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Multivariate correlation of infrared fingerprints and molecular weight distributions with bioactivity of poultry by-product protein hydrolysates

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ABSTRACT

Characterization of protein hydrolysates is a vital step in developing peptide-based bioactive ingredients. Multivariate correlation of chemical fingerprints and bioactivity of poultry by-product protein hydrolysates is explored as a potential analytical strategy for characterization and quality control. Chemical fingerprints of sixty hydrolysates were acquired using Fourier-transform infrared spectroscopy (FTIR) and size exclusion chromatography (SEC). Bioactivities (2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and angiotensin-1-converting enzyme (ACE-1) inhibition) were measured *in vitro*. Partial least squares regression models based on FTIR fingerprints or SEC chromatograms showed a better prediction performance for ACE-1 inhibition (coefficients of determination (R^2) = 0.91, root mean square error of prediction (RMSECV) = 2.8; R^2 = 0.85, RMSECV = 3.5, respectively) than for DPPH radical scavenging (R^2 = 0.74, RMSECV = 0.3; R^2 = 0.75, RMSECV = 0.3, respectively). Such models are promising tools for rapid prediction of bioactivities and as a quality control technology in production of bioactive peptides.

1. Introduction

Enzymatic protein hydrolysis (EPH) is a versatile processing technology where proteases are used to cleave proteins into peptides of various lengths under moderate conditions of pH and temperature. EPH does not deteriorate the nutritional quality of the proteins and allows to control relevant properties of the product, such as sensory attributes, functional property and bioactivity (Aspevik et al., 2017). A variety of bioactive properties has been reported for EPH-derived peptides from foods or food processing by-products, such as antihypertensive, antioxidant, antidiabetic, antithrombotic, antimicrobial, opioid, and satiety regulating activities (Lafarga & Hayes, 2014; Romero-Garay et al., 2022; Xing et al., 2019; Zamora-Sillero et al., 2018).

In this study, antihypertensive and antioxidant properties of poultry by-product protein hydrolysates were studied. One of the important therapeutic targets for dietary protein-derived bioactive peptides is angiotensin-1-converting enzyme (ACE-1). ACE-1 is a crucial component of the renin-angiotensin-aldosterone system, which is involved in the pathogenesis of cardiovascular disease (Putnam et al., 2012). There are several clinically approved prescription drugs for inhibition of ACE-1, however, they have adverse side effects (Israili & Hall, 1992; Lahogue et al., 2010; Sánchez-Borges & González-Aveledo, 2010). Therefore, the search for alternative sources of ACE-1 inhibitors in the form of nutraceuticals has become a major area of research in recent years. Several studies have shown the potential of protein hydrolysates as promising sources of ACE-1 inhibitors (Lee & Hur, 2017; Mas-Capdevila et al., 2019; Onuh et al., 2013). Another example of bioactivity attributed to food-derived peptides is antioxidant activity (Di Bernardini et al., 2011; Lorenzo et al., 2018; Samaranayaka & Li-Chan, 2011). Oxidative stress causes damage of essential biomolecules (i.e., proteins, lipids, DNA) and this damage can initiate for example inflammation, cardiovascular disease, diabetes, neurodegeneration, or tumorigenesis (Lorenzo et al., 2018; Pisoschi et al., 2021). Studies show that bioactive peptides from hydrolysates can neutralize radicals by hydrogen transfer, electron transfer (Romero-Garay et al., 2022) and metal chelating (Chakka et al., 2015).

Despite several evidence of *in vivo* and *in vitro* ACE-1 inhibitory and antioxidant effects of protein hydrolysates, development of nutraceuticals for such applications remains a challenging task. This is partly due to the chemical complexity of crude hydrolysates and the resulting

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challenges associated with characterization and discovery of the bioactive peptides. Identification and characterization of the bioactive peptides in a given hydrolysate is a vital step in process- and product development and documentation (Chalamaiah et al., 2019; Li-Chan, 2015). Processing parameters, such as choice of enzyme and hydrolysis time, can affect the hydrolysate's chemical composition and hence it's bioactivity. *In vitro* bioactivity screening of crude hydrolysates in arbitrary doses can lead to false positives due to, for example, bone mineral content. One of the solutions for screening for potent hydrolysates is to use analytical strategies to correlate chemical fingerprints with biological effects (i.e., bioactivity). Correlations of chemical fingerprints with bioactivity can also serve as a platform to ensure reproducible production of bioactive peptides. Process control is particularly important in EPH of by-products (e.g., poultry processing by-products) where raw materials are highly varying in composition, which can result in undesirable quality changes in the final hydrolysates (Wubshet et al., 2018).

Methods for chemical characterization of hydrolysates include size exclusion chromatography (SEC), degree of hydrolysis (DH%) and Fourier-transform infrared spectroscopy (FTIR). Molecular weight distribution (MWD), derived from SEC, has been used for comparing hydrolysates produced under different processing conditions and to monitor the hydrolysis process (Damgaard et al., 2015; Lindberg et al., 2021; Silvestre, 1997). Similarly, FTIR has been demonstrated as an effective tool for monitoring changes in the secondary and primary structure induced by enzymatic cleavage of single proteins (Güler et al., 2011; Ruckebusch et al., 1999) and complex biological tissues (Böcker et al., 2017). Wubshet et al. (2017) demonstrated that weight average molecular weight (M_w) of protein hydrolysates is correlated with FTIR fingerprint, so multivariate statistical models based on FTIR fingerprint can be used to predict M_w. In contrast to SEC, FTIR is a rapid technique with great potential for monitoring the hydrolysis process in an industrial setup (Wubshet et al., 2017) and as quality assessment tool for protein hydrolysates (Måge et al., 2021).

Classical bioactivity screening is a laborious and time-consuming process. Therefore, predictive methods are needed to facilitate screening of complex protein hydrolysates. *In silico*-based integrated '-omics' approaches are alternatives, which allow high throughput screening and enable narrowing down potential bioactive peptides for subsequent *in vitro* screening (Agyei et al., 2016). However, *in silico*-based techniques require knowledge of protease specificity and the raw material protein composition. This limits its use, as industrial enzyme preparations are often a mixture of both predominant enzymes and minor enzymes which give side-activities (FitzGerald et al., 2020) and the protein composition of by-products is varying. The literature shows that bioactivity of peptides is closely related to their chemical structure. Since FTIR fingerprints and SEC chromatograms of protein hydrolysates have been successfully used to predict the chemistry of EPH, these analytical techniques most likely also contain relevant information related to bioactivity. Therefore, the main aim of this study was to develop and evaluate FTIR- and SEC-based models for prediction of antioxidant or hypertensive potential of protein hydrolysates. For this purpose, a library of 60 hydrolysates from mechanically deboned chicken residues (MDCR) was produced using ten industrial protease preparations and six hydrolysis times. Partial least squares regression (PLSR) models based on FTIR- and SEC- data of the crude hydrolysates were developed, and performance of the models in predicting the bioactivates (i.e., ACE-1 inhibition and antioxidant activity) was evaluated. This study represents a first example of direct bioactivity prediction from chemical fingerprints of protein hydrolysates.

2. Materials and methods

2.1. Raw material and chemicals

MDCR were provided by a Norwegian slaughterhouse (Nortura, Hærland, Norway). Protease from *Bacillus licheniformis* (Alcalase, 2.4 U/g) was from Sigma-Aldrich (St. Louis, MO, USA); Endocut 01, Endocut 02 and Endocut 03 from Tailorzyme ApS (Søborg, Denmark); FoodPro PNL and FoodPro 30L from DuPont Danisco (Copenhagen, Denmark); MaxiPro NPU from DSM Food Specialties (Delft, the Netherlands); Promod 950 L and Promod 144P from Biocatalyst Ltd. (Cardiff, UK); and Veron L10 was from AB Enzymes GmbH (Darmstadt, Germany). Analytical grade acetonitrile, trifluoracetic acid (TFA) and monosodium phosphate used for SEC were purchased from Sigma-Aldrich. Sulfanilamide used for Dumas analysis; 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, methanol used for DPPH assay; and ACE from rabbit lung (≥2 U/mg, EC 3.4.15.1), N-Hippuryl-His-Leu hydrate (HHL), hippuric acid (HA), captopril, boric acid, hydrochloric acid, sodium hydroxide, sodium chloride used for ACE-1 assay were analytical grade and purchased from Sigma-Aldrich. Water was prepared by deionization and membrane filtration (0.22 μm) using a Millipore Milli-Q purification system (Merk Millipore, USA).

2.2. Production of hydrolysates

The hydrolysis of MDCR was performed according to the method described by Wubshet et al. (2017). First, MDCR were homogenised using a food processor, vacuum packed into plastic bags and stored at −20 °C until further use. The hydrolysis was performed in a Reactor-Ready™ jacketed reaction vessel (Radleys, Saffron Walden, Essex, United Kingdom) connected to a JULABO circulator pump (Julabo GmbH Seelbach, Germany). Water in the vessel jacket was kept at a selected temperature (±1°C) for individual enzymes (Table 1). The homogenized MDCR (500 g) were suspended in 1 L of purified water and mixed at 300 rpm until the suspension reached the selected temperature for the hydrolysis. At that point, a selected enzyme was added, enzyme loading percent (relative to 500 g of MDCR) is specified in Table 1. The hydrolysis was performed for 120 min, and samples (40 mL) were collected at 10, 30, 45, 60, 90 and 120 min. After the sample collection, the enzyme was thermally inactivated by rapid increase of temperature in a microwave oven (ACP, IA, USA) for several seconds followed by heating in a water bath at 90 °C for 15 min. After the enzyme inactivation, samples were centrifuged for 15 min at 4400 rpm and 25 °C, to separate three phases: fat, water, and sediment. The separated water phase was filtered with a Seitz® T 2600 depth filter sheet (Pall Corporation, Fribourg, Switzerland) and lyophilized using a Gamma 1–16

Table 1
An overview of enzymes and hydrolysis conditions. Individual temperatures and enzyme loadings were selected based on the optimal conditions specified by the manufacturers or previous study. The enzymes in powder form were dissolved in purified water.

Enzyme	Code	Enzyme loading (w/w) %	Temperature (°C)	Production organism or biological source
Alcalase	Alc	1	50	*Bacillus licheniformis*
Endocut 01	E01	1	55	*Bacillus subtilis*
Endocut 02	E02	1	60	*Bacillus licheniformis*
Endocut 03	E03	1	62.5	*Bacillus clausii*
FoodPro 30L	FP30	5	55	*Bacillus subtilis*
FoodPro PNL	PNL	5	60	*Bacillus amyloliquefaciens*
MaxiPro NPU	NPU	3	45	*Bacillus amyloliquefaciens*
Promod 144P	P144	2	50	*Carica papaya*
Promod 950L	P950	1	55	microbial
Veron L10	V10	3	50	*Carica papaya*

LSCplus freeze dryer (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). A similar procedure was performed for each of the 10 enzymes (listed in Table 1), resulting in 60 different samples.

2.3. Moisture and protein content

Moisture content of the freeze-dried hydrolysates was determined by overnight weight loss after oven drying at 105 °C. Freeze-dried hydrolysates (ca. 5 mg) were packed into tin foils and combustion was performed using a Vario EL cube (Elementar, Langenselbold, Germany) according to Rieder et al. (2021). The instrument was operated in CNS mode and sulfanilamide was used as a standard for correction. Protein content was calculated from total nitrogen using the protein conversion factor 6.25.

2.4. Size exclusion chromatography

SEC was performed as described by Wubshet et al. (2017). The hydrolysates were prepared in ultrapure water at a concentration of 10 mg/mL and filtered through a Millex-HV PVDF syringe filter with pore size 0.45 mm (Merck Millipore, Billerica, MA). Peptides were separated on a BioSep-SEC-s2000 column (Phenomenex, Værløse, Denmark, 300 × 7.8 mm) coupled with a Dionex UltiMate 3000 HPLC system (Thermo Scientific, Waltham, Massachusetts, U.S.). An injection volume of 10 μL was used for all analyses. The mobile phase was acetonitrile (30% v/v) in ultrapure water (70% v/v) containing 0.05% TFA. The flow rate was 0.9 mL/min, and the UV absorption was monitored at 214 nm. Chromatographic runs were controlled using Chromeleon 6.80 software. MWD and M_w of the hydrolysates were calculated using PSS winGPC UniChrom V 8.00 software (Polymer Standards Service, Mainz, Germany). For calculation of MWD and M_w, similar peptide standards were used as described in Wubshet et al. (2017).

2.5. Dry-film FTIR analysis

Dry-film FTIR analysis was performed according to Wubshet et al. (2017). The freeze-dried hydrolysates were dissolved in ultrapure water to 50 mg/mL, followed by filtration. Each of the filtered samples (5 μL) was deposited on to a 96-slot Si-microtiter plate (Bruker Optik GmbH, Germany) and dried at room temperature to form dry films. Each sample was made in five replicates and measured by a High Throughput Screening eXTension unit coupled to a Tensor 27 spectrometer (both Bruker Optik GmbH, Germany). 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. For each spectrum, 40 interferograms were collected and averaged. Data acquisition was controlled using Opus v 6.5 software (Bruker Optik).

2.6. Radical scavenging (antioxidant) activity

The radical scavenging activity of the hydrolysates were determined using a spectrophotometric method described by López et al. (2007) with some modifications. The freeze-dried hydrolysates were dissolved in 50% methanol to obtain concentration of 0.94 mg/mL. DPPH and quercetin were also dissolved in 50% methanol to obtain 0.2 mM and 80 μM, respectively. An aliquot of a hydrolysate (100 μL) was mixed with 100 μL of 0.2 mM DPPH (sample measurement) or with 100 μL 50% methanol (sample blank measurement). The negative control was 200 μL of 0.1 mM DPPH in 50% methanol. Absorbance (Abs) was measured at 515 nm after incubation at 30 °C for 30 min in a microplate reader Synergy H1 (BioTek, Winooski, VT, USA). The radical scavenging capacity was calculated as given in Eq. (1):

$$\text{Radical scavenging activity}(\%) = \left[\left(Abs_{negative\ control} - \left(Abs_{sample} - Abs_{sample\ blank}\right)\right)/Abs_{negative\ control}\right] \times 100 \quad (1)$$

The antioxidant activity of the hydrolysates was expressed as quercetin equivalents (Q Eq). For calculation of Q Eq, a calibration curve was created based on measured activities of 0.62, 1.25, 2.5, 5, 10, 20, 40, 80 μM quercetin (start concentrations). Activities for all hydrolysates were measured in triplicates and reported as averages with standard deviation. Due to poor solubility of samples in the assay conditions, antioxidant activity of hydrolysates from Alcalase and Endocut 03 could not be acquired.

2.7. ACE-1 inhibition activity

ACE-1 inhibitory activity of the hydrolysates was determined according to a protocol by Lahogue et al. (2010) with some modifications. The hydrolysates, ACE-1, HA, and captopril were dissolved in 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl. The dissolved hydrolysates (1.75 mg/mL) were filtered through a Millex-HV PVDF syringe filter with pore size of 0.45 mm. The 50 mU/mL ACE-1 solution (50 μL) was mixed with 25 μL of sample, borate buffer (negative control) or 3.5 μM captopril (positive control) and incubated in a 48 well plate at 37 °C for 10 min. After incubation, 100 μL of 2.5 mM substrate HHL was added, and the samples were further incubated at 37 °C for 60 min. The reaction was stopped by addition of 1 M HCl (210 μL). The product HA and the substrate HHL were separated on a Luna C18 column (Phenomenex, 4.6 × 150 mm, 3 μm) coupled with a Dionex UltiMate 3000 HPLC system at room temperature. The mobile phase consisted of 0.05% TFA in ultrapure water (solvent A) and 0.05% TFA in acetonitrile (solvent B). A solvent gradient was applied. The mobile phase composition was 15% B for 15 min, increased to 55% B (from 15 to 21 min), increased to 100% B (21 to 35 min) and returning to 15% B (35 to 45 min). The injection volume was 50 μL. The flow rate was 0.5 mL/min and the UV absorption was measured at 228 nm. The inhibition percentage was calculated as given in Eq. (2):

$$\text{ACE-1 inhibition}\ (\%) = [1 - (\text{Area of HA for sample})/\text{Area of HA for negative control}] \times 100 \quad (2)$$

Activities for all hydrolysates were measured in triplicates and reported as averages with standard deviation.

2.8. Statistics

Correlation between univariate variables (% moisture, % protein, M_w, antioxidant activity (Q Eq), and ACE-1 inhibition) were studied by calculating Pearson's correlation coefficients (r) and p-values. Pearson's covariance matrix was calculated in MATLAB (R2018a, The MathWorks, Inc., Natick, MA, USA). Prior to multivariate analysis, SEC and FTIR raw data were pre-processed. SEC chromatograms were normalized against total area and the chromatographic region 5–15 min was chosen for analysis. The five technical replicates of the FTIR spectra were averaged to create a single spectrum per hydrolysate. The averaged FTIR spectra were transformed into second derivative spectra using the Savitzy-Golay algorithm with a polynomial degree of two and a window size of 13

points. Afterwards, the second derivative spectra were normalized using extended multiplicative signal correction and the spectral region 1800–700 cm^{-1} was chosen for analysis. Principal component analysis (PCA) of the FTIR and SEC data was performed to study the overall variation in the SEC and FTIR datasets. Validation of PCA was performed using leave-one-out cross-validation (LOOCV). Correlation between PC scores and bioactivities were studied by fitting linear regression model. PLSR models based on SEC and FTIR were developed for prediction of % ACE-1 inhibition and DPPH radical scavenging (μM Q Eq) of the protein hydrolysates. Cross validation of the PLSR models was performed using both LOOCV and leave-one-group-out cross-validation (LOGOCV) (Baumann, 2003; Montesinos López et al., 2022). In LOGOCV, a group consisted of six samples produced by the same enzyme was held out at a time. Coefficients of determination (R^2), root mean square error of prediction (RMSECV) and number of factors were used for model evaluation. Multivariate analysis was performed using Unscrambler 11 software (CAMO ASA, Oslo, Norway).

3. Results and discussion

A total of sixty hydrolysates were produced from MDCR using different processing conditions. Subsequently, ACE-1 inhibitory and DPPH radical scavenging activity of the hydrolysates were measured. PLSR models based on FTIR fingerprint and SEC were developed for prediction of ACE-1 inhibitory and DPPH radical scavenging activities of the hydrolysates.

3.1. Effect of processing parameters on antioxidant activity and ACE-1 inhibition

The hydrolysates showed varied DPPH radical scavenging and ACE-1 inhibitory properties (Fig. 1). The observed DPPH radical scavenging activity ranged from 0.08 μM Q Eq (P144 120 min) to 2.8 μM Q Eq (NPU 10 min) measured at hydrolysates' concentration of 0.47 mg/mL. The results show that both enzyme choice and time of hydrolysis influence the DPPH radical scavenging capacity of the hydrolysates (Fig. 1 A). Our observation agrees with previous studies showing that enzyme and hydrolysis time influence antioxidant activity of protein hydrolysates from blue mussel (Wang et al., 2013), barley hordein (Bamdad et al., 2011) and silver carp (Malaypally et al., 2015). The hydrolysates made by FP30 and NPU have overall higher activity than the other hydrolysates, when comparing in accordance with hydrolysis time. The variation in antioxidant activity depends on enzyme since various proteases have different specificities and can result in peptides with different sequences.

Another specific trend was a decrease in radical scavenging capacity of the hydrolysates with increasing hydrolysis time (Fig. 1 A). Hydrolysis time is inversely correlated to M_w of hydrolysates. Our results indicated that samples with lower hydrolysis time (higher M_w) have higher DPPH radical scavenging activity (Fig. 1 A). However, an inconsistent relationship between MW and antioxidant activity has previously been reported. For example, Jamdar et al. (2012) showed that antioxidant activity of poultry viscera protein hydrolysate did not depend on MW of the peptides. In contrast, Li et al. (2013) demonstrated that DPPH radical scavenging activity of fish collagen hydrolysates is negatively correlated with the average MW of the peptides. The raw material used in the current study (i.e., chicken deboning residue) is also rich in collagen (Kristoffersen et al., 2022).

The ACE-1 inhibition of the hydrolysates varied from 35% (P144 90 min) to 74% (PNL 45 min) measured at hydrolysates' concentration of 0.25 mg/mL. The results show that the choice of enzyme has a stronger effect on the inhibitory potential than the hydrolysis time (Fig. 1 B). No consistent trend was observed for changes in activity in the course of the hydrolysis time. The hydrolysates can be roughly divided into three groups (Fig. 1 B). One group contains the hydrolysates with no strong dependency of the hydrolysis time on the activity (FP30, PNL, P950 and E01). Another group includes the hydrolysates that showed decrease in activity with the increasing hydrolysis time (Alc, NPU, P144 and V10). The third group comprises the hydrolysates that demonstrated some increase of ACE-1 inhibition with hydrolysis time (E02 and E03). While it is important to have a relatively short peptide (range 2–12 amino acids) for having an adequate ACE-1 inhibitory activity (Hernández-Ledesma et al., 2011), the peptide chain length alone does not result in increased ACE-1 inhibition. This is reflected in the lack of consistent trend between hydrolysis time and ACE-1 inhibitory activity. A specific inhibitor of a therapeutic target such as ACE-1 requires, in addition to being a small molecule, a specific pharmacophore with a strong binding affinity (i.e., small dissociation constant, Kd). Previous *in vitro* and *in silico* studies have indicated that ACE-1 inhibitory potential of peptides is connected to their specific amino acid sequence (Iwaniak et al., 2014; Wu et al., 2006; Zhang et al., 2020). ACE-1 inhibitory peptides have been reported to have competitive, noncompetitive, or mixed modes of action (Ahn et al., 2012; Lee & Hur, 2017; Udenigwe & Aluko, 2012). Our observation agrees with the previous studies showing that several factors influence the ACE-1 inhibitory properties of the hydrolysates such as specific amino acid sequences and peptide length.

Covariance analysis was performed (Fig. 2) to study the correlation of observed bioactivities with gross composition parameters of the hydrolysates. No strong correlation between the bioactivities (i.e., ACE-1 inhibition and antioxidant activity) and protein content, moisture content or M_w of the hydrolysates was found. However, there was a moderate correlation between M_w and antioxidant activity ($r = 0.41$ and p-value $= 0.0041$). Overall, the absence of strong correlation with single variables indicates that a multivariate correlation based on a detailed fingerprinting of constituting peptides is required to establish a relationship with bioactivities.

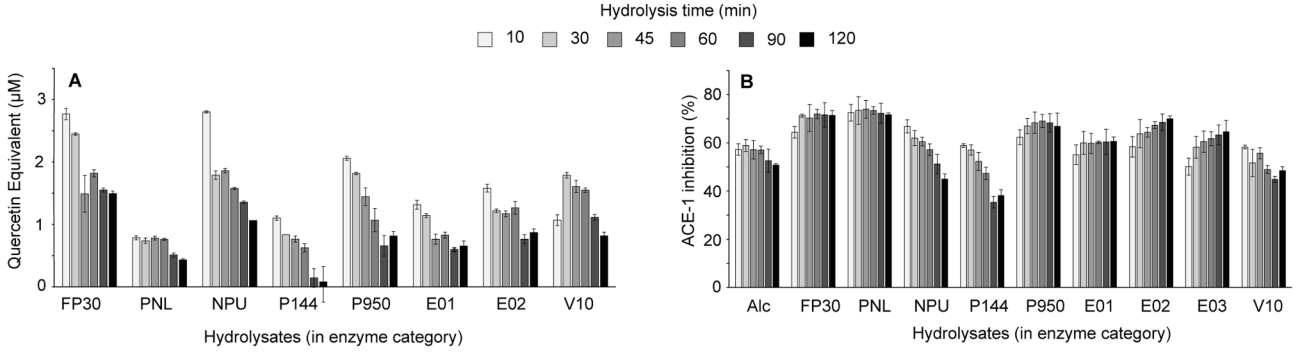

Fig. 1. Bioactive properties of the hydrolysates: (A) DPPH radical scavenging activity (Q Eq in μM). The hydrolysates were tested at a concentration of 0.47 mg/mL. (B) ACE-1 inhibition (%). The hydrolysates were tested at a concentration of 0.25 mg/mL. Standard deviations are shown.

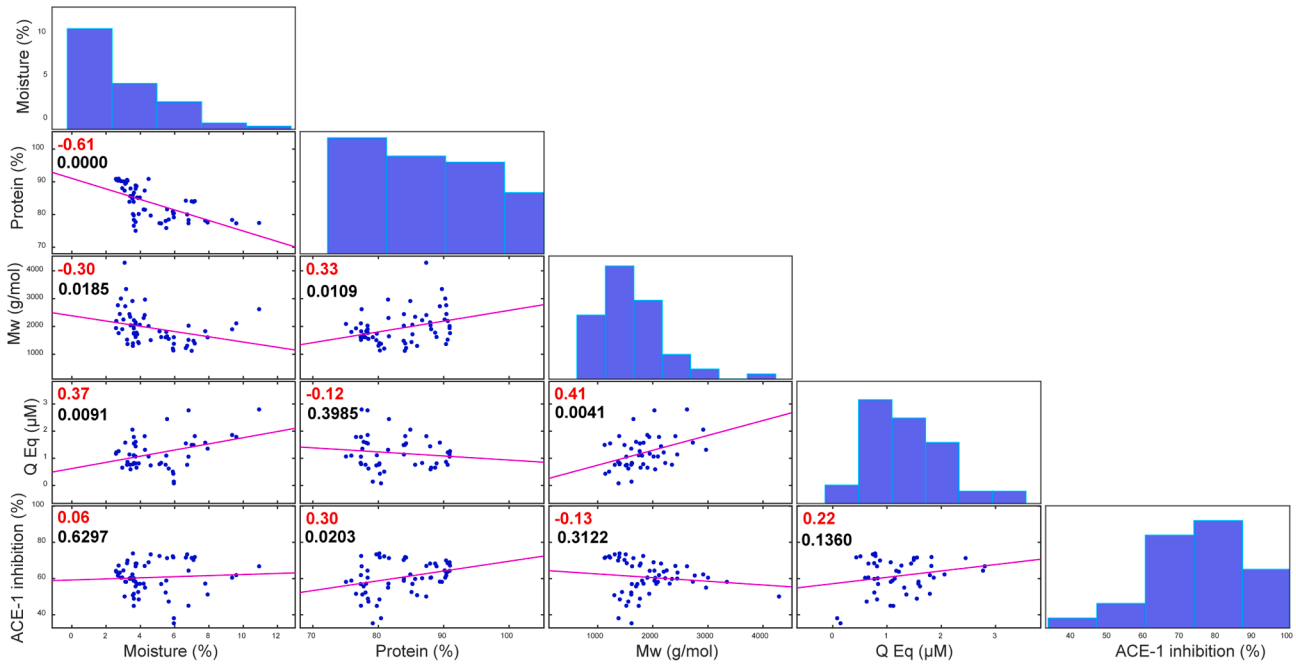

Fig. 2. Pearson's correlation coefficients between all pairs of variables (% moisture, % protein, M_w, antioxidant activity (Q Eq), and ACE-1 inhibition) measured for chicken protein hydrolysates. Histogram showing distribution of the data in each of the variables are presented in the diagonal sub-plot. Inserted to the top-left corner of the off-diagonal subplots are r (red font) and p-values (black font) for each pair of coefficients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

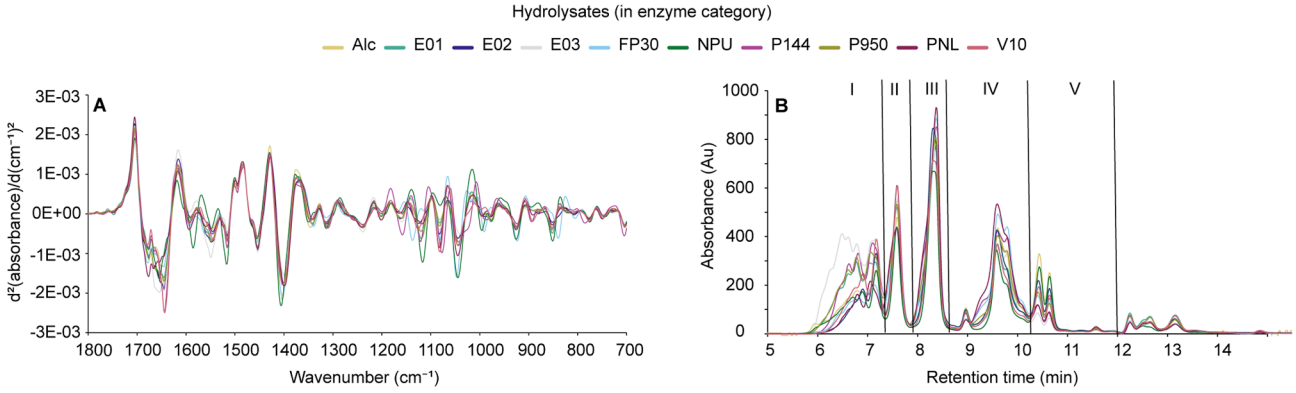

Fig. 3. Chemical characteristics of the hydrolysates produced by ten enzymes after 30 min. (A) Second derivative of FTIR spectra (1800–700 cm^{-1}). (B) SEC chromatograms of the samples measured at 214 nm (from 5 to 15 min).

3.2. Effect of processing parameters on the chemical fingerprints of hydrolysates

3.2.1. FTIR fingerprints of the hydrolysates

Fig. 3 A shows ten representative FTIR spectra of the hydrolysates prepared with the 10 different proteases (sampled after 30 min of hydrolysis). The spectra of different samples show clear differences in certain areas (e.g., around 1676, 1643, 1585, 1549, 1516, 1454, 1402, 1238, 1118, 1080, 1041 cm^{-1}). These spectral regions have been previously attributed to features of secondary protein structure, peptide backbone, terminal groups of peptides and side chains of amino acids (Barth, 2000; Böcker et al., 2017). Similarly, the influence of hydrolysis time on the hydrolysates prepared by P950 is shown in Fig. S1 A. An increase in the hydrolysis time resulted in a decrease in the absorption regions around 1645 and 1547 cm^{-1}. These absorption areas have been previously assigned to alpha-helical structures (amide I and amide II, respectively) (Böcker et al., 2017). Additionally, an increase in the absorption regions around 1516 and 1402 cm^{-1} was observed. These regions are considered to be characteristic for free amino- and carboxyl-termini (Böcker et al., 2017). Both the decrease in the absorption areas characteristic for secondary structure and the increase in the absorption regions assigned to terminal groups of peptides are consistent with changes during hydrolysis process, such as loss of secondary structure and increasing number of peptides.

PCA of the FTIR spectra was carried out to evaluate the variation between the 60 hydrolysates and study the influence of processing conditions on hydrolysates composition. The first principal component (PC-1) (Fig. 4 A) explained 44% of the variance and the grouping of the samples indicates that time of hydrolysis (within the time series produced by each enzyme) is the main factor. The loadings for PC-1 (Fig. S2 A) show that the sample variance is related to changes around 1645, 1583, 1548, 1518 and 1410 cm^{-1}. These absorption regions were previously assigned to alpha-helices in amide I region (1645 cm^{-1}) and amide II region (1548 cm^{-1}), free carboxyl- (1583 and 1410 cm^{-1}) and amino-groups (1518 cm^{-1}) (Böcker et al., 2017). PC-2 explained 20% of the sample variance and seemed to group samples according to enzyme

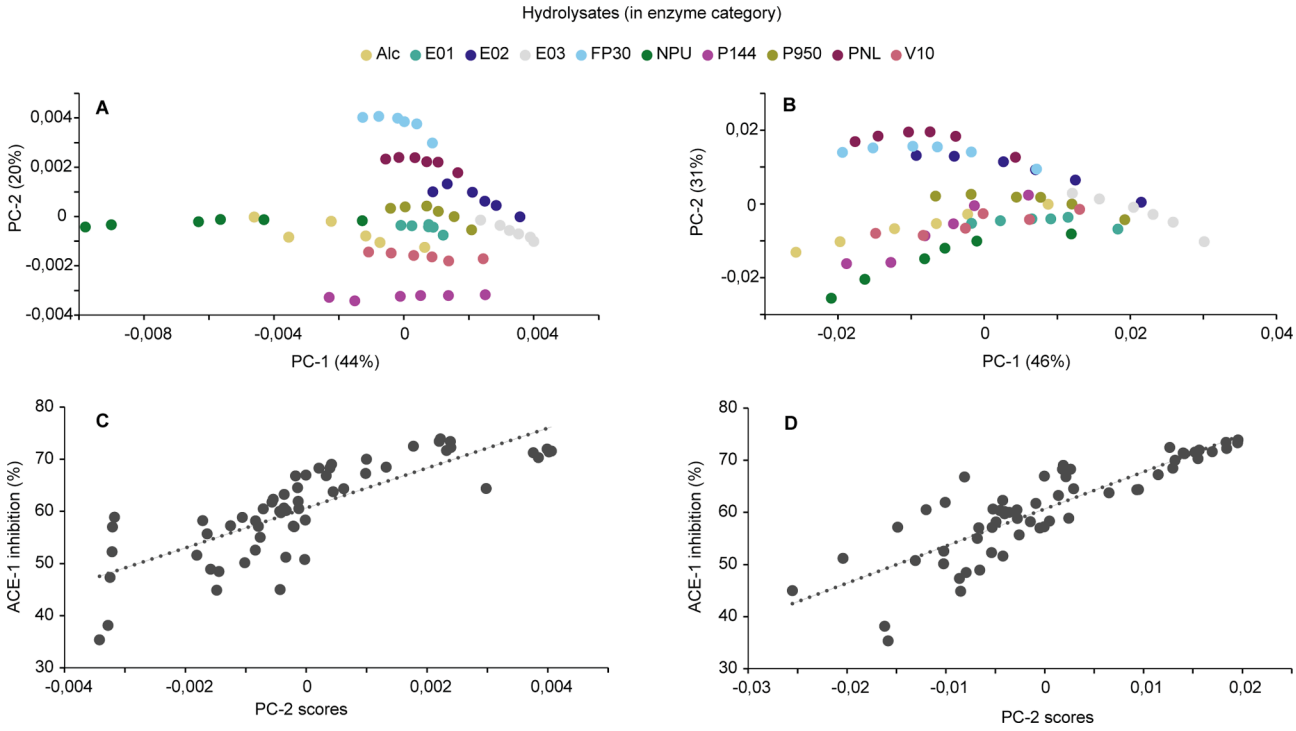

Fig. 4. PCA scores plot PC-1 vs PC-2 (A) for FTIR spectra and (B) for SEC. PC-2 scores in relation to the ACE-1 inhibition of the hydrolysates (C) for FTIR spectra and (D) for SEC.

type. The hydrolysates made by the enzymes P144 and V10, both produced from papaya, are grouped relatively together on one end of the axis in relation to the other hydrolysates made by enzymes produced by microorganisms (Table 1). The loadings for PC-2 (Fig. S2 A) show that the most prominent features are around 1678, 1643, 1155, 1120, 1070, 1049, 1040 and 1026 cm^{-1}. These features are characteristic for secondary structure (1678 and 1643 cm^{-1}), peptide backbone (1120 and 1049 cm^{-1}) and side chains of amino acids (1155, 1040, 1070 and 1026 cm^{-1}) (Barth, 2000; Böcker et al., 2017). Since there is a distinct enzyme-based grouping of hydrolysates along PC-2, the loadings for PC-2 could potentially be related to the specificity of protease. Interestingly, PC-2 scores were shown to have a correlation (R^2 = 0.64) with ACE-1 inhibitory activity of the hydrolysates (Fig. 4 C). This observation indicates that FTIR signatures can have a quantitative relationship with bioactivity of the hydrolysates.

3.2.2. SEC chromatograms of the hydrolysates

Representative SEC chromatograms of samples produced by the ten different proteases (hydrolysis time = 30 min) and samples hydrolysed for six time periods (enzyme = P950) are shown in Fig. 3 B and Fig. S2 B, respectively. The chromatograms are divided into five areas: (I) retention time (RT) 5–7.3 min corresponds to protein fragments larger than 2660 Da or 24 amino acids (calculated using M_w of 113); (II) RT 7.3–7.9 min corresponds to peptides of 2660–1500 Da or 24–13 amino acids; (III) RT 7.9–8.6 min – 1500–770 Da or 13–7 amino acids; (IV) RT 8.6–10.2 min – 770–230 Da or 7–2 amino acids and (V) RT 10.2–12 min – less than 230 Da or less than 2 amino acids. The absorbance intensities in each area are different depending on the enzyme type (Fig. 3 B) and the hydrolysis time (Fig. S2 B). For example, E03 has the most peptides at large MW (RT 5–7.9 min), while PNL has the most peptides at low MW (RT 7.9–10.2 min) compared to the other enzymes. When the chromatograms of hydrolysates produced by P950 are compared between different hydrolysis times (Fig. S2 B), the areas with high MW decreased and the areas with low MW increased as a function of hydrolysis time.

PCA of SEC chromatograms of the hydrolysates (Fig. 4 B) showed that PC-1 explained 46% of the sample variance, which could be attributed to the progress of hydrolysis or hydrolysis time. The loadings for PC-1 (Fig. S2 B) show that the main feature is the change in the areas' ratio of the highest MW (area I) and the lowest MW (area V). PC-2 explained 31% of the sample variance. An explanation for the sample grouping, as shown by the loadings for PC-2 (Fig. S2 D), is in the increase of the areas with peptides of 230–1500 Da (areas III and IV). In addition, PC-2 scores were found to have a strong correlation (R^2 = 0.72) with ACE-1 inhibition of hydrolysates (Fig. 4 D).

3.3. FTIR- and SEC-based prediction of bioactivity

PLSR models based on FTIR fingerprints and SEC chromatograms were developed for prediction of antioxidant and ACE-1 inhibition activity of the hydrolysates. The FTIR-based PLSR afforded an adequate model for prediction of DPPH radical scavenging activity with R^2 = 0.74 and RMSECV = 0.3 (Fig. 5 A, Table 2). The regression coefficients identified nine features as the most influential for the model (Fig. 5 C). These features can be attributed to peptide backbone (1049 cm^{-1}), protein secondary structure (1676, 1655, 1626 cm^{-1}) or amino acid side chains (1676, 1626, 1425, 1390, 1070, 1049, 1028 cm^{-1}) according to Barth (2000) and Böcker et al. (2017). FTIR spectra have previously been successfully used for prediction of antioxidant capacity of different products containing phenolic compounds (Leopold et al., 2012; Versari et al., 2010). The PLSR for prediction of ACE-1 inhibition afforded a model with R^2 of 0.91 and RMSECV of 2.7 (Fig. 5 B, Table 2). The regression coefficients indicated that five distinct features have the most influence in the model (Fig. 5 D). These features can be related to the peptide backbone (1412 cm^{-1}), protein secondary structure (1680, 1660 cm^{-1}) and amino acid side chains (1680, 1630, 1392 cm^{-1}) according to Barth (2000) and Böcker et al. (2017). The interpretations of the regression coefficients for DPPH radical scavenging and for ACE-1 inhibition suggest that the peptides' length and amino acid sequence are important for both activities.

PLSR models for prediction of bioactivities (i.e., DPPH radical scavenging and ACE-1 inhibition) were also developed using SEC chromatograms of the hydrolysates. The PLSR model for prediction of

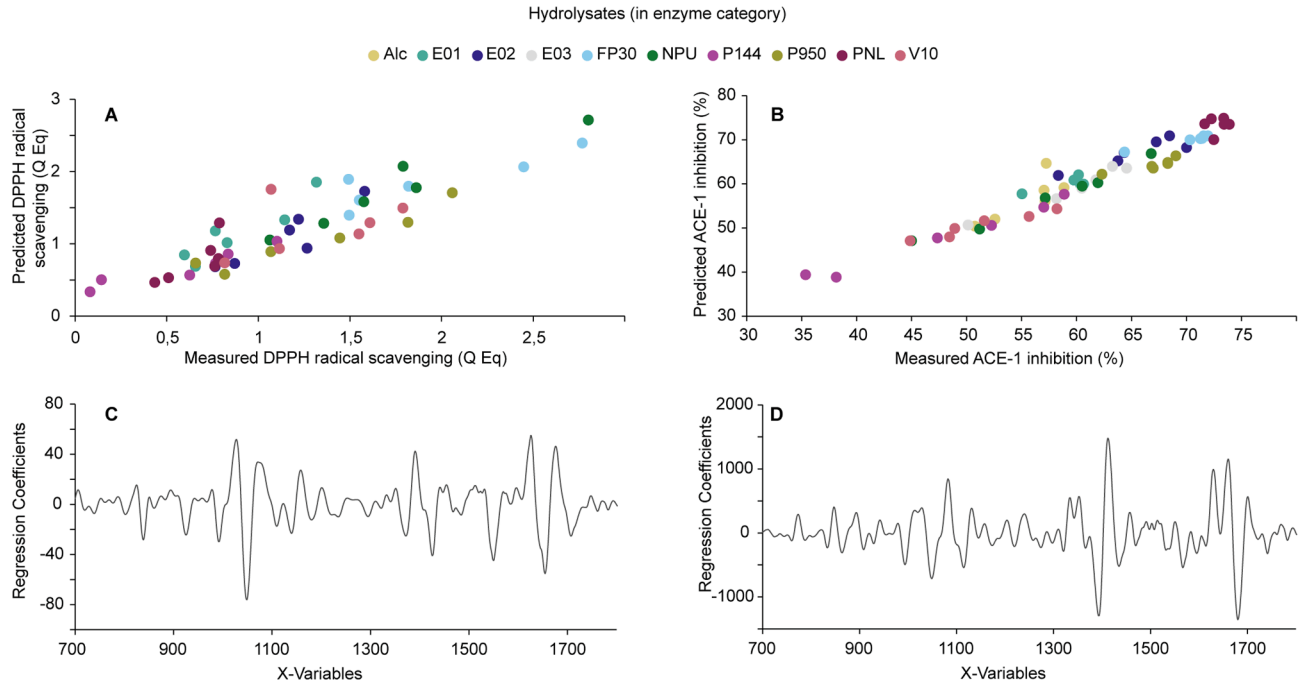

Fig. 5. PLSR models based on FTIR fingerprint of the hydrolysates: (A) PLSR model for prediction of DPPH radical scavenging, (B) PLSR model for prediction of ACE-1 inhibition and regression coefficients for prediction of (C) DPPH radical scavenging and (D) ACE-1 inhibition.

Table 2
Parameters of the PLSR models based on FTIR fingerprint and SEC chromatogram for prediction of DPPH radical scavenging and ACE-1 inhibition. PLSR models for DPPH radical scavenging were made using the results from 48 samples and PLSR models for ACE-1 inhibition were made using the results from 60 samples.

	FTIR fingerprint				SEC chromatogram			
	DPPH radical scavenging		ACE-1 inhibition		DPPH radical scavenging		ACE-1 inhibition	
	LOOCV	LOGOCV	LOOCV	LOGOCV	LOOCV	LOGOCV	LOOCV	LOGOCV
R-square	0.74	0.57	0.91	0.83	0.75	0.33	0.85	0.73
RMSECV	0.3	0.4	2.8	3.9	0.3	0.5	3.5	5.0
Number of factors	3	7	6	5	6	4	5	5

DPPH radical scavenging performed relatively similar to FTIR-based model with R^2 = 0.75 and RMSECV = 0.3 (Fig. 6 A, Table 2). The regression coefficients showed that the peak of the area III (i.e., 1061 Da) and the second peak of area IV (i.e., 405 Da) were the main variables with the largest influence on the prediction model (Fig. 6 C). Similarly, the SEC-based PLSR for prediction of ACE-1 inhibition afforded an adequate model with R^2 of 0.85 and RMSECV of 3.5 (Fig. 6 B, Table 2). The regression coefficients indicated that the peak in area III has the highest influence on prediction of ACE-1 inhibition (Fig. 6 D). A link between peptide M_w and ACE-1 inhibitory activity (Hernández-Ledesma et al., 2011) or antioxidant activity (Centenaro et al., 2014; Fernando et al., 2020; Liu et al., 2015) has previously been indicated, but this is the first study presenting a direct prediction of bioactivities from SEC chromatograms.

The number of factors, R^2 and RMSECV for all PLSR models are summarized in Table 2. The performance of models based on FTIR and SEC to predict the bioactivities of hydrolysates were relatively similar, when LOOCV was used. LOGOCV was performed to further test the robustness of the models. When comparing model performances after LOGOCV, the model based on FTIR fingerprint had higher R^2 compared to the models based on the SEC chromatograms, indicating a higher robustness of the FTIR-based models. FTIR fingerprints contain information on secondary structure, peptide backbone and side chains of amino acids (Barth, 2007). While SEC chromatograms contain information on hydrodynamic volume of a peptide, this volume is a function of molar mass, conformation and molecular configuration (Lubomirsky et al., 2021). Our results indicated that FTIR fingerprints possess more valuable information for prediction of bioactivities than the SEC chromatograms. The prediction models for ACE-1 inhibition showed a slightly better performance than for DPPH radical scavenging. This difference is likely due to the lower number of samples and lower range of values in the DPPH radical scavenging data set in comparison to ACE-1 inhibition data set.

3.4. General discussion

In the present study we demonstrated direct multivariate correlation between chemical fingerprints (SEC and FTIR) and bioactivities (ACE-1 inhibition and DPPH radical scavenging) of poultry by-product protein hydrolysates. Moreover, promising PLSR models for predicting bioactivity of the protein hydrolysates from their chemical fingerprints were developed. Such models can provide a quick insight into variables (a reflection of chemical constituents) important for a given activity of a hydrolysate. Both SEC and FTIR were in several previous studies used for chemical characterization of protein hydrolysates (Lindberg et al., 2021; Wubshet et al., 2017) and were used to predict parameters such as DH% (Kristoffersen et al., 2020). The current study, for the first time, directly predicted the bioactivities of protein hydrolysates using the two chemical fingerprints. Such prediction models (especially the FTIR-based models) can serve as an industrially relevant analytical solution to control quality of a given bioactive product. However, the reported models in this study are based on one type of raw material and two types

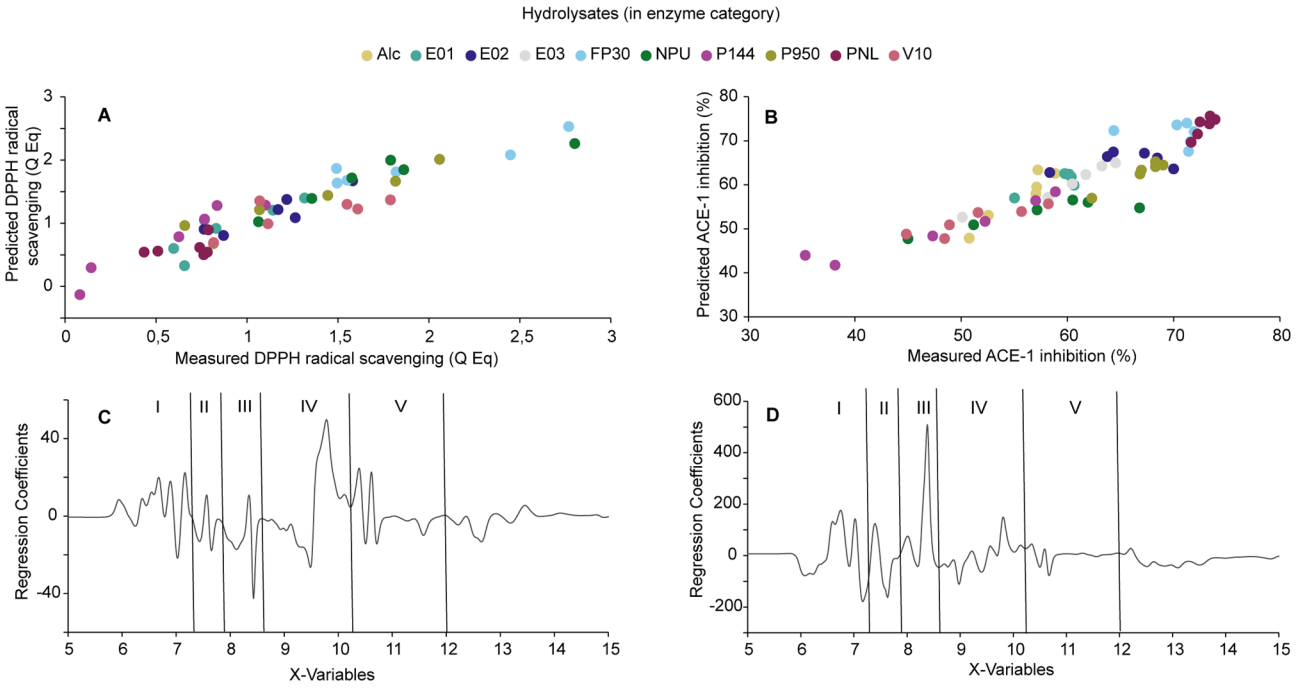

Fig. 6. PLSR models based on SEC chromatograms of the hydrolysates: (A) PLSR model for prediction of DPPH radical scavenging, (B) PLSR model for prediction of ACE-1 inhibition and regression coefficients for prediction of (C) DPPH radical scavenging and (D) ACE-1 inhibition.

of bioactivities. Further studies with larger calibration- and validation data sets, incorporating relevant raw material variations, are needed to make the model more robust. The present study suggests a potential that PLSR models of FTIR and SEC fingerprints can be expanded to other proteinaceous materials, such as by-products from marine products (e.g., fish) or novel protein sources (e.g., insects and algae) to predict bioactivities of their resulting hydrolysates/peptides.

Ensuring stable quality over time is an essential aspect for products with health-promoting effects. Due to the inherent raw material variation, bioactive products based on enzymatic hydrolysis of by-products are prone to product quality variations. This aspect is one of the major technological hurdles hampering development of bioactive peptides from complex by-products such as poultry residues. Therefore, analytical technologies to monitor variations in bioactivities of protein hydrolysates are essential elements in process and quality control. The FTIR-based model presented here can serve as such technology by providing a quick prediction tool for bioactivity. A recent study by Måge et al. (2021) based on a database of more than 1300 FTIR spectra of hydrolysates demonstrated that FTIR signatures can serve as an industrial tool to capture and monitor quality variations. The authors used the FTIR signature as a "quality" specification without direct correlation to attributes such as bioactivity. Our study suggests that such databases can further be expanded by providing a direct measure of the desired characteristics (i.e., bioactivities) and, hence, serve as quality control tool. However, the presented models must be expanded to include larger calibration datasets and independent validation sets before they can be used as a robust technology for quality control in the industry.

4. Ethics statement

Ethical approval was not required for this study.

CRediT authorship contribution statement

Liudmila Sorokina: Investigation, Methodology, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Anne Rieder:** Conceptualization, Investigation, Software, Writing – review & editing, Supervision. **Shiori Koga:** Conceptualization, Investigation, Writing – review & editing, Supervision. **Nils Kristian Afseth:** Conceptualization, Investigation, Software, Writing – review & editing. **Rita De Cássia Lemos Lima:** Conceptualization, Methodology. **Steven Ray Wilson:** Writing – review & editing. **Sileshi Gizachew Wubshet:** Conceptualization, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing, Supervision.

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105170.

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