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From laboratory to industrial use: Understanding process variation during enzymatic protein hydrolysis with dry film fourier-transform infrared spectroscopy

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ABSTRACT

Industries are continuously looking for dedicated sensor solutions for evaluating the chemical composition of products that can provide valuable insights into process control, process optimization, and product quality. A rapid and robust analytical method, Fourier-Transform Infrared (FTIR) spectroscopy, holds significant potential in this regard as it provides detailed compositional values of food nutrients. The main aim of the present study was to use dry film FTIR spectroscopy as an analytical tool to characterize products from an industrial enzymatic protein hydrolysis process and link this to understand industrial process variations. For this purpose, 463 protein hydrolysate samples were obtained from an industrial enzymatic protein hydrolysis plant. In the same period, systematic variations in process parameters such as raw material composition, enzyme type, and water addition, were performed. All samples were analyzed using dry film FTIR spectroscopy. Two subsets containing 200 and 68 hydrolysate samples were chosen for measuring average molecular weight (AMW) and collagen content respectively, providing reference data for constructing calibration models based on partial least squares regression (PLSR). The percentage of low molecular weight constituents was derived from the molecular weight distribution of size exclusion chromatograms of protein hydrolysates and also used in the modeling. Calibration models for the prediction of AMW, low molecular weight constituents, and collagen content were obtained with a good fit and lower estimation errors. Principal component analysis (PCA) of protein hydrolysates' FTIR spectra effectively differentiated process variations (enzyme types and raw materials) without extensive reference analysis. One-factor analysis of variance (ANOVA) tests was used to observe the impact of process variation on product quality. FTIR proved to be a sensitive method for liquid protein analysis and process control with a significant potential in process optimization approaches. To the authors' knowledge, this is the first time dry film FTIR spectroscopy has been used to evaluate an industrial bioprocess with designed process variations.

1. Introduction

Recovering valuable ingredients from food processing by-products is a key industrial approach to reducing food waste that will ultimately contribute to future food sustainability. A major challenge lies in effectively handling and utilizing bioresources with varying origins and quality. Integration of smart sensors for monitoring, controlling, and optimizing industrial processing is one key step in solving this challenge. Fourier-Transform Infrared (FTIR) spectroscopy holds great promise in this respect.

FTIR spectroscopy is a rapid and non-destructive technique with significant potential for applications in the food, bioprocess, and pharmaceutical industries, and within medical diagnostics (Burmistrov et al., 2021; Fahelelbom et al., 2022; Gengler et al., 2016; Lohumi et al., 2015; Theakstone et al., 2021). FTIR spectroscopy provides signature spectra or spectral fingerprints of chemical compounds based on their molecular

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vibrations, enabling structural identification, and qualitative and quantitative analysis. Two common sampling approaches in FTIR spectroscopy are transmission and attenuated total reflectance (ATR). The latter approach is particularly flexible since it can be linked to ATR sampling in the tip of fiber-optic cables and thus enables flexible FTIR analysis of powders and liquid streams (McFearin et al., 2011). Another recent development that is expected to pave the way for future industrial IR applications is laser-based IR systems that can be used in low-cost portable and handheld infrared systems for quality measurements (López-Lorente et al., 2016; Schwaighofer et al., 2021). Consequently, these approaches are gaining interest in the food and bioprocess industries. However, industrial infrared analysis is still usually confined to high-throughput measurements in industrial laboratories using non-portable benchtop FTIR systems for quality analysis. Thus, regardless of the optical sampling approach used, there is a need to develop and showcase applications where FTIR spectroscopy can be used to monitor and understand actual industrial processes. These examples are to a large degree lacking today.

The increasing interest in valorizing industrial by-products emphasizes the ability to adapt and fine-tune process parameters to meet specific product characteristics. One of the emerging process industries with a potentially significant impact on food sustainability is enzymatic protein hydrolysis (EPH). EPH is a process where enzymes are used to refine proteins and peptides from co-streams of marine and animal processing industries (Aspevik et al., 2017). Thus, upcycling these proteins to markets in the feed-, food or nutraceutical areas will to a large part depend on efficient product characterization of the proteins and peptides. The degree of hydrolysis (i.e., the extent of protein hydrolysis) (Kristoffersen et al., 2022; Poulsen et al., 2016), protein contents, and peptide size distributions (usually characterized using size exclusion chromatography (SEC)) are established laboratory-based methods for the analysis of hydrolyzed proteins (Lapeñ et al., 2018; Liaset et al., 2000). Moreover, quality parameters such as average molecular weights (AMW) can be linked to product features such as solubility, bioactivity, and sensory attributes (Arteaga et al., 2020; Li et al., 2013; Sorokina et al., 2022; Wubshet et al., 2017). Additionally, collagen is found in collagen-rich co-streams such as skin, tendons, and cartilage (Kristoffersen et al., 2019, 2022) and can potentially add functional properties to products for pharmaceuticals, nutraceuticals, cosmeceuticals, and biomedical purposes (Avila Rodríguez et al., 2018; Gómez-Guillén et al., 2011; Rezvani Ghomi et al., 2021; Tang et al., 2022). Factors such as raw material composition (Lindberg et al., 2021; Vázquez et al., 2019), choice of enzymes, enzyme-substrate ratios (Lapeñ et al., 2018), and other parameters such as temperature and hydrolysis times are known to affect product qualities (Liaset et al., 2000; Steinsholm et al., 2020; Wubshet et al., 2018).

FTIR spectroscopy is a sensitive probe for protein analysis and secondary structures (Barth, 2007; Carbonaro & Nucara, 2010; Kong & Yu, 2007; Yang et al., 2015). The change in absorption peaks such as amide I (~1650 cm^{-1}) and amide II (~1550 cm^{-1}) and specific bands originating from NH_3^+ deformation (amino terminals) (~1516 cm^{-1}) and carboxylate (COO^-) groups (~1400 cm^{-1}) can also be used as indicators of hydrolyzed protein quality and progress of an enzymatic hydrolysis process (Böcker et al., 2017). In recent years, the quantitative analysis of FTIR spectra of hydrolyzed proteins has been thoroughly studied, and it has been shown that the FTIR fingerprints carry information both on size distributions (Kristoffersen et al., 2019, 2020; Wubshet et al., 2017) and collagen contents (Kristoffersen et al., 2023). Among different sampling approaches, the so-called dry film FTIR approach is particularly interesting in proteins since multiple protein-related infrared absorbances could be "buried" when water is present in the sample (Haris & Severcan, 1999). Kafle et al. recently showed that this approach is the method of choice for liquid protein solutions where the analysis is highly dependent on the amide I band (Kafle et al., 2023).

The FTIR fingerprint contains more information than quantitative compositional information on hydrolyzed proteins. In a recent study, Måge et al. showed that using a large database of FTIR fingerprints of laboratory-produced protein hydrolysates, information related to raw material composition and other processing factors (e.g., enzyme type) could be obtained. Moreover, the database could also be used to evaluate industrial protein hydrolysates (Måge et al., 2021). In the current study, the main aim was to use dry film FTIR spectroscopy as an analytical tool to characterize products from an industrial EPH process and link this to understand industrial process variations. In the large-scale industry experiment, frequent product sampling and dry film FTIR analysis from an industrial poultry hydrolysis process with controlled variations in raw material composition and process parameters were performed. In six weeks, 463 protein hydrolysate samples were obtained. In the same period, systematic variations in process parameters such as raw material composition, enzyme type, and water addition, were performed. FTIR spectra of all samples were obtained, and from a subset of samples, AMW, low molecular weight constituents, and collagen content were estimated. The FTIR fingerprints could subsequently be used both qualitatively and quantitatively to evaluate process variations. For this purpose, statistical analyses using multivariate regression models, principal component analysis (PCA), and analysis of variance (ANOVA) were performed. To the authors' knowledge, this is the first-time dry film FTIR spectroscopy has been used to evaluate an industrial bioprocess with designed process variations. Moreover, the current study provides significant evidence of the potential of taking FTIR analysis from a laboratory environment toward in-field industrial applications.

2. Materials and methods

2.1. Materials

Molecular weight standards (bovine serum albumin, 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, angiotensin II human, bradykinin fragment 1–7, [D-Ala2]-leucine enkephalin, Val-Tyr-Val, and L-tryptophan) ranging from 66,000 g/mol to 204 g/mol were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from 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. Industrial design

Enzymatic hydrolysis of the rest raw materials from chicken and turkey was done in the Bioco enzymatic protein hydrolysate production plant (Hærland, Norway). Samples were collected during November and December 2022. The hydrolysis process included grinding, water addition, preheating, enzyme addition, and enzyme deactivation. Typically, an uncontrolled mixture of chicken and turkey carcasses was used as the feed for hydrolysis, and the set points for enzyme concentration, water addition, and flow rate may vary depending on the product category and availability of raw materials. In this study, the aim was to test how different enzyme types, raw material composition, and raw material/water ratio affected product quality. For this purpose, four different enzymes were used, denoted A1, A2, B, C, and D, where A1 and A2 represent low and high concentrations of the same enzyme. Enzymes B, C, and D had constant concentration levels.

The experiment was planned so that one of the enzymes A1, B, C, or D was tested weekly, and adjustments of process parameters were done according to a pre-defined experimental schedule: on Mondays, the raw material was an uncontrolled mix of turkey and chicken, and the raw material/water ratio was set to a low level. On Tuesday mornings, the raw material/water ratio was shifted to a high level. During two production hours on Tuesdays, the raw material type was controlled so that

1 h was pure turkey and the following hour was pure chicken. On Wednesdays, the enzyme was changed to "A2", and the operators were free to adjust the process parameters as they saw fit for the remainder of the week. In addition to this setup, data was collected for two more weeks using enzyme A, without adhering to the weekly schedule for controlling the process parameters. The hydrolysate samples were collected from the inlet point of the holding tank after enzyme inactivation. Sampling occurred at 5-min intervals during the 2-h raw material variation on Tuesdays, and every 15 min the rest of the working day. During nighttime operations, sampling was conducted once every hour. The number of hydrolysate samples collected for each of the combinations of enzyme, raw material type, and raw material/water ratio is provided in Table 1. Other process parameters such as temperature and hydrolysis time were held constant throughout this production period. A total of 463 protein hydrolysate samples were collected. After each sampling, centrifugation for 4 min at 4100 rpm (MEGA STAR 600, VWR, Oslo, Norway) was carried out for phase separation. The phase-separated samples were frozen down to −20 °C and were kept frozen until further analysis.

2.3. Protein hydrolysate characterization

2.3.1. Dry film FTIR analysis

Before FTIR analysis, frozen protein hydrolysate samples were thawed using a hot water bath for 30 min at 50 °C and then cooled using a water bath of 25 °C for 15 min. The samples were centrifuged at 4400 rpm and 25 °C for 10 min (Heraeus Multifuge 4 KR Centrifuge, Thermo Fisher). The samples were left at room temperature to equilibrate for 15 min. The samples were then filtered in a 2 mL Eppendorf tube using Millex-HV PVDF 0.45 μm, 33 mm. The rest of the unfiltered samples were frozen at −20 °C immediately and stored for further 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. Three aliquots from each sample were deposited on the well plate to allow replicate measurements. The silicon plates were introduced to a High Throughput Screening eXTension (HTS-XT) unit coupled to an Invenio R spectrometer (Bruker Optics, Billerica, MA, USA), and the FTIR measurements of each well were performed in transmission mode. 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).

Table 1
Major in-process variations during industrial EPH and corresponding number of samples collected.

Week	Enzyme	Raw Material Type	Raw Material/Water Ratio	Number of Samples
44	B	Unknown mix	Low	15
44	B	Unknown mix	High	34
44	B	Turkey	High	12
44	B	Chicken	High	6
44	B	Unknown mix	Uncontrolled	3
44	A2	Unknown mix	Uncontrolled	44
45	C	Unknown mix	Low	18
45	C	Unknown mix	High	30
45	C	Turkey	High	10
45	C	Chicken	High	5
45	C	Unknown mix	Uncontrolled	7
45	A2	Unknown mix	Uncontrolled	29
47	A1	Unknown mix	Uncontrolled	23
47	A2	Unknown mix	Uncontrolled	17
48	D	Unknown mix	Low	6
48	D	Unknown mix	High	33
48	D	Turkey	High	13
48	D	Chicken	High	5
48	D	Unknown mix	Uncontrolled	6
48	A2	Unknown mix	Uncontrolled	12
49	A1	Unknown mix	Low	12
49	A1	Unknown mix	High	18
49	A1	Turkey	High	17
49	A1	Chicken	High	7
49	A1	Unknown mix	Uncontrolled	9
49	A2	Unknown mix	Uncontrolled	9
50	A2	Unknown mix	Low	8
50	A2	Unknown mix	High	22
50	A2	Turkey	High	9
50	A2	Chicken	High	4
50	A2	Unknown mix	Uncontrolled	20
			Total	463

2.3.2. Size exclusion chromatography

SEC was performed using a published protocol with some modifications (Wubshet et al., 2017). Molecular weight standard solutions (2 mg/mL) were prepared in ultrapure water. The mobile phase was a 30:70 (V/V) mixture of acetonitrile and ultrapure water with 0.05 % trifluoroacetic acid (TFA). The injection volume was 10 μL for both standards and samples, and separation was performed using a BioSep SEC-S2000 column, 300 mm long with an inner diameter of 7.8 mm (Phenomenex, Torrance, CA, USA). 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. The sample compartment was kept at a temperature of 40 °C. The detection of the eluents was done by monitoring absorbance at 214 nm. 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.

Molecular weight distributions were derived from the raw chromatograms using known calibration standards as mentioned in Wubshet et al. (2017). From these weight distributions, the average molecular weight (AMW) and the percentage area of six specific molecular weight ranges were calculated. The six weight fractions were defined based on visual inspection of the chromatograms following retention times: (I) > 2979 g/mol, (II) 2979-1800 g/mol, (III) 1800-900 g/mol, (IV) 900-680 g/mol, (V) 680-277 g/mol, and (VI) < 277 g/mol. All these calculations were carried out using the openly available MATLAB toolbox SEC2MWD (Måge et al.).

2.3.3. Collagen content

The collagen content was quantified by measurement of the amino acid Hyp using a Hydroxyproline assay and based on the assumption that the collagen contains 13.5 % Hyp per weight (Kristoffersen et al., 2023). A subset of 68 protein hydrolysate samples was selected for Hyp content analysis using the Hydroxyproline assay kit MAK008-1 KT (Sigma-Aldrich, St. Louis, Mo, USA). The Hyp assay was performed using the methodology described by Kristoffersen et al. (2022). The analysis was performed in triplicates. Freeze-dried samples were weighed and dissolved in 1.0 M HCl to prepare a 1 mg/mL solution. The samples were further diluted with 50 % v/v of concentrated HCl (~12 M) in Wheaton sample vials with PTFE solid caps. The samples were hydrolyzed for 3 h at 110 °C in a heating block from VWR (Radnor, PA, USA). After hydrolysis, 10 μL of the samples were transferred to a Pierce 96-well Polystyrene plate (Thermo Fisher Scientific, Waltham, MA, USA) together with different concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 μg/well of Hydroxyproline standards. The samples were heated at 60 °C for around 30–40 min before adding 100 μL of the reagent mix containing Chloramine T in oxidation buffer to all samples and standards wells. After mixing and incubating for 5 min, 100 μL of Dimethylamine borane (DMAB) reagent containing 50 % of Perchloric acid, was added to the wells and mixed. The plate was again incubated in a heated oven at 60 °C for 90 min. The absorbance was measured at 560 nm using a BioTek Synergy H1 spectrophotometer (BioTek Instruments, VT, USA).

The Hydroxyproline standard curves were used to determine the Hydroxyproline content as described in the Sigma-Aldrich protocol. Some of the samples with a standard deviation higher than 2 percent were excluded.

2.4. Data analysis

FTIR spectra were imported to the statistical software The Unscrambler® version 11 (CAMO Process AS, Oslo, Norway) for data processing. The spectra were pre-processed using the 2nd derivative Savitzky-Golay algorithm with a 2-degree polynomial and a window size of 13. The derivatized spectra were subsequently normalized by applying standard normal variate (SNV) corrections in the region from 1800 to 700 cm^{-1}, which were used for further analysis.

Two subsets of samples were selected employing a space-filling algorithm (i.e., the Kennard-stone algorithm) on two principal components from the FTIR spectra and process conditions. This is a commonly used method for selecting a representative subset from a larger dataset (Kennard & Stone, 1969). One of these subsets, consisting of 200 samples, was analyzed using size exclusion chromatography (SEC). The derived AMW and six weight range fractions were used as reference values to construct calibration models from the FTIR spectra. The second subset, consisting of 68 samples, was analyzed for collagen, and utilized for developing a model to predict collagen content. The calibration models were built using partial least squares regression (PLSR). The optimal numbers of PLSR factors were selected based on 10-fold systematic segmented cross-validation. The performances of the models were evaluated based on root mean square error of cross-validation (RMSECV), coefficients of determination (R^2), and examination of scores, loadings, and regression coefficients. Furthermore, a principal component analysis (PCA) was conducted on the FTIR spectra data, and score plots were color-coded to represent different enzyme types and concentrations, raw materials, average molecular weight ranges, and collagen contents. This visualization technique helped in understanding patterns and relationships among these variables in the dataset.

Effects of the controlled factors *enzyme*, *raw material type,* and *raw material/water ratio* were assessed by analysis of variance (ANOVA). The pure turkey and chicken were run only with the high raw material/water ratio, meaning that not all factor combinations of the three factors are available. Two separate ANOVAs were therefore performed: First, the effects of raw material type and enzyme were analyzed using the 88 samples with known raw material type (see Table 1). Then, the effects of raw material/water ratio and enzyme were analyzed using the 196 samples where the ratio was either low or high, and the raw material type was an uncontrolled mix (see Table 1). In both cases, the ANOVA included both main effects and their interactions. Type III sums-of-squares were used since the data is unbalanced (i.e., different number of samples for each factor combination). Results are given as ANOVA tables with overall effect sizes, accompanied by interaction plots for comparison of factor levels.

3. Results and discussion

3.1. FTIR profiling and reference analysis

FTIR analysis was performed on all hydrolysate samples using a dry film approach. A flowchart of the FTIR spectroscopic analysis, reference analysis, and subsequent data analysis is provided in Fig. 1. The obtained FTIR spectra were pre-processed, and a specific region of the FTIR spectra (1800-700 cm^{-1}) rich in protein-related information was chosen for subsequent data analysis. The original FTIR spectra (1800-700 cm^{-1}) are shown in Supplementary Fig. 1, and the preprocessed FTIR spectra are provided in Fig. 2. From the figure it can be seen that the amide I band (1700-1600 cm^{-1}), clearly exhibited significant variations (Glassford et al., 2013). The bands within the amide I region at 1690-1670 cm^{-1}, 1656 cm^{-1}, and 1631 cm^{-1} indicated differences in the protein secondary structure between the hydrolysates. Additional bands of interest were observed around 1548 cm^{-1} and 1242 cm^{-1}, corresponding to the amide II and III regions, respectively. Significant variations were also noticed in the terminal bands of peptides, specifically the amino-terminal ($NH3^+$ ~1516 cm^{-1}) and carboxyl-terminal (COO^- ~1593 cm^{-1} and ~1400 cm^{-1}) bands. Several studies suggest that a noteworthy reduction in amide bands and an increase in these terminal bands indicate hydrolysis of peptide bonds in a protein backbone (Böcker et al., 2017; Güler et al., 2011). Furthermore, bands in the lower region around 1124 cm^{-1}, 1083 cm^{-1}, and 1045 cm^{-1} indicated vibrations of functional groups such as C–NH_3^+ rocking vibrations and C–O stretching vibrations in the protein backbones of polypeptides. The variation in these bands indicates conformational changes in the protein backbones (Taga et al., 1997). The FTIR profiling of all the bands in the specified region is summarized in Supplementary Table 1 (Barth, 2007; Böcker, Wubshet, Lindberg, & Afseth, 2017, Jul 24).

3.2. Multivariate calibration for prediction of AMW and collagen content

3.2.1. AMW

AMW of a subset of 200 protein hydrolysate samples was obtained from SEC analysis. The AMW values ranged from 3249 g/mol to 13,318 g/mol. To develop a calibration model for AMW, a PLSR model was created using FTIR spectra and AMW data. As can be seen in Fig. 3A, this model has a good fit with R^2 values of 0.95 and RMSECV of 555 g/mol, using only three PLS components. Another significance is that the model can predict within a wide range of AMW. The regression coefficient of the calibration model reveals a strong dependence on the amide I region,

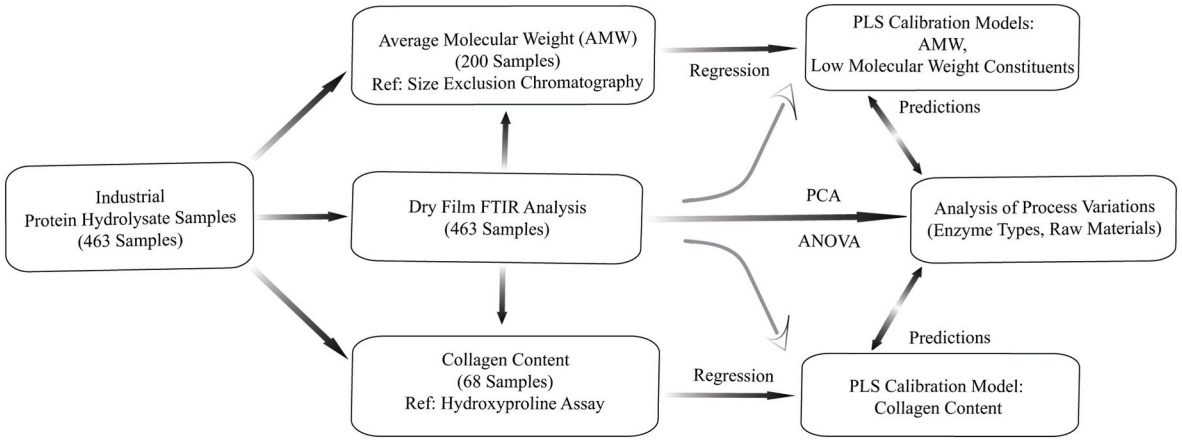

Fig. 1. An experimental journey: exploring industrial process using dry film FTIR.

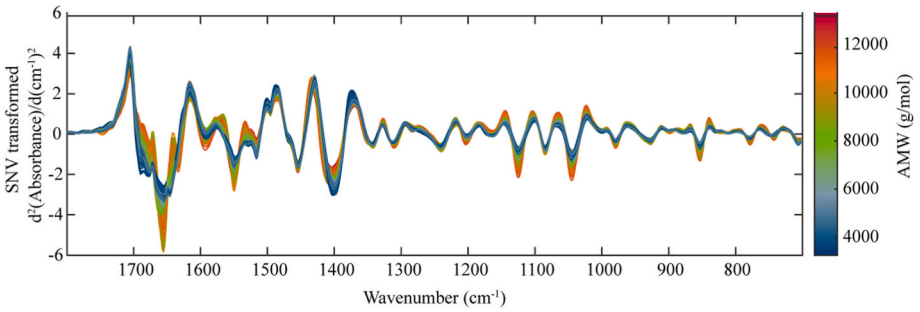

Fig. 2. Preprocessed FTIR spectra (1800-700 cm^{-1}) of hydrolysate samples. The spectra are color-coded according to their corresponding AMW values.

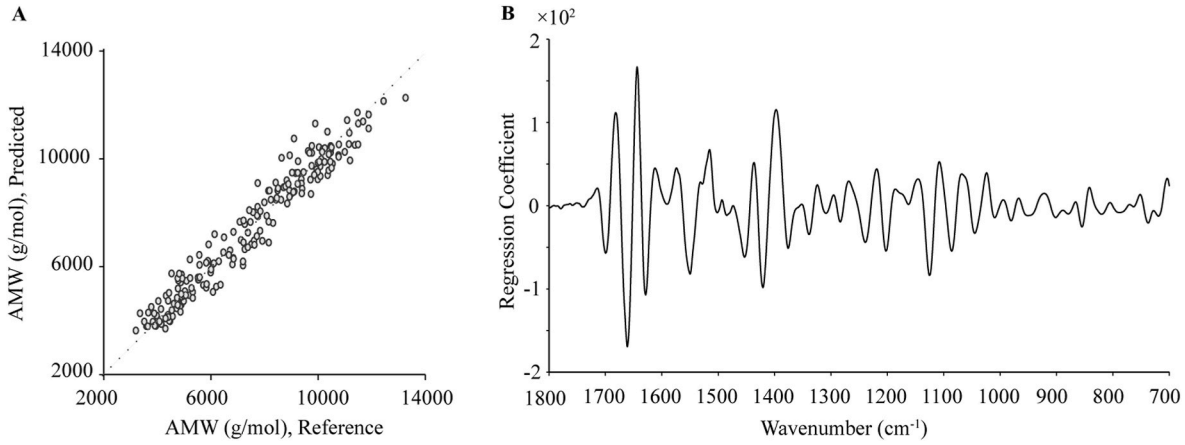

Fig. 3. (A) Predicted vs. reference plot of PLSR model for calibration of AMW in protein hydrolysates, with corresponding regression coefficients (B).

particularly bands around 1662 cm^{-1} and 1631 cm^{-1} as depicted in Fig. 3B. These bands play a crucial role in the model's predictive performance for AMW estimation. It can thus be stated that the huge variation observed in the amide I region in Fig. 2 corresponds to the differences in AMW of protein hydrolysate samples. This observation aligns with earlier experiments on a comparable substrate, where the calibration models heavily relied on amide I bands. However, in the current study, it is evident that these models are feasible even for a broader range of higher average molecular weight proteins (Kafle et al., 2023).

3.2.2. *Molecular weight range fractions*

Molecular weight fractions provide insights into the distribution of high and low-molecular-weight constituents. These different fractions might be correlated with their functional and bioactive properties (Sorokina et al., 2022) The regression models of the current study were constructed for each of the six weight ranges derived from the SEC chromatograms. The results of these models are presented in Supplementary Table 2, providing information on the models' performances and predictive capabilities.

A cross-correlation matrix including AMW, and all six SEC fractions (Supplementary Table 3) reveals that AMW has a strong positive correlation of 0.99 with fraction 1 (>2979 g/mol) and a significantly weaker correlation of 0.24 with fraction 6 (<277 g/mol). This is expected as AMW is a weight average molecular weight where contributions of molecular weight fractions are weighted by their corresponding molar mass. Therefore, the low molecular weight constituents eluted as fraction 6 have the lowest contribution in the calculated AMW and hence also the lowest correlation. Hence, in addition to AMW, a percentage of low molecular weight constituents were used in subsequent process analysis. Such low molecular weight constituents can contribute to small peptides, free amino acids, metabolites as well as modifiers used in enzyme preparations. The calibration model for the prediction of fraction 6 showed a high R^2 value and lower standard errors. The regression coefficient of fraction 1 shows similarities with the regression coefficient of AMW, and both are dominated by the band at 1660 cm^{-1} (Supplementary Fig. 2). Conversely, the regression coefficient of fraction 6 is dependent on the amide I band around 1660 cm^{-1} and a band around ~1400 cm^{-1} assigned to the carboxylate terminal (COO^-), as shown in Fig. 4B.

3.2.3. *Collagen content*

The collagen content of a subset of 68 protein hydrolysate samples was measured using the Hydroxyproline reference assay. The collagen content of the calibration ranged from 10.79% to 35.16%. The regression analysis produced an adequate model with an R^2 of 0.66 and an RMSECV of 3.0% (Fig. 5A) with only four PLSR components. The lower R^2 value could be attributed to the high estimation errors of the Hydroxyproline reference assay. However, the RMSECV is relatively low compared to the collagen variation range, indicating that collagen content can be predicted with reasonable accuracy using FTIR spectra. The regression coefficients for the PLSR models showed that the model heavily relies on the band at 1047 cm^{-1} (related to CO, CC, and CN stretching) which is associated with the protein backbone structure (Fig. 5B), whereas a recent study by Kristoffersen et al., showed that a comparable model relied on the bands in the amide I region (between 1700 and 1600 cm^{-1}). More specifically, the band at 1631 cm^{-1} is suggested to be associated with the presence of collagen (Júnior et al., 2015; Kristoffersen et al., 2023).

3.3. *Process analysis using the FTIR spectra*

In industrial processes, flow design and operating protocols are vital. While parameters such as temperature, flow rate, hydrolysis time,

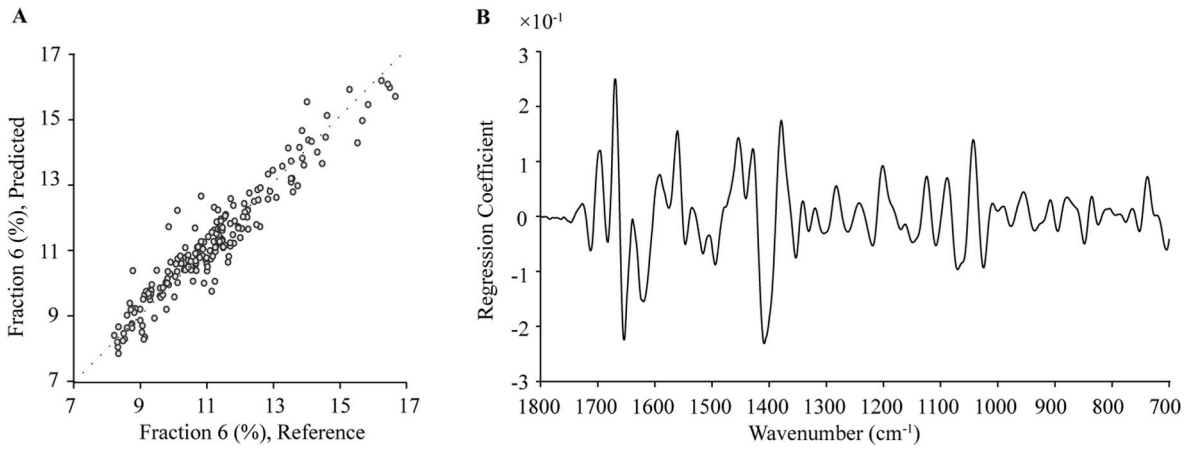

Fig. 4. (A) Predicted vs. reference plot of PLSR model for calibration of low molecular weight constituents (fraction 6) in protein hydrolysates, with corresponding regression coefficients (B).

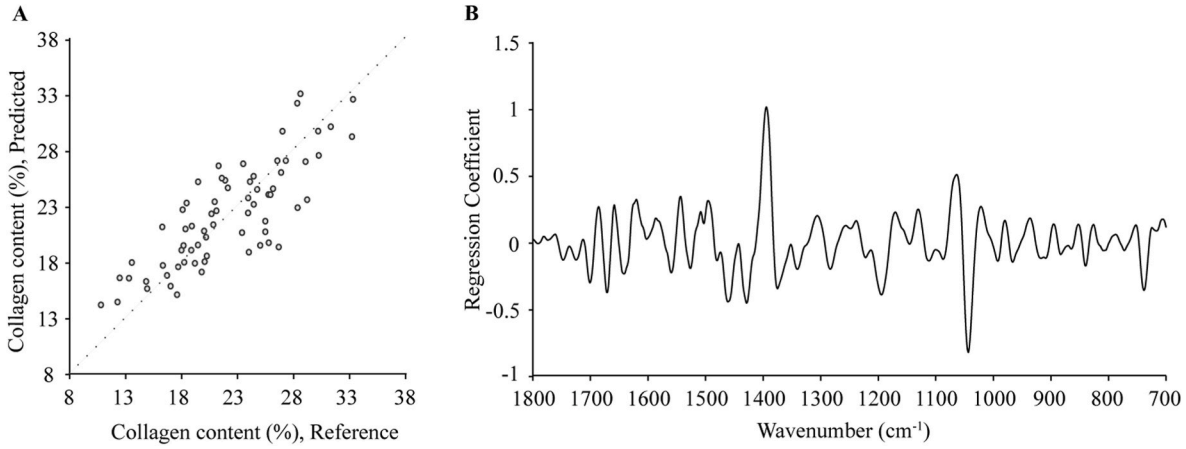

Fig. 5. (A) Predicted vs. reference plot of PLSR model for calibration of collagen content in protein hydrolysates, with corresponding regression coefficients (B).

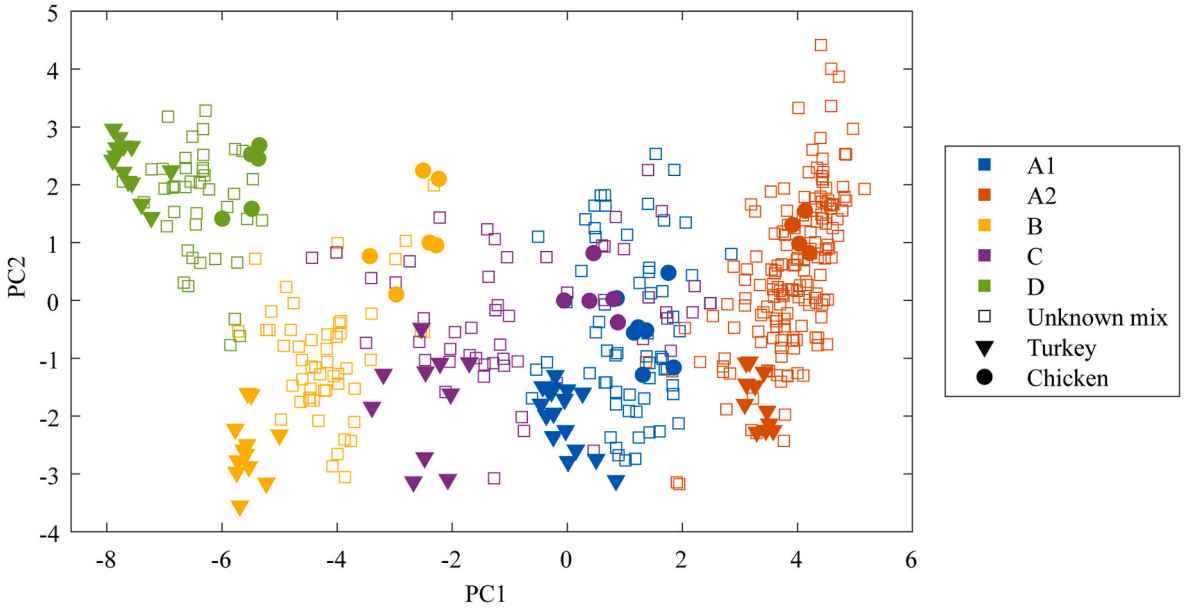

Fig. 6. PCA score plot based on all FTIR spectra. Color-coding is performed according to enzyme type, whereas different shapes correspond to raw material variations.

enzyme type, and concentration can be controlled, the raw materials are less controllable but also have a significant impact on the product characteristics. In previous work, we have shown that FTIR can be used to predict AMW in industrial poultry hydrolysate samples which were produced using a single enzyme type (Kafle et al., 2023). Now, we have developed these models and verified the FTIR can capture vital product quality parameters in such industrial samples produced with more variations. This allowed a novel opportunity to further use FTIR spectra for understanding the correlation between the process parameters and product quality in industrial settings. Thus, here we first explore if FTIR spectra of protein hydrolysates can capture essential process variations including changes in enzymes, raw material composition, and high or low raw material to water ratio in the process lines.

3.3.1. FTIR spectra and enzyme variations

A PCA was performed on the FTIR spectra. Fig. 6 shows the score plot of the PC1 versus PC2, with colors denoting enzyme types. The first and second principal components (PC1 and PC2) accounted for around 72 % and 11 % of the variance, respectively. Distinct groupings based on enzyme type are evident. Notably, varying concentrations of enzyme A (A1 and A2) are distinctly separated. Since the FTIR spectra of protein hydrolysates are highly dependent on the concentration and size of the proteins and peptides after hydrolysis, we can infer that different enzymes have different tendencies for protein breakdown and, therefore, might have a significant influence on the average molecular weight and collagen content of the protein hydrolysates (Kristoffersen et al., 2019). In a recent study by Måge et al. (2021), a comprehensive dataset of dry film FTIR spectra from protein hydrolysates measured over time was analyzed suggesting correlations of FTIR fingerprints to factors such as the composition of the raw material composition, enzyme activity, and hydrolysis time. The loading plots of PC1 indicate that the significant variation between hydrolysates derived from different enzyme types primarily originates from the amide I band. The loadings from PC2 show an additional band in ~1400 cm^{-1}, indicating this variation is due to the formation of more terminal bands (carboxylate) caused by protein degradation (Supplementary Fig. 3).

3.3.2. FTIR spectra and raw material variations

Variation in raw materials significantly impacts the AMW and collagen content of protein hydrolysates, causing inconsistencies in the final product (Lindberg et al., 2021). Intentional alterations using pure chicken, pure turkey, and a mixture of both highlight the extremes of raw material variation. The PCA of FTIR spectra (Fig. 6) shows the distinct classification of products from the different enzymes across the first principal component. In addition, moderate group separation can be seen in the PCA based on the type of raw material, this is "suppressed" by the dominating effect of enzymes in the PCs. However, for each enzyme, a clear separation between turkey and chicken can be seen. The distinct traits of turkey and chicken might be difficult to distinguish when analyzing protein hydrolysates obtained from a heterogeneous blend of turkey and chicken carcasses. Therefore, analyzing the FTIR spectra of protein hydrolysates separately obtained from turkey and chicken sources, and making comparisons would provide a clearer understanding of specific characteristics, qualities, and attributes that distinguish turkey from chicken. A significant association in turkey samples with higher average molecular weight and collagen content is also expected (Kristoffersen et al., 2019).

3.3.3. FTIR and level of raw material/water ratio

Typically, a normal water flow is incorporated into the process line regardless of the chosen raw material or enzyme to have good flowability and to avoid clogging. Occasionally, this water flow is adjusted to a higher or lower flow. Water addition might compromise enzyme effectiveness. It is therefore important to know if it also affects product quality parameters. From the FTIR results, differentiating between samples with high and low water flow through simple PCA visualization seems challenging (Supplementary Fig. 4). The observed limitations might be reasonable from factors like slight water flow variations and the dominant impact of enzymes and raw materials on average molecular weight and collagen content. However, a systematic controlled study can be performed to uncover the true effectiveness of water addition on product quality.

3.4. Process analysis using the FTIR calibrations

The PLSR calibration models previously described were used to predict AMW, the content of low molecular weight constituents (i.e., SEC fraction 6), and the collagen content of the remainder of the protein hydrolysate samples. The obtained values together were then used to assess whether the enzyme types, raw material types, and level of water flow have a significant impact on AMW, low molecular weight constituents (i.e., SEC fraction 6), and collagen. ANOVA tables investigating these relationships are presented in Table 2 and Table 3. The statistical analysis reveals a significant impact of enzyme types and raw materials on all three responses. Enzyme type contributes to 50–60 % of variance in AMW whereas 24 % influence on collagen. Correspondingly, raw materials type contributes 64 % of the variability in collagen content (main effect and interaction, Table 2). While water flows' impact on AMW and collagen content is statistically significant, its role in generating variation is relatively small.

The interaction plots (Fig. 7) illustrate the factor effects on AMW, low molecular weight constituents (i.e., fraction 6), and collagen content. Enzymes B and D produced hydrolysates with larger AMW compared to enzymes A and C. The enzyme concentration has a significant effect on cleaving the proteins which can be seen by looking at enzymes A1 and A2. Enzyme D, rich in exo-enzymes, generated a higher share of small peptides (included in fraction 6). This variation in different sizes of protein hydrolysates produced by different enzymes can be explained by the fact that enzymes (endo and exo) have different mechanisms for cleaving the peptide chain at different sites (Merz et al., 2016; Xu et al., 2020). Enzyme D also led to higher collagen content followed by enzyme A1 with lower concentration. There is also an interesting interaction effect between enzyme and raw material on collagen, suggesting that the differences between enzymes are larger for chicken than for turkey. There is a slight biological correlation between AMW and collagen content (a cross-correlation of 0.58). This can be observed in plots where pure turkey-derived hydrolysates contain peptides with higher AMW and also have higher collagen content compared to pure chicken-derived hydrolysates (Lindberg et al., 2021). However, studies have shown that the collagen content can be predicted independently of the AMW (Kristoffersen et al., 2023). The interaction plots also show that a large portion of low molecular weight constituents (fraction 6) were obtained from chicken. The water flow has minimal impact on the quality parameters shown by interaction plots and the effect sizes in Table 3. The ANOVA results further validate the results presented in Fig. 6.

Table 2
ANOVA table for factors Enzyme and Raw material type. Effect sizes are given as explained variances. All effects were statistically significant with $p < 0.01$.

Effect	Df	AMW	Fraction 6	Collagen
		Expl. Var (%)	Expl. Var (%)	Expl. Var (%)
Enzyme	4	53	38	24
Raw material Type	1	29	43	58
Enzyme x Raw Material Type	4	2	2	6
Error	78	1 $R^2 = 0.99$	3 $R^2 = 0.97$	7 $R^2 = 0.92$

*Df = Degree of freedom, Expl. Var = Explained variance.

Table 3
ANOVA table for factors Enzyme and Raw material/water ratio. Effect sizes are given as explained variances. All effects were statistically significant with $p < 0.01$, except for fraction 6 which had a p-value of 0.05 for the main effect of raw material ratio.

Effect	Df	AMW	Fraction 6	Collagen
		Expl. Var (%)	Expl. Var (%)	Expl. Var (%)
Enzyme	4	59	39	16
Raw Material/Water Ratio	1	1	1	8
Enzyme x Raw Material/ Water Ratio	4	4	2	9
Error	186	17 $R^2 = 0.83$	27 $R^2 = 0.72$	58 $R^2 = 0.40$

*Df = Degree of freedom, Expl. Var = Explained variance.

3.5. General discussion and future outlook

The current study shows, for the first time, that dry film FTIR spectroscopy is a feasible tool for the characterization of an industrial enzymatic protein hydrolysis process. Moreover, the study points in two potential directions for future industrial use of dry film FTIR spectroscopy: 1) as an at-line process development tool, or 2) as an at-line/in-line tool for process monitoring and process optimization. The variation in FTIR spectra (Fig. 2) reflects differences in protein secondary structures and molecular vibrations of peptides and amino acids generated during hydrolysis. Calibration models built using FTIR spectra provide precise predictions of product characteristics like protein composition, i.e., average molecular weight (AMW) and collagen content. The model developed for predicting AMW has a high coefficient of determination (R^2) and a correspondingly low prediction error. These results align with the previous studies performed at the laboratory scale by (Kristoffersen et al., 2019; Wubshet et al., 2017), and our earlier analysis of industrial protein hydrolysate samples in terms of coefficient of determination ($R^2 = 0.95$) for predicting AMW (Kafle et al., 2023). In addition, the current study shows that predictions of low molecular weight constituents (fraction 6) and subsequent process interpretations can be performed. While the calibrations for low molecular weight constituents were initially introduced by Måge et al. (2024), this study marks the first extensive application of this method to industrial samples. Similarly, the collagen content can be predicted with lower estimation errors. Kristoffersen et al. (2023), observed similar trends in predicting collagen content in protein hydrolysates, yet the regression coefficients differed from those from the present study. This variation could be attributed to several factors, including differences in collagen content, the degree of correlation between collagen and AMW, and variations in the degree of hydrolysis within studied samples. The FTIR-based PLSR approach proves to be a powerful and reliable method for characterizing protein hydrolysates of industrial relevance and understanding their composition and molecular weight distributions. Moreover, a straightforward PCA of the FTIR spectra gives insights into relationships between process parameters and the major spectral variations.

The value of FTIR analysis is clearly visualized when the FTIR spectra are linked to industrial design parameters. Even a simple PCA of FTIR spectra reveals clear differences in different enzymes and types of raw materials used and it consists of considerable information that can be utilized without any calibration models developed (Beattie & Esmonde-White, 2021). However, the real advantage of applying this FTIR analysis industrially is revealed when quantitative calibrations are also used. The enzyme type and concentration are shown to influence AMW and collagen content of protein hydrolysates. Different enzymes have distinct mechanisms for breaking down proteins, i.e. degree of hydrolysis, resulting in different AMW ranges (Liaset et al., 2000). Raw material variations due to varying protein compositions can be observed within each enzyme group, and pure turkey hydrolysates tend to have higher AMW and collagen content compared to chicken hydrolysates. The overall conclusion would be that the selection of either enzyme type or raw materials will help to fine-tune the enzymatic hydrolysis process to obtain protein hydrolysates with desired AMW and collagen content. Understanding these relationships aids in process optimization and product development. Such studies could of course be done in more

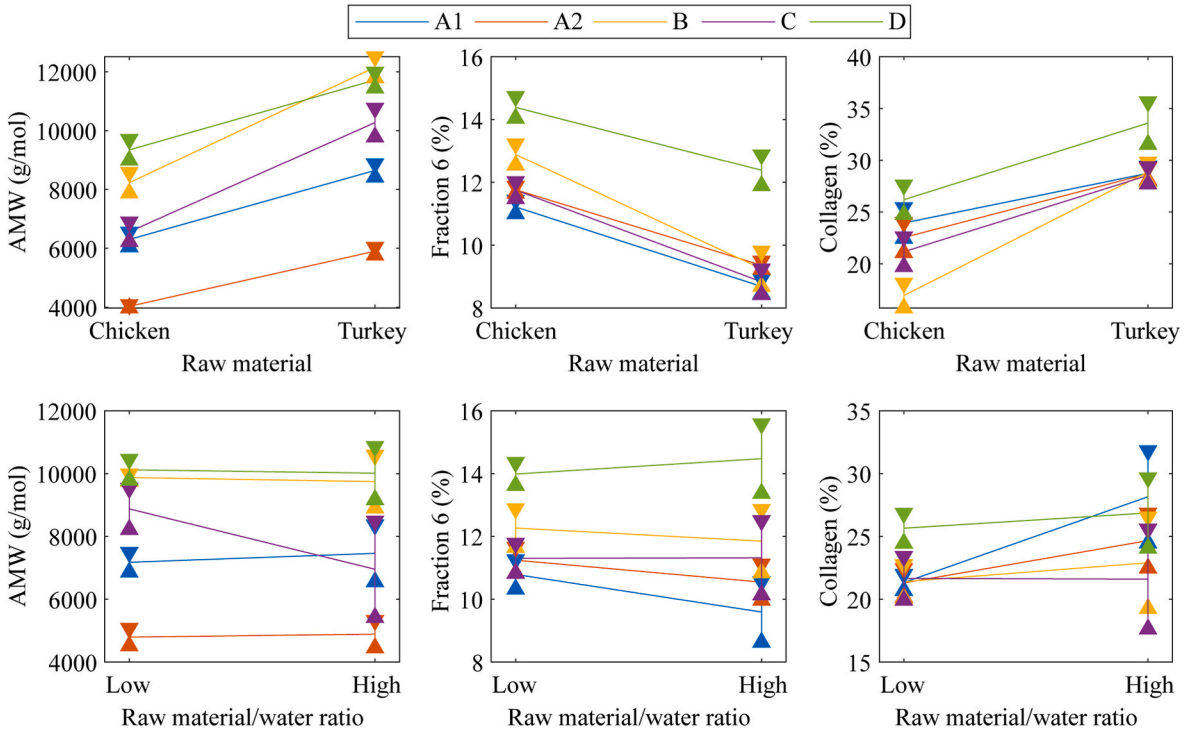

Fig. 7. Interaction plots showing the interactions between enzymes and raw material (top row) and water flow (bottom row). The whiskers represent ±1 standard deviation of the means.

controlled laboratory environments, but it is only by performing studies in relevant industrial environments that such relationships can be validated. There exist commercial FTIR systems such as Milkoscan based on liquid transmission used by dairy industries, the recently launched ProcessScan 2 system (Foss) and IRmadillo (Keit Spectrometers) for real-time process analysis, and hand-held and portable systems based on FTIR-ATR for in-line measurements tailored to describe industrial processes (Cebi et al., 2023; Coitinho et al., 2017; Koch et al., 2016). However, the application of dry film FTIR for exploring industrial processes has not been reported. This work therefore represents the first example of using dry film FTIR to understand quality variations of protein hydrolysates and process variations in an industrial environment.

The ability of FTIR to predict quality parameters and capture process variations clearly demonstrates its potential as a highly relevant tool in industrial settings. This study not only provides qualitative insights into industrial protein hydrolysate samples but also establishes strong correlations between FTIR spectral signatures and various process parameters and variations. This information could enable operators to effectively monitor and optimize the process according to their requirements by adjusting process parameters. This flexibility in modifying the characteristics of protein hydrolysates is crucial for targeting products for specific populations and optimizing the utility of raw materials while minimizing losses and costs. The results demonstrated that FTIR has the potential to be used industrially, however, the experiment described here was performed on a lab instrument. To realize the full potential of the dry film approach beyond the lab, the development of a low-cost, portable FTIR spectrometer with sufficient signal-to-noise and spectral resolution would be required. An advancement would also be to automatize the sampling and deposition of samples on silicon plates using robotics and use targeted approaches for accelerating the drying process. Such innovation would enhance convenience and accessibility, enabling widespread use of the FTIR technology in various industrial settings.

4. Conclusions

The present study highlights the effectiveness of dry film FTIR spectroscopy for characterizing liquid protein solutions obtained from an industrial enzymatic protein hydrolysis process. The FTIR-based calibration models demonstrated sufficiently low standard errors for the prediction of AMW, low molecular weight constituents, and collagen content. Subsequently, the FTIR data was successfully used to comprehend industrial process variations. The FTIR analysis allows for a straightforward detection of process variations, making it easier to monitor how process parameters like enzyme type and raw material choices affect product quality. This, in turn, aids in fine-tuning the enzymatic hydrolysis process enabling the operators to make real-time adjustments. To the authors' knowledge, this is the first time dry film FTIR spectroscopy has been used to evaluate an industrial bioprocess with designed process variations, but the versatility of FTIR technology extends beyond the protein hydrolysate industry with potential applications in different sectors. Looking ahead, the development of low-cost portable FTIR spectrometers based on dry film holds promise to enhance industrial accessibility and utility.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Bijay Kafle reports equipment, drugs, or supplies was provided by Bioco As. If there are other authors, they 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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110577.

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