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Determination of molecular weight of hyaluronic acid by near-infrared spectroscopy

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ABSTRACT

This paper attempted the feasibility to determine the molecular weight of hyaluronic acid with near-infrared (NIR) diffuse reflectance spectroscopy. In this work, 46 experimental samples of hyaluronic acid powder were analyzed by partial least square (PLS) regression multivariate calibration method in the selected region of NIR spectra. The leave-one-out cross-validation method was used for the PLS model selection criterion. The accuracy of the final model was evaluated according to correlation coefficient of prediction set (Rp) and root mean square error of prediction set (RMSEP). The repeatability was verified through repeated measurement of spectra coupled with an appropriate chi-square test. Finally, the optimal calibration model was obtained with Rp = 0.9814 and RMSEP = 88.32 when using Savitzky-Golay first (SG-1st) derivative with 9 smoothing points spectral preprocessing method. The parameters above and repeatability of NIR spectroscopy obtained from chi-square test were both within the range of permissible error in factories. This study demonstrated that NIR spectroscopy was superior to conventional methods for the fast determination of molecular weight of hyaluronic acid.

1. Introduction

Hyaluronic acid (HA) is a high molecular weight biopolysaccharide, discovered in 1934, by Karl Meyer and his assistant, John Palmer in the vitreous of bovine eyes. It is found in most connective tissues and is particularly concentrated in synovial fluid, the vitreous fluid of the eye, umbilical cords and chicken combs [1]. HA is a naturally occurring biopolymer whose molecular structure is highly consistent between mammalian species. It has been used across a wide variety of medical fields as diverse as neurosurgery and cutaneous wound healing. Presently it has reached prominence in cosmetic practice where it is now the injectable dermal filler of choice for most surgeons [2].

Molecular weight is one of the most fundamental parameters characterizing HA. HA of different molecular weight displays different physical and chemical properties which decide its application fields including medical fields, cosmetics and health-care food industries [3]. Various methods can be applied to determine the molecular weight of HA, such as intrinsic viscosity, capillary electrophoresis (CE), high performance gel permeation chromatography (HPGPC), and multi-angle laser light scattering combined with size-exclusion chromatography (SEC-MALLS) [4–7]. However, all these methods are time-consuming and reagent-consuming. In contrast to these conventional methods, near-infrared (NIR) spectroscopy is a fast, accurate and non-destructive technique that can be a candidate as a replacement of conventional analysis.

The NIR technique is becoming recognized extensively in agricultural, nutritional, petrochemical, textile and pharmaceutical industries [8–15]. The NIR region (780–2500 nm) is situated between the red-band of the visible light and mid infrared (mid-IR) region. The NIR signal is a consequence of the absorbance of light due to molecular vibrations (over-tone and combinations of fundamental vibrations) of hydrogen bonds like C–H, N–H and O–H [16]. Many of these hydrogen bonds are present in HA molecules; therefore, NIR spectroscopy is suitable to study the physical or chemical properties of HA. In recent years, a number of attempts had been made to investigate the probability of monitoring or controlling polymer molecular weight by using NIR spectroscopy [17,18], nevertheless all these studies did not make any theoretical explanations on why molecular weight can be determined by NIRS. Based on the studies about molecular weight determination by infrared spectroscopy coupled with an end-group concentration analysis [19,20], we speculate that HA of different molecular weight possesses a different amount of hydroxyl end-group per unit which will be reflected in the NIR spectra. Thus, molecular weight can be determined through the changes of NIR spectra.

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Table 1
The viscosity-average molecular weight of the 46 self-designed samples.

Sample number	Molecular weight(kDa)	Sample number	Molecular weight(kDa)
1	2.722E+03	24	1.566E+03
2	2.582E+03	25	1.646E+03
3	2.567E+03	26	1.580E+03
4	2.442E+03	27	1.677E+03
5	2.478E+03	28	1.471E+03
6	2.418E+03	29	1.601E+03
7	2.339E+03	30	1.389E+03
8	2.196E+03	31	1.435E+03
9	2.277E+03	32	1.402E+03
10	2.080E+03	33	1.370E+03
11	2.097E+03	34	1.303E+03
12	1.967E+03	35	1.325E+03
13	2.134E+03	36	1.328E+03
14	1.929E+03	37	1.299E+03
15	1.946E+03	38	1.255E+03
16	1.855E+03	39	1.244E+03
17	1.917E+03	40	1.205E+03
18	1.756E+03	41	1.166E+03
19	1.868E+03	42	1.160E+03
20	1.716E+03	43	1.083E+03
21	1.809E+03	44	1.096E+03
22	1.633E+03	45	1.100E+03
23	1.809E+03	46	2.710E+03

This paper discusses a fast and complete method to determine the molecular weight of HA by NIR spectroscopy and data evaluation with using the partial least square (PLS) regression multivariate calibration method. Intrinsic viscosity was used to acquire reference data. In this research, we also systematically presented the accuracy and repeatability of NIR spectroscopy method for molecular weight determination of HA. In comparison with the conventional methods, the proposed method was applied easily, because it requires no treatment of samples and the process of model building is simple.

2. Materials and methods

2.1. Sample preparation

One batch of HA powder sample (96.80% purity, with average molecular weight of 2.710E+03 kDa, previously determined using intrinsic viscosity) was obtained from Shandong Freda Biochemical Limited Company of Shandong province (High-tech Industrial Development Zone, Jinan, Shandong Province, China). To acquire more homogeneous molecular weight gradient, we developed 46 samples of different molecular weight by heating degradation at 110 °C in a heating and drying oven, which was the modification of the method described by Choi [21]. The molecular weights of the 46 self-designed samples were shown in Table 1.

2.2. Chemical analysis

The viscosity-average molecular weight ($M\eta$) was measured by intrinsic viscosity according to China pharmacopoeia (page 39–40, the third method of appendix VI G, 2nd revision 2005) [4]. The determination of molecular weight was carried out with SYD-265D-1 kinematic viscosity measuring equipment for petroleum products equipped with a Peltier heating system. The solution of HA was prepared as the test solution, of which the concentration was 1 mg ml^{-1} and the solvent was 0.2 M sodium chloride. A clearly washed Ubbelohde type of capillary viscometer (inside diameter is 1.0 ± 0.05 mm) was used for the measurement of flow time for solvent and test solution at 25 ± 0.1 °C, and flow time measurements were conducted with a stopwatch. The measurement was repeated at least twice to insure results accurately. In this process, the ratio of t_1/t_0 should be between 1.3 and 1.5 while t_0 should be greater than 100 s [22], where t_1 is the flow time of test solution and t_0 is the flow time of solvent. The viscosity-average molecular weight is calculated by the following equations [23]:

$$[\eta] = \frac{1}{c} \times \sqrt{2\left[\left(\frac{t_1}{t_0} - 1\right) - \ln\frac{t_1}{t_0}\right]} \quad (1)$$

$$M\eta = \sqrt[0.78]{\frac{[\eta]}{3.6 \times 10^{-4}}} \quad (2)$$

where c is the concentration of HA (g ml^{-1}).

In this process, HA samples were allowed to completely dissolve at room temperature for at least 24 h prior to next operation. The time for viscosity measurement needs approximately 1 h for one single sample. Compared with the proposed NIR method which measurement time for each sample was only about 5 s, this method is time-consuming and destructive for samples.

2.3. NIR spectroscopy measurement

A Luminar 5030 AOTF-NIR spectrometer (Brimrose Co., USA) with an InGaAs detector was used to collect the NIR spectra in ratio mode. A home-made sample cup which could contain approximately 5 mg sample was used, and there was an optical reflector at the bottom of the cup, which could reflect back NIR radiation to the detector. Each spectrum was the average of 300 scans with a wavelength increment of 2 nm over the wavelength range 1100–2300 nm [24]. The sample was compressed by a cap, and in the center of the cap there is a hole, thus NIR radiation could pass into the sample through the cap.

For sample 2, sample 10, sample 20, sample 30 and sample 45, 10 readings of each were measured without moving the sample. For other samples, two spectra of each sample were collected to eliminate the error caused by sample loading and then the average spectrum was calculated. Therefore, the spectra of 46 samples were obtained and each spectrum has 601 data points. The temperature was kept around 25 °C and the humidity was kept at a steady level in the laboratory.

2.4. Spectral preprocessing

Spectral preprocessing are mathematical corrections that reduce, eliminate or standardize the effect of variable physical sample properties or instrumental effects on the spectra [25]. Correct selection of spectral data preprocessing can significantly improve the accuracy, precision and repeatability of a model. In this study, three spectral preprocessing methods were applied comparatively. These methods are Savitzky-Golay first (SG-1st) derivative with 9 smoothing points, standard normal variate (SNV) and multiplicative scatter correction (MSC).

2.5. Models calibration and validation

PLS method was used to construct calibration models. The molecular weight of HA obtained from intrinsic viscosity analysis was used as the molecular weight matrix for PLS model. The leave-one-out cross-validation method was used for the model selection criterion. The "leaving-one-out" method is leaving one sample out and using the rest of the samples to build the model. Then the model is used to predict the samples being left out. This step is repeated for every sample in the calibration set. Root mean square error of cross-validation (RMSECV) is obtained by leave-one-out cross-validation via the set of calibration samples, which gives an estimate of the models' performance [26]. The optimal number of PLS components is determined by the lowest RMSECV. Finally the model with the lowest RMSECV and the optimal number of PLS components will be selected as final model.

In order to corroborate the predictive ability of the model, 18 samples of prediction set were predicted by the established PLS model and then the molecular weight values obtained using NIR were compared with the results of intrinsic viscosity analysis. In the course of this process, the root mean square error of prediction (RMSEP) and the correlation coefficient of prediction set (Rp) were obtained. Accuracy can be characterized by these two parameters.

2.6. Repeatability tests

Repeatability tests were performed by repeated measurement of spectra [27]. Sample 2, sample 10, sample 20, sample 30 and sample 45 were chosen from the prediction set, each sample was scanned 10 times without moving as described above. The molecular weights of these five samples were predicted using the corresponding model, and then the mean of each sample's predicted values and repeatability standard deviations were calculated by

$$\overline{\hat{y}_i} = \frac{\sum_{j=1}^{r_i} \hat{y}_{ij}}{r_i} \tag{3}$$

$$\sigma_i = \sqrt{\frac{\sum_{j=1}^{r_i} (\hat{y}_{ij} - \overline{\hat{y}_i})^2}{r_i - 1}} \tag{4}$$

$$\sigma = \sqrt{\frac{1}{r} \sum_{i=1}^{z} r_i \sigma_i^2} \tag{5}$$

where

$$r = \sum_{i=1}^{z} r_i \tag{6}$$

$\hat{y}_{ij}$ is the j-th spectrum of sample i and r_i represents the spectral totality of sample i.

A chi-square (χ^2) test was used to investigate whether these repeatability standard deviations belong to the same population [27], according to Eqs. (7) and (8)

$$\chi^2 = \frac{2.3026}{c} \left(r \log \sigma^2 - \sum_{i=1}^{r_i} r_i \log \sigma_i^2 \right) \tag{7}$$

where

$$c = 1 + \frac{1}{3(z-1)} \left(\sum_{i=1}^{z} \frac{1}{r_i} - \frac{1}{r} \right) \tag{8}$$

and z is the total number of samples for repeated measurements.

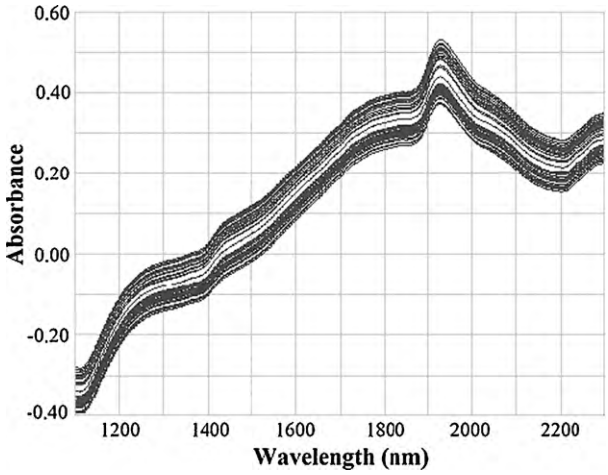

Fig. 1. Original spectra of 46 samples.

2.7. Software

The Snap! 2.03 software was used for spectra acquisition and the Snap 32! software for preprocessing of original spectral data. The PLS modeling was performed in the Unscrambler software, version 7.8 (Camo process AS).

3. Results and discussion

3.1. Spectra investigation

Fig. 1 shows the original spectra of all 46 samples including 92 spectra. In general, it is difficult to find specific bands in NIR spectra since NIR bands are composed of overtones and combinations of fundamental vibrational groups. However, the strong absorption around 1400 nm and 1900 nm were easily assignable as that of water in the sample [27]. The extremely strong absorption of water has a significant influence on the PLS model created. Therefore, exclusion of water absorption bands in the NIR spectra for PLS regression becomes a required general procedure for quantitative analysis [25,28–30]. In this study, the absorption bands at 1365–1450 nm and 1850–1950 nm were removed prior to construction of the calibration model. Comparing with the results obtained by three spectral preprocessing methods mentioned above, SNV method was as good as MSC and SG-1st derivative with 9 smoothing points which would be used in the next analysis was slightly better than SNV and MSC methods. The reason is that the spectral noise can be eliminated by nine-points Savitsky-Golay smoothing and the baseline shift can be minimized by first derivative. SNV and MSC were employed to reduce the scattering effect caused by different particle size. All these samples were sieved by a 40 mesh screen. And for this reason, both SNV and MSC did not give significant effects. Fig. 2 shows the spectral data of 46 samples after SG-1st derivative with 9 smoothing points preprocessing method.

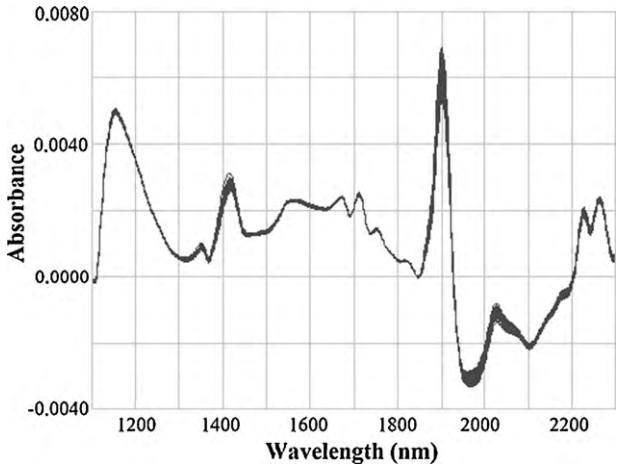

Fig. 2. Spectra of 46 samples after SG-1st derivative with 9 smoothing points preprocessing method.

Table 2
The reference measurements and sample numbers in calibration set and prediction set.

Set	n[a]	Maximum value (kDa)	Minimum value (kDa)	Mean value (kDa)	SD[b]
Calibration set	28	2.722E+03	1.083E+03	1.741E+03	4.962E+02
Prediction set	18	2.582E+03	1.100E+03	1.790E+03	4.611E+02

[a] n = number of samples.
[b] SD = standard deviation.

3.2. Sample selection

To obtain suitable multivariate models, the division of sample set is a crucial step. All 46 samples were divided into two subsets. One of subset was called calibration set, which was used to build model; the other was called prediction set, which was used to verify the prediction capacity of the model (external validation set). In order to improve the predictive ability of the model, a wide range of molecular weight was covered, from low molecular weight to high molecular weight (1.083E+03 kDa ~ 2.722E+03 kDa), which was the sampling criterion for calibration set. Two spectra of every five samples were divided into the prediction set in order to get the ratio of calibration/prediction was 3/2. In the sample dividing step all samples were selected randomly and the scope of prediction set was smaller than calibration set's scope [26]. Therefore the calibration set contained 28 samples, the rest 18 samples constituted the prediction set, see in Table 2.

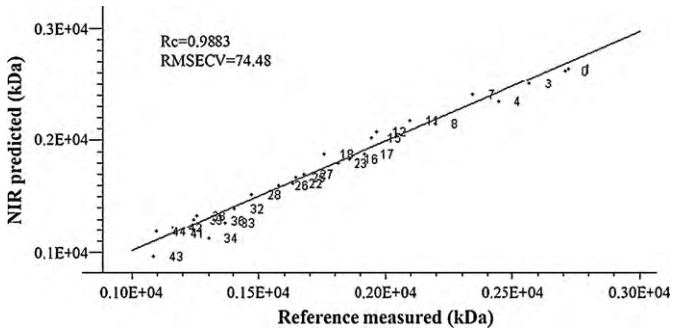

Fig. 3. Reference measured versus NIR predicted by PLS in calibration set.

3.3. Calibration models

After leave-one-out cross-validation, the lowest RMSECV was 74.48 when 2 PLS components were included in calibration model with the selected ranges of wavelength (1100–1364 nm, 1450–1850 nm and 1950–2300 nm). Fig. 3 is the scatter plot showing a correlation between reference measured obtained from intrinsic viscosity analysis and NIR predicted in calibration set by PLS model. It is clear from Fig. 3 that the correlation coefficient of calibration set (Rc) between the measured and predicted values is 0.9883.

3.4. External validation

The external validation of the method was performed with the samples of prediction set. All samples of prediction set were predicted by the established PLS model and the molecular weight results obtained were compared with the results of intrinsic viscosity analysis. When optimal principal components selected were 2, the RMSEP was 88.32 and Rp was 0.9814 after the calculation. These results can satisfy the needs of 100kDa error in factories.

3.5. Repeatability proof

The predicted values and statistical results see in Table 3. For a given 95% confidence level, the critical value of $\chi^2_{(0.05,4)}$ is 7.81. The χ^2 of NIR model of molecular weight was 0.7288, lower than the critical value, which indicated that the repeatability standard deviations belonged to the same population.

The average standard deviation σ could be perceived as the standard deviation of NIR spectroscopy measurement and the repeatability of NIR spectroscopy for molecular weight determination could be calculated by $z \times \sqrt{2} \times \sigma$ [27]. Ultimately, the standard deviation and repeatability of NIR spectroscopy measure-

Table 3
Repeatability results of NIR spectroscopy for molecular weight determination.

No.	Sample 2 (kDa)	Sample 10 (kDa)	Sample 20 (kDa)	Sample 30 (kDa)	Sample 45 (kDa)
1	2.592E+03	2.031E+03	1.576E+03	1.458E+03	1.109E+03
2	2.568E+03	2.004E+03	1.560E+03	1.446E+03	1.139E+03
3	2.554E+03	2.005E+03	1.547E+03	1.436E+03	1.137E+03
4	2.554E+03	1.992E+03	1.548E+03	1.442E+03	1.132E+03
5	2.557E+03	1.991E+03	1.539E+03	1.430E+03	1.125E+03
6	2.560E+03	1.985E+03	1.543E+03	1.423E+03	1.121E+03
7	2.550E+03	1.990E+03	1.533E+03	1.424E+03	1.119E+03
8	2.549E+03	1.992E+03	1.534E+03	1.413E+03	1.116E+03
9	2.548E+03	1.980E+03	1.532E+03	1.418E+03	1.118E+03
10	2.559E+03	1.992E+03	1.537E+03	1.420E+03	1.105E+03
Mean value	2.559E+03	1.996E+03	1.545E+03	1.431E+03	1.122E+03
Standard deviation	13.03	14.36	13.89	14.25	11.27
χ^2	0.7288				
Repeatability	9.481E+01				

ment showed values of 13.41 and 94.81, respectively. They were both within the range of permissible error in factories.

4. Conclusions

The overall results sufficiently verify feasibility that molecular weight of HA can be determined non-destructively by NIR spectroscopy with the combination of the PLS multivariate regression calibration method. The proposed method is fast, accurate and precise in comparison to the conventional methods and the deviations are all within the permissible error limits in pharmaceutical factories. Since there are still a few differences between the samples after thermal treatment and the actual samples, the model established is not applicable to actual samples. We suggest that the actual samples must be used if we want to establish a practical model in production. After enrichment of the actual samples, the NIR spectroscopy maybe a potential method for lot release test in QC laboratory.

Due to quick test and non-destruction of samples, this method may be useful to the fast analysis of molecular weight in production process, and also for parameter optimization of HA fermentation process combined with feedback control in factories. As a result of the strong moisture absorption of HA, we suggest that the presented strategy is best used in GMP environments like the pharmaceutical industry where the environment is consistent. Undoubtedly, more research on the use of NIR within the field of molecular weight determination of HA is to come.

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