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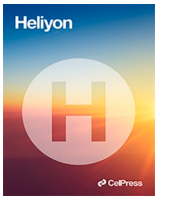

Oxidation of whey protein isolate after thermal convection and microwave heating and freeze-drying: Correlation among physicochemical and NIR spectroscopy analyses

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ABSTRACT

This study investigated the oxidative susceptibility of whey protein isolate (WPI) dispersions treated by microwave or thermal convection before freeze-drying. WPI (20 mg protein/mL) in distilled water (DW) was heated at 63 ± 2 °C for 30 min by microwave (WPI-MW) or convection heating (WPI–CH) and freeze-dried. Untreated WPI (WPI–C), WPI solubilized in DW and freeze-dried (WPI-FD), and WPI solubilized in DW, heated at 98 ± 2 °C for 2 min and freeze-dried (WPI–B) were also evaluated. Structural changes (turbidity, ζ potential, SDS-PAGE, and near-infrared spectroscopy (NIR)) and protein oxidation (dityrosine, protein carbonylation, and SH groups) were investigated. WPI-FD showed alterations compared to WPI-C, mainly concerning carbonyl groups. Microwave heating increased carbonyl groups and dityrosine formation compared to conventional heating. NIR spectrum indicated changes related to the formation of carbonyl groups and PCA analysis allowed us to distinguish the samples according to carbonyl group content. The results suggest that NIR may contribute to monitoring oxidative changes in proteins resulting from processing.

1. Introduction

Whey protein is widely used in food formulations. Whey protein isolate (WPI) contains more than 90% of proteins, which are composed of β-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins, which represent, respectively, 50–55%, 20–25%, 10–15%, and 5–10% of total proteins [1].

Apart from its techno-functional properties such as hydration, gelification, emulsification, and foaming, whey protein can improve the nutritional value of processed foods, for example, dairy beverages, meat sausages, and bakery products; however, these properties may be affected by food processing [2].

Proteins are susceptible to oxidation during food processing, such as heat treatment, dehydration, and storage. Oxidation can alter the protein's physical-chemical and structural characteristics by forming carbonylated compounds, dityrosine, and disulfide bonds, among other oxidation products, and, consequently, reflect on the functional and nutritional techno properties. In addition, consuming foods with high amounts of oxidized protein has been associated with the prevalence of some diseases, such as cancer [3,4].

The effect of heat treatment on proteins has been extensively studied in the food processes. Even at temperatures below 40 °C,

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heating can induce protein oxidation, mainly by forming reactive oxygen or nitrogen species during the Maillard reaction, auto-oxidation of sugars, or lipid peroxidation [5]. On the other hand, there are few reports on the consequences of freeze-drying – alone or combined with other techniques - on the oxidation of proteins in food.

Freeze-drying is carried out below −20 °C, and, consequently, thermal damage is minimized; thus, heat-sensitive nutrients, such as vitamins, are not degraded. However, freezing the food matrix at temperatures below – 20 °C and the dehydration conditions during this process can cause conformational changes in the protein structure and consequently modify its bioactivity and functionality [6].

Protein oxidation markers, such as carbonyl groups and dityrosine, reduction of free SH groups, can be detected or quantified by conventional analytical techniques, such as physical analyses combined with visible-ultraviolet spectroscopy (UV–vis) or liquid chromatography coupled to mass spectrometry (LC-MS) [7]. Non-destructive rapid methods, such as vibrational spectroscopy in the near-infrared region (NIR spectroscopy), have been mainly used to identify the chemical composition of organic samples, with few applications to investigate protein oxidation markers in food matrices [8].

NIR has low absorption and molar dispersion. The NIR spectra provide physical and chemical information related to the behavior of molecular vibration of *C*–H, *O*–H, and *N*–H bonds. It is a non-destructive technique for food analysis that requires little or no prior sample preparation, preserving the integrity of the evidence and allowing the material to be analyzed again after some time or, if necessary, by an alternative technique. NIR spectroscopy has been used effectively to investigate changes in the secondary structural conformation of proteins induced by freeze-drying and temperature transitions [9].

However, to the best of our knowledge, no studies in the literature associate physicochemical and NIR spectral analyses with protein oxidation. This study aimed to investigate the oxidative susceptibility of whey protein isolate (WPI) when heated by microwave or thermal convection before freeze-drying, correlating the results of physicochemical analyses with NIR spectra.

2. Material and methods

2.1. Material

Whey protein isolate (WPI) (PROVON®, Glanbia Nutritionals, Kilkenny, Ireland) was acquired in the local commerce. The protein concentration of WPI was 89.5 ± 0.2 g/100 g, determined by the micro-Kjeldahl method, using 6.38 as the conversion factor [10]. The total lipid content of WPI was 1.67 ± 1.5 g/100 g, determined by the method of Bligh & Dyer [11]. The WPI presented ash and moisture contents of 2.61 ± 1 g/100 g and 1.90 g/100 g, respectively. The main reagents were 5,5 dithiobis (2-dinitrobenzoic acid) (DTNB), BSA, and guanidine hydrochloride acquired from Sigma-Aldrich® (St. Louis, MO, USA); 2,4-dinitrophenylhydrazine (DNPH) obtained from Panreac Quimica, SL® (San Fernando De Henares, Madrid, Spain), and Bio-Rad molecular mass (MM) standard (Hercules, CA, USA, code 161–0304). All other reagents were of analytical or chromatographic grade.

2.2. Sample preparation

WPI (not submitted to additional treatment) was considered the control sample (WPI–C). To investigate the effect of freeze-drying and heat treatment before freeze-drying, WPI was dispersed in distilled water (20 mg protein/mL) at room temperature under magnetic stirring for 30 min. The WPI dispersions were then submitted to the following treatments: 1) freeze-drying (Liotop L101, Liobras, Prismalab, Brazil) for 72 h at a chamber pressure below 100 μmHg at −55 ± 2 °C (WPI-FD); 2) heating at 98 ± 2 °C under magnetic stirring for 2 min (WPI–B); 3) heating at 63 ± 2 °C under magnetic stirring for 30 min (WPI–CH) and 4) microwave heating at 63 ± 2 °C under magnetic stirring for 30 min (Start-E, Milestone, Sorisole, Italy) (WPI-MW). After heat treatment, the samples were immediately cooled at 4 °C in an ice bath and then freeze-dried under the conditions described for WPI-FD. The heat treatment conditions applied in WPI-CH and WPI-MW are equivalent to the slow pasteurization of dairy beverages, according to Normative

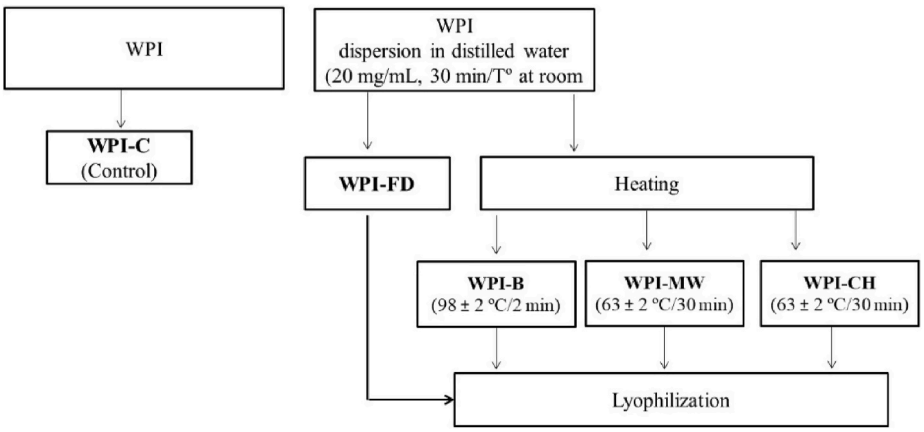


Fig. 1. General flowchart of sample preparation and treatment conditions.

Instruction No. 16 of August 23rd, of the Brazilian Ministry of Agriculture, Livestock and Supply [12] and *Codex Alimentarius* [13]. WPI-CH and WPI-MW thermal curves are available in the Online Resource and are shown in Online Resource 1.

All samples were vacuum-packed in plastic bags after freeze-drying and stored at 4 °C for further analysis. Fig. 1 shows the general flowchart of sample preparation and treatments.

2.3. Dityrosine

The method adapted from Cui et al. [3] was used to investigate the formation of dityrosine. For this, the sample was dispersed (1 mg protein/mL) in sodium phosphate buffer (20 mmol/L, pH 7.0) containing 0.6 mol/L potassium chloride (KCl) for 1 h under magnetic stirring at room temperature. Dityrosine formation was monitored by the increase in fluorescence using excitation at 325 nm and emission at 410 nm at 25 °C in a PC-1 fluorimeter (ISS Inc., Illinois, EUA) equipped with a xenon lamp and 10 nm bandwidth. In parallel, the decrease in tyrosine fluorescence was monitored using λ_{exc} 260 nm, and the maximum fluorescence λ_{em} spectra were recorded from 300 to 400 nm [14]. Corrected fluorescence for dityrosine and tyrosine was obtained by dividing the fluorescence measured by the protein concentration determined by the biuret method using WPI-C as standard. The results were expressed as fluorescence intensity/mg protein.

2.4. Carbonyl groups

The quantification of carbonyl groups was performed according to the method described by Oliver et al. [15], using DNPH to detect carbonyl groups in the form of protein hydrazones. The samples were dispersed in 3 mL of distilled water (20 mg protein/mL) for 30 min under magnetic stirring, and 1 mL of 10 mmol/L DNPH in 2 mol/L HCl was added. The controls were treated with 1 mL of 2 mol/L HCl. After 1 h of reaction at room temperature in the dark under magnetic stirring, the samples and their controls were added 1 mL of trichloroacetic acid (TCA) (50 g/100 mL) and centrifuged (20.000 *g*/15 min, 25 °C). The precipitates were washed three times with 1 mL of ethanol: ethyl acetate (1:1 (v/v)), dissolved in 4 mL of 6 mol/L guanidine hydrochloride and kept at rest for 24 h at 4 °C. Subsequently, the samples were heated to 37 °C/30 min under agitation in a thermomixer (AG 22331 comfort Eppendorf, Hamburg, Germany) and centrifuged (20,000 *g*/15 min, 25 °C). The concentration of carbonyl groups was determined by absorbance at 370 nm using the molar extinction coefficient of 22,000 M^{-1} cm^{-1}. The total content of carbonyl groups was expressed as nmol carbonyl group per milligram of soluble protein, determined by the biuret method, using WPI-C as standard.

2.5. Free SH groups

The free SH groups were determined according to the modified Ellman method [16] using DTNB. The samples were dispersed (1 mg protein/mL) in tris-glycine buffer (0.08 mol/L) containing urea (8 mol/L) and SDS (0.002 mmol/L) (pH 8.0) and subsequently added 100 μL of the Ellman reagent (0.01 mol/L). After incubation for 30 min in the dark, absorbance was read at 412 nm using tris-glycine buffer (pH 8.0) added 100 μL of Ellman reagent as blank. The results were expressed in nmol of free SH groups/mg of protein using the absorption coefficient of 13,600 $M^{-1}cm^{-1}$.

2.6. Turbidity

Samples were dispersed (5 mg protein/mL) in 0.1 mol/L sodium phosphate buffer (pH 7.0). After magnetic stirring at room temperature for 30 min, the turbidity of the dispersions was measured at room temperature and expressed as absorbance at 600 nm [3].

2.7. ζ potential

The average particle diameter and ζ potential were evaluated in a ZetaSizer Nano ZS 90 (Malvern Instruments Ltd., U.K). The ζ potential of the treated samples and WPI-C were determined according to an adaptation of the method described by Nourbakhsh and collaborators [17]. Samples were dispersed in distilled water (1 mg protein/mL) under magnetic stirring for 1 h. Smoluchowski's mathematical model was used to convert electrophoretic mobility measurements into ζ potential values expressed in millivolts (mV).

2.8. SDS-PAGE

The electrophoretic profiles of the samples were obtained in a polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) under reducing conditions [18]. A Mini Protean II equipment (Bio-Rad, Hercules, California, USA) was used, and separation and stacking gels with 12% and 4% acrylamide, respectively. The samples were diluted (1%) in reducing buffer (62.5 mM Tris-HCl, 2% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.1% Coomassie Blue G250, pH 6.8) and heated at 95 °C for 4 min. Five microliters of each sample were applied. A kit of standard MM markers from 14.4 to 97.0 kDa (Bio-Rad, Hercules, CA, USA) was used to estimate the molecular mass of the protein fractions. The analyses were performed in duplicate.

2.9. Near-infrared vibrational spectroscopy (NIR spectroscopy)

The NIR spectra of the samples were collected at room temperature using a portable spectrometer (DLP NIRscan Nano Evaluation Module, EVM, Texas Instruments, Dallas, USA), in absorbance mode, in the range of 900 to 1700 nm at 3.9 nm intervals, using a diffuse reflectance standard as white reference. The samples were placed in a quartz cuvette, and the spectra were obtained with the sensors in contact with the samples. A total of 15 spectra were acquired for each sample at random locations on the sample surface to investigate the dispersion of the spectral points concerning each different sample treatment. Thus, a total of 75 spectra were acquired to investigate the difference among sample treatments in the NIR range.

2.10. Statistical analysis

Except for NIR and electrophoresis, all analyses were performed in triplicate, with at least three measurements made by repetition. The results were presented as average ± standard deviation. The results were evaluated by the unidirectional analysis of variance (ANOVA), and Tukey's test determined the significant differences ($P < 0.05$). Statistical analysis was performed using the GraphPad Prism 6 statistical package (GraphPad Software Inc., La Jolla, USA, version 6.01). Principal Component Analysis (PCA) was used to explore the NIR spectral variance due to the effect of treatments. The spectra were analyzed using data analysis software (Pirouette, Infometrix Inc., Woodinville, Washington, USA, version 3.11).

3. Results

Proline, valine, histidine, lysine, cysteine, methionine, tyrosine, and tryptophan are among the amino acids most susceptible to oxidation. Tyrosine may oxidize by covalent bonding between tyrosine residues and be monitored by increased fluorescence intensity; tyrosine oxidation by reactive species favors the formation of tyrosyl radical, which is stable due to its aromatic nature and may still be long-life in some proteins - such as BSA - and continue oxidative reactions in the protein [7,19].

All samples showed higher intensity of dityrosine fluorescence when compared to the control (WPI–C) (Fig. 2a). Among the treated samples, WPI-B presented higher fluorescence intensity throughout the spectrum range, followed by WPI-MW, WPI-CH, and WPI-FD. As expected, there was a decrease in the maximum fluorescence intensity for tyrosine (327 nm) concomitant with the increase in dityrosine in all samples (Fig. 2b). A red shift of 10 nm was also observed in the WPI-B spectrum, resulting in the maximum fluorescence emission at 337 nm. The higher fluorescence intensity of WPI-MW for dityrosine, when compared with WPI-CH, suggests that dityrosine formation was higher when WPI was heated by microwave before being freeze-dried.

Cysteine residues are susceptible to oxidation by several reactive species [20]. The content of free SH groups can be used to monitor the susceptibility of proteins to oxidation because the decrease of free SH supported the formation of disulfide bonds. WPI-B decreased by 22% in the content of free SH groups when compared with WPI-C ($P < 0.05$), while the other samples showed no differences when compared to WPI-C (Table 1). These results indicate that convective thermal and microwave treatments before freeze-drying and only freeze-drying did not significantly change the content of free SH groups in the WPI.

Protein carbonylation has been a common and important indicator for evaluating oxidative damage in proteins [3,7]. WPI-FD showed twice as much carbonyl protein as WPI-C ($P < 0.05$), suggesting that freeze-drying increased the susceptibility to carbonyl protein formation. When compared to WPI-C, WPI-CH showed an increase of 32% in the carbonylated compound content, while WPI-MW showed an increase of 98%. However, WPI-B presented the carbonyl group content three times higher than WPI-C ($P < 0.05$),

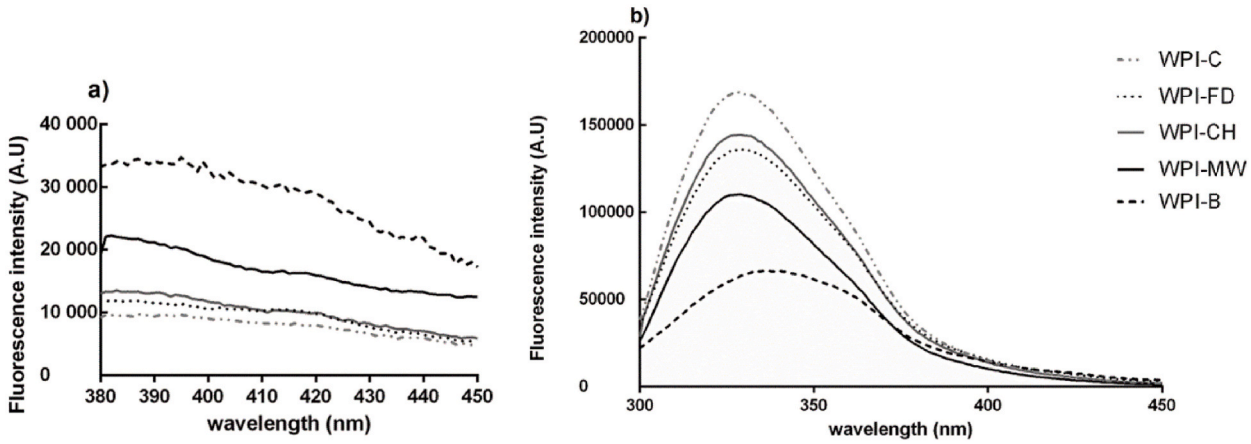


Fig. 2. Fluorescence spectra of dityrosine (a) and tyrosine fluorescence (b): WPI-C untreated (control); WPI-FD freeze-dried sample; WPI–CH heated at 63 ± 2 °C under magnetic stirring for 30 min; WPI-MW microwave heated at 63 ± 2 °C under magnetic stirring for 30 min; and WPI-B heated at 98 ± 2 °C under magnetic stirring for 2 min. All samples were lyophilized after thermal treatment and diluted in 20 mM sodium phosphate buffer (pH 7.0) containing 0.6 M KCl for analysis. Results expressed as fluorescence intensity/mg protein.

Table 1
Content of free SH groups, carbonyl groups, turbidity and ζ of WPI samples.

Sample	Free SH groups (nmol/mg protein)	Carbonyl groups (nmol/mg protein)	Turbidity (A600nm)	ζ potential (mV)
WPI-C	25.06 ± 0.48^{a,b}	2.09 ± 0.02^{d}	0.26 ± 0.006^{b}	−32.81 ± 1.25^{b}
WPI-FD	25.46 ± 0.38^{a}	4.24 ± 0.30^{b}	0.16 ± 0.003^{c}	−33.40 ± 1.33^{b}
WPI-CH	24.58 ± 0.50^{b}	2.75 ± 0.06^{c}	0.26 ± 0.006^{b}	−32.41 ± 1.26^{b}
WPI-MW	25.44 ± 0.70^{a}	4.14 ± 0.36^{b}	0.27 ± 0.002^{b}	−32.11 ± 2.21^{b}
WPI-B	19.57 ± 0.70^{c}	5.23 ± 0.35^{a}	0.54 ± 0.03^{a}	−35.80 ± 0.53^{a}

Different letters in the same column mean significant difference ($P < 0.05$).
WPI-C untreated (control); WPI-FD freeze-dried sample; WPI–CH heated at 63 ± 2 °C under magnetic stirring for 30 min; WPI-MW microwave heated at 63 ± 2 °C under magnetic stirring for 30 min and WPI-B heated at 98 ± 2 °C under magnetic stirring for 2 min.

suggesting that the high temperature of the treatment played a fundamental role in forming these compounds.

Turbidity analysis provides qualitative results regarding the extent of protein aggregation caused by the effect of treatments on WPI due to the formation of particles that disperse light and make the solution appear turbid [21]. Carbonyls, disulfide, and dityrosine bridges may cause polymerization and contribute to aggregate formation and noncovalent interactions [4]. WPI-FD presented the lowest turbidity value ($P < 0.05$) (Table 1), indicating that freeze-drying without previous heat treatment favored dispersion with a lower degree of aggregation than the control (WPI–C) and the other samples. However, WPI-B turbidity, which underwent the most severe heat treatment before freeze-drying, increased by around 50% in relation to the other samples ($P < 0.05$).

ζ Potential analysis are used to determine changes in the surface charge of particles in solution, due to treatments in WPI [3,22]. The ζ potential determined at pH 7.0 showed no difference ($P > 0.05$) among the samples, except for WPI-B, which increased around 10% in relation to the other samples ($P < 0.05$) (Table 1), which suggest an increased exposition of hydrophilic amino acid residues.

In the electrophoretic profiles obtained under reducing conditions (Fig. 3), the bands related to the BSA (66.2 kDa), β-Lg (18.3 kDa), and α-La (14.2 kDa) were well-defined for all samples (columns 2, 3, 5 and 6); however, with lower intensity for WPI-B (Fig. 3, column 4). These results suggest no change in the molecular weight of proteins perceptible by this technique, regardless of the treatment. However, in the WPI-B profile (Fig. 3, column 4), a blurred band between ~20 and 45 kDa and the presence of material that did not penetrate the separation gel suggest the presence of high MM aggregates.

The spectra obtained by spectroscopy in the NIR interval (900–1700 nm) were pretreated with standard normal variate (SNV) (Fig. 4) to minimize light scattering effects on samples due to the irregularity of particle size. Spectra treated with SNV showed more

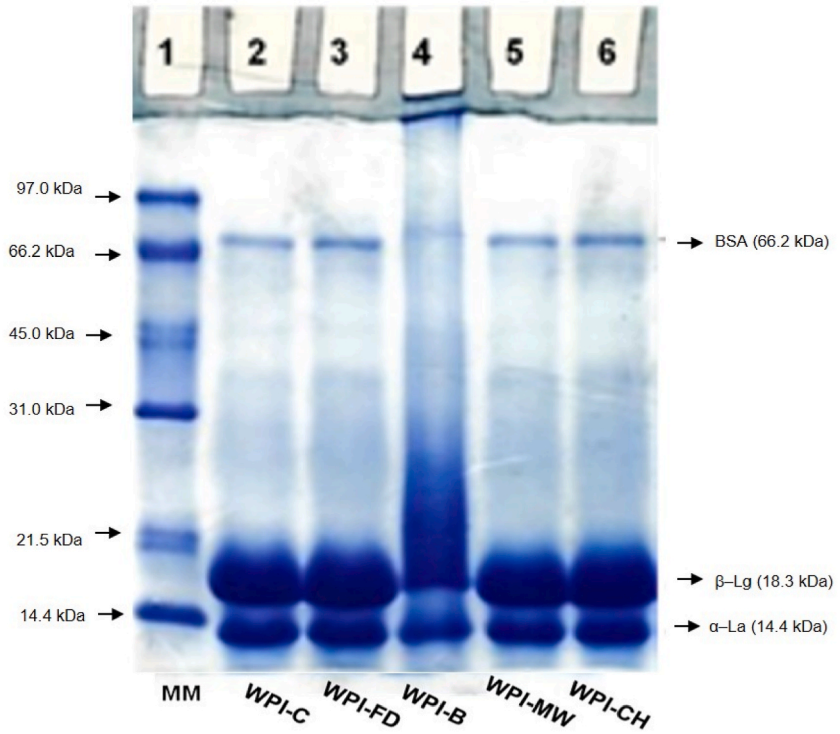


Fig. 3. SDS-PAGE electrophoresis profile under reducing conditions of WPI samples (columns 2–6) and standard molecular mass marker (column 1). WPI-C untreated (control); WPI-FD freeze-dried sample; WPI-B heated at 98 ± 2 °C under magnetic stirring for 2 min; WPI-MW microwave heated at 63 ± 2 °C under magnetic stirring for 30 min; and WPI–CH heated at 63 ± 2 °C under magnetic stirring for 30 min.

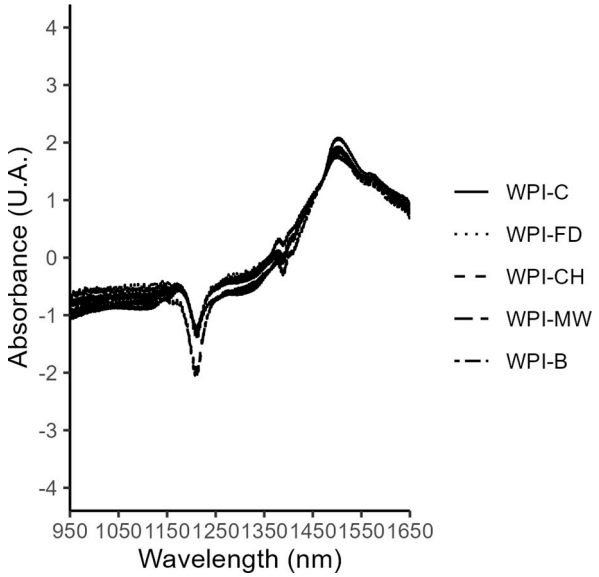


Fig. 4. NIR spectra after application of SNV (standard normal variation): WPI-C untreated (control); WPI-FD freeze-dried sample; WPI–CH heated at 63 ± 2 °C under magnetic stirring for 30 min; WPI-MW microwave heated at 63 ± 2 °C under magnetic stirring for 30 min; and WPI-B heated at 98 ± 2 °C under magnetic stirring for 2 min.

pronounced variations in the absorption peaks around 1200 nm, corresponding to the 2nd overtone of the *C*–H bond stretch, between 1380–1440 nm, which corresponds to the 2nd overtone of the *C*–H bond stretch and the 1st stretch of the *O*–H bond, and 1510 nm related to the stretch of the 1st overtone of the *N*–H bound [23].

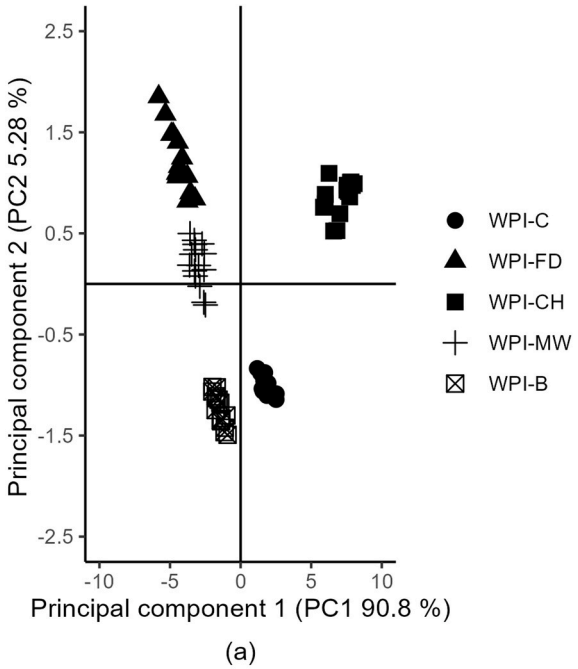
The PCA scores obtained from spectra pre-processed with SNV were used to investigate the spectral difference between samples (Fig. 5a). The first two main components (PCs) were responsible for 96.1% of the spectral variation, PC1 was responsible for 90.80%, and PC2 for 5.28% of data variation. Separately, PC1 was accountable for identifying differences between the WPI-C and WPI-CH, which are individually grouped and separated from the samples WPI-FD, WPI-B, and WPI-MW. PC2 accounted for differences between WPI-B, WPI-MW, and WPI-FD. Hence, the scores plot of PC1xPC2 shows that the first two PC were responsible for separating the samples according to the type of treatment based on the NIR spectral information.

Loadings from PCA (Fig. 5b) show that the main information is around 1,210, 1380–1410 nm, and 1510 nm, for the first four principal components. A strong influence around 1210 nm can be observed, mainly related to the stretching of the *C*–H bond, the second overtone associated with the CH_2 bond (Osborne, Fearn, Hindle 1986). This region influenced PC1, responsible for the difference between WPI-CH, WPI-C, and the remaining samples. This region also strongly influenced PC2, in addition to the region around 1,400 nm. PC2 was responsible for the difference between samples WPI-B, WPI-MW, and WPI-FD. The region between 1380–1,410 nm is associated with CH_2 and *O*–H stretching, first overtone, while the region around 1,510 nm is associated with *N*–H stretching, first overtone of proteins [23].

4. Discussion

WPI-B, the sample that received the severest heat treatment before freeze-drying, presented more pronounced changes than the control and other heat-treated samples. Above 80 °C, irreversible changes in the native structure of WPI may occur, leading to extensive aggregation, as was shown by the highest turbidity results. This treatment condition favored oxidative changes (dityrosine, free SH groups, carbonylation) due to the exposure of susceptible amino acids previously located inside the protein [24] and non-covalent interactions by the exposure of hydrophobic amino acids residues, which in turn led to protein-protein interaction. The samples treated at 63 °C/30 min by conventional (WPI–CH) or microwave heating (WPI-MW) before freeze-drying showed different oxidative index values, which suggest they have other effects on protein structure. It is worth noting that the freeze-drying alone increased some of the oxidative indexes compared to the control sample.

Despite the samples having some differences in their oxidative indexes, the carbonylated protein was the characteristic that allowed us to statistically differentiate the samples into three groups: WPI-FD, WPI-MW > WPI-CH > WPI-C. WPI-FD showed alterations compared to the control, WPI-C, mainly in relation to the formation of carbonyl groups. During the freeze-drying stages, the protein can unfold and expose side chains originally located inside its structure, favoring chemical changes since the unfolded conformation is more reactive than the native structure. Freeze-drying is a dehydration process consisting of three steps - freezing, primary drying, and secondary drying - during which the frozen material is dehydrated by sublimation below −50 °C and dried at pressures below 100 μm Hg [25]. Changes in aqueous dispersed proteins may begin with freezing when the water is gradually removed by crystallization. After water removal, proteins undergo structural rearrangement, and this can disrupt their structure. This structural rearrangement appears



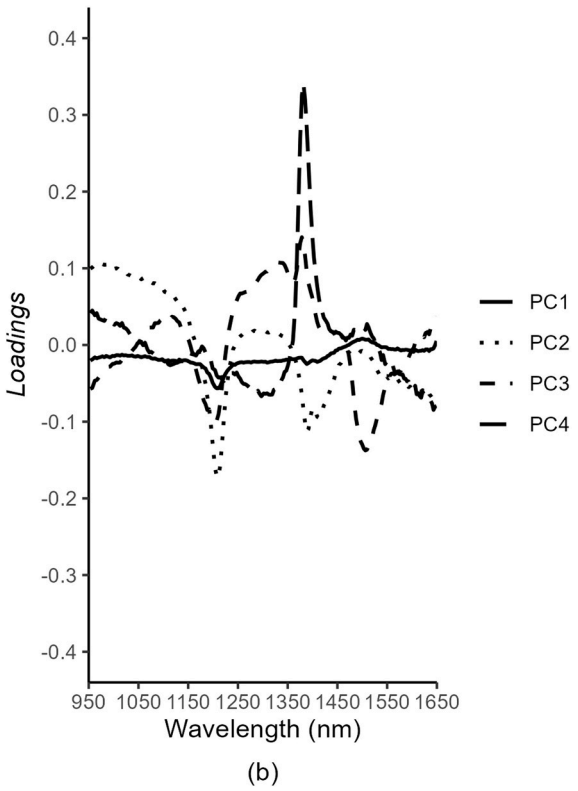


Fig. 5. Score graph (PC1 vs PC2) of pre-processed NIR spectra with SNV (a) and loadings graph of the first four main components (b) of samples: WPI-C untreated (control); WPI-FD freeze-dried sample; WPI–CH heated at 63 ± 2 °C under magnetic stirring for 30 min; WPI-MW microwave heated at 63 ± 2 °C under magnetic stirring for 30 min; and WPI-B heated at 98 ± 2 °C under magnetic stirring for 2 min.

to result from the protein's attempt to compensate for the hydrogen bonds lost with water removal, leading to the formation of intra- and intermolecular hydrogen bonds [6].

Thermal treatments performed before freeze-drying resulted in samples with different oxidative modifications, confirming that the physical-chemical properties of proteins are determined by their history of heating before freeze-drying [6]. WPI-CH presented the lowest carbonyl group levels among the treated samples, suggesting that the changes promoted by convective heat protected the sample from carbonylation due to the freeze-drying process. In the convective heat treatment, the temperature increase causes thermal agitation, vibrations, and disturbances that break down hydrogen bonds, van der Waals and polar bonds, launching groups against each other at random, forming new interactions or bonds and structure, which may contribute to protect the susceptible groups against oxidation [6].

On the other hand, microwave heating, compared to conventional heating, resulted in increased carbonyl groups and dityrosine formation. This result may be related to the effect of microwave heating, which involves two main properties: dielectric constant and ion exchange [26]. When this radiation is absorbed by polar molecules or ions inside the food, these are moved according to the alternating electromagnetic field, and heat is generated, raising the temperature of the food matrix [27]. Thus, microwave heating favors changes in the redox state of ions of ionic compounds or charged molecules, such as the basic amino acids lysine, histidine, and arginine, which can generate reactive species that, in turn, interact with other amino acid residues in proteins and can lead to the formation of stable organic radicals involved in oxidative reactions [26].

Oxidative changes can also be favored by forming reactive species generated by the Maillard reaction [28]. Intermediate carbonyl compounds generated during the Maillard reaction are highly reactive, following glycoxidation and lipoxidation reactions and reacting directly with protein. In addition, numerous reactive oxygen species can be generated through the Maillard reaction [28].

The NIR spectra indicated changes in the *C*–H, *O*–H, and *N*–H bonds related to the secondary structure of WPI stabilized by hydrogen bonds between the *N*–H and C═O groups of the main chain [23], which may be associated with the formation of carbonyl groups in the samples. The analysis of PC1xPC2 (Fig. 5a) showed that the samples were separated into four groups: 1) WPI-C, 2) WPI-CH, 3) WPI-FD and WPI-MW, and 4) WPI-B. The same grouping was obtained by statistical analysis of the carbonylated protein results (Table 1).

Loadings from PCA (Fig. 5b) suggest that WPI-CH and WPI-C suffered significant influence related to the stretching of the *C*–H bond, second overtone associated with the CH_2 bond [23]. On the other hand, WPI-B, WPI-MW, and WPI-FD differentiation from the other samples was associated with CH_2 and *O*–H stretching and *N*–H stretching [23], reinforcing that food proteins are prone to different oxidation reaction profiles, depending on the amino acid composition of the protein, as well as food processing conditions [29]. It is also interesting to point out that the fluorescence spectra (Fig. 2b) showed a decrease in the fluorescence intensity and a redshift, which indicates the opening of the tertiary structure of the protein [30] in the inverse order of the carbonyl group values.

PCA scores obtained from the NIR spectra could reflect the main chemical changes of the sample to some extent. The proximity or distance of the objects reflects similarities or differences in the properties of the samples [31]. Pabari et al. [32] reported that NIR in the range of 1500 to 2200 nm provided reliable and rapid monitoring of structural changes in proteins from infant formulas, casein, and whey powder, resulting from heating at 80 and 105 °C and may be used to improve the quality and nutritional value of the product. In the present study, even though NIR analysis has been performed in a narrow wavelength range (900–1700 nm), it was possible to separate the samples according to their carbonylated protein contents and observe changes resulting from the different types of processing to which the WPI was submitted. Therefore, our results suggest that NIR spectroscopy may contribute to monitoring oxidative changes in proteins resulting from processing. Most works using NIR spectroscopy have been applied to determine chemical composition in organic samples, mainly major compounds such as protein, moisture, and carbohydrate contents. The main novelty of the current work is the investigation of how changes in protein conformation associated with oxidation can be identified in the NIR spectra.

5. Conclusion

In this study, after freeze-drying a WPI solution (2%), the formation of carbonyl and dityrosine groups was verified. Conventional heat treatment (63 ± 2 °C/30 min) performed before freeze-drying protected the protein from oxidative damage. However, under the same conditions, microwave heating favored the formation of dityrosine and carbonyl groups in WPI. The most severe heat treatment (98 °C/2 min) led to the most pronounced oxidative changes.

The PCA analysis of the NIR spectra (900–1700 nm) allowed us to distinguish the samples processed by different heat treatments. Moreover, it was possible to evidence structural changes and to identify differences among the samples, indicating that this technique can be used for rapid and nondestructive preliminary analysis. In addition, the apparatus, a portable NIR spectroscopy equipment, is accessible and has the potential to be used to process control and enhance the quality of food products by supporting the definition of new strategies to prevent or minimize the oxidation reaction.

Author contribution statement

Juliany Cristiny Sonda Bordignon: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Amanda Teixeira Badaró: Performed the experiments; Analyzed and interpreted the data. Douglas Fernandes Barbin, Lilian Regina Barros Mariutti, Flavia Maria Netto: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Data availability statement: Data will be made available on request.

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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