Chemometric analysis of Near-Infrared spectra for determining molecular distribution of gelatin extracted from pork rinds

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1. Introduction

Gelatin is a natural biopolymer obtained by denaturation and partial hydrolysis of fibrous collagen, which is a fibrous protein present in the connective tissues of all species in the animal kingdom. Gelatin is used in many different industries, such as food, pharmaceutical, photographic, and cosmetics. Its widespread use is mainly due to its multifunctional properties. The characteristics of gelatin depend on its origins. For example, fish gelatin has a lower melting temperature than pork gelatin (≈ 35–37 °C) due to its amino acid composition. However, the origin of a gelatin is not the only factor that determines its properties. Extraction conditions (time, temperature, acid hydrolysis, and basic hydrolysis) also strongly influence the physicochemical properties by affecting the size of the final collagen fragments.

Asymmetric flow field flow fractionation (AF4) is a useful tool for simultaneous separation and characterization of polydisperse macromolecules or colloidal nanoparticles. This technique allows the fractionation of proteins ranging from 1×10^4 to 1×10^7 g/mol whereas, for the same sample, size-exclusion chromatography gives sizes up to only 1×10^6 g/mol. In this work, the molecular-weight distribution of pork gelatin is studied by AF4 coupled with multi-angle light scattering.

Near-infrared (NIR) spectroscopy is ubiquitous in the food industry and is commonly used to determine the fat, protein, water, and carbohydrate content. In 1954, Eldridge & Ferry (1954) found that the physicochemical properties of gelatin are related to its molecular-weight distribution. More recently, Segtnan used NIR spectroscopy to determine the physicochemical characteristics of gelatin (V. Segtnan, Kvaal, Rukke, Schüller, & Isaksson, 2003; V. H. Segtnan & Isaksson, 2004).

The aim of the present study is to investigate the use NIR spectroscopy to determine the molecular distribution of various pork gelatins.

2. Materials and methods

1. Preparation of gelatins

Preparation of pork rinds

Pork (Sus Scrofa) rinds were provided by the Weishardt group and all came from the same batch. Rinds were transported fresh and stored at −40 °C for 14 days prior to pretreatment.

Pretreatment, acidification step

The pork rinds were cut into 1-cm-thick slices. Pieces of rind were soaked in sulfuric acid solution (pH = 1.74, 19 °C) for five hours. The sample/solution ratio was 1/10 (w/w). After this pretreatment phase, the liquid and solid phases were separated by using a nylon membrane.

Collagen extraction, hydrolysis step

After pretreatment, the rinds were separated into nine groups. Three groups were placed in each of three extraction baths (Milli-Q water) held at 60, 70, and 80 °C. After 2, 4, 6, 8, 10, 12, and 14 h, 5 ml gelatin

samples were extracted from each bath. The Ph of each extraction bath was verified before each extraction. After extraction, the gelatin samples were frozen, lyophilized, and then stored at −40 °C until analyzed.

2. AF4 analysis

Theory

For AF4 analysis, the sample is first injected in asymmetrical cell that forms a channel. The sample is carried by a laminar-flow aqueous or organic solvent. Next, a force field is generated by applying a flow perpendicular to the laminar flow (called the "cross flow"). This technique allows macromolecules to be separated during elution as a function of their diffusion coefficient.

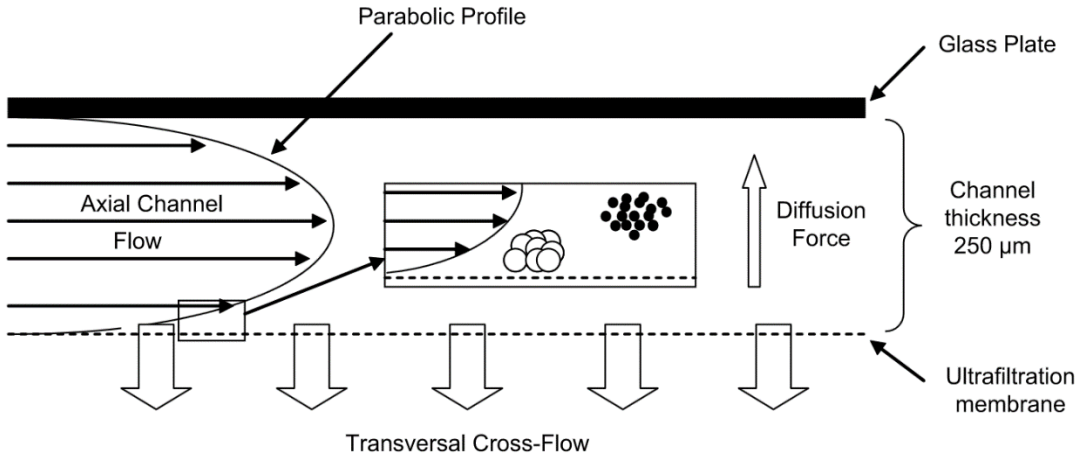

Figure 1 : Separation of samples in AF4 (● small sample component; ○ large sample component) (Rbii, 2010)

Eluent

The eluent for the gelatin consisted of 2 mM sodium phosphate in a 14 mM sodium chloride buffer. The pH of the eluent was adjusted to 6.0 by adding phosphoric acid (85% v:v) and was filtered before use through a vacuum filtration system with 0.1µm Gelman filters (Rbii, Violleau, Guedj, & Surel, 2009). Lyophilized gelatin was dissolved in the buffer solution at a 1/3 w:w ratio and 50 µl were injected.

Instrumentation

The AF4 analysis was done with a Dual Tech System (Wyatt Technology Europe, Dernbach, Germany). The AF4 channel had a trapezoidal geometry and was 19.5 cm long with an initial (final) width of 1.65 (0.27) cm. A 250-µm-thick Mylar spacer was placed between the ultrafiltration membrane and the upper glass plate. The accumulation wall consisted of an ultrafiltration membrane of regenerated cellulose with a 5 kDa cutoff (Wyatt Technology Europe, Dernbach, Germany). Samples were injected into the AF4 channel by a Dionex® HPLC ultimate 3000 Series (LC-Packings, Dionex, Amsterdam, The Netherlands) with an in-line vacuum degasser and carrier flow. A 0.1 µm in-line filter (VVLP, Millipore, Germany) was installed between the pump and the AF4 channel. The AF4 was connected to an 18-angle multi-angle light scattering (MALS) Heleos II instrument (Wyatt Technology, Santa Barbara, USA), an OptilaRex refractometer (Wyatt Technology, Santa Barbara, USA), and a ultraviolet (UV) detector Ultimate 3000 RS (λ = 214 nm). The MALS instrument used a wavelength of 690 nm and the detectors were normalized by using bovine serum albumin. Filtered toluene (HPLC grade) was used to calibrate the scattering intensity. The UV detector served as a quantitative detector and gave a UV extinction coefficient of 1.27×10^4 ml g^{-1} cm^{-1} (Rbii et al., 2009).

Elution method

For separation, the channel flow rate was fixed at 1 mL/min and the cross-flow rate was varied. The separation program started 3 min after sample injection. The cross-flow rate started at 0.2 mL/min, then decreased linearly for 7 min, following which elution proceeded at 0.2 mL/min for 40 min. This

separation method yielded good sample fractionation. The flow rate through the detectors was held constant at 1 mL/min (Rbii et al., 2009).

Data analysis
The data were treated by the Astra 6.1 software (Wyatt Technology, Santa Barbara, USA).

3. Near-infrared spectroscopic analysis

Near-infrared spectroscopy
NIR spectra were acquired by using a Fourier-transform NIR spectrometer in transmission mode (MPA, Bruker Optics, Ettlingen, Germany). The spectrum of each sample was collected over the range 12 500–5500 cm^{-1} at room temperature (21 °C) with a resolution of 16 cm^{-1} (10 kHz scanner velocity, 32 scans). OPUS software (v.6.5 Bruker Optics, Ettlingen, Germany) was used to acquire the spectra.

Data analysis
The data were preprocessed by using standard normal variate and nine-point smoothing. Next, a principal component analysis (PCA) was applied to the data by using the NIPAL algorithm. A partial least squares (PLS) regression (Savitzky & Golay, 1964) was developed from the 51 spectra, which were verified by cross validation (25%). Spectra were preprocessed by applying a second-derivative Savitzky-Golay filter with a nine-point gap. The calculation was done by using The Unscrambler® v10.3.0.89 (CAMO ASA, Trondheim, Norway) and MATLAB® R2015a (The MathWorks, Inc., Nattick, USA)

3. Results and discussion

AF4 analysis
Figure 2 shows the transmitted UV intensity, 90° light-scattering intensity, and molar mass for gelatin

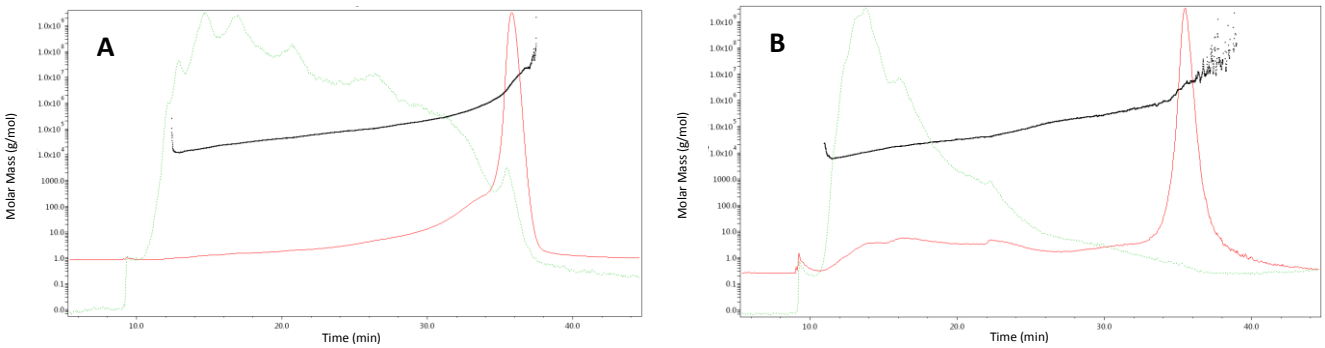

Figure 2 : Fractogramms from gelatins samples: (A) sample extracted at 60 °C after 4 hs, (B) sample extracted at 80 °C after 12 h. Transmitted UV intensity (green dotted line) and light-scattering intensity (red solid line) are expressed in arbitrary units. The calculated molar mass is represented by the black dots.

as function of extraction time. The fractogram in Fig. 2A is for a gelatin extracted at low temperature (60 °C) after 4 h and the fractogram in Fig. 2B is for a gelatin extracted at high temperature (80 °C) after 12 h. The light-scattering intensity increases with the size of the gelatin aggregates and peaks for large aggregates with a mass of approximatively 10×10^7 g/mol. The evolution of the molar mass is calculated with the aid of the light-scattering data. For a given range of calculated molecular weight in both fractograms, the transmitted UV intensity in Fig. 2A remains relatively strong over the entire time range, whereas, in Fig. 2B, the UV intensity is strong only for the time corresponding to small molecular weight. The short interval in Fig. 2A between the drop in UV intensity and the rise in the light-scattering intensity indicates a large polydispersity of the gelatin. Polydispersity is higher in the gelatin sample extracted at low temperature after 4 h than in the gelatin sample extracted at high temperature after 12

h. These results confirm that the majority of hydrolysis occurs on longer timescales and at high temperature, and that most collagen fragments are hydrolyzed as short chains.

The effect of temperature and extraction time on the total number-average molecular weight (Mn) and the polydispersity highlights a decrease in Mn as a function of temperature and time, and a decrease in polydispersity as a function of time (see Table 1). In addition, Mn decreases strongly at the highest temperature (80 °C). After 8 h, Mn and polydispersity become rather stable.

Table 1 : Number-average molecular weight (Mn) and polydispersity determined by AF4 analysis and the corresponding temperature and extraction time (SD means standard deviation).

Time (h)	Temperature (°C)	Mn (Kda) total	SD	polydispersity
	60	Ø	Ø	Ø
2	70	54.63	5.6	5.5
	80	28.63	7.6	9.5
	60	45.50	6.5	6.2
4	70	45.10	1.6	6.4
	80	24.63	3.0	6.9
	60	33.75	1.5	5.4
6	70	30.35	6.7	8.6
	80	20.53	2.0	8.9
	60	27.55	0.6	5.3
8	70	29.60	0.7	5.5
	80	27.36	6.8	8.2
	60	31.20	Ø	6.4
10	70	25.53	0.9	6.1
	80	20.05	0.5	3.1
	60	31.27	0.7	8.1
12	70	24.40	1.7	4.2
	80	19.03	2.2	2.9
	60	33.70	Ø	3.7
14	70	29.20	3.7	4.4
	80	24.15	0.8	4.1

The AF4 analysis thus reveals the differential distribution of molar mass from gelatin samples extracted under different conditions.

Principal Component Analysis of near-infrared spectral data

The NIR spectra of gelatins were studied by applying a PCA. Although the spectral range spanned from 5500 to 12 500 cm^{-1}, the best range for explaining the variation is from 5800 to 7500 cm^{-1}.

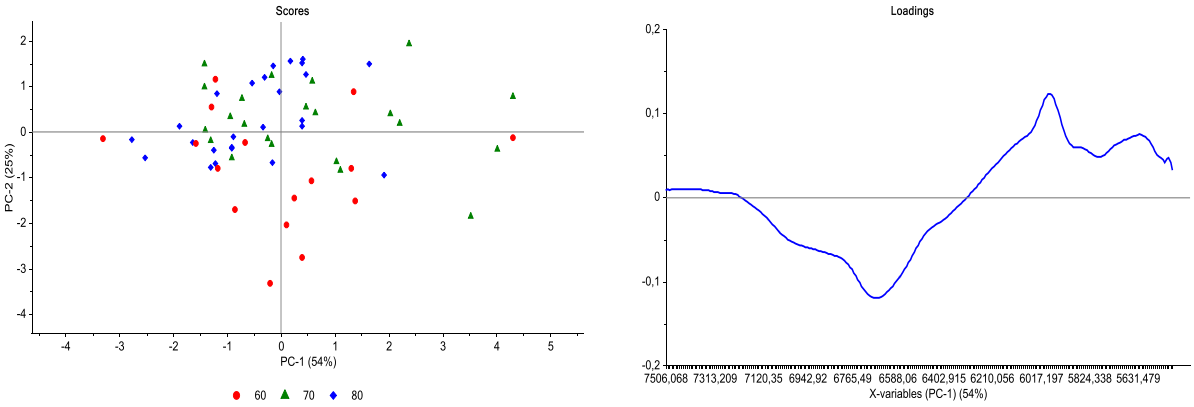

Figure 3 : PCA results (scores for PC1 and PC2, loadings of PC1) for gelatin over the 5800–7500 cm^{-1} range.

For samples grouped according to their extraction temperature, Fig. 3 shows the scores for the first two principle components (PC1 and PC2) obtained by the PCA and the loadings for PC1. Although the

groups are difficult to clearly define, a trend with extraction temperature is nevertheless possible to discern. However, no trend with extraction time appears. These results are attributed to the fact that the gelatins were extracted at very different times and temperatures, and that these parameters produce a synergistic effect.

Near-infrared determination of average molecular weight:

The interesting spectral range spans from 5500 to 8500 cm^{-1} (Fig. 4A) where the spectrum varies the most (Fig. 4B). However, the average molecular weight was deduced from the less-noisy range between 6000 and 8500 cm^{-1}.

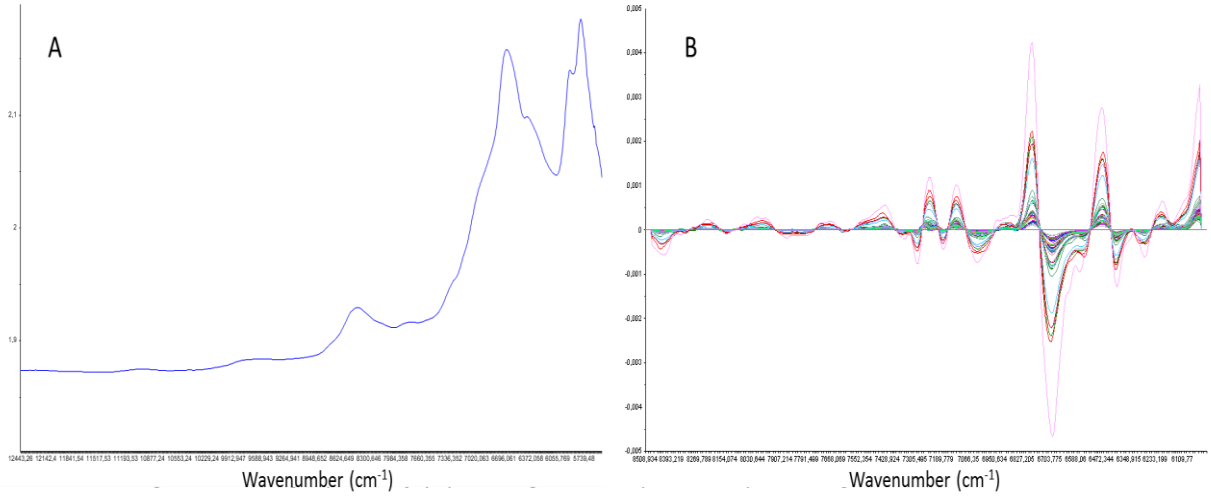

Figure 4 : A – Average spectra of lyophilized gelatin samples (5500-12500cm^{-1}), B – Second derivate and smoothing spectrums of lyophilized gelatin samples (6000-8500cm^{-1})

The gelatin samples extracted from the same pork rinds have the same amino acid composition. The only difference that appears involves the molar distribution. Within this spectral range, the maximum variability is explained by seven PLS components. Figure 5 and Table 2 show the calibration and validation models obtained by PLS. The results indicate that the molar distribution may be determined by NIR spectroscopy. The root-mean-square error of prediction (RMSEP) is 5.59 kDa, which corresponds to 16.43% of the range of average molecular weight. The RPD cross validation (data not

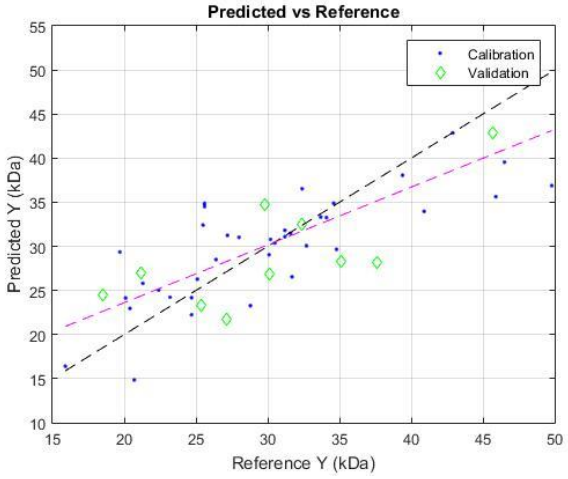

Figure 6 : Mn determined by NIR plotted against reference values for average Mn of extracted gelatin

Table 2 : Overview of results for average Mn of extracted gelatin.

Parameters	Calibration	Validation
Unit	kDa	kDa
N	39	12
Outliers	1	1
Min	15,9	18,5
Max	49,80	45,7
Mean	30,48	31,47
RMSEP	Ø	5,59
LVs	7	7
SD	8,47	8,53
SEP	Ø	5,57
SEC	4,91	Ø
RPD	1,70	1,53
R-square	0,76	0,53

presented in Table 2) is 2.06, which suggests that a good quantitative determination of Mn is possible. Both gelatin extraction and AF4 analysis strongly affect the molar distribution, which suggests that the model can be greatly improved with less analytical variability. In addition, models must be developed with samples of greater diversity of gelatin (different origin of rinds, different species, acidic or basic extraction, etc.).

4. Conclusion and perspectives

This work constitutes a preliminary study that focuses only on the total number-average molecular weight obtained by AF4, which is an innovative technology for studying molecular distributions in detail. We use this technology here to study the molecular-weight profiles of various gelatins.

The results indicate that the near-infrared transmission spectrum varies as a function of total number-average molecular weight, although the cross validation predictions are not optimal. This means that differences other than in chemical composition may be detectable with NIR spectroscopy.

In forthcoming work, we shall study the molar distribution as a function of molecular weight and apply other types of spectroscopy, such as Raman spectroscopy. Although the present work only used gelatins from acid extraction on pork skin, gelatins from fish and beef with acidic or basic extraction should also be considered. The ultimate goal is to develop a tool that allows us to characterize and understand industrial gelatin by using spectroscopy techniques to determine molecular mass distribution.

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