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Thao T. Le: Conceptualization, Methodology, Supervision, Investigation, Data curation, Visualization, Project administration, Funding acquisition, Writing - Review & Editing.

Effect of pH and heat treatment on physicochemical and functional properties of spray-dried whey protein concentrate powder

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ABSTRACT

Effects of pH and heating on deamidation of whey protein concentrate (WPC) solution and functional properties of resultant spray-dried WPC powder were investigated. Temperature and heating time affected deamidation rates with the highest reactivities for WPC solutions heated at 120 °C for 15 min and 145 °C for 120 s. Deamidation sites were pH dependent: pH 3 induced more glutamine deamidation; pH 10 induced more asparagine deamidation. The functional properties of spray-dried WPC powders were also pH dependent. WPC solution adjusted to pH 3 and heated at 145 °C for 120 s (prior to spray drying) exhibited a reduction in solubility and foamability, but markedly improved foam stability of the resultant powders; meanwhile, the properties of powders were not significantly impacted by pH adjustment to 10.0 and heating at 145 °C for 120 s. However, pH 3 and 10 with and without heating significantly improved emulsifying properties of spray-dried WPC.

1. Introduction

Whey powder is mainly derived from whey produced during cheese manufacture; therefore, its main components are water (moisture), lactose and whey proteins. Although whey is a co-product, it has been used in many parts of the food industry because of its low price, and desirable functional and nutritional properties (Khaire & Gogate, 2019). For example, whey protein concentrate (WPC) powder, one of the major types of dried whey products, is used to fortify cereals, beverages, infant formulae and sports supplements. It is also used to improve functional properties such as emulsifying, foaming, thickening and water-binding in a range of food products (Lizarraga, Vicin, González, Rubiolo, & Santiago, 2006; Ramos et al., 2016). The functionality of whey powder is generally attributed to whey proteins. In addition, WPC contains 35–80% (w/w) whey proteins (Guo & Wang, 2019), thus, any changes or modifications to the whey proteins may influence the quality of WPC powder.

Previous studies have shown that deamidation using the protein-glutaminases improves functionalities (e.g., solubility, viscosity and emulsifying properties) of skim milk as well as producing a more coherent and thicker yoghurt gel (Miwa, Nio, & Sonomoto, 2014). Enzymatic deamidation has also been applied in cereals to counter their poor solubility in water due to the high proportion of non-polar amino acid residues in the cereal proteins resulting in high surface hydrophobicity (e.g., oats and rice) (Jiang et al., 2015). The improved solubility of cereal proteins is the result of an increased net negative charge of proteins, because deamidation converts the amide groups of the glutamine (Q) and asparagine (N) residues in proteins to carboxyl groups.

Moreover, deamidation via heat treatment or pH adjustment has been reported; this non-enzymatic approach can prevent the occurrence of side reactions such as proteolysis and

cross-linking due to the presence of impurities in the enzyme used in the enzymatic deamidation. Heat-induced deamidation has been studied in soy protein, egg white lysozyme, casein and gliadin in a restricted water environment (Zhang, Lee, & Ho, 1993), caseinate (Metwalli & Van Boekel, 1998), and canine milk lysozyme under mild conditions (Nonaka et al., 2008). In addition, deamidated wheat and barley proteins obtained through pH adjustment (e.g., citric and hydrochloric acids) displayed an increase in water solubility, emulsifying properties and stability of emulsion (Qiu, Zhao, Sun, Zhou, & Cui, 2013; Zhao, Tian, & Chen, 2011). The degree of deamidation was reported as the ratio of ammonia released from the deamidated (treated) sample to that of the native (untreated) sample; however, a direct measurement of deamidated proteins and characterisation of deamidation sites have not been carried out in these food applications. To the best of the authors' knowledge, and following a literature search, pH and heat-induced deamidation and its potential influences on functional properties have not been explored for milk proteins.

This study investigated the effect of both pH adjustment and heat treatment on deamidation of whey protein and the subsequent impact on protein functionality including solubility, emulsifying and foaming properties.

2. Materials and methods

2.1. Materials

Commercially manufactured WPC powder was purchased from Maxum Foods Pty. Ltd. (Victoria, Australia). According to the specification provided by the supplier, WPC powder is produced from fresh cheese whey by ultrafiltration and spray drying, and contains 76.8% (w/w) protein, 8.9% (w/w) lactose, 3.5 mg calcium g^{-1} powder and 4.5 mg potassium

g^{-1} powder. Triethylammonium bicarbonate (TEAB), dithiothreitol (DTT), iodoacetamide (IA) and all other chemicals used in this study were analytical grade and were purchased from Sigma Aldrich (New South Wales, Australia).

2.2. pH adjustment and heat treatment of WPC solutions

WPC powder was dissolved in distilled water to prepare 7% (w/w) WPC solution under continuous stirring conditions (400 rpm for 30 min, overhead stirrer, Heidolph RZR 2050, Kelheim, Germany). The pH of the prepared WPC solution was measured at 6.2 and then adjusted to 3 and 10 by 0.2 N HCl and 0.2 N KOH, respectively. The preliminary experiments were done to estimate the volume of HCl and KOH that would need to be added to the WPC solutions (e.g., 7.5 mL HCl and 10 mL KOH added to 500 mL of WPC solution to achieve pH 3.0 and 10, respectively), and that amount was subtracted to the amount of distilled water used to prepare 7% (w/w) WPC solution. The resulting pH-adjusted solutions were decanted into 10 mL vials, and 20 vials were simultaneously heated in an oil bath at 95 °C and 120 °C with total heating times of 3 and 15 min. As a large volume of WPC solution (> 500 mL) was required for spray drying, multiple batches (20 vials/batch) of the same pH and heat treatment were combined. Treatment at 145 °C with total heating time of 30, 60, 90, and 120 s was also carried out in a similar manner. All sample solutions were kept at 4 °C for 18 h before deamidation analysis and spray drying.

2.3. Deamidation analysis

The degree of deamidation in pH- and heat-treated WPC solutions was measured by liquid chromatography coupled to a high resolution QExactive Focus Hybrid Quadrupole-

Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The major protein component, β-lactoglobulin (β-Lg) (55–65% of whey protein content) was quantified using a targeted peptide approach. Thirteen deamidated peptides (Table 1) obtained from trypsin digestion of β-Lg in WPC solutions were selected and quantified, based on full scan MS/MS experimental data from the QExactive. These selected peptides cover 9 out of 14 deamidated sites which are at N and Q in the β-Lg sequence.

Briefly, a 5 µL aliquot of WPC solutions (7%, w/w) was diluted with 95 µL of 40 mM TEAB, pH 8, to obtain an approximate 2.7 mg mL^{-1} protein solution. The protein solution (100 µL) was reduced with 5 µL of DTT (20 mg mL^{-1}) and alkylated with 5 µL of IA (50 mg mL^{-1}) before digested with 100 µL of trypsin (10 µg mL^{-1}) at 37 °C for 16 h. The solution digests were spiked with 1 ppm of C^{13} and N^{15} phenylalanine labelled dermorphin (Auspep Pty Ltd., Victoria, Australia) (used as internal standard). The digests were analysed by ultra-performance liquid chromatography coupled with hybrid quadrupole Orbitrap mass spectrometry (UPLC Orbitrap MS/MS) in a full scan MS/MS mode with an inclusion list of targeted peptides (Table 1). The data was analysed using TraceFinder™ 5.1 SP1 software (Thermo Fisher Scientific, Bremen, Germany). The level of deamidation was normalised by multiplying peak areas of precursor ions by 100 and dividing them by the corresponding peak areas of non-deamidated peptides as in eq. 1.

$$Normalised\ deamidation\ level = \frac{Peak\ areas\ of\ deamidated\ peptides}{Peak\ areas\ of\ nondeamidated\ peptides} * 100 \quad (1)$$

2.4. Spray drying of WPC solutions

Seven percent of non-treated WPC solution (control sample), pH-treated WPC solutions (pH 3 and 10), and pH and heat-treated WPC solutions (pH 3 and 10, 145 °C/120 s) were prepared and stored at 4 °C for 18 h before spray drying. The heating condition (e.g.,

145 °C/120 s) was selected for the powder production because it exhibited the highest level of deamidation (more details in Section 3.1). Spray drying was carried out at inlet and outlet air temperature of 180 °C and 70 °C, respectively (Mini Spray Dryer B-290, Buchi Corporation, New Castle, USA). The collected powder (estimated yield of 60–70% of solid content) was kept at –18 °C in airtight containers for further analyses of physiochemical and functional properties. The powders used for these tests were commercial WPC powder (WPC), spray-dried WPC solution (WPC-SD), spray-dried WPC solution subjected to pH adjustment to 3.0 (WPC-pH3-SD), or 10.0 (WPC-pH10-SD) and spray-dried WPC solution subjected to pH adjustment to 3 or 10, and heating at 145 °C/120 s (WPC-pH3-H-SD and WPC-pH10-H-SD, respectively).

2.5. Determination of physiochemical properties of spray dried WPC powders

Moisture content of spray-dried WPC powders was determined by following the method reported by AOAC 925.45 (AOAC, 1996). Water activity (a_w) of samples was measured using an AquaLab 3 Water Activity Meter (Decagon Devices Inc., Pullman, USA) at 25 °C. True density of samples was determined using a nitrogen pycnometer (Multipycnometer, MVP-6DC, Scientific Solutions, New South Wales, Australia). The colour of samples was measured for L*, a* and b* using a Chroma meter (CR-400, Konica Minolta, New Jersey, USA). Whiteness of WPC powders was calculated from the LAB colour system (Ho & Noomhorm, 2011) as in eq. 2.

$$Whiteness = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{\frac{1}{2}} \qquad (2)$$

The conformational changes of protein in spray-dried WPC powders were analysed by Fourier-transform infrared (FTIR) spectroscopy using a FTIR Spectrometer Attenuated Total Reflectance (ATR) Spectrum 100 (PerkinElmer Ltd, Beaconsfield, UK), over a scan range of

166 4000 to 700 cm^{-1} with 32 scans per spectrum, and 4 cm^{-1} spectral resolution, as previously
167 described by Ho et al. (2019). The obtained FTIR spectra were deconvoluted at amide I band
168 (1700–1600 cm^{-1}), as the most intense absorption band in proteins, by Fourier self-
169 deconvolution program (OriginPro 2018 Software, Hearne Scientific Software Pty Ltd,
170 Victoria, Australia). The secondary structure compositions or the percentages (%) of
171 secondary structures of proteins were determined based on the area under each deconvoluted
172 peak against the total area.
173
174 *2.6. Determination of functional properties of spray-dried WPC powders*
175
176 Solubility, foaming and emulsifying properties of WPC powders were determined.
177 From the preliminary experiment, WPC solutions prepared from non-treated WPC powder
178 (WPC and WPC-SD), pH-treated WPC powder (WPC-pH3-SD and WPC-pH10-SD) and pH
179 and heat-treated powders (WPC-pH3-H-SD and WPC-pH10-H-SD) showed different pH
180 levels (Table S1), which might contribute to differences in functional properties of the
181 powders. Therefore, the pH of all WPC solutions was standardised to 6.20, which was the
182 same pH as the commercial WPC powder solution, using 0.2 N HCl and 0.2 N KOH before all
183 the functionality measurements.
184
185 *2.6.1. Solubility*
186 Solubility of WPC powders at 25 °C was determined by following the method of Ho
187 et al. (2019) with a slight modification. Aqueous solutions of WPC powders (5.5%, w/w)
188 were stirred using an overhead stirrer (400 rpm, Heidolph RZR 2050, Kelheim, Germany) for
189 30 min to completely disperse the powders into water. The dispersions were adjusted to pH
190 6.2 and distilled water was added to make a final concentration of 5% (w/w). The dispersions

were stirred for another 30 min before being centrifuged at 1000 × *g* for 15 min at 20 °C using an Eppendorf Centrifuge 5702 (Eppendorf South Pacific Pty. Ltd., New South Wales, Australia). During stirring, temperature of the solutions was maintained at 25 °C in a water bath. The insoluble solids were flushed with 5 mL distilled water and transferred to pre-weighed moisture pans which were then dried in a Thermoline vacuum oven (Scientific Equipment, New South Wales, Australia) at 105 °C for 16 h (absolute pressure 80 kPa). The increase in the weight of the moisture pan was the content of insoluble solids. Total solids in the dispersion before centrifugation were determined from the precisely-measured amount of whey powder and water initially used to prepare the dispersion. The solubility (S, %) of WPC powders was calculated using following eq. 3.

$$S\ (\%) = \frac{W_{ts} - W_{is}}{W_{ts}} * 100 \qquad (3)$$

where, W_{ts} is the weight of total solids (soluble and insoluble) in the solution (g), W_{is} is the weight of insoluble solids (g).

2.6.2. Foaming properties

The foaming properties of WPC powders were evaluated by following the method reported by Liao et al. (2016b) using 5% (w/w) WPC solution. WPC powders were dissolved into distilled water (5.5%, w/w) under stirring (300 RPM) for 30 min. The solutions were equilibrated at 4 °C for 18 h, and then subjected to pH standardisation (~ pH 6.2) and water addition to make a final concentration of 5% (w/w) prior to foaming. A hundred mL of WPC solution was poured into a graduated plastic jug (250 mL, polypropylene, Genetics Australia Co-operative Ltd., Victoria, Australia) and was then homogenised via a T25 digital Ultra-Turrax® (IKA, Bio-Strategy Pty Ltd., Victoria, Australia) at 10,000 rpm for 1 min at 25 °C. Foamability was determined as the percentage increase in volume of WPC solution upon

214 mixing. Foam stability was expressed as the percentage of foam volume that remained after
215 30 min.
216
217 *2.6.3. Emulsifying properties*
218 The emulsifying activity and stability of WPC powders were determined using the
219 method of Shilpashree, Arora, Chawla, Vakkalagadda and Sharma (2005) with a minor
220 adjustment. About 40 mL WPC solution (1%, w/w), which was initially standardised to pH
221 6.2. was sonicated with 20 mL soybean oil (Coles, Queensland, Australia) using a 24 KHz
222 sonicator (Model UP 400S, Hielscher Ultrasonics GmbH, Teltow, Germany). Sonication was
223 performed with 95% amplitude for 30 s. About 10 mL of the sonicated solution was
224 centrifuged at 1100 × g for 5 min at 20 °C using an Eppendorf Centrifuge 5702 (Eppendorf
225 South Pacific Pty. Ltd.). The height of the emulsified layer and that of the total contents in the
226 tube were measured. The emulsifying activity (EA) was calculated as eq. 4.

$$\text{EA}\ (\%) = \frac{\text{Height of emulsified layer in the tube (mm)}}{\text{Height of the total content in the tube (mm)}} * 100 \qquad (4)$$

227 Emulsion stability (ES) was determined by heating the emulsion at 80 °C for 30 min
228 before being centrifuged at 1100 × g for 5 min at 20 °C using an Eppendorf Centrifuge 5702
229 (Eppendorf South Pacific Pty. Ltd.) and calculated as equation (5).

$$\text{ES}\ (\%) = \frac{\text{Height of emulsified layer after heating (mm)}}{\text{Height of emulsified layer before heating (mm)}} * 100 \qquad (\text{eq. } 5)$$

230
231 *2.7. Experimental design and statistical analysis*
232
233 The experiments were performed following a fully randomised design with three
234 replications. Statistical analysis of the data was conducted using the Minitab Express
235 statistical program (Minitab Inc., State College, PA, USA). A one-way analysis of variance

(ANOVA) was used. Tukey's multiple comparison test was employed to determine significant differences in treatment means at $p < 0.05$.

3. Results and discussion

3.1. Deamidation degree

The effects of pH and heat treatment on deamidation of whey protein were investigated in WPC solutions adjusted to pH 3 and 10, and heated at 95 and 120 °C for 3 and 15 min. The degree of deamidation in whey protein was determined by quantifying deamidated β-Lg as the most abundant protein in WPC. Fig. 1 shows normalised deamidation of four representative deamidated peptides of β-Lg, WEnDECAQK, WENDECAqK, IDALnENK and LIVTqTMK, with small letters n and q indicating the deamidation sites. Of the 14 available deamidation sites (N and Q) in β-Lg, 9 sites (present in 13 deamidated peptides) were characterised and quantified in this study. Two obvious trends can be observed with N and Q deamidation in WPC solutions: there was a preference for Q deamidation sites at pH 3 and N deamidation sites at pH 10, and this preference was statistically significant (Fig. 1). The rapid occurrence of N deamidation under the mild conditions has been reported as analytical artifacts during sample preparation of protein digest; shortened digestion time and digestion at lower temperature and at lower pH were suggested to reduce the N deamidation (Liu, Wang, Xu, May & Richardson, 2013). This earlier hypothesis is supported by our findings with increased N deamidation at pH 10 and significantly reduced deamidation at pH 3. It can be seen that N site is more predominant than Q site under non-enzymatic conditions; for example, the highest normalised deamidation was 16.2% for the peptide WENDECAQK deamidated at N and 0.86% for deamidation at Q

site. Q deamidation is known to happen at a much slower rate than N (Bischoff & Kolbe, 1994), however, as peptides respond differently in MS, an absolute quantification approach would be more accurate to determine the differences between N and Q deamidation.

In addition, heating time and temperature influenced the reactivity of deamidation, as can be seen in Fig. 1; higher temperatures support higher reactivity at both Q and N sites. In fact, the treatment condition pH 3, 120 °C and 15 min induced the most deamidation at Q, while treatment conditions pH 10, 120 °C and 15 min induced the most deamidation at N (Fig. 1). However, pH or heat alone had little effect on the normalised deamidation level of these peptides. The results are similar for all 13 investigated peptides (Fig. 1; Supplementary material Fig. S1). Hence, the combination of pH, temperature, and heating time may have a synergistic effect on the deamidation reaction in whey proteins, particularly β-Lg. It can be noted that the rate of deamidation also depends on neighbouring amino acid residues (e.g., N-Glycine > N-Serine > N-Alanine) and the higher order structure of the unfolded protein (Wright, 1991). The rate of deamidation in α-lactalbumin (α-La) might be different from that in β-Lg due to the variation in their amino acid sequences, particularly those around N and Q, for example, neighbouring serine (S) and threonine (T) increase deamidation, however, the known deamidation motifs (N–S and N–T) are not present in α-La as can be found in β-Lg. Importantly, the unfolding of whey protein (e.g., denaturation) as well as other chemical reaction (e.g., Maillard reaction) could take place under heating and high pH treatment. Miwa, Yokoyama, Wakabayashi, and Nio (2010) observed a partial disruption of the tertiary structures of proteins, mainly β-Lg and α-La in whey protein isolate resulted from deamidation; they also noted that deamidation causes less severe denaturation compared with heat denaturation. Further studies are required to look at the effects of protein structure and/or relative impact of chemical reactions (e.g., denaturation, Maillard reaction) on deamidation or vice versa of whey protein induced by heat and pH.

As N and Q reacted differently at two pH conditions, both pH 3 and 10 were chosen for a follow-up experiment where a higher temperature (145 °C) and shorter heating times (30, 60, 90 and 120 s) were used to reflect the industrial method of powder production and to investigate the effects of heat and pH on the functional properties of WPC powders. The four representative peptides, WEnDECAQK, WENDECAqK, IDALnENK and LIVTqTMK, showed comparable results with the initial experiments (Supplementary material Fig. S2), where the longer heating time (e.g., 120 s) at 145 °C resulted in the greatest amount of deamidation. Therefore, 145 °C and 120 s were chosen as the optimal conditions to produce powders for a test of functional properties.

3.2. Physiochemical properties

3.2.1. Moisture content, water activity, true density and colour

As shown in Table 2, WPC-pH3-SD and WPC-pH3-H-SD samples had slightly lower moisture content (4.75–5.89%, w/w) than the other samples which had similar values in moisture content (6.39–7.01%, w/w). A similar trend was also observed for water activity. Similar spray drying conditions were employed for all WPC powders; thus, the differences in moisture content and water activity among these samples resulted from the changes in sample compositions during pH adjustment and heating, probably lactose degradation. It is known that treating of whey solutions at low pH and high temperature induces lactose hydrolysis (Zadow, 1992). Hence, lactose hydrolysis could possibly occur in WPC solutions heated at 145 °C/120 s and/or spray dried (e.g., 180 °C inlet and 70 °C outlet) and adjusted to pH 3.0 (e.g., WPC-pH3-SD and WPC-pH3-H-SD), reducing the water-holding capacity of resultant WPC powders.

The true density of WPC powders was 0.883–1.084 g cm^{-3}, which was highly comparable with values reported by de Carvalho-Silva, Vissotto, and Amaya-Farfan (2013). Although all spray-dried WPC powders had lower true density than commercial WPC powder ($p < 0.05$), the comparison can only be relative as the commercial WPC powder was produced from a large-scale dryer which is different from the small Buchi dryer used in this study. The lower true density in all spray-dried WPC powders could also possibly be due to the lower feed solids concentration (7%, w/w) of these powders before spray drying as compared with approximately 10% used to produce the commercial ones. As reported by Nguyen, Nguyen, Mounir, and Allaf (2018), an increase in feed solids concentration of soymilk during spray drying increased the true density of the powders produced. Another possibility is that other components in WPC powders (e.g., lactose) could change from a crystalline to an amorphous structure during spray drying, which could affect the true density of the powder. Unlike the production of commercial WPC in which lactose is crystallised prior to spray drying, direct spray drying of WPC in this study led to the presence of amorphous lactose in the final product. A lower true density in amorphous solids than crystalline counterparts was also reported by Bookwala, DeBoyace, Buckner, and Wildfong (2020). Among spray-dried WPC powders, samples adjusted to pH 3 (e.g., WPC-pH3-SD and WPC-pH3-H-SD) had lowest true density values. This could be because of lactose hydrolysis occurring in these samples. Aguilar and Ziegler (1994) reported that true density of whole milk powder gradually increased as lactose concentration in the powders was increased. In any case, since WPC-pH3-SD and WPC-pH3-H-SD had lowest not only true density but also moisture content and water activity, it is necessary to analyse and confirm whether these are caused by lactose degradation in the future.

For colour, it is noted that in the LAB colour system, L* indicates the lightness/darkness coordinate, a* is the red/green coordinate, and b* is the yellow/blue

coordinate. Whiteness values account for all L*, a* and b*, which correlates the visual ratings of whiteness for certain white and near-white surfaces. For instance, the powders with high L* do not necessarily have high whiteness, as it also depends on a* and b* values. As indicated in Table 2, all spray-dried WPC powders had much more lightness and whiteness, but less yellowness than commercial WPC. These differences could be observed from images of WPC powders shown in Supplementary material Fig. S3. Compared with WPC-SD, WPC-pH10-SD and WPC-pH10-H-SD were lower in lightness and whiteness.

Overall, the application of pH (3.0 and 10) and heating treatment (145 °C/120 s) to WPC solutions prior to spray drying did not cause marked effects on physiochemical properties (e.g., moisture content, water activity, true density and colour) of spray-dried WPC powders. Notably, the unchanged colour could also imply that the browning was not developed in these powders during pH and heat treatment. Browning is one of the common ways to investigate progression of the Maillard reaction, especially the advanced or late stage of the reaction, and the b* values were used as an indicator for browning in all types of milk powders upon storage (Le, Bhandari, Holland, & Deeth, 2011). Although WPC solutions were treated at high temperature (145 °C) and low and high pH (3 and 10), the short heating time (120 s) might not be enough to cause browning.

3.2.2. FTIR

FTIR spectra of WPC powders, and a list of FTIR band assignments are shown in Supplementary material Fig. S4 and Table S2, respectively. Secondary structure of proteins including α-helix, unordered, β-sheet, β-turn and loop structures can be studied in the amide region of the FTIR spectrum, particularly amide I band (1700–1600 cm^{-1}) due to its high sensitivity to infrared spectroscopy (Barth, 2007; Yazdanpanah & Langrish, 2013). However, due to overlapping signals, α-helix and unordered structures could not be well-defined,

regardless of multiple attempts at changing deconvolution and peak fitting. Some studies on secondary structure of proteins showed that, in amide I, vibration for α-helical and random-coil structure occurred at about the same frequency (Anderle & Mendelsohn, 1987) and that the band linked to random structure is too small to be separated from the α-helix structure (Dong, Huang, & Caughey, 1990). The analytical results of secondary structure of proteins in WPC powders are shown in Fig. 2; it can be interpreted from Fig. 2 that peaks at ~1609–1620 cm^{-1} represent adsorption of amino acid side chains, peaks at ~1625–1635 cm^{-1} represent β-sheets, those at ~1642–1652 cm^{-1} represent α-helices and/or unordered, and the remaining peaks represent β-turns (Barth, 2007; Yang, Yang, Kong, Dong, & Yu, 2015).

The percentages (%) of protein secondary structures in WPC powders are shown in Table 3. Spray drying of reconstituted WPC powder resulted in changes in the secondary structure of proteins, as the WPC-SD sample had a significantly higher percentage of α-helix/unordered, but markedly lower percentage of β-sheet and β-turn than the WPC sample. The protein secondary structure in the powders produced by spray drying is known to exhibit more percentages of α-helix and less β-turn than that in the powders produced from freeze drying and that in liquid samples (Hou, Wang, Song, Wu, & Zhang, 2019). A comparison among spray-dried WPC powders revealed that pH and heating had a great impact on the secondary structure of proteins. All spray-dried WPC powders subjected to pH adjustment and heating exhibited a marked reduction in percentages of α-helix/unordered structure, or a high portion of β-sheet and β-turn structure altogether was present, as compared with WPC-SD powder (Table 3). This indicates that pH and heating treatment induced the unfolding of proteins and pH 10 had a more profound effect than pH 3.0. The result is consistent with the study of Tomczynska-Mleko et al. (2014) where, at pH 3, the secondary structure of whey protein based on circular dichroism (CD) spectra had little change between non-heated and heated whey protein isolate solutions, while an increased pH caused a loss in the helical

structure of protein in heated samples. Heating reduced percentages of α-helix, β-sheet and β-turn structures and increased percentages of unordered structures of whey protein isolate solutions; this suggests the results were linked to protein aggregation. These changes were more pronounced with increased pH, with highest percentages of unordered structure obtained at pH 10 (Tomczynska-Mleko et al., 2014). In this study, the pH and heat-treated WPC powder showed the opposite trend, such as an increase in percentages of β-sheet (except WPC-pH3-SD) and β-turn (except for WPC-pH3-H-SD) as compared with WPC-SD. This could be due to differences in e.g., techniques used (CD *vs.* FTIR), physical state (solution vs. power) and heating temperature and time between the two studies (145 °C/2 min versus 80 °C/30 min). However, both studies indicated the highest unordered structure obtained at pH 10.

Similar results were also reported by Liao et al. (2016a) for wheat gluten deamidated by a carboxylic acid/heat water solution, and by Wong et al. (2012) for wheat gliadin deamidated by HCl. Both studies found that deamidation of proteins resulted in increased percentages of β-sheet/β-turn and decreased percentages of α-helix. In addition, it was reported that the ratio of α-helix to β-sheet (α/β) represents the molecular flexibility of proteins by which proteins with the smaller ratio were the more flexible and more open conformation (Liao et al., 2016a). From Table 3, as compared with the WPC-SD sample (α/β ≈ 1.7), pH 10 and heating treated samples had a much lower ratio (α/β ≈ 0.3–0.5) while the ratio of pH 3.0 and heating treated samples was slightly smaller (α/β ≈ 1.2–1.6). Higher flexibility of proteins in pH and heat-treated samples, especially for those at pH 10, could result from deamidation of whey proteins induced by pH and heating (Fig. 1, Supplementary material Figs. S1 and S2), or protein denaturation/unfolding. In the study of Tomczynska-Mleko et al. (2014), α/β ≈ 0.6 was calculated from the reported values of pH 3 and 10 of heat-treated whey protein isolate dispersions.

3.3. Functional properties

3.3.1. Solubility

The solubility of WPC powders is presented in Fig. 3a. As can be seen, commercial WPC powder dissolved almost completely in water with solubility about 99.01%, and concurs with the solubility values reported by Luck et al. (2013). Interestingly, the solubility of WPC powder in this study is approximately 10% higher than that shown by Tunick et al. (2016). These differences could be explained by variation in WPC sources or measurement technique of solubility.

Overall, the solubility of all WPC powders in this study is high (above 97%). WPC and WPC-SD had similar solubilities (Fig. 3a), confirming further spray drying did not affect the solubility of whey powder. Among WPC powder samples subjected to pH and heating treatment, only the WPC-pH3-H-SD sample exhibited a decline in solubility ($p < 0.05$). The reduction in the solubility of the WPC-pH3-H-SD sample possibly could be due to the powder characteristics (e.g., the lowest moisture content and true density). Among them, there is a possibility of lactose hydrolysis as previously mentioned. It has been reported that rehydration and solubility of milk powder were greatly affected by the degree of lactose hydrolysis prior to spray drying. The higher degree of lactose hydrolysis led to the greater decrease in solubility of milk powders (Torres et al., 2017). As previously mentioned, lactose hydrolysis possibly occurred in the WPC-pH3-H-SD sample, reducing its solubility.

In addition, the factors of the reduction in the solubility of the WPC-pH3-H-SD sample are considered in terms of protein unfolding. It was found that changes in the secondary structure of proteins in milk powders (e.g., protein unfolding) are detrimental to their solubility (Pugliese et al., 2017). In this study, as indicated in Table 3 and discussed in

435 the FTIR results, pH adjustment and heat treatment prior to spray drying induced the
436 unfolding of proteins. Compared with WPC-SD, the percentages of α-helix in WPC-pH3-SD,
437 WPC-pH3-H-SD, WPC-pH10-SD and WPC-pH10-H-SD decreased while percentages of β-
438 sheet/β-turn increased. A greater alteration in samples at pH 10 than those at pH 3.0 was also
439 observed. These results indicated that the changes in secondary structure of proteins could not
440 be the reason for the lowest solubility of the WPC-pH3-H-SD sample. In other words, the
441 degree of protein denaturation is not a decisive factor in the solubility of spray-dried WPC
442 powder. A comparison of the FTIR results (Table 3; Fig. 2) between WPC and WPC-SD
443 indicates that spray drying changed the secondary structure of proteins, but this change did
444 not cause solubility reduction. Oldfield, Taylor, and Singh (2005) reported that
445 denaturation/unfolding of whey protein components (e.g., β-Lg, α-La, bovine serum albumin
446 and immunoglobulin) in skim milk occurred mostly at the preheating stage, and spray drying
447 conditions (160–200 °C and 89–101 °C inlet and outlet air drying temperature, respectively)
448 did not significantly denature whey proteins. Thus, the effect of spray drying on the
449 denaturation of whey protein is not consistent with past findings. This could be because of the
450 difference in spray drying conditions which possibly induces different degrees of structural
451 changes. This study showed that spray drying processes without pH or preheating have little
452 effect on the solubility of WPC, but a more detailed investigation is needed on the association
453 between protein structure and solubility.
454
455 *3.3.2. Foaming properties*
456 The foaming properties of WPC solutions (5%, w/w) prepared from various WPC
457 powders were tested and the results are presented in Fig. 3b. WPC-pH3-H-SD samples
458 possess significantly lower foamability than WPC and WPC-pH10-SD ($p < 0.05$). The result
459 indicated that spray drying and pH treatment (e.g., pH 3 and 10) did not affect foamability,

460 but heating in combination with pH 3 treatment significantly reduced foamability. Regarding
461 foam stability, the spray-dried WPC sample (WPC-SD) when treated at pH 3 (WPC-pH3-SD)
462 did not show any improvement of foam stability, but it was doubled when heating was
463 applied (WPC-pH3-H-SD) ($p < 0.05$). The opposite trend was seen for WPC samples treated
464 at pH 10. Foam produced from WPC samples treated at pH 10 alone (WPC-pH10-SD) was
465 much more stable than that prepared from WPC-SD samples ($p < 0.05$), while foam stability
466 of WPC samples subjected to both heating and pH treatment (WPC-pH10-H-SD) was not
467 different to that of WPC-SD. It was found that foaming properties of WPC solutions were
468 affected by the solubility of WPC, and removal of large insoluble particles improved foaming
469 properties of WPC solutions (Hawks, Phillips, Rasmussen, Barbano & Kinsella, 1993;
470 Onwulata, Konstance, & Tomasula, 2004). These findings agree with our study results in
471 which the WPC-pH3-H-SD sample had the lowest solubility and foamability.

472 Foaming properties of proteins are greatly affected by protein deamination. Liao et al.
473 (2016b) found that while foaming properties of wheat gluten were dependent on the degree of
474 deamidation, an excessive increase in deamidation (> 40%) did not result in a further increase
475 in foaming properties. Also, it was reported that deamidation of oat protein isolate in acidic
476 condition (0.5 N HCl), in combination with heating at 70 °C for 2 h, increased foaming
477 capacity as solubility increased, but depressed foam stability, because deamidation increases
478 protein net charges which reduce the intermolecular interaction of proteins (Mirmoghtadaie,
479 Kadivar, & Shahedi, 2009). Along with the effects of protein deamidation, protein
480 conformational changes (e.g., the unfolding of proteins) induced by pH and heating of whey
481 proteins markedly improves foaming properties. However, in this study, foaming properties
482 of WPC powders were not well correlated with the conformational changes of proteins based
483 on the FTIR results (Table 3). Compared with WPC-SD, only WPC-pH3-H-SD and WPC-
484 pH10-SD exhibited changes in foaming properties while the structural changes of proteins

485 occurred in all samples to different extents. Foaming properties might depend on the level of
486 protein secondary structural alteration. However, foaming is a very complicated process,
487 depending on multiple factors (Huppertz, 2010). Heating of protein solutions at low and high
488 pH levels affected not only lactose hydrolysis but also the mineral equilibrium state,
489 particularly Ca^{2+} ions (Zadow, 1992), leading to changes in foaming properties of protein
490 solutions. Thus, the interesting correlation between foaming properties, the degree of
491 deamidation and solubility of whey protein under heat and pH treatment requires further
492 studies.
493
494 *3.3.3. Emulsifying properties*
495 The impact of pH and heat on emulsion properties of spray dried WPC was
496 investigated. As shown in Fig. 3c, spray drying alone did not affect emulsion ability (EA) and
497 emulsion stability (ES) of WPC powders ($p > 0.05$) as both EA and ES of WPC and WPC-
498 SD were similar. pH treatment or pH treatment followed by heating significantly improved
499 emulsion ability and emulsion stability of WPC powders ($p < 0.05$). The improvement of
500 emulsifying properties is due to the net result of deamidation extent, peptide bond cleavage,
501 and protein unfolding that took place during the deamidation process caused by pH and
502 heating. Similarly, Fachin and Viotto (2005) reported that the emulsifying properties of WPC
503 produced by ultrafiltration were greatly affected by pH and heat treatments (prior to
504 ultrafiltration), which determined the degree of protein denaturation. A slight degree of whey
505 protein denaturation (e.g., pH 6.0–7.0 and 75 °C/2 min) enhanced the emulsifying properties,
506 due to an exposure of hidden hydrophobic groups of the globular proteins, while excessive
507 protein denaturation (e.g., pH 7.0 and 80 °C/2 min) declined emulsifying properties because
508 of the decrease in surface hydrophobicity. Improved emulsifying properties due to
509 deamidation have been reported for different proteins such as barley glutelin (Zhao et al.,

2011), rice proteins (Paraman, Hettiarachchy & Schaefer, 2007) and skim milk (Miwa et al., 2010). There might be a combination effect of pH and heat-induced denaturation and deamidation on emulsifying properties of whey protein powder. However, whether denaturation comes first and influences deamidation or vice versa is a challenging question and requires a model study to follow up.

4. Conclusion

This study presents the first investigation of non-enzymatic deamidation in whey protein powder using high resolution mass spectrometry. The degree of deamidation of WPC was dependent on temperatures, heating time and pH in which N deamidation increased significantly at pH 10 compared with pH 3. The pH (3 and 10) and heating (145 °C/120 s) did not influence marked physical properties (colour, moisture content, water activity, and true density) of spray-dried WPC powders, but caused protein unfolding. In terms of functional properties (solubility, foaming properties and emulsifying properties), while the samples treated at pH 10 did not show any effect in solubility and foaming properties, those treated at pH 3 exhibited a reduction in solubility and foamability but markedly improved foam stability. Interestingly, the emulsifying properties of spray-dried WPC powders were significantly improved under all pH and heat treatment conditions. It is noteworthy that the results imply that pH treatment and spray drying could be an effective way to improve functional properties of whey powders. Therefore, it is considered that WPC having the intended functional characteristics can be prepared by optimising the treatment conditions (e.g., pH, temperatures and possibly protein concentration).

Further research is needed on the structural changes of proteins on the functional properties of spray-dried WPC. In particular, it is necessary to analyse the effect of the degree

of non-enzymatic deamidation and hydrolysis on structural changes and functional characteristics. It has also been suggested that factors other than proteins in WPC such as lactose and salts may also affect functional properties, so comparative studies using desalted whey ingredient may also be useful. To develop applications to food, it is helpful to evaluate the effects on various functional properties such as gel formation and thermal stability in addition to solubility, foaming, and emulsification. Furthermore, by conducting comparative studies with past studies on enzymatic deamidation of whey proteins (e.g., measurement of ammonia release, analysis of circular dichlorism, size exclusion chromatography and gel electrophoresis), it can be considered the significance of non-enzymatic deamidation in more depth.

In summary, deamidation and structural changes of whey proteins by pH and heat treatment were confirmed in this study, nevertheless these changes did not have any correlation with the functional characteristics of WPC. In fact, the WPC sample such as WPC-pH10-H-SD, which had the greatest degree of change in FTIR, had no significant difference in functional characteristics (solubility, foaming, emulsification) with other samples. It is inferred that the preparation conditions of spray dried WPC samples in this study did not bring about sufficient non-enzymatic deamidation to significantly improve the functional properties of WPC. In the future, quantitative analysis is necessary to determine the extent to which non-enzymatic deamidation affects the functional properties of whey protein powders.

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696

Figure legends

Fig. 1. Normalised deamidation (%) of β-Lg in WPC solutions (7%, w/w) subjected to pH adjustment to 3.0 and 10.0 and heating at 95 and 120 °C for 3 and 15 min. Four deamidated peptides represented N (A, C) and Q deamidation (B, D). In x-axis, C6.2, C3 and C10: control samples at pH 6.2, 3.0 and 10, respectively without heating; 95 and 120: heating temperatures (^{o}C); 3 and 15: heating time (min).

Fig. 2. Deconvolution of the amide I band in the FTIR spectra of WPC powders. WPC, commercial WPC powder; WPC_SD, powder produced by spray drying of WPC solution (7.0%, w/w); WPC_pH3_SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 3.0; WPC_pH3.0_H_SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 3.0 and heating at 145 °C/120 s; WPC_pH10_SD, powder produced by spray drying of WPC solution (7.0%. w/w) subjected to pH adjustment to 10.0; WPC_pH10_H_SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 10 and heating at 145 °C/120 s. The back continuous curves (almost overlapped with red dashed curves) are FTIR spectra of amide I. The deconvolution and peak fitting resulted in sum (red dashed curves) and individual peaks (blue continuous curves).

Fig. 3. Solubility (a), foaming properties (b: hatched bars, foamability; solid bars, foam stability) and emulsifying properties (c: hatched bars, emulsion ability; solid bars, emulsion stability) of WPC powders. WPC, commercial WPC powder; WPC_SD, powder produced by spray drying of WPC solution (7.0%, w/w); WPC_pH3_SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 3.0;

WPC_pH3.0_H_SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 3.0 and heating at 145 °C/120 s; WPC_pH10_SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 10.0; WPC_pH10_H_SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 10 and heating at 145 °C/120 s.

Table 1

Deamidated peptides identified and quantified in β-Lg from WPC solutions. [a]

Sequence	Residues	Charge	*m/z*	RT (min)
LIVTqTMK	17–24	2	467.7675	8.39
LIVTqTmK	17–24	2	475.7650	6.95
WEnDECAQK	77–85	2	590.7324	5.89
WENDECAqK	77–85	2	590.7324	5.68
WEnGECAQK	77–85*	2	561.7297	5.60
WEnGECAqK	77–85*	2	562.2217	5.93
IDALnENK	100–107	2	459.2324	6.62
IDALnEnK	100–107	2	459.7244	6.89
CMEnSAEPEQSLVCQCLVR	122–140	3	770.9989	11.05
CMENSAEPEqSLVCQCLVR	122–140	3	770.9989	11.23
LSFnPTQLEEQCHI	165–178	2	858.8985	12.46
LSFNPTQLEEqCHI	165–178	2	858.8985	12.18
LSFnPTQLEEqCHI	165–178	2	859.3905	12.74

[a] n, q, deamidation; m, oxidation; RT, retention time; C, carbamidomethylated cysteine. An asterisk indicates variant B of β-Lg.

Table 2

Moisture content (MC), water activity (a_w), true density and colour of WPC powders. [a]

Samples	MC, % (w/w)	a_w	True density (g cm^{-3})	L*	a*	b*	Whiteness
WPC	6.46 ± 0.19^{ab}	0.33 ± 0.02^{a}	1.084 ± 0.003^{a}	90.29 ± 0.04^{d}	-0.70 ± 0.07^{cd}	15.88 ± 0.09^{a}	81.38 ± 0.08^{d}
WPC-SD	6.39 ± 0.32^{ab}	0.26 ± 0.02^{ab}	0.976 ± 0.009^{b}	96.48 ± 0.03^{b}	-0.54 ± 0.03^{ab}	5.72 ± 0.28^{c}	93.26 ± 0.24^{ab}
WPC-pH3-SD	5.89 ± 0.99^{ab}	0.29 ± 0.05^{ab}	0.883 ± 0.029^{c}	97.52 ± 0.05^{a}	-0.83 ± 0.02^{d}	5.44 ± 0.19^{c}	93.96 ± 0.17^{a}
WPC-pH3-H-SD	4.75 ± 0.34^{b}	0.21 ± 0.01^{b}	0.873 ± 0.002^{c}	97.40 ± 0.11^{a}	-1.12 ± 0.11^{e}	6.08 ± 0.38^{bc}	93.29 ± 0.35^{ab}
WPC-pH10-SD	7.63 ± 1.51^{a}	0.31 ± 0.08^{ab}	0.955 ± 0.019^{b}	96.24 ± 0.32^{bc}	-0.47 ± 0.02^{a}	6.67 ± 0.29^{b}	92.33 ± 0.33^{c}
WPC-pH10-H-SD	7.01 ± 0.91^{ab}	0.27 ± 0.03^{ab}	0.969 ± 0.032^{b}	95.97 ± 0.19^{c}	-0.68 ± 0.03^{bc}	5.80 ± 0.35^{c}	92.90 ± 0.30^{bc}

[a] Superscript lowercase letters indicate statistically significant differences between samples in a column ($p < 0.05$). WPC, commercial WPC powder; WPC-SD, powder produced by spray drying of WPC solution (7.0%, w/w); WPC-pH3-SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 3.0; WPC-pH3-H-SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 3.0 and heating at 145 °C/120 s; WPC-pH10-SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 10.0; WPC-pH10-H-SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 10 and heating at 145 °C/120 s.

Table 3

The percentages (%) of protein secondary structures of WPC powders produced from different treatment conditions. [a]

Samples	β-sheet	α-helix/unordered	β-turn	Side chain
WPC	38.08 ± 1.14[b]	32.59 ± 0.99[d]	25.52 ± 1.70[b]	3.82 ± 0.46[b]
WPC-SD	30.22 ± 1.51[c]	51.76 ± 0.30[a]	13.50 ± 1.73[a]	4.52 ± 0.14[b]
WPC-pH3-SD	25.84 ± 1.46[c]	41.36 ± 0.56[c]	27.38 ± 2.22[b]	5.42 ± 0.53[b]
WPC-pH3-H-SD	39.27 ± 0.82[b]	47.65 ± 0.44[b]	11.68 ± 0.47[a]	1.40 ± 0.29[c]
WPC-pH10-SD	42.21 ± 2.28[b]	21.61 ± 0.47[e]	23.83 ± 2.47[b]	12.35 ± 1.28[a]
WPC-pH10-H-SD	52.95 ± 3.04[a]	16.49 ± 0.62[f]	26.40 ± 3.01[b]	4.16 ± 1.08[b]

[a] Protein secondary structures determined from amide I FTIR peak, 1700–1600 cm^{-1}. Different letters superscript lowercase letters in the same column indicate significant differences between samples ($p < 0.05$). WPC, commercial WPC powder; WPC-SD, powder produced by spray drying of WPC solution (7.0%, w/w); WPC-pH3-SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 3.0; WPC-pH3-H-SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 3.0 and heating at 145 °C/120 s; WPC-pH10-SD, powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 10.0; WPC-pH10-H-SD: powder produced by spray drying of WPC solution (7.0%, w/w) subjected to pH adjustment to 10 and heating at 145 °C/120 s.

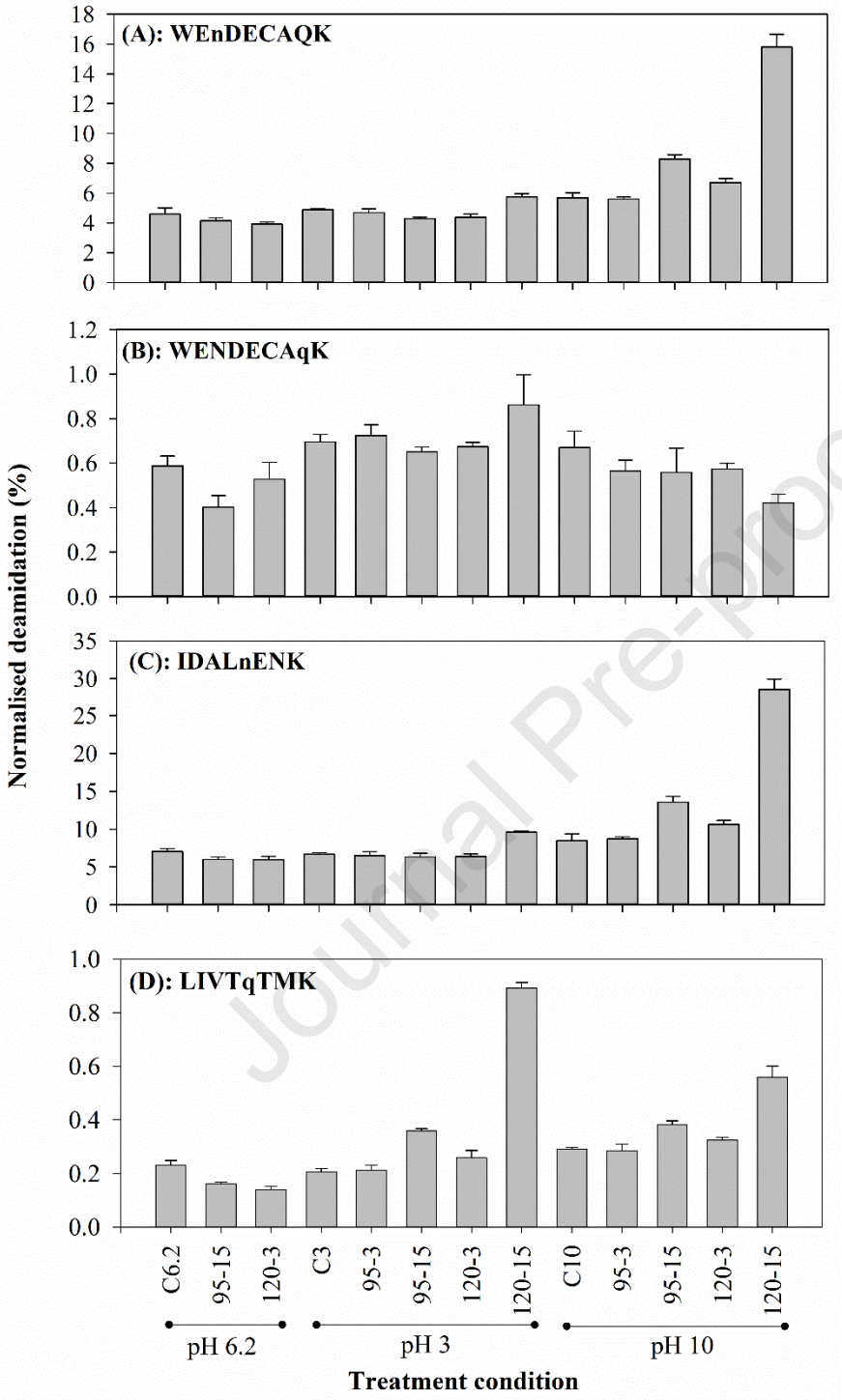
(A): WEnDECAQK
(B): WENDECAqK
(C): IDALnENK
(D): LIVTqTMK
Normalised deamidation (%)
C6.2
95-15
120-3
C3
95-3
95-15
120-3
120-15
C10
95-3
95-15
120-3
120-15
pH 6.2
pH 3
pH 10
Treatment condition

Figure 1.

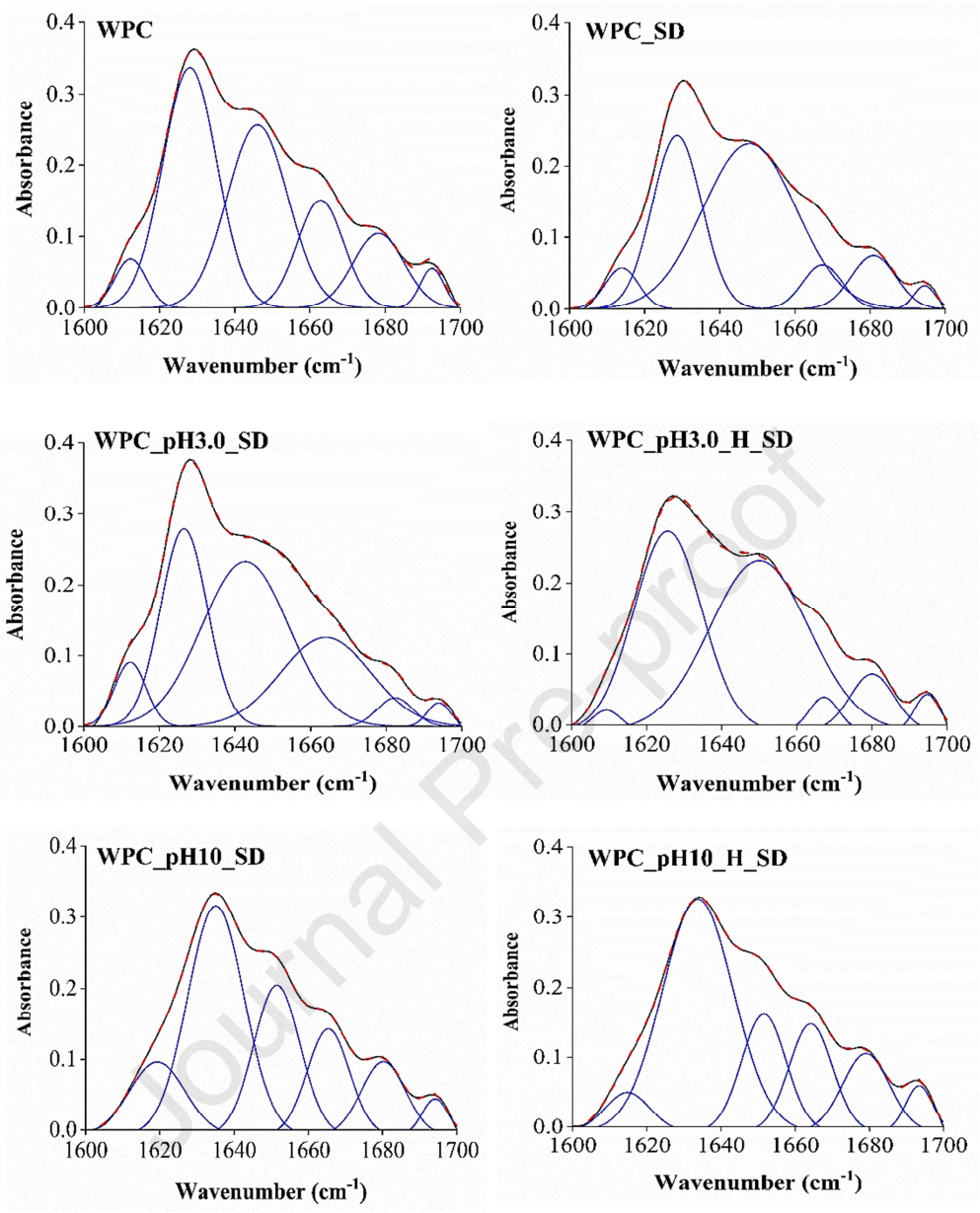
WPC
WPC_SD
WPC_pH3.0_SD
WPC_pH3.0_H_SD
WPC_pH10_SD
WPC_pH10_H_SD
Absorbance
Wavenumber (cm-1)
0.0
0.1
0.2
0.3
0.4
1600
1620
1640
1660
1680
1700

Figure 2.

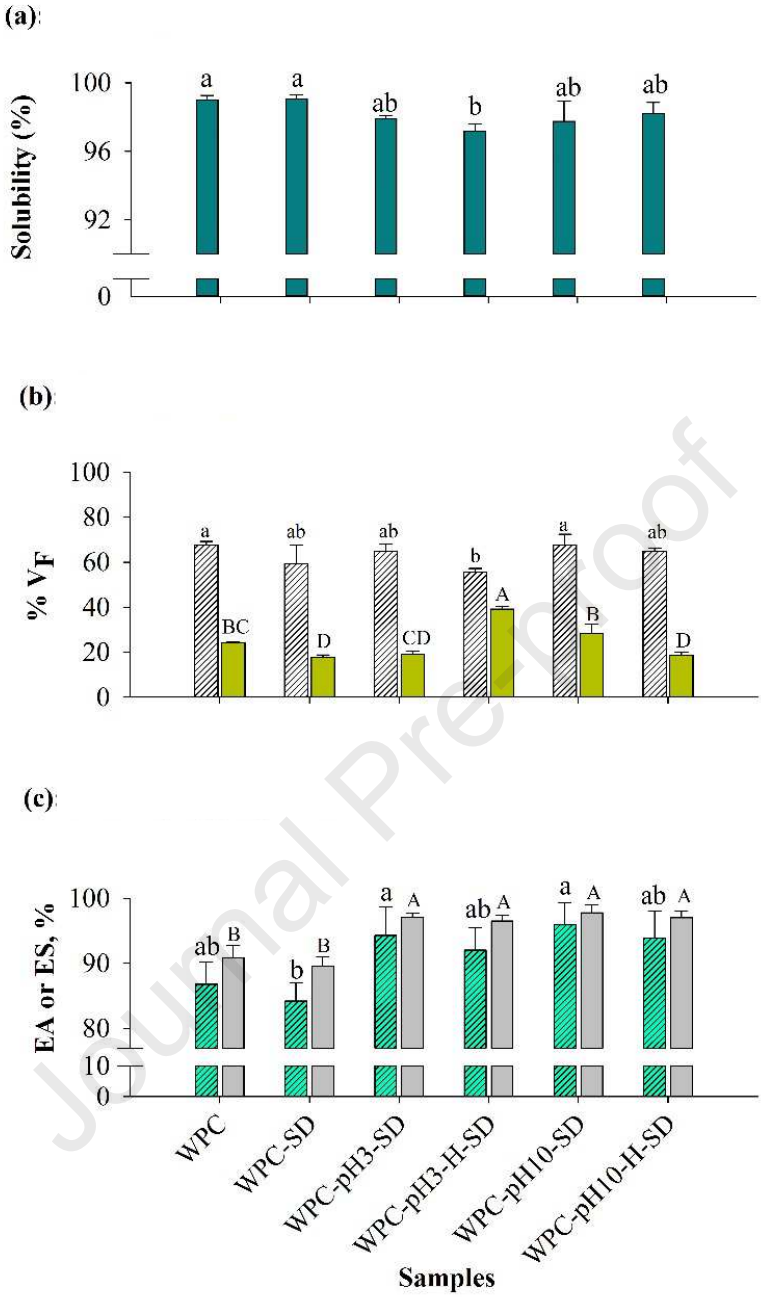
(a)
Solubility (%)
100
96
92
0
a
a
ab
b
ab
ab
(b)
% V_F
100
80
60
40
20
0
a
BC
ab
D
ab
CD
b
A
a
B
ab
D
(c)
EA or ES, %
100
90
80
10
0
ab B
b B
a A
ab A
a A
ab A
WPC
WPC-SD
WPC-pH3-SD
WPC-pH3-H-SD
WPC-pH10-SD
WPC-pH10-H-SD
Samples

Figure 3.

Declaration of interests

☒ 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.

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