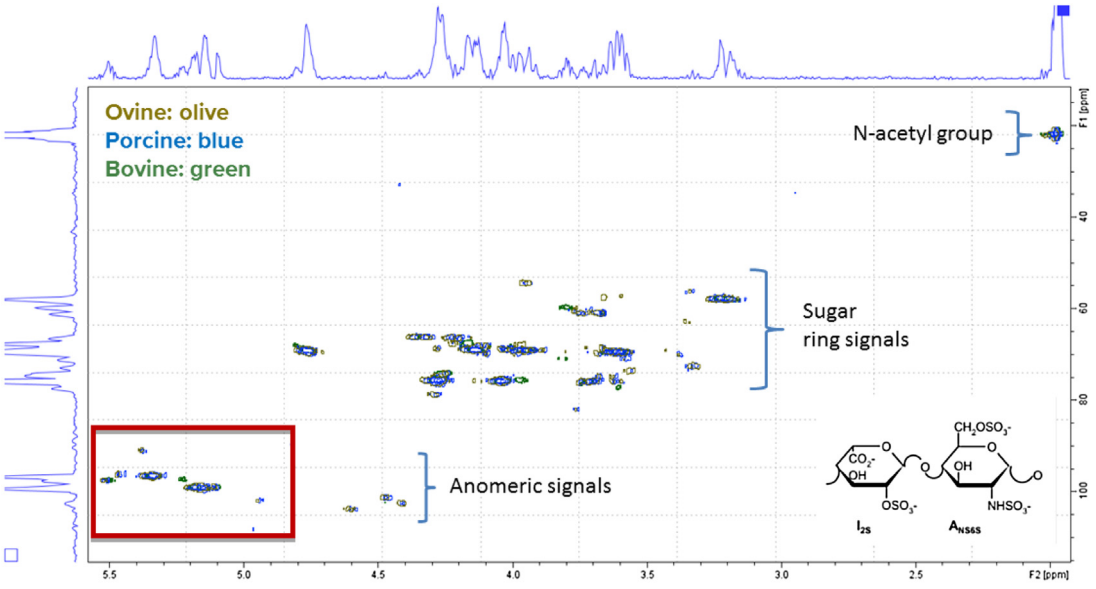

Fig. 3. Representative HMQC spectra of ovine (olive), porcine (blue) and bovine (green) heparin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As the next step, the predictive power of different chemometric classification methods was assessed. Several data analysis approaches (LDA, FDA, and PLS- DA) were evaluated for predicting class membership of heparin samples from the test set consisted of 19 objects (approximately 20% of the whole data set, see Materials and methods section). All 19 samples were correctly attributed to the "ovine", "bovine", "porcine", as well as to M1 and M2 groups by LDA and PLS-DA. On the contrary, one 5 w/w% ovine blend in porcine heparin was wrongly assigned as ovine heparin by FDA (classification accuracy was, therefore, about 95%). Therefore, HMQC combined with chemometric methods provides information about a heparin sample/blend with less overlap than observed with 1D NMR data.

The same strategy was applied to the analysis of HMQC profiles of LMWH samples (Fig. 5). A HMQC spectrum of LMWH contained some additional signals in comparison with heparin due to the presence of residuals generated by the depolymerization process (Figs. 3 and 5) [6,9,26]. In comparison with heparin, additional cross-peaks at δ 5.93/106 ppm, δ 5.55/101 ppm and at δ 5.50/101 ppm were recognized (Fig. 5). These intense signals belong to non-reducing unsaturated units of uronic acid linked to a *N*,6-disulfated glucosamine (GlcN6diS), which is the predominant GlcN type seen in LMWHs [6,9,26]. Similarly to heparin, a close similarity between LMWHs from ovine and porcine starting material was observed (Fig. 5).

The data from the region of anomeric signals between δ 4.8–6.0 ppm (^{1}H) and δ 85–110 ppm (^{13}C) was selected for statistical calculations. The PCA scores showed that 81.8% of variance was described by the LMWH model built from HMQC NMR spectra (Fig. 6). Fig. 6 showed that the samples were separated according to their animal identity: ovine species group was in the region of negative PC1 and PC2, whereas porcine cluster was in the region of positive PC2 and PC3 values. The scores corresponding to ovine blends in porcine material were located in the region of positive PC3 and PC1 values. Due to the small amount of LMWH samples in our dataset, independent set validation was not performed. How-

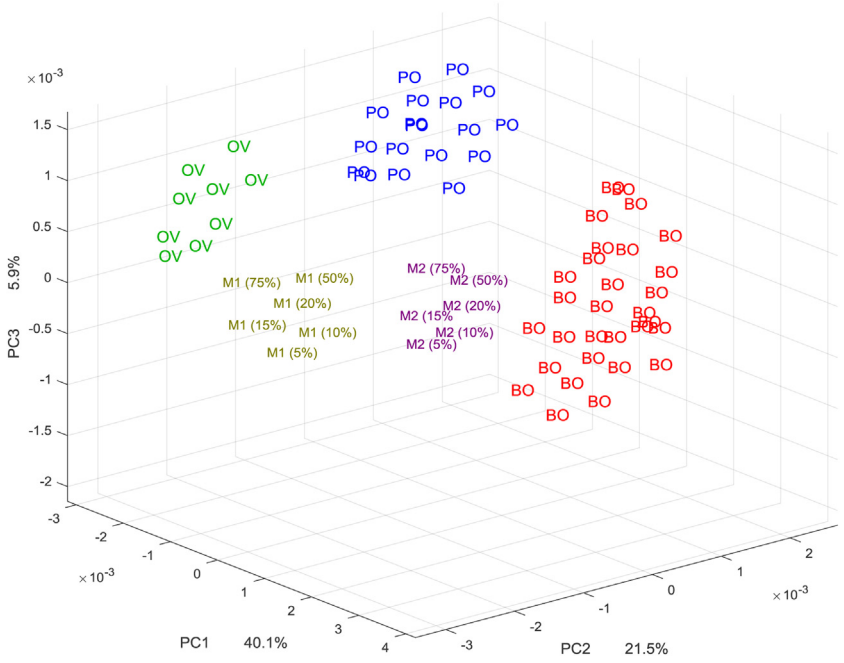

Fig. 4. 3D scatter plot of the PCA scores values for the authentication of heparin animal origin BO – bovine, OV – ovine, PO-porcine, M1–porcine-ovine blends, M2–bovine-porcine blends. The percentage of porcine material in blends is shown in brackets.

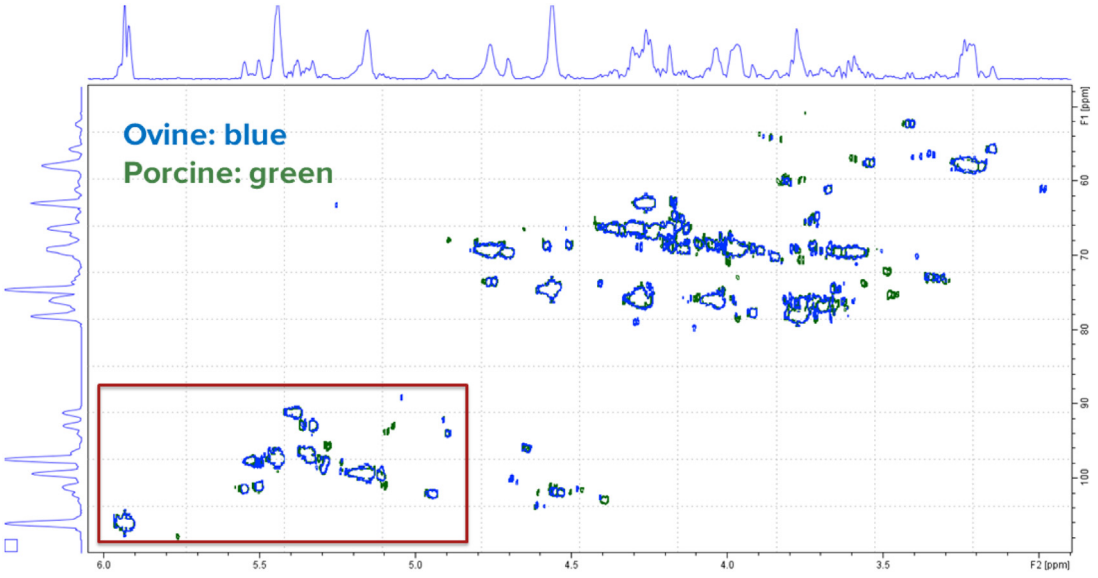

Fig. 5. Representative HMQC spectra of porcine (green) and ovine (blue) LMWH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ever, the results indicated that LMWH samples can be differentiated by their species of origin based on chemometric analysis of HMQC data. We are currently creating libraries of 2D spectra with sufficient reference heparin and LMWH samples with proven animal origin for detailed validation.

3.3. Independent component analysis for modelling of HMQC heparin spectra

In the literature several chemometric methods have been described for multivariate modelling of 2D heteronuclear NMR data [27–33]. For example, decomposition of 2D total correlation (TOCSY) and 2D [1H, 15N] HSQC spectra was achieved using multivariate curve resolution and band-target entropy minimization (BTEM) method [27–30]. General discriminant analysis (GDA) was applied to integral values of specific signals in Heteronuclear Multiple Bond Correlation (HMBC) spectra to assess honey authenticity [31]. Orthogonal partial least squares (OPLS) analysis of HSQC spectra was utilized to different plant pectin samples and serum from cancer patients [32,33].

In this study, apart from classical classification methods (PCA, LDA, FDA, and PLS-DA), independent components analysis (ICA) was also applied as an alternative chemometric tool for HMQC profiling of heparin. The primary goal of this method is to extract the pure signals from a data set of mixed signals by finding a transformation that minimizes dependences between “pure” sources

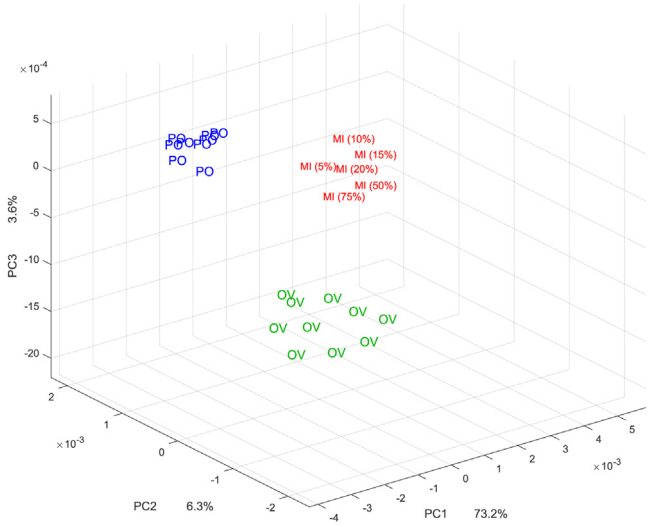

Fig. 6. 3D scatter plot of the PCA scores values for the authentication of LMWH animal origin: OV – ovine, PO-porcine, MI – porcine-ovine blends. The percentage of porcine material in blends is shown in brackets.

(called ICs) [17]. This technique has been extensively used for multicomponent spectroscopic analysis of different matrices as well as for classification [18,34–37].

Regarding 2D NMR data, the first problem, where ICA could be helpful, is the determination of the number of significant components in a complex data set. It is important for experimental design as well as for construction of classification models with the optimum number of factors. For this purposes, ICA-by-Blocks method was proven successful to estimate the number of significant components in chemical data [19,37].

Fig. 7A shows average correlation coefficients between pairs of resolved signals for the ICA models with up to 10 ICs for the matrix containing individual 40 heparin samples (HMQC data). The data were evaluated using 30 random splittings in two blocks. As can be seen, in models with up to three ICs, the correlations between corresponding ICs from each block were high, indicating that similar ICs are extracted in each segment. Therefore, it can be concluded that these ICs were significant. Addition of more ICs to the model led to a significant decrease of correlations between ICs from the two blocks, indicating that the fourth extracted IC was not valid and was almost exclusively based on background noise. In contrast, four factors were found to be optimal for the LDA and PLS-DA models. This means that ICA model was more compact and, therefore, more reliable than that based on supervised classification methods. Similar results were also obtained for the LMWH data set, where two significant components were resolved (porcine and ovine species).

HMQC NMR spectra of heparin samples from three species were evaluated by the ICA method. A good model for individual heparin samples was obtained using the three-component ICA model (Fig. 7B). ICA showed similar to the classical PCA discriminative ability (Figs. 4, 7B). Moreover, regarding the animal origin of the heparin samples, 100% correct classification rate was achieved according to the previously proposed formula [35]. These results were comparable with those of supervised classification methods. ICA was previously applied only to functional magnetic resonance imaging (FMRI) data in neuroscience applications and had not been utilized for analytical problems in spectroscopy [38].

4. Conclusions

The complexity of drugs, especially those based on animal derived polymer material, warrants the need for higher sensitivity

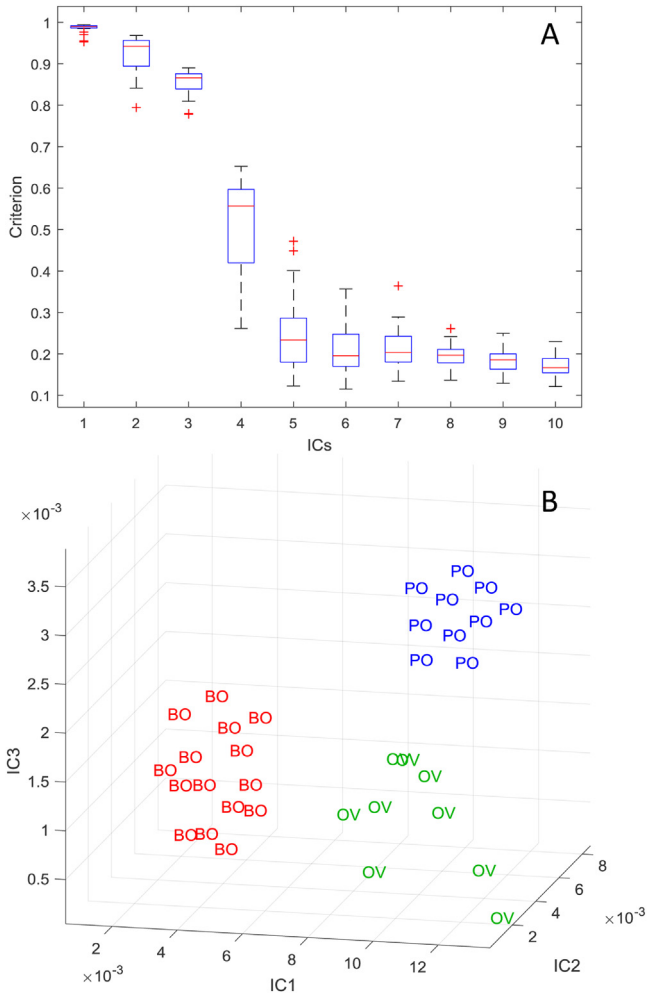

Fig. 7. A: Average correlation coefficients between pairs of signals (experimental/resolved) for the ICA models with different number of components for the heparin data set; B: 3D scatter plot of the ICA scores values for the authentication of heparin animal origin: OV – ovine, PO-porcine, BO – bovine.

and reliability for the determination of their critical quality parameters. Thus, powerful and versatile analytical approaches such as NMR spectroscopy are needed to assure the quality of complex drugs. In our previous studies it was demonstrated that by using ^{1}H NMR coupled with chemometrics, heparin samples from various origin and producers could be differentiated [14,25].

In the present study our hypothesis that the application of 2D NMR techniques (in particular, HMQC) would result in improved classification results regarding heparin animal origin was validated. In case of heparin, the switch from 1D to 2D NMR provides a wealth of additional information for multivariate modeling, which results in better classification models.

We also expanded the range of possible applications of ICA to include 2D NMR spectra. It should be noted that, despite being an unsupervised method, ICA demonstrated only slightly inferior performance compared to the classical supervised classification methods (LDA and PLS-DA), thus again demonstrating its applicability for solving classification in spectroscopy.

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