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NMR Spectroscopy as an Alternative Analytical Method for Biopolymers Without Chromophore: Example of Hyaluronic Acid in Dietary Supplements

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Abstract

To respond to the increasing demand for hyaluronic acid (HA) in dietary supplements (DSs) and nutricosmetics marketed for the treatment of osteoarthritis or moistening, it is essential to have an accurate and reliable method for its analysis in the final products. The study aimed to develop and validate alternative method for the quality control of HA in DSs using low-field (LF) and high-field (HF) nuclear magnetic resonance (NMR) spectroscopy at 80 MHz and 600 MHz, respectively. Moreover, chondroitin sulphate (CH), another active ingredient in DSs, can be simultaneously quantified. The ^{1}H-NMR methods have been successfully validated in terms of limit of detection (LOD) and limit of quantitation (LOQ), which were found to be 0.1 mg/mL and 0.2 mg/mL (80 MHz) as well as 0.2 mg/mL and 0.6 mg/mL (600 MHz). Recovery rates were estimated to be between 92 and 120% on both spectrometers; precision including sample preparation was found to be 4.2% and 8.0% for 600 MHz and 80 MHz, respectively. Quantitative results obtained by HF and LF NMR were comparable for 16 DSs with varying matrix. HF NMR experiments at 70 ℃ serve as a simple and efficient quality control tool for HA and CH in multicomponent DSs. Benchtop NMR measurements, upon preceding acid hydrolysis, offer a cost-effective and cryogen-free alternative for analyzing DSs in the absence of CH and paramagnetic matrix components.

1 Introduction

Hyaluronic acid (HA), a natural polysaccharide and glycosaminoglycan (GAG), consists of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine connected by alternating ß-1,3 and ß-1,4 glycosidic bonds. It is found in the extracellular matrix of skin, joint, and cartilage tissues [1, 2]. The global HA market has been experiencing continuous growth, with a valuation of USD 9.4

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billion for 2022 [3]. One of the primary factors contributing to this growth is the increasing demand for HA due to the ageing demographic and the rising demand for minimally and non-invasive cosmetic treatments [3]. Additionally, HA's high biocompatibility, biodegradability, and non-toxicity have led to its widespread use in various sectors, including dietary supplements (DSs), medicine, pharmaceuticals, cosmetics, and nutricosmetics [1, 4, 5].

One of the applications of HA is DSs promoted for osteoarthritis treatments and nutricosmetics [3, 6]. While HA often serves as a minor component in such supplements, the primary components are glucosamine and another GAG chondroitin sulphate (CH). These (poly-)saccharides are frequently utilized in supplements due to their natural presence in cartilage tissue. Controlling the quality of the active ingredients used in such formulation is crucial, as DSs are far less strictly regulated than pharmaceuticals [7].

Besides low concentrations, the broad molecular weight distribution, high viscosity in solution and low UV absorption, are further critical aspects in the analysis of HA. Quantitative analysis of HA in DSs is challenging also due to matrix components, such as further structurally similar polysaccharides, proteins, vitamins, and minerals. As a result, analytical procedures based on high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, capillary electrophoresis (CE), as well as gel permeation chromatography (GPC) were usually performed after complex derivatization or enzymatic hydrolysis [8–13]. Regarding spectroscopic methods, Mirzayeva et al. performed qualitative analysis of HA in various DSs by Fourier-transform infrared spectroscopy (FT-IR) after time-consuming sample purification and final precipitation of HA with copper(II)-cations [14]. Nuclear magnetic resonance (NMR) is primarily used in the characterization of HA-based hydrogels in novel drug-delivery systems [15–18]. Furthermore, it is used for the characterization of synthesized or extracted HA [19].

Since high-field NMR spectroscopy has been proven a powerful tool in the analysis of multicomponent samples, and DSs in particular, the objective of this study was the development and validation of a simple and fast high-field NMR method for ensuring the simultaneous quality control of HA and CH in multicomponent dietary supplements [20–22]. The structures of two polysaccharides were shown below.
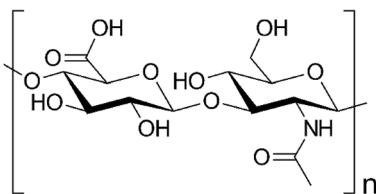
Hyaluronic acid (HA):   Chondroitin sulphate (CH):
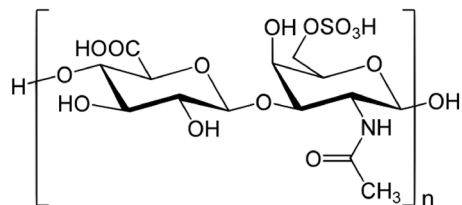
As a cheaper alternative, low-field NMR spectroscopic method based on acid hydrolysis was proposed. Advantages and disadvantages of both instrumental approaches were discussed.

2 Experimental

2.1 Samples and Chemicals

In total, 16 dietary supplements in form of capsules and tablets were investigated (see Table 1 for details). The samples were purchased from June 2022 to October 2023 from online retailers and local drugstores in Germany. Ethylenediaminetetraacetic acid (EDTA) buffer in D_2O was prepared as described by Monakhova et al. [23]. In detail, Cs-EDTA solution was prepared by weighting approximately 2.9 g EDTA and 6 g Cs_2CO_3 dissolved in 100 mL D_2O. The pH was adjusted to 7.0. Nicotinamide (NSA) of 99% purity and the reference substance sodium hyaluronate (95% purity) with a molecular weight distribution between 1500 and 2200 kDa was purchased from Thermo Fisher Scientific (Kandel, Germany). Chondroitin sulfate A sodium salt from bovine trachea was obtained from Sigma Aldrich Chemie GmbH (Steinheim am Albuch, Germany). Sodium hydroxide (pellets of 98% purity) and hydrochloric acid (1 M, 98% purity) were provided by Carl Roth GmbH (Karlsruhe, Germany).

2.2 High-Field NMR Measurements

High-field NMR experiments were performed using 600 MHz NMR spectrometer AVANCE NEO 600 with TCI probe (Bruker BioSpin GmbH, Ettlingen, Germany). ^{1}H-NMR spectra were collected at 70 °C with an acquisition time (AT) of 4.5 s, relaxation delay (RD) of 1 s, 8 scans (NS), and pulse angle (PA) of 30°. 1D stimulated echo experiments were performed at 70 °C using bipolar gradient pulses for diffusion. The sequence stebpgp1s1d was applied with a diffusion time big delta (d_{20} in Bruker language) of 60 ms. Increased temperatures were required to drastically reduce the viscosity of HA in solution. For capsules, the shell was removed, and the powder finely ground, and tablets finely crushed. Samples with a HA content of less than 5 w/w% were prepared with a HA target concentration of 0.25 mg/mL. The remaining samples with a higher HA content were prepared with a target concentration of 1 mg/mL. Ground samples were dissolved in 1 mL of a solution containing the EDTA buffer with 20 mg/mL NSA as internal standard. To ensure complete dissolution, samples have been sonicated at 50 °C for 30 min. After quantitative transfer into plastic tubes, samples were centrifuged at 13,000 rpm for 10 min. For analysis, a volume of 0.6 mL was transferred to an NMR tube.

2.3 Benchtop NMR Measurements

Benchtop NMR experiments were performed on 80 MHz Carbon Spinsolve benchtop NMR spectrometer equipped with autosampler and Spinsolve software version

Table 1 Composition of analytes and first three matrix ingredients of examined dietary supplements declared by manufacturers

Sample	Form	Analytes mg per capsule/tablet	Matrix ingredients[a]
H1	Capsule	Hyaluronic acid 5 mg Chondroitin sulphate 75 mg	Glucosamine sulphate Gelatine Magnesium oxide
H2	Capsule	Hyaluronic acid 5 mg Chondroitin sulphate 75 mg	Glucosamine sulphate Gelatine Turmeric Root Extract
H3	Tablet	Hyaluronic acid 5 mg Chondroitin sulphate 100 mg	Glucosamine sulphate Cellulose L-ascorbic acid
H4	Capsule	Hyaluronic acid 7.5 mg Chondroitin sulphate 40 mg	Glucosamine sulphate Collagen hydrolysate Iron oxide
H5	Capsule	Sodium hyaluronate 7.5 mg Chondroitin sulphate 40 mg	Glucosamine sulphate Gelatine Methylsulfonylmethane
H6	Capsule	Sodium hyaluronate 22.5 mg Chondroitin sulphate 100 mg	Glucosamine sulphate Methylsulfonylmethane Cellulose
H7	Tablet	Sodium hyaluronate 100 mg	Cellulose Ascorbic acid Coenzyme Q_{10}
H8	Capsule	Sodium hyaluronate 400 mg	Collagen hydrolysate L-Ascorbic acid Coenzyme Q_{10}
H9	Capsule	Sodium hyaluronate 500 mg	Grapefruit extract Vitamin C Zinc gluconate
H10	Capsule	Sodium hyaluronate 500 mg	Hydroxypropyl methylcellulose
H11	Capsule	Sodium hyaluronate 500 mg	Hydroxypropyl methylcellulose
H12	Capsule	Sodium hyaluronate 500 mg	Zinc bisglycinate Hydroxypropyl methylcellulose
H13	Capsule	Sodium hyaluronate 525 mg	Rice bran extract Hydroxypropyl methylcellulose
H14	Capsule	Hyaluronic acid 600 mg	Hydroxypropyl methylcellulose
H15	Capsule	Hyaluronic acid 650 mg	Hydroxypropyl methylcellulose
H16	Tablet	Sodium hyaluronate 500 mg	Calcium hydrogen phosphate Cellulose Silicon dioxide

[a]First three matrix ingredients declared by manufacturer

2.2.3 (Magritek, Aachen, Germany). ^{1}H-NMR spectra were collected at room temperature with an AT of 3.2 s, a repetition time (RT) of 30 s, NS of 64, and PA of 90°. The viscosity of the samples was reduced by means of acid catalysis hydrolysis. For capsules, the shell was removed, and the powder was then finely ground, and tablets were finely mortared. Supplements containing less than 35 w/w% of HA were

prepared with a HA target concentration of 2 mg/mL. Samples with a higher HA content were prepared with a target concentration of 10 mg/mL. NSA used as internal standard was dissolved in EDTA buffer with a concentration of 20 mg/mL. The ground samples were dissolved in 1 mL of 0.1 M hydrochloric acid, hydrolyzed in an oven at 80 °C for 2 h, and then neutralized with the equivalent volume of equimolar sodium hydroxide (1 mL 0.1 M). Subsequently, the samples were completely evaporated in a sand bath at 80 °C and redissolved in 1 mL of internal standard buffer solution. The solution was sonicated for 30 min at 50 °C and afterwards centrifuged at 13,000 rpm for 10 min (Hettich Universal 320, Tuttlingen, Germany). A volume of 0.6 mL was then transferred into an NMR tube for analysis.

2.4 ^{1}H-NMR Spectra Processing and Quantitative Analysis

NMR spectra were processed manually in MestReNova version 14.1.2 (Mestrelab Research, Santiago de Compostela, Spain). Spectra were exponentially apodised with a factor of 0.2 Hz. To improve spectra quality zero-filling of 64 K was applied. For comparability, all spectra were referenced to TSP at δ 0.0 ppm. The phase as well as the baseline were corrected manually for the entire spectrum. The NSA signal of the aromatic proton at δ 7.57 ppm and the HA acetyl signal at δ 2.0 ppm were integrated manually (sum integration mode in MestReNova). In samples, which also contained CH, the interfering acetyl signals were deconvoluted (global spectral deconvolution mode in MestReNova).

Since the compounds of interest were polysaccharide with variable molecular weight distribution, the quantification scheme described by Monakhova et al. was applied [24]. Calibration was performed by preparing the HA reference substance using the EDTA buffer solution with an NSA concentration of 20 mg/mL. For the low-field NMR method, the HA concentration was 10 mg/mL. For the high-field method, the concentrations of HA and CH were each 1 mg/mL. For qNMR, the integrated HA acetyl signal was normalized to 100.00. The NSA signal in the NMR spectra of the supplements was then normalized to the resulting NSA value. Thereby, the HA percentage in the sample was determined relative to the calibrated reference standard.

Since such scheme was used for NMR quantification, different measuring conditions (NS, AQ, RD, pulse angel) can be applied on HF and LF NMR spectrometers. Moreover, the condition RT = 5*T1 does not have to be fulfilled. It is only important that the same acquisition parameters were applied for calibration sample and DSs.

To account for day-dependent measurement variability, a quality control (QC) sample with the HA reference substance was prepared daily for calibration of both NMR methods. Benchtop QC samples were hydrolyzed under the conditions described above.

2.5 ^{1}H-NMR Validation Studies

The validation of both NMR methods was performed through the following measurements: measurement precision was asserted by analyzing two representative

samples five times during 1 day. Limits of detection (LOD) and quantification (LOQ) were determined in matrix as signal-to-noise ratios (SNR) of 3 and 9, respectively. The reproducibility of acid hydrolysis was validated by analyzing five preparations of two representative samples. The recovery rate was determined by spiking two representative samples with 2 mg, 4 mg, and 6 mg of HA reference substance. Two representative samples were stored at room temperature and were measured repeatedly over a period of 5 days for hydrolyzed samples and 20 days for non-hydrolyzed samples to assess sample stability. T_1 was determined for target signals of NSA and HA in hydrolyzed samples prior to quantitative analyses. The longest T_1 of 4.0 s was determined for NSA.

3 Results and Discussion

3.1 High-Field NMR

^{1}H-NMR spectra of a representative DS in comparison to the HA reference standard, as well as the corresponding signal assignment of HA and the internal NSA, are shown in Fig. 1. The identification of HA was accomplished through the assessment of the specific intensity ratio of the anomeric hydrogen signal (F) at δ 4.6 ppm to the acetyl signal (A) at δ 2.0 ppm. In samples H1–H6, the hyaluronic acetyl signal (A) was masked by acetyl signals of chondroitin sulfates A and C (G + G') (Fig. 2a, c). In these samples, HA was identified using stimulated echo diffusion experiments in combination with bipolar gradients. This approach allowed the detection of HA while excluding smaller molecules, such as CH, NSA, and other low-molecular-weight matrix components (Fig. 2b, d). The decreased mobility of HA limited its diffusion, making this natural polymer detectable. As a result, echo diffusion ^{1}H-NMR spectra were considerably less complex (Fig. 2). CH, which formed significantly shorter polymer chains (MW = 15–70 kDa in comparison with HA MW = 1500–2200 kDa), was not detectable by echo diffusion experiments in the EDTA buffer used (Fig. 2). Echo diffusion experiments performed on supplements containing HA and CH allowed the unambiguous identification of HA.

The quantitative results for HA and CH contents determined by high-field ^{1}H-NMR are listed in Table 2. Paramagnetic matrix compounds, such as iron oxide, iron hydroxide, and copper sulphate, were sufficiently masked by the EDTA buffer used. NMR possessed enough specificity for the simultaneous quantitation of structurally related polysaccharides HA and CH, which CH:HA ratio varied between 3.8 and 17.8. The determination of the GAGs was enabled by deconvolution (Fig. 3). The acetyl signal of CH showed a double peak caused by the variations in the sulfation pattern (Fig. 3a). The specific ratio of the two CH peaks to each other was determined in a separate QC sample. Subsequently, it was subtracted from the joint signal of a synthetic CH and HA mixture as well as DSs containing both polysaccharides (Fig. 3c, d). For most samples, the labelled and found concentrations of both GAGs were comparable. The maximum discrepancy between the declared specifications and the experimentally determined contents was 13.6 w/w% (sample H12,
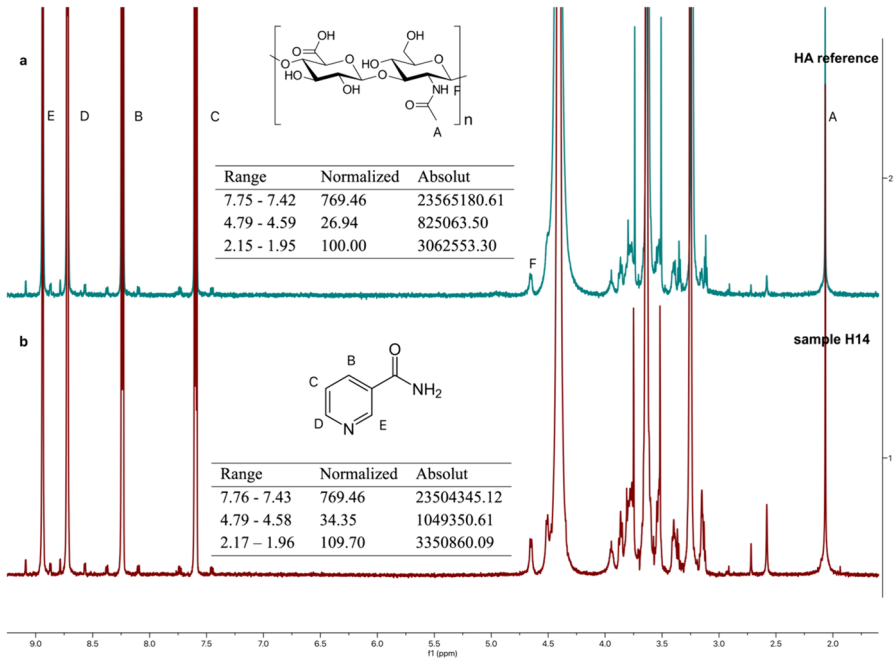
Fig. 1 600 MHz ^{1}H-NMR spectra of HA QC reference (**a**) and dietary supplement H14 (**b**) with corresponding signal assignment of analyte HA and internal standard NSA. The percentage of HA in H14 directly referenced to HA QC is 110%. The ratio of the anomeric hydrogen signal at δ 4.6 ppm to the acetyl signal at δ 2.0 ppm is 1:3

Table 2), which exceeded the determined measurement variability (refer to results of NMR validation studies in Table 3).

3.2 Benchtop NMR

The quantitative HA results determined by low-field NMR are also listed in Table 2. Preliminary tests showed that the hydrolysis products formed did not correspond to the molecular size of a single HA disaccharide unit. However, the hydrolysis ended always at the same point. The presented benchtop NMR method was sufficiently sensitive and provided well-resolved ^{1}H-NMR spectra for samples H8–H16 (Fig. 4). However, the ^{1}H-NMR spectra of samples H1–H7 were not sufficiently resolved, showed peak overlap and distortions caused by paramagnetic substances. These compounds disturbed the shimming process and led to an inhomogeneous magnetic field. Other reasons for poor resolution could be low HA content for samples H1–H6 or the high viscosity for the sample H7.

Consequently, quantitative analysis was not possible for these samples. In contrast to the high-field NMR method, a distinction between the acetyl signals of HA and CH was not possible after acid-catalyzed hydrolyzation. The HA content determined by low-field NMR analysis was in line with the manufacturer's specification for the
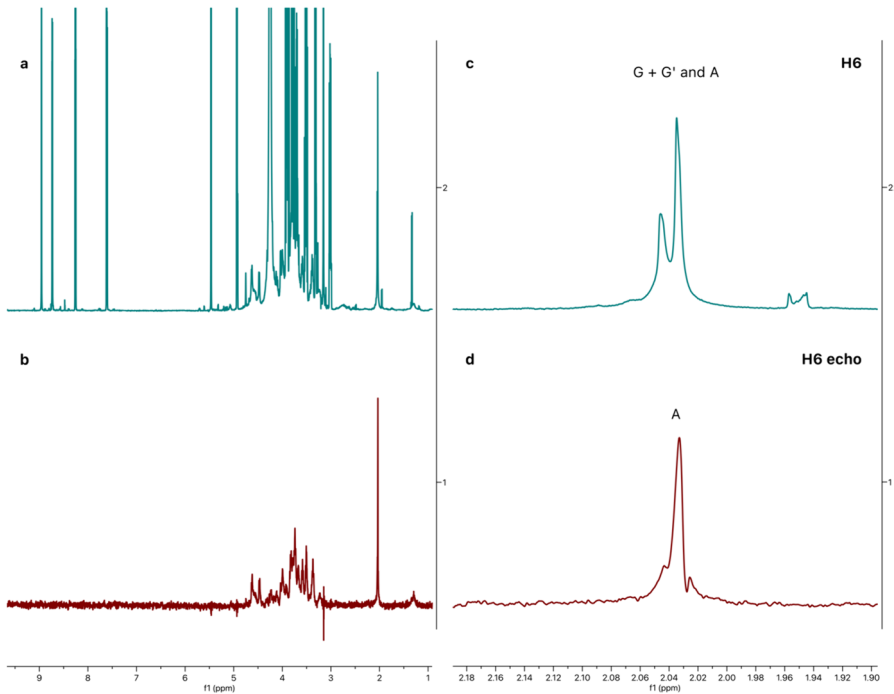
Fig. 2 600 MHz ^{1}H-NMR spectra of supplement H6 measured at 70 °C as standard [1]H (**a**) and at 70 °C using stimulated echo sequence (**b**). Magnified sections of the spectra from δ 1.90 to δ 2.18 ppm are shown in (**c**) and (**d**), respectively

remaining samples. The maximum discrepancy of 27.0 w/w% between the declared specifications and the experimentally determined contents was found for the sample H16 (Table 2). The probable explanation could be not complete hydrolysis.

To conclude, the described low-field NMR approach was considered suitable for the determination of HA in the absence of other GAGs and paramagnetic compounds. Acid hydrolysis is a precise and effective method for preparing biopolymers for further analysis. In contrast to the benchtop method, high-field ^{1}H-NMR enabled accurate content determinations of DSs containing HA as a minor component. Additionally, CH was simultaneously quantified in the presence of paramagnetic substances using the high-field ^{1}H-NMR method (Table 2, H1–H7).

3.3 NMR Method Validation

Samples H8 (64.4 w/w% HA) and H14 (91.0 w/w% HA) were selected for validation studies (Table 3). The limit of detection (LOD) and limit of quantitation (LOQ) were determined for HA in matrix for concentrations exceeding the SNR of 3 and 9, respectively. The LOD were 0.2 mg/mL and 0.1 mg/mL and the LOQ were 0.6 mg/mL and

Table 2 Quantitative overview of HA and CH in examined dietary supplements by 80 MHz and 600 MHz NMR spectroscopy. All contents are expressed in w/w%

Sample	Hyaluronic acid			Chondroitin sulphate	
	Labelled	Found		Labelled	Found
		80 MHz	600 MHz		600 MHz
H1	0.5	a	0.5	7.0	6.9
H2	0.5	a	0.5	7.6	8.9
H3	0.4	a	0.4	3.1	3.6
H4	0.7	a	0.9	3.9	4.1
H5	0.7	a	0.9	3.9	3.4
H6	3.1	a	3.2	13.8	12.5
H7	34.2	a	43.3	b	b
H8	64.4	67.7	71.3	b	b
H9	81.4	79.5	90.0	b	b
H10	70.0	75.8	75.6	b	b
H11	89.0	87.2	95.0	b	b
H12	77.1	84.1	88.3	b	b
H13	75.5	77.9	89.1	b	b
H14	91.0	87.2	93.0	b	b
H15	87.3	90.0	90.0	b	b
H16	76.2	49.2	75.6	b	b

[a] Quantitative evaluation of spectra not possible due to poor resolution and peak overlap

[b] CH was not labelled on and was not detected in dietary supplement (refer to Table 1)

0.2 mg/mL for HA by benchtop and high-field NMR, respectively (Table 3). It should be mentioned that these LOD and LOQ levels determined for different measuring conditions and, therefore, cannot be compared with each other.

The measurement precision showed comparable results for both methods, with coefficients of variation (CV) of 2.4% and 2.5% for multiple measurements of the same NMR tubes. The presented hydrolysis procedure was considered reproducible, with a CV of 8.0% ($n = 5$). In contrast to enzymatic hydrolysis of HA [10, 13], the presented acid hydrolysis was significantly faster and less expensive, since no specific enzyme kits were required. The hydrolysis process could also be stopped in a targeted manner by adding sodium hydroxide. The validation studies demonstrated good reproducibility for the acid hydrolysis. Stability measurements revealed that hydrolyzed samples had a maximal stability of 2 days, while non-hydrolyzed samples remained stable for more than 7 days. Recovery rates of 93% to 120% were obtained for benchtop NMR, and 94% to 106% for high-field NMR. Based on these results, both low and high-field 1H-NMR spectroscopy were found to be effective methods for controlling the presence of HA in dietary supplements.
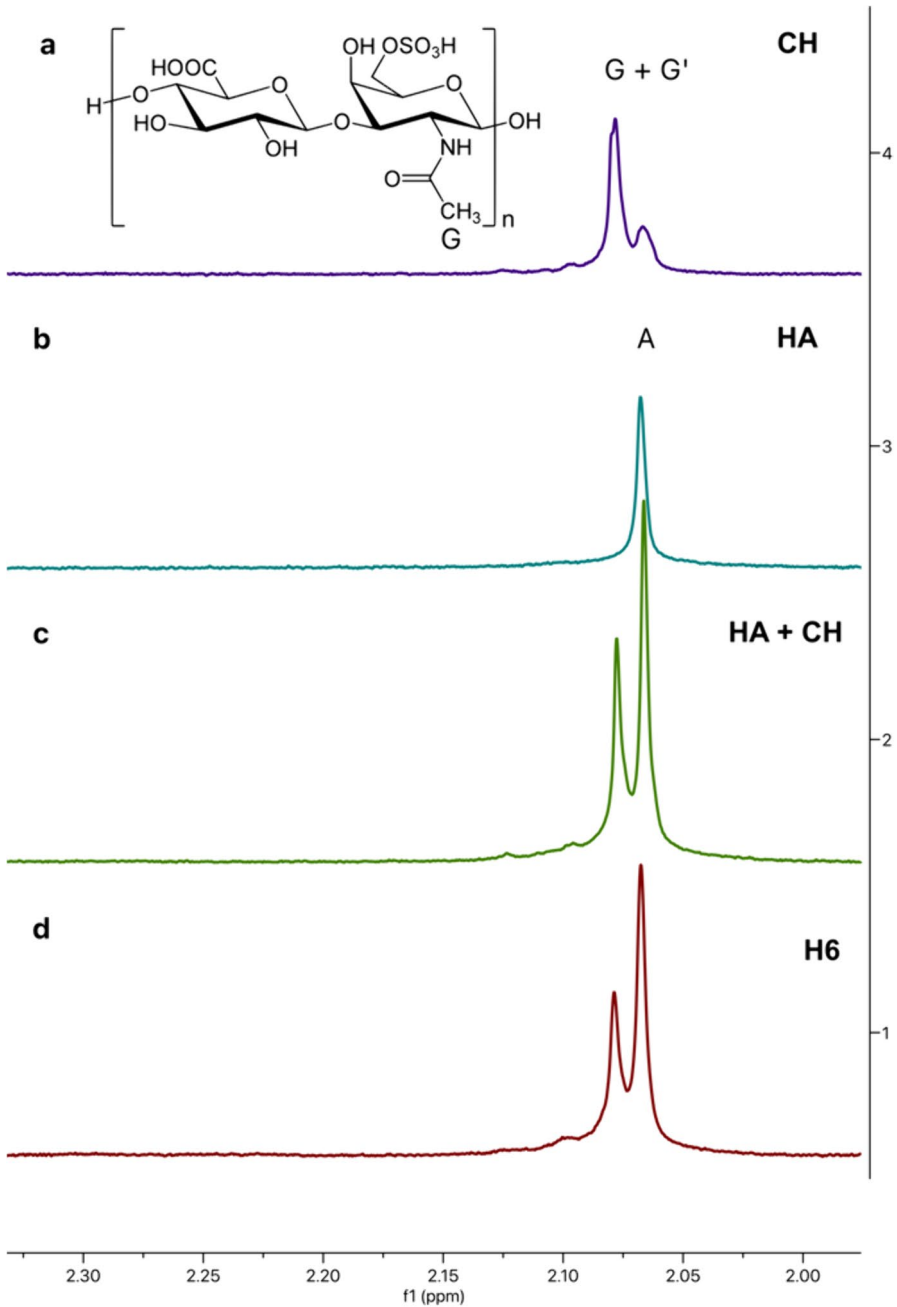
Fig. 3 600 MHz spectra obtained for CH (**a**) with signal assignment of acetyl signals G and G' corresponding to CH salts C and A, HA (**b**), a mixture of both GAGs (**c**), and sample H6 (**d**) which also contains CH and HA. The overlaying signals of present GAGs are sectioned by means of deconvolution allowing quantitative analysis of both compounds

Table 3 Validation results for the quantitative analysis of hyaluronic acid by NMR spectroscopy

Parameter		80 MHz			600 MHz		
LOD [mg/mL]		0.2			0.1		
LOQ [mg/mL]		0.6			0.2		
Precision [CV %] n = 5	multiple measurements of the same NMR tube	2.4			2.5		
	multiple sample preparations	8.0			4.2		
Stability [d]		2			at least 7		
Average recovery [%]		at 2 mg	at 4 mg	at 6 mg	at 2 mg	at 4 mg	at 6 mg
	Sample 1 (64.4 w/w%)	120	103	110	97	97	106
	Sample 2 (91.0 w/w%)	93	93	102	97	94	104
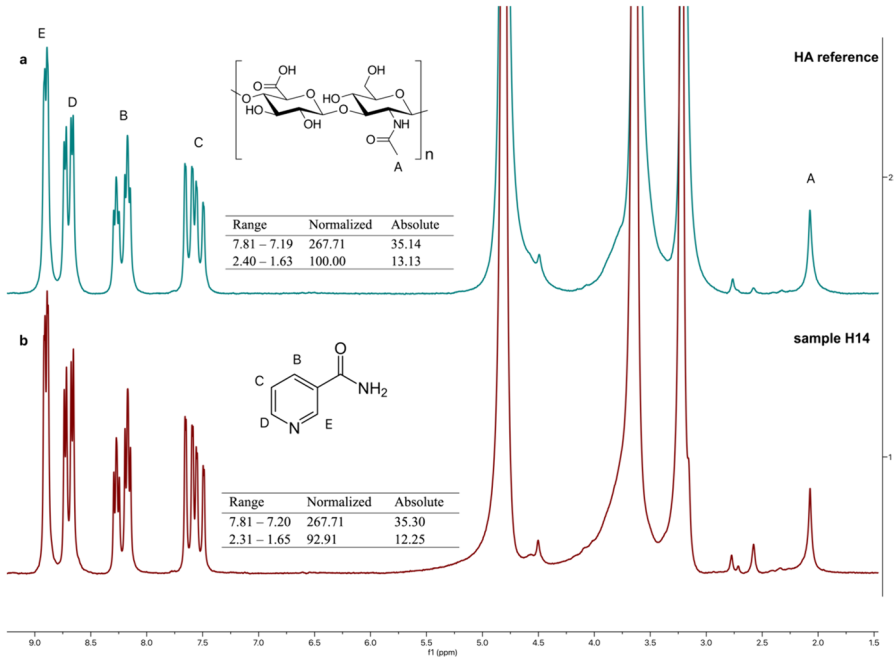
Fig. 4 80 MHz ^{1}H-NMR spectra of hydrolyzed HA QC reference (**a**) and hydrolyzed dietary supplement H14 (**b**) with corresponding signal assignment of analyte HA and internal standard NSA. Residual water and EDTA signal are between δ 3.0 and δ 5.0 ppm. The percentage of HA in H14 directly referenced to HA QC is 93%

4 Conclusion

This study presented validated ^{1}H-NMR methods for the qualitative and quantitative analyses of HA and CH in dietary supplements. Moreover, high-field ^{1}H-NMR also enabled simultaneous determination of CH. Experiments conducted at 70 °C reduced sample viscosity to an acceptable level. Measurements at elevated temperatures for instance enabled reaction monitoring in process analytics and allowed for the examination of temperature-dependent structural changes, such as phase transitions [25, 26]. Furthermore, raising the sample temperature can shift temperature-dependent signals, such as HDO, thereby enhancing the method's sensitivity and selectivity [27]. Such measurements were not used to increase the sensitivity of qNMR.

Echo diffusion experiments were found appropriate to confirm the presence of HA in complex matrices containing other biopolymers. A considerable positive effect was that diffusion ^{1}H-NMR spectra were significantly less complex, as low-molecular matrix components are not recorded due to their increased diffusion. Previously, diffusion NMR experiments using pulsed gradient stimulated echo (PGSTE) or spin echo (PGSE) were performed for structural characterization of (bio-) polymers [28–30]. It would be interesting to develop quantitative approaches based on such experiments in the future.

Benchtop NMR was found to be a more cost-effective and cryogen-free alternative that yielded acceptable results for HA content in DSs. However, this method was limited to supplements that do not contain CH or paramagnetic matrix components, which were not sufficiently masked by EDTA buffer. Acid-catalyzed hydrolysis was required to reduce the sample viscosity.

The approach used for data evaluation was considered suitable for the determination of HA and CH, which are natural biopolymers with variable molecular weight depending on their origin. The study's findings encourage transfer of NMR methods to cosmetics, such as serums, creams, and injections due to the increasing demand for HA-containing matrices.

Data Availability Some raw data generated during the current study are available at https://doi.org/10.5281/zenodo.11217980.

Declarations

Conflict of Interest The authors declare no competing interests.

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