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Fourier-transform infrared spectroscopy for characterization of liquid protein solutions: A comparison of two sampling techniques

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ABSTRACT

Whereas industrial laboratory systems for Fourier-Transform Infrared (FTIR) spectroscopic characterization have been available for decades, dedicated FTIR solutions for in-line process control or for industrial at-line analysis of protein quality are still scarce. Thus, the present study aimed to compare two industrially viable sampling techniques, namely attenuated total reflectance (ATR) and dry film FTIR spectroscopy, for qualitative and quantitative analysis of liquid protein solutions. For this purpose, two sample sets were acquired: set 1 consisted of 95 protein hydrolysate samples produced in the laboratory from salmon processing by-products, and set 2 consisted of 133 protein hydrolysate samples obtained from an industrial processing plant of poultry by-products. Average molecular weights (AMW) and Brix measurements of protein hydrolysates were used as reference values for obtaining regression models. AMW was predicted with higher accuracy and lower estimation errors using dry film FTIR compared to ATR measurements for both sample types. The difference in predictive performance was higher in poultry hydrolysates because the protein and tissue complexities are higher than in salmon hydrolysates, and information from the amide I region in the FTIR spectra is needed to provide good calibration results. ATR, on the other hand, was more reliable for the prediction of Brix values of protein hydrolysates. The study also showed that FTIR spectroscopy can be used to predict protein quality features of industrially produced protein hydrolysates with a sensitivity of high industrial relevance. Therefore, developing industrially viable portable instruments for at-line process analysis in the food, feed, and biotech industries is a natural next development stage.

1. Introduction

Over the last decades, Fourier-Transform Infrared (FTIR) spectroscopy has become an established technique for the rapid characterization of proteins and peptides. Proteins and peptides give rise to nine distinct infrared absorption bands (i.e., amide A, amide B, and amide I-VII), with the amide I (~1650 cm^{-1}) and amide II (~1550 cm^{-1}) bands being the most prominent [1,2]. These bands are well known for providing information on protein secondary structure, protein, and peptide size, but they can also be used to study related parameters such as hydration, solvent, and pH effects on proteins [2–6]. With the current focus on sustainable food systems and the future need for proteins to feed an increasing global population, huge research efforts are put into the development of alternative protein sources, from vegetable proteins to cellular agriculture. Therefore, the concept of protein quality is also gaining increasing attention, and parameters such as average molecular weight (AMW) and protein composition are regarded as some of the key protein quality indicators [7,8]. Measuring such parameters in an industrial setting could thus be valuable for process control and optimization purposes. Today, however, there are no or limited analytical solutions for rapid at-line protein and peptide quality measurements in the industry. FTIR spectroscopy is one of the techniques that can be expected to meet this demand.

A special case in protein ingredient production is the processing of food processing by-products. Currently, such by-products are processed using, e.g., enzymes to obtain value-added peptides and amino acids, subsequently mainly utilized as feed, food ingredients, or nutraceuticals [9]. It has been shown that FTIR can be used as a rapid tool for

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monitoring enzymatic protein hydrolysis and for the characterization of protein hydrolysates formed by these reactions [10–12]. Studies have also proven the potential of FTIR for the prediction of AMW or the degree of hydrolysis of protein hydrolysates. In these models, the amide I band and the bands originating from NH_3^+ deformation (amino terminals) at ~1516 $cm^{-1,}$ and carboxylate (COO^-) groups at ~1400 cm^{-1} were shown to play important roles [13]. Moreover, the depolymerization of proteins due to heat and pressure [14], and peak shifts due to the presence of collagen and gelatine [15] can also be observed in FTIR spectra. Recently, Måge et al., [16] showed that the FTIR fingerprint of proteins and peptides can be directly used to give insight into enzyme action, processing time, and raw material composition in enzymatic protein hydrolysis of food processing by-products.

Industrial laboratory systems based on FTIR characterization have been available for decades, frequently used for the measurement of, e.g., protein content and other components in milk [17]. However, dedicated FTIR solutions for in-line process control or for industrial at-line analysis of protein quality are still scarce. A major challenge in FTIR analysis of protein-containing liquids is related to interference from water. Since water absorbs IR light in the region between 1600 cm^{-1} and 1700 cm^{-1} and above 3000 cm^{-1}, the amide I band of proteins at ~1640 cm^{-1} is often obscured or hidden [2,4]. A closer investigation of feasible sampling approaches that can potentially be used in industrial process lines is thus highly relevant. In classical FTIR transmission sampling, where liquid samples are measured using transmission cells, very short optical path lengths and high protein concentrations are needed, and spectral information from the water absorption regions is often lost [2]. However, there are two sampling approaches, namely attenuated total reflectance (ATR) and dry film FTIR, that have been commonly employed to reduce the influence of water on the spectra of aqueous protein samples. While these two sampling approaches have been independently evaluated for measuring protein-based samples, a systematic comparison of the quantitative prediction performances for protein quality has yet to be explored.

In ATR, an infrared beam is guided into and internally reflected through a high refractive index crystal (e.g., diamond or zinc selenide crystals). This internal reflection creates an evanescent wave that penetrates and interacts with the sample, giving an infrared spectrum of the sample. Since ATR allows direct measurements of liquids as well as solid samples, the technique has been frequently used for protein characterization in recent years, including applications for biofluid characterization [18,19]. Infrared light has very limited sample penetration depth and sample inhomogeneity can make a huge impact on the ATR measurements [20]. During ATR measurements, the spectrum of either air or water is normally used for background correction [4,21,22]. Similarly, liquid samples can be placed on top of the ATR crystal for drying into thin films before analysis. Working on the rapid analysis of disease state in liquid human serum, Sala and co-workers showed that such a drying procedure improved classification results compared to ATR measurements of liquid serum [3]. There are only a few studies performed using ATR for the analysis of protein hydrolysates. For instance, Schmidt et. al., [23] successfully used ATR in the characterization of collagen hydrolysates from mechanically separated chicken meat residue.

Dry film FTIR is a sampling technique where liquid samples are deposited on an IR transparent material (i.e., silicon) and left to dry and form a thin film. Subsequent FTIR measurements are performed in transmission mode, and the approach can be a viable solution to minimize water interferences. One of the first applications of dry film FTIR was related to the characterization of microorganisms [24], but today it is used on a range of sample types, like liquid foods and biofluids [13,25]. During drying, the analytes of interest are concentrated, which improves the sensitivity of the method. This was shown in a study where estimated errors for fatty acid determination of milk samples using dry film FTIR were lower overall when compared to liquid transmission measurements [26]. A study showed that dry film measurements were very similar to the results obtained with ATR measurements, with a high coefficient of determination (R^2 >0.96) for predicting glucose concentrations (concentrations up to 80 g/L) and citric acid during microbial bioprocessing [27]. In contrast, the ATR method provided better predictions of crude proteins, lactose, fat, and urea in milk compared to the dry film approach [20]. Thus, both sampling techniques will have their advantages and disadvantages [28], therefore there is a need to carefully compare the prediction performance of the two approaches when examining applications for protein quality characterization.

The present study aimed to compare ATR and dry film FTIR for qualitative and quantitative analysis of liquid protein solutions. For this purpose, two sample sets were acquired that both provided different complexities in the protein composition and originated from either laboratory experiments or industrial processing. Sample set 1 consisted of 95 samples of salmon protein hydrolysates produced in the laboratory from salmon processing by-products. Sample set 2 consisted of 133 protein hydrolysate samples obtained from an industrial processing plant of poultry by-products. Average molecular weights were estimated using size exclusion chromatography (SEC) and degrees Brix measured using a Brix refractometer were used as reference values for obtaining regression models. To our knowledge, this is the first study to compare ATR and dry film FTIR spectroscopy for quantitative analysis of liquid protein solutions. Moreover, in the study, FTIR spectroscopy for the prediction of AMW of industrial protein hydrolysates is presented for the first time.

2. Materials and methods

2.1. Materials

Molecular weight standards for SEC analysis (bovine serum albumin (BSA), albumin from chicken egg white, carbonic anhydrase from bovine erythrocytes, lysozyme, cytochrome c from bovine heart, aprotinin from bovine lung, insulin chain B oxidized from bovine pancreas, renin substrate tetra decapeptide porcine, angiotensin II human, bradykinin fragment 1–7, [$DAla^2$]-leucine enkephalin, Val-Tyr-Val, and L-tryptophan) ranging from 66,000 g/mol to 204 g/mol were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). BSA for FTIR analysis was obtained from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). Similarly, the enzymes for salmon hydrolysis, Promod 439 L, and Promod 950 L were obtained from Biocatalysts Inc (Chicago, IL, USA). HPLC-grade acetonitrile was provided by VWR (Radnor, PA, USA). All the chemicals used were of HPLC grade. Water was prepared by deionization and membrane filtration (0.22 μm) using a Millipore Milli-Q purification system (Merck Millipore, Burlington, MA, USA).

2.2. Protein hydrolysates

2.2.1. Salmon hydrolysates

The salmon hydrolysates were produced in the laboratory based on a full factorial design constituting 12 by-products, 2 enzymes, 2 reaction temperatures, and 2 hydrolysis reaction times, resulting in 96 samples. One hydrolysate sample was excluded, leaving 95 samples for the final analysis. By-products from salmon production (two samples from each of the following by-product groups: heads, backbone, belly flap, trimmings, skin, and whole fish) were obtained from Biomega Norway AS (Skogsvåg, Norway). Two enzymes, Promod 439 L and Promod 950 L were used for hydrolysis. The enzymatic hydrolysis was carried out using a 2-liter, Reactor-Ready jacketed reaction vessel with a heating mantle (Radleys, Essex, United Kingdom). The temperature was controlled using a water flow system with a JULABO circulation pump (Julabo GMBH, Seelbach, Germany) connected to the reactor. The temperature of the reactions was kept at 50 °C. Approximately 500 g of homogenized salmon by-product and 990 mL of distilled water were added to the reactor. The mixture was stirred at 360 rpm and heated to a controlled temperature (either 45 or 50 °C) before enzyme addition. 5 g of enzyme suspended in 5 mL water was added to the individual

hydrolyzes. Approximately 15 mL of sample was collected at two different time points, i.e., after 45 min and 60 min of hydrolysis time. The enzymes were then inactivated using a microwave oven (Menumaster, ACP Inc., Iowa, USA) for about 15 s, followed by 15 min in a water bath maintained at a temperature of 90 °C. The samples were centrifuged at 25 °C for 30 min at 4400 rpm (Heraeus Multifuge 4KR Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) to get phase separation of solid particles, lipids, and the water phase. The aqueous phase containing proteins and peptides was extracted and filtered with a Millex-HV Polyvinylidene Difluoride (PVDF) 0.45 μm, 33 mm syringe filter unit (Merck Millipore, Burlington, MA, USA). The samples were stored at − 40 °C before further analysis.

Prior to FTIR analysis, frozen samples were thawed in a sonic bath holding 50 °C for 30 min and cooled in a water bath at 22 °C for 30 min. The samples were centrifuged at 25 °C for 10 min at 4400 rpm. The samples were left at room temperature for equilibration for 30 min. The samples were then filtered using a Millex-HV PVDF 0.45 μm, 33 mm, before further analysis.

2.2.2. Poultry hydrolysates

The protein hydrolysate samples (133 samples) (raw material: by-products of chicken and turkey processing) were obtained from the Bioco enzymatic protein hydrolysis production plant (Hærland, Norway). The samples were collected at different time points in autumn (October) 2020 and spring (March) 2021. The hydrolysis process includes grinding, preheating, enzyme addition, and enzyme deactivation. In addition, centrifugation for 4 min at 4100 rpm (MEGA STAR 600, VWR, Oslo) was carried out for phase separation. The phase-separated samples were frozen down to − 40 °C and sent to Nofima and kept frozen until further analysis. Prior to FTIR analysis, the same procedure was adopted as for the salmon hydrolysates mentioned above. In addition, all poultry hydrolysate samples were diluted to approximately 2.5°Bx prior to SEC and dry film FTIR analysis. This dilution was performed to keep within the linearity range of the detector of the FTIR instrument.

2.3. Brix analysis (for estimation of dry matter content)

The Brix value of protein hydrolysate samples was approximated as degree Brix (°Bx) measured using a Brix refractometer (Reichert AR200 Digital Hand-held Refractometer, New York, USA). The Brix value was measured on 100 μL of samples and calibrated against 100 μL Milli-Q purified (Merck Millipore, Burlington, MA, USA) water after every five samples. All samples were measured twice. The Brix values provide an approximation of the dry matter content of the protein solutions.

2.4. Size exclusion chromatography

The SEC and AMW calculations were performed using a published protocol with some modifications [10]. The size exclusion chromatographic traces were acquired using a Dionex Ultimate 3000 instrument (Thermo Scientific, Waltham, MA, USA) equipped with a quaternary pump and photodiode array detector and fitted with a security guard, HPLC guard cartridge system, and a BioSep SEC-s2000 column, 300 mm long with an inner diameter of 7.8 mm (Phenomenex, Torrance, CA, USA). For SEC analysis, a 2 mg/mL standard solution was prepared in ultrapure water. The mobile phase was prepared as a 30:70 (V/V) mixture of acetonitrile and ultrapure water with 0.05% trifluoroacetic acid (TFA). The injection volume was 10 μL for the standards and 15 μL for the samples. Isocratic elution was performed at a 0.9 mL/min flow rate for 20 min before the mobile phase was changed to NaH_2PO_4 (0.1 M) for 3 min (for cleaning) and finally, the column was equilibrated for 27 min before the next run. Chromatographic runs were controlled from Chromeleon Chromatography Data System (CDS) software (Thermo Fisher Scientific). The detection of the eluents was done by measuring their absorbance at 214 nm. The chromatographic data were baseline corrected using PSS WinGPC UniChrom V 8.33 (Polymer Standards Service, Mainz, Germany).

From chromatographic runs of both the standards and hydrolysates, an Ultraviolet (UV) trace of 214 nm was used in the average molecular weight calculations. A molecular weight calibration curve was built by fitting a proprietary third-order polynomial regression (PSS poly 3 in WinGPC) (Polymer Standards Service, Mainz, Germany) to the retention times plotted against the common logarithm (log10) of the molecular masses of the calibration standards. The included data consists of the raw chromatographic traces which have been baseline corrected in WinGPC. The trace from 5 to 20 min was used for analyses. The average molecular weight (AMW) was calculated from the calibrations. The calculation from the software was based on a slicing method.

2.5. Fourier-transform infrared spectroscopy

FTIR analysis of pure BSA solutions, salmon hydrolysates, and poultry hydrolysates was performed using two different sampling approaches as mentioned below. This was done directly after the Brix measurements of the samples.

2.5.1. FTIR ATR measurements

A Nicolet iS5 FTIR spectrometer equipped with a diamond crystal ATR iD7 accessory with a Deuterated Triglycine Sulfate (DTGS) detector was used for data collection (Nicolet iS5, Thermo Scientific, Madison, WI, USA). A volume of 15 μL of the sample was directly applied to the ATR surface and analyzed. All FTIR spectra were recorded from 4000 to 600 cm^{-1}, co-adding 50 interferograms at a spectral resolution of 4 cm^{-1} and with an approximate interval of 0.5 cm^{-1} (digital resolution). Before each sample scan, a background reference measurement was performed by applying 15 μL water to the ATR crystal. The absorbance was calculated using the standard log transform of the sample and reference single beam spectra, respectively, to eliminate spectral features related to water. A new water background reference spectrum was taken for each sample. Three replicates were measured for each sample.

2.5.2. Dry film FTIR analysis

Dry films of protein hydrolysate samples were made by depositing 7.5 μL of sample solution on a 96-well Si-microtiter plate (Bruker Optics, Billerica, MA, USA) and subsequently drying at room temperature for 45 min. Five aliquots from each sample were deposited on the well plate to allow replicate measurements. FTIR measurements were performed using a High Throughput Screening eXTension (HTS-XT) unit coupled to a Tensor 27 spectrometer (Bruker Optics, Billerica, MA, USA). The spectra were recorded in the region between 4000 and 400 cm^{-1} with a spectral resolution of 4 cm^{-1} and an aperture of 5.0 mm with approximately 2 cm^{-1} intervals (digital resolution). For each spectrum, 40 interferograms were collected and averaged. Data acquisition was controlled using Opus v6.5 (Bruker Optics, Billerica, MA, USA).

2.6. Data analysis

FTIR spectra were imported to the statistical software The Unscrambler version 11 (AspenTech, Oslo, Norway) for data processing. Spectral preprocessing was optimized for each sampling approach. The dry film FTIR spectra were pre-processed using the 2nd-derivative Savitzky-Golay algorithm with a 2nd-degree polynomial and a window size of 13. Similarly, for FTIR ATR spectra, a Savitzky-Golay 2nd-derivative algorithm with a 2nd-degree polynomial and a window size of 101 was used. The higher window size used for FTIR ATR spectra was chosen due to digital resolution and lower signal/noise ratios compared to the dry film FTIR spectra. The derivative spectra from both approaches were subsequently normalized by applying extended multiplicative signal correction (EMSC) in the region from 1700 to 850 cm^{-1}. After preprocessing, all replicate spectra were averaged so that every sample was represented by one spectrum (per sampling approach).

Multivariate regression analysis was performed for the prediction of AMW and Brix values by applying partial least squares regression (PLSR). The optimal number of PLSR factors was found using 10-fold segmented cross-validation. The prediction performance of the models was evaluated using root mean square error of cross-validation (RMSECV), coefficients of determination (R^2), and inspection of scores, loadings, and regression coefficients.

3. Results and discussions

In the present study, ATR and dry film FTIR were compared for the characterization of protein solutions. Initially, FTIR analyses of pure BSA solutions were performed to see how the presence of water affects the FTIR spectra. Spectra of BSA solutions at 3 different concentrations measured with ATR and dry film FTIR are provided in Fig. 1. The ATR spectra have been background corrected for water, whereas dry film FTIR spectra are not corrected. The figure shows two important features: First, for both techniques, the protein concentrations are revealed, as expected. Secondly, the presence of water (and the subsequent background correction) affects the amide I region (~1700–1600 cm^{-1}) of the ATR spectra and thereby the ratio between the amide I and amide II regions. From the figure, it is clearly observed that the relative intensity of the amide I region is reduced after background correction of water (i.e., ATR). One natural explanation for this is the prominent water band found around ~1645 cm^{-1} (i.e., H-O-H bending), overlapping the amide I band completely. To illustrate the dominating water signals, supplementary Fig. 1 compares an ATR spectrum of water to a protein hydrolysate ATR spectrum without background correction. Here it is clearly shown to what extent water dominates an ATR spectrum of aqueous proteins. Thus, one could expect that the amide I intensity most likely is altered slightly after background correction of the ATR spectra. In the current study, alternative water subtraction approaches were tested, but with similar results (data not shown). Aernouts et. al. [20] also experienced a similar effect in the ATR spectra of milk after water background correction. Most important, however: since the water subtraction approach used here is standardized for all samples, the quantitative information in the ATR spectra is retained.

3.1. Salmon hydrolysates

A total of 95 salmon hydrolysates were analyzed using both sampling approaches. The AMW of salmon hydrolysates varied between 1496 and 2451 g/mol. Similarly, the Brix values varied between 4.6°Bx to 6.4°Bx. Preprocessed spectra of representative protein samples are provided in Fig. 2. In the spectra, the most prominent protein bands constitute amide I (1700–1600 cm^{-1}), amide II (1600–1500 cm^{-1}), and the carboxylate band at around ~1400 cm^{-1}. These bands were also associated with the most significant variation in the spectra. It is also noticeable that the dry film spectra showed more distinct features in both the amide I and amide II regions compared to the ATR method, indicating that more information potentially can be obtained from the former approach.

PLSR models were developed linking ATR and dry film spectra to AMW and Brix values, respectively. Regression results are provided in Table 1 and Fig. 3. The table reveals that for AMW, both sampling approaches provide good results, with dry film performing slightly better than ATR. Also, the model complexity (i.e. number of factors used) was similar for both techniques, which is visually confirmed in the predicted vs. reference plots of Fig. 3. This figure also reveals two outliers in the ATR model. Since neither reference nor spectral data could give an explanation for why they show outlying behavior, the samples were not removed from the model. Table 1 also shows that while ATR provides good models for Brix values, the Brix value estimates using dry film FTIR is slightly worse, with higher model complexity. This is related to the fact that water is removed in the dry film analysis and thus water cannot contribute to providing stable estimates of the dry matter contents. Apparently, since no enzyme-related grouping could be seen in the regression models, the two different types of enzymes used for the production of the salmon hydrolysates did not affect the performance of the regression models (data not shown).

The regression coefficients obtained from regression models of AMW are provided in Fig. 4. As seen in the figure, for both measurement techniques, the amide I region is important, but the dominating spectral feature is related to the COO^- stretching at ~1400 cm^{-1}. Similar findings were shown in a study done on salmon hydrolysates, stating that fish muscle has a relatively "simple" composition, compared to, for instance, poultry raw materials and other land-based mammals [29]. For ATR, this is favorable, since the models will rely less on the region in the spectra that are affected the most by water. The regression coefficients also still reveal that the amide I region is better resolved in the dry film coefficients than in the ATR coefficients.

3.2. Poultry hydrolysates

A total of 133 poultry hydrolysates were analyzed using both sampling approaches. The AMW of poultry hydrolysates varied between 2819 and 6973 g/mol. Similarly, the Brix value varied between 3.7°Bx to 6.7°Bx. It is interesting to note that the poultry hydrolysates have significantly higher AMW values than the salmon hydrolysates, and the variation in the AMW values is also considerably higher. In general, poultry carcasses contain more collagen and thus reveal a higher protein complexity than salmon by-products, and are, therefore, considered more difficult to degrade enzymatically than salmon by-products [29]. In addition, sampling from an industrial process might lead to more variation due to different raw materials than obtained in a controlled laboratory environment. Spectra of representative poultry hydrolysates are provided in Fig. 5. Here the complexity of the protein composition is confirmed by a large variation in the amide I and II region of the dry film FTIR spectra, which is not seen to the same extent in the ATR spectra. Thus, as also seen in Fig. 2, more information on protein characteristics can potentially be obtained from the dry film spectra compared to the

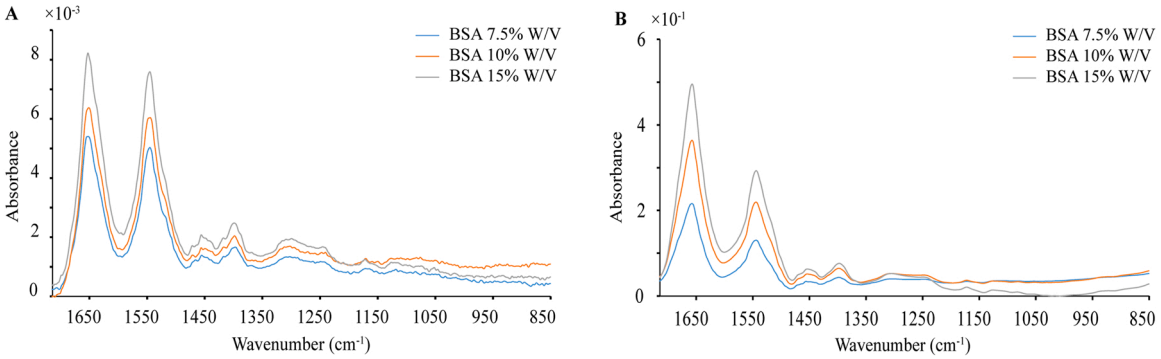

Fig. 1. FTIR spectra of pure BSA using (A) ATR and (B) The dry film approach (1700–850 cm^{-1}).

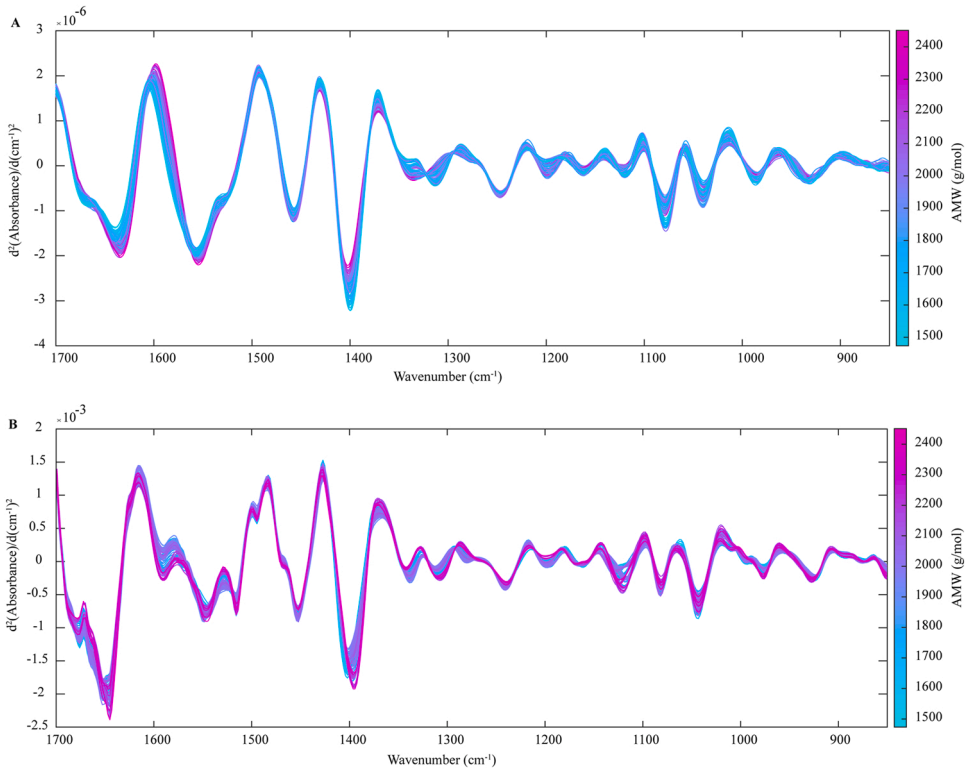

Fig. 2. Preprocessed salmon hydrolysate FTIR spectra obtained using (A) ATR and (B) The dry film approach in the spectral region of 1700–850 cm^{-1}. All spectra are color-coded according to the average molecular weight of the protein hydrolysates.

Table 1
FTIR regression results for AMW and Brix values of salmon hydrolysates (95 samples) using the spectral region of 1700–850 cm^{-1}.

FTIR method	Parameters	R^2	RMSECV	Factors	Preprocessing
ATR	AMW	0.89	70.1[a]	7	2nd derivative + EMSC
Dry films	AMW	0.94	54.1[a]	7	2nd derivative + EMSC
ATR	Brix value	0.88	0.15[b]	4	2nd derivative
Dry films	Brix value	0.84	0.18[b]	6	2nd derivative + EMSC

[a] AMW (g/mol), Mean ± SD: 1885 ± 215.
[b] Brix value (°Bx), Mean ± SD: 5.6 ± 0.4.

ATR spectra.

The coefficient of determination (R^2) and root mean square error of cross-validation (RMSECV) of the prediction models for AMW, and Brix value was obtained (Table 2). Table 2 reveals that for AMW, both sampling approaches provide good results, with dry film FTIR performing better than ATR. A major difference in these models is the model complexity: for the ATR model, four factors are used while the dry film FTIR model requires seven factors. However, when inspecting the corresponding predicted vs. reference plots of Fig. 6, there is a clear curvature in the ATR plot, indicating a non-linear behavior of this model, and the challenge is particularly visible in the higher molecular weight region. Increasing the number of factors of the ATR AMW model did not improve the coefficient of determination or the curvature challenges (data not shown). It is known that the uncertainty of the reference SEC AMW measurements increases with increased molecular weight, especially for poultry hydrolysates due to the high contents of collagen [29]. However, since we do not see a similar non-linear trend in the dry film model, this uncertainty is unlikely a valid explanation for the non-linearity in the ATR model. For the prediction of the Brix value, ATR provided moderate results (R^2 of 0.72 and RMSECV of 0.34°Bx). For dry film FTIR, Brix value models were not obtained due to a dilution step performed before FTIR analysis.

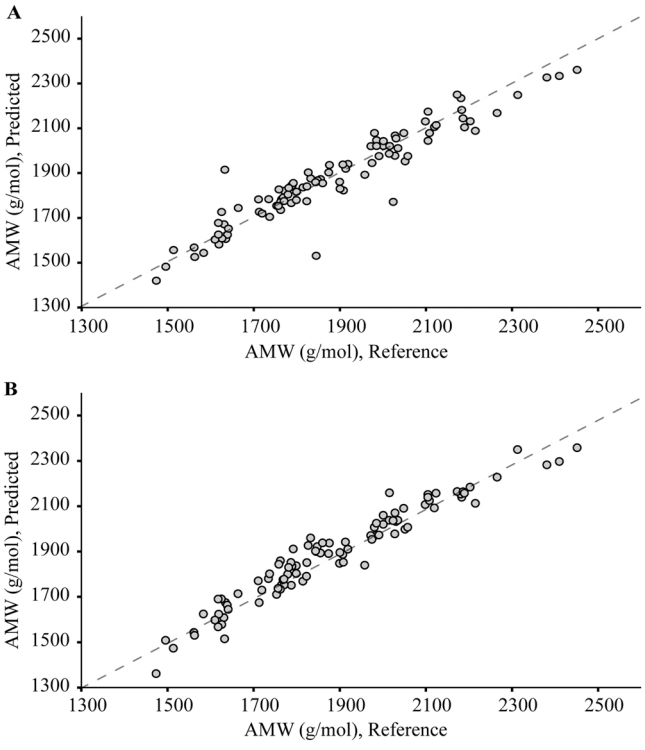

Fig. 3. PLSR models for prediction of AMW of salmon hydrolysates using (A) ATR and (B) The dry film approach.

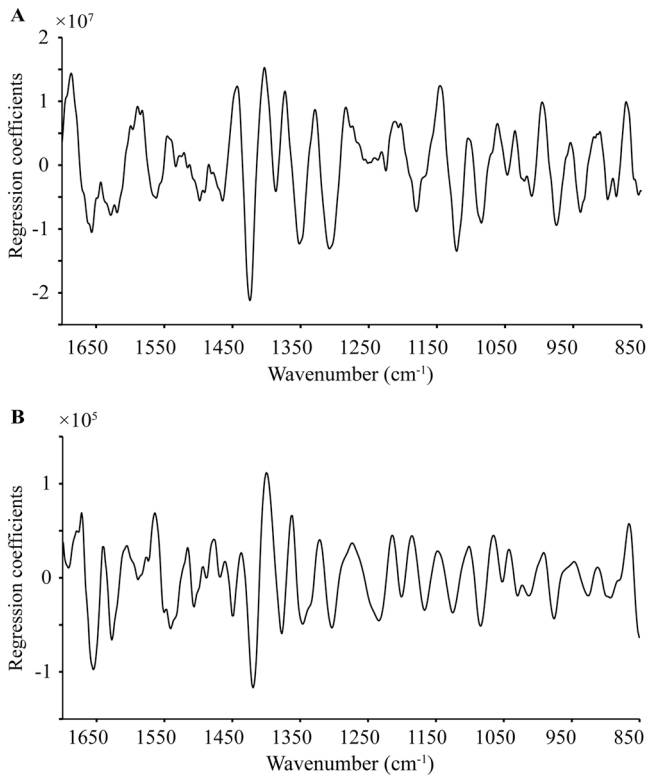

Fig. 4. Regression coefficients of PLSR models for prediction of AMW of salmon hydrolysates: (A) ATR and (B) The dry film approach.

The regression coefficients of the AMW models of poultry hydrolysates provided in Fig. 7 reveal that the ATR and the dry film models are different and that there are differences between the salmon hydrolysate (Fig. 4) and poultry hydrolysate models. For the ATR model, the band around 1083 cm^{-1} is the most prominent band. In the literature, this band has been assigned to C-O stretching vibrations and could thus be related to glycosylated proteins [30,31]. Based on previous research studies, this band is peculiar as a strong contributor to an AMW prediction model, and the current model can therefore potentially be the result of variables indirectly linked to protein degradation. In turn, this could result in unstable models and could thereby compromise model interpretability for protein quality. In the regression coefficients, there is also an important contribution from the COO^- stretching around 1400 cm^{-1}, as also seen for the salmon hydrolysates. For the dry film model, the major contribution is coming from the amide I band around 1660 cm^{-1}, with less emphasis on the COO^- stretching around 1400 cm^{-1}. This is expected based on previously published studies and is related to the increasing complexity of the proteins and tissues in poultry carcasses [29]. Thus, the poultry hydrolysate models indicate that when the protein complexity of the raw material increases and the models need to rely more heavily on the amide I region, the ATR sampling approach provides poorer results since the amide I region is less resolved due to the presence of water.

3.3. General discussions

There is no doubt that water influences infrared spectroscopic analysis, and the two sampling approaches studied here meet this challenge in different ways. In ATR the effect of water is diminished due to the measurement principle and can subsequently be corrected if needed. In dry film FTIR, water is physically removed by drying. Thus, the choice of preprocessing and correction approaches is an important aspect in this regard. For the dry film FTIR spectra, a standard preprocessing approach based on 2nd-derivatives using a Savitkzy-Golay algorithm and subsequent normalization using EMSC was used. Especially the thickness of the dried films will usually vary from sample spot to sample spot, and maybe also within one sample spot, and the current preprocessing regime used is known to correct for this and other physical effects [32]. The scaling effect of the normalization approach also means that the information on protein contents is partly removed. Another feature frequently occurring in dried sample spots is chemical inhomogeneities like e.g., the coffee ring effect. In the current study, however, we expect these effects to be of minor importance since proteins and peptides are known to have good film-forming abilities.

For ATR the literature is less clear on a standard preprocessing approach, and often no correction is performed. In the current study, however, the same approach as for dry film spectra was used. This was based on a preprocessing optimization study performed using different approaches (data not shown). For the Brix value calibration, on the other hand, no normalization procedure was used due to the risk of removing important information related to protein contents, which sometimes can be directly related to the normalization constant used, as visualized in Fig. 1. In the ATR measurements, the water background was taken before each analysis and corrected from the sample spectrum during data acquisition within the instrument (Fig. 1) [1,33]. The alternative for this approach is to use air as the background, and then correct for water digitally afterward. In the current study, both approaches were tested on a subset of samples, and no significant differences could be seen from the spectra (see supplementary Fig. 2). Digital water correction is a research field by itself and can contribute to improving calibration performance [3], but an extensive elaboration of these possibilities are considered outside the scope of the present study. It should also be noted that there were other differences in the acquisition parameters used for obtaining ATR and dry film FTIR spectra, respectively. For practical reasons, more replicates were used in the dry film analysis compared to the ATR analysis. However, using an equal number of replicates for the two sampling approaches did not significantly alter the predictive performance (data not shown). In addition, an increased window size in the derivative calculations of the ATR spectra was used, which could be attributed to higher digital resolution and generally lower signal/noise ratios compared to the dry film spectra.

Even though the present study was performed on two specific types of protein hydrolysates, there are some generic trends observed that are applicable to the infrared analysis of liquid protein solutions in general. The difference between ATR and dry film as a sampling approach can be regarded on two different levels, one related to the chemistry measured, and one related to practical aspects of the FTIR analysis. For the AMW-based protein quality measurements, the dry film approach expectedly provided more precise regression models with a higher coefficient of determination and lower RMSECV values compared to ATR. For the salmon hydrolysates, where the complexity of the protein composition can be regarded as low, the regression results are comparable. However, when the protein complexity increases, and when information from the amide I region is needed, the dry film approach works clearly better. Thus, even though ATR measurements provide good-quality spectra of liquid proteins, protein complexity must be considered when choosing the preferred sampling approach for a given application. In the case of the Brix value, this can be best modeled using ATR, whereas, for dry film analysis, only relative amounts of protein contents (related to dry matter contents) could potentially be measured. All in all, these results are in concordance with a recent study on the rapid evaluation of nutritional parameters in liquid foodstuffs. Here, ATR measurements on intact liquid foodstuffs were shown to provide the best predictions of main constituents like proteins, fat, carbohydrates, and water, whereas a drying procedure was needed to provide sufficient predictions of fractions of fat and carbohydrates [28].

Regarding the practical aspects of FTIR sampling approaches, there are clear differences between the two sampling approaches studied here. ATR requires no sample preparation, and the liquid samples can be measured very close to real-time in each process. The approach is thus

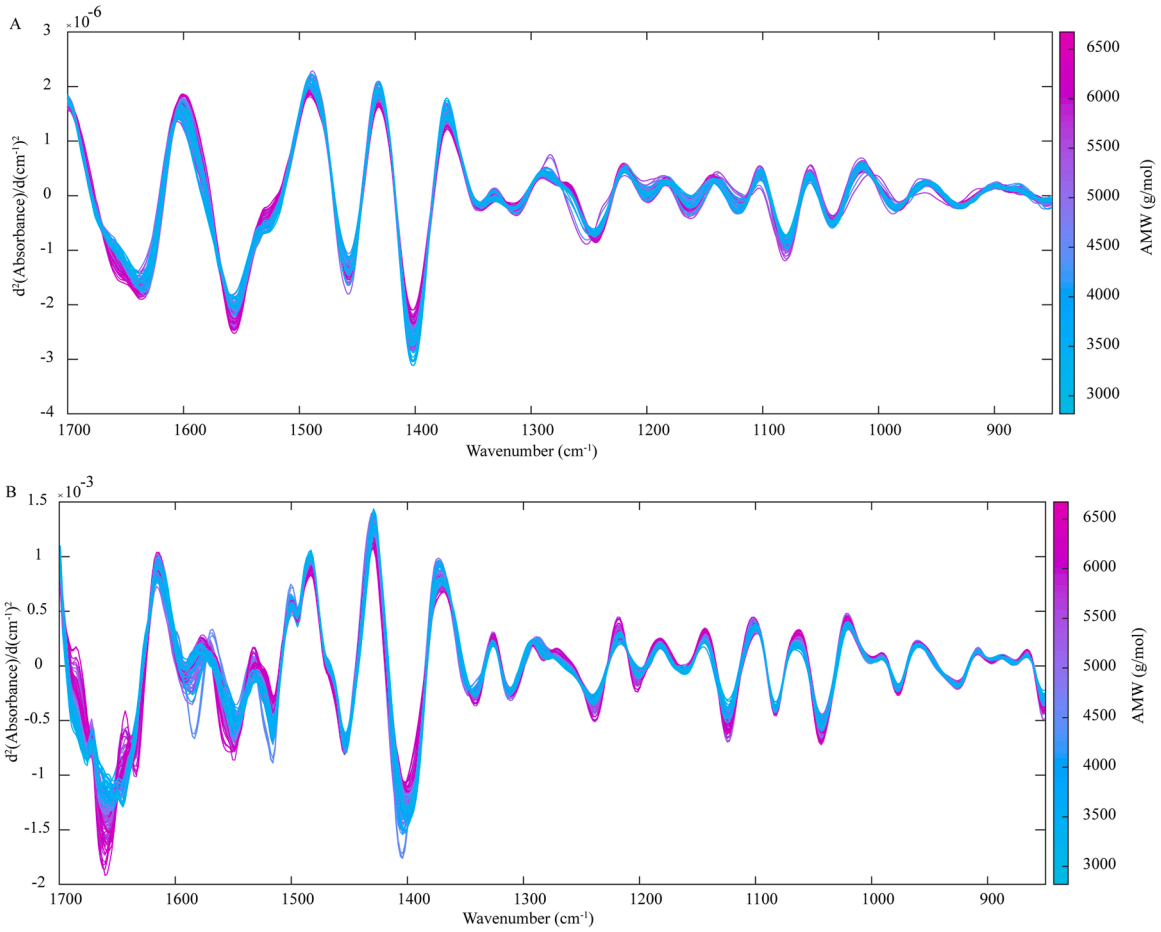

Fig. 5. Preprocessed salmon hydrolysate FTIR spectra obtained using (A) ATR and (B) The dry film approach in the spectral region of 1700–850 cm^{-1}. All spectra are color-coded according to the average molecular weight of protein hydrolysates.

Table 2
FTIR regression results for AMW and Brix values of poultry hydrolysates (133 samples) using the spectral region of 1700–850 cm^{-1}.

FTIR method	Parameters	R^2	RMSECV	Factors	Preprocessing
ATR	AMW	0.87	343.7[a]	4	2nd derivative + EMSC
Dry films	AMW	0.95	219.5[a]	7	2nd derivative + EMSC
ATR	Brix value	0.72	0.34[b]	1	2nd derivative

[a] AMW (g/mol), Mean ± SD: 4889 ± 940.
[b] Brix value (°Bx), Mean ± SD: 5.4 ± 0.6.

ideal when a real-time analysis is needed, i.e., in kinetic analysis and when the analysis does not require high sensitivity in the amide I region. The ATR method has lower sample throughput, is generally more time-consuming when hundreds of samples are to be tested and would require the handling and washing of a key expensive component, which is not ideal in an industrial setting, compared to an easily mass-produced silicon plate (i.e., dry film FTIR analysis). In biofluid analysis, a drying procedure is often performed prior to ATR measurements [34]. However, for industrial purposes, since one would usually be confined to using only one ATR crystal, dried ATR analysis was considered too time-consuming for the current study [35]. For the dry film approach, the drying step might take a few minutes, and sometimes also a dilution step is needed to avoid non-linear detector responses. However, the approach is highly suited for high-throughput analysis using multi-well transmission plates, and it has been shown that the procedure from sample to dry film formation and subsequent FTIR analysis can be readily automated [36]. Thus, there is a potential for the development of this approach as an automated at-line solution for measurements of protein quality in industrial processes.

To the authors' knowledge, this is the first time that FTIR has successfully been used to predict protein quality features of industrially produced protein hydrolysates (e.g., poultry protein hydrolysates). Since the samples were provided from an ongoing process, it is clear that the FTIR technique has sufficient sensitivity to predict protein quality variations that are highly relevant for industrial purposes. This shows that FTIR has the potential to be developed into a technique that can be used for process monitoring, process optimization, and product documentation in industrial environments.

4. Conclusions

The present study shows that ATR and dry film FTIR spectroscopy provides different opportunities for the analysis of liquid protein solutions. AMW was predicted with higher accuracy and lower estimation errors using dry film FTIR compared to ATR measurements for both sample types. The difference in predictive performance was higher in poultry hydrolysates compared to salmon hydrolysates because poultry hydrolysates consisted of higher protein and tissue complexities and the calibration models were based on the information from the amide I region in the FTIR spectra, which is needed to provide good calibration results. ATR, on the other hand, showed to be a more reliable method for the prediction of Brix values in the hydrolysates. This study also showed that the protein quality features of industrially produced protein

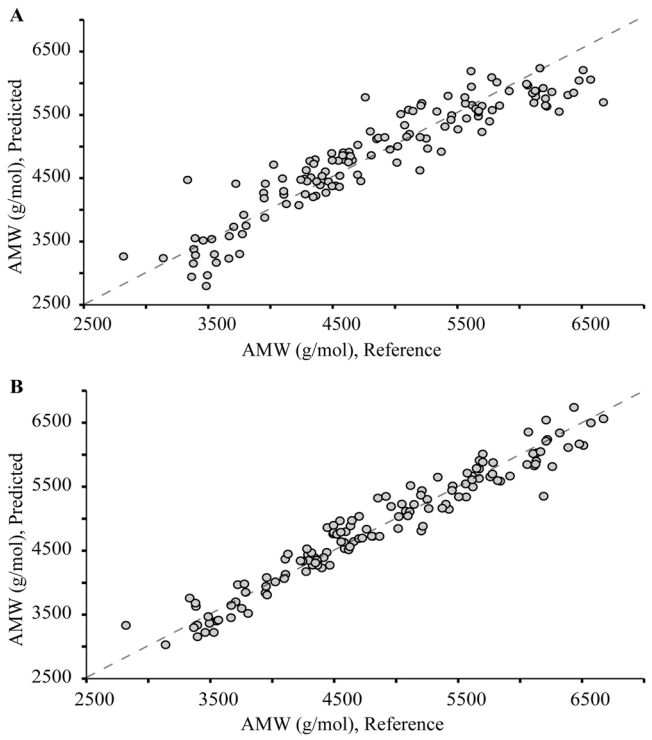

Fig. 6. PLSR models for prediction of AMW of poultry hydrolysates using (A) ATR and (B) The dry film approach.

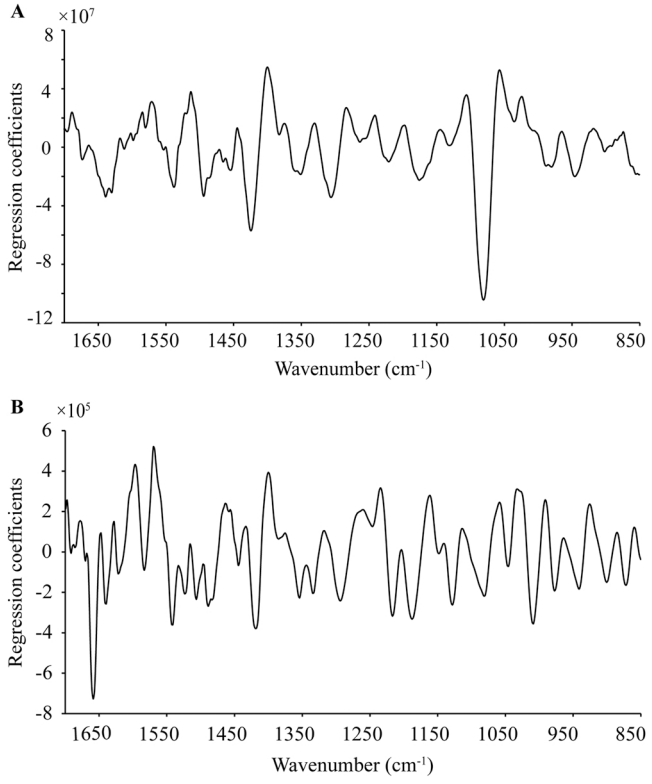

Fig. 7. Regression coefficients of PLSR models for prediction of AMW of poultry hydrolysates using (A) ATR and (B) The dry film approach.

hydrolysates can be predicted with a sensitivity of high industrial relevance using FTIR spectroscopy. Therefore, developing industrially viable portable instruments for at-line process analysis in the food, feed, and biotech industries is a natural next development stage.

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CRediT authorship contribution statement

Bijay Kafle: Writing – original draft, Data curation, Conceptualization, Visualization, Investigation, Methodology, Formal analysis. **Ulrike Böcker**: Data curation, Conceptualization, Writing – review & editing. **Sileshi Gizachew Wubshet**: Conceptualization, Writing – review & editing. **Katinka Dankel**: Data curation, Conceptualization, Writing – review & editing. **Ingrid Måge**: Data curation, Conceptualization, Writing – review & editing. **Marion O`Farrell**: Data curation, Conceptualization, Writing – review & editing. **Nils Kristian Afseth**: Writing – original draft, Writing – review & editing, Conceptualization, Investigation, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vibspec.2022.103490.

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