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Corresponding Author:	Stephanie P Bull, Ph.D. University of Reading Reading, UNITED KINGDOM
First Author:	Stephanie P Bull, Ph.D.
Order of Authors:	Stephanie P Bull, Ph.D.
	Vitaliy V Khutoryanskiy, Ph.D.
	Jane K Parker, Ph.D.
	Marianthi Faka, Ph.D.
	Lisa Methven, Ph.D.
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Oral retention of thermally denatured whey protein: *in vivo* measurement and structural observations by CD and NMR

Stephanie P. Bull [a,*], Vitaliy V. Khutoryanskiy [b], Jane K. Parker [a], Marianthi Faka [c], Lisa Methven [a,]

[a] Department of Food and Nutritional Sciences, University of Reading, Whiteknights, Reading, Berks, RG6 6AD, United Kingdom

[b] Department of Pharmacy, University of Reading, Whiteknights, Reading, Berks, RG6 6AD, United Kingdom

[c] Volac International Ltd, 50 Fishers Lane, Orwell, Royston, Hertfordshire, SG8 5QX, United Kingdom

* Corresponding author. Tel.: +44 118 378 8593. E-mail address: s.p.bull@reading.ac.uk

Author email addresses: v.khutoryanskiy@reading.ac.uk, j.k.parker@reading.ac.uk, marianthi.faka@volac.com, l.methven@reading.ac.uk

Abstract

This study investigated structural changes and the *in vivo* retention in the oral cavity of heated whey protein concentrate (WPC). Heated WPC was shown to have both a higher retention time in the oral cavity compared to unheated whey protein up to 1 minute post swallow, and a concomitant increase in free thiol concentration. Nuclear magnetic resonance and circular dichroism demonstrated structural changes in the secondary and tertiary structures of the WPC upon heating. Structural loss of the β-barrel was shown to increase during heating, leading to the exposure of hydrophobic regions. The increase in free thiols and hydrophobic regions are two factors which are known to increase mucoadhesive strength and hence increase oral retention of heated whey protein which may subsequently increase the perception of mouthdrying.

1 Introduction

Whey protein is often heated during processing; this can be to a mild extent during the production of a spray dried powder, or to a greater extent when manufactured for incorporation into drinks and snacks. The heating of whey protein at temperatures over 70 °C can cause denaturation (Dewit & Swinkels, 1980; Etzel, 2004), which is linked to an increase in the perception of mouthdrying (Bull et al., 2017). Whey protein provides an important source of protein to patients at risk of sarcopenia; however, an increase in mouthdrying has been linked to a reduction in compliance (Gosney, 2003; Withers, Gosney, & Methven, 2013). Mouthdrying in whey protein has been attributed to interactions with salivary proteins (Vardhanabhuti & Foegeding, 2010; Ye, Streicher, & Singh, 2011), and more recently to interactions with the oral mucosa, a phenomenon known as mucoadhesion (Withers, Cook, Methven, Gosney, & Khutoryanskiy, 2013; Ye, Zheng, Ye, & Singh, 2012). An *in vitro* dynamic model found an increase in turbidity associated with the addition of whey protein isolate to artificial or whole human saliva, which was related to higher scores of astringency (Andrewes, Kelly, Vardhanabhuti, & Foegeding, 2011), supporting mucoadhesion as the cause of mouthdrying in whey protein beverages.

Whey protein concentrate (WPC) is a spray-dried powder of 80% protein, which additionally contains lactose, calcium salts and lipids. As a complex mixture, there are many factors which affect WPC denaturation. When heating WPC, the different components are able to interact and influence the denaturation of proteins: for example, β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and bovine serum albumin (BSA) form both homopolymers and heteropolymers when heated together (Havea, Singh, & Creamer, 2001). These biopolymers form large particles, which have been suggested to lead to an increase in astringency (Ye et al., 2011).

The presence of calcium ions in WPC also affects thermal denaturation and aggregation through calcium bridges between negative charges on proteins, shielding of negative charges, and increasing ion-specific hydrophobic interactions (Havea, Singh, & Creamer, 2002). The increased interactions due to calcium ions can lead to less disulfide bonding during aggregation, however the increased interactions due to charge shielding and hydrophobic interactions lead to larger particle sizes overall (Riou, Havea, McCarthy, Watkinson, & Singh, 2011), both of which can increase mucoadhesive strength.

The denaturation of proteins can alter the number of free accessible thiol groups due to protein unfolding, disulfide bond formation, and aggregation. The total thiol content of β-LG increases upon heating at pH 3, but decreases at pH 5 – 7; this could be due to unfolding and fragmentation of β-LG exposing more thiol groups at low pH and high temperature, and participation of free thiol groups in thiol-thiol oxidation or thiol-disulfide exchange occurring at higher pH leading to aggregation (Rahaman, Vasiljevic, & Ramchandran, 2015), due to the unfolding of the protein, revealing a buried cysteine residue (Zeiler & Bolhuis, 2015). Polymers containing thiol groups can form disulfide bonds with mucosal surfaces, leading to increased mucoadhesive strength (Bernkop-Schnürch, 2005); therefore altering the number of free accessible thiols in WPC through thermal denaturation could affect mucoadhesive interactions.

Mucoadhesion can occur through many mechanisms: covalent bonding, including formation of disulfide bridges; non-covalent interactions, such as Van der Waals forces, hydrogen bonding and hydrophobic interactions; and electrostatic forces. Descriptions of these mechanisms have been covered in reviews elsewhere (Cook, Bull, Methven, Parker, & Khutoryanskiy, 2017). Mucoadhesion can be measured using a range of techniques: physical techniques, such as rheology, change in particle size and charge, and change in structure observed by circular dichroism (CD) (Celebioglu et al., 2015; Thirawong, Kennedy, & Sriamornsak, 2008); *in vitro* methods, such as measuring wash-off and tribological methods (Cave, Cook, Connon, & Khutoryanskiy, 2012; Dresselhuis, de Hoog, Stuart, & van Aken, 2008); and *in vivo* oral retention methods (Cook, Woods, Methven, Parker, & Khutoryanskiy, 2018).

CD has been used to study whey protein structure by measuring the effect of chiral molecules on circularly polarised light to predict secondary structural features. CD has been used in the literature to study both individual whey proteins (Celebioglu et al., 2015; Chandrapala, Zisu, Kentish, & Ashokkumar, 2012; Wada, Fujita, & Kitabatake, 2006; Wijesinha-Bettoni et al., 2007), and whey protein mixtures, such as isolate or concentrate (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Liu & Zhong, 2013; Tomczynska-Mleko et al., 2014). Effects of thermal treatment on whey protein has been observed using CD, showing a decrease in α-helical structure and an increase in

unfolding upon heating (Tomczynska-Mleko et al., 2014; Wada et al., 2006; Wijesinha-Bettoni et al., 2007).

Nuclear magnetic resonance (NMR) has been used previously to study various structural changes in β-LG (Belloque & Smith, 1998; Celebioglu et al., 2015, Indrawati et al., 2007). The study of the structural denaturation of β-LG at 75 °C showed unfolding of the protein at neutral pH, with suggestions that the α-helix unfolds and exposes a reactive thiol group (Belloque & Smith, 1998). Celebioglu et al used high-resolution NMR to detect interactions between β-LG and bovine serum mucin, with suggestions of hydrogen bonding and hydrophobic effects as the mechanisms of interaction (2015).

The factors affecting mucoadhesion are varied, especially in a system as complex as WPC. This study aims to investigate the effect of thermal processing on the retention time of whey protein concentrate beverages in the oral cavity, and the structural and physicochemical characteristics which may underpin the oral retention.

The aim of this study is to determine a relationship between thermal denaturation and mucoadhesion by measuring the oral retention of thermally treated model whey protein concentrate beverages. It is hypothesised that WPC exposed to longer heating times will have higher oral retention, which may be caused by either increased free ionic calcium, increased accessible thiol groups, or a change in secondary or tertiary structure leading to increased intermolecular interactions. The structural and physicochemical characteristics of the samples were analysed to investigate the underlying mechanism of adhesion.

2 Materials and methods

The whey protein concentrate (WPC) used was Volactive Ultrawhey 80 Instant (Volac International Limited, Orwell, Royston, UK), a dry powder with a minimum protein content of 80% and containing soy lecithin (0.5% maximum) as an emulsifying agent. The remaining 20% contained moisture (5%), fat (7%), lactose (4%), and minerals.

DTNB (5,5-dithio-bis-(2-nitrobenzoic acid), deuterium oxide, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, and L-cysteine hydrochloride monohydrate were supplied by Sigma-Aldrich (Dorset, UK).

2.1 Preparation of whey protein beverages

Model WPC beverages were prepared by addition of WPC powder to deionised water (10% w/v). All samples were stirred for 30 min at room temperature to hydrate the powder (25 ± 2 °C). A native sample was then stirred for a further 60 min at room temperature (WPC00). Three samples were stirred while being heated in a water bath at 70 °C for 5, 10 and 20 min once the sample had reached 70 °C, which took 10 – 12 min to reach temperature (WPC05, WPC10, and WPC20 respectively). These heating times were selected as aggregation occurs after 20 min of heating at 70 °C, and sensory differences in drying were previously observed between samples heated for 5, 10 and 20 min (Bull et al., 2017).

The samples were cooled in a cold water bath until they reached room temperature, then allowed to hydrate overnight at 4 °C. The pH of all samples ranged from 6.5 to 6.7 (Mettler Toledo SevenEasy, Switzerland; 22 ± 3 °C).

2.2 In vivo protein retention method

An *in vivo* retention study was used as a measure of mucoadhesion. Five healthy volunteers were recruited; four males and one female, aged between 25 and 30. Before the session, each volunteer rinsed their mouth with a salt solution (1% w/v) to clear the mouth of any particulate matter, before rinsing with water and waiting for 2 min. Saliva was collected, as described in section 2.2.1, for each sample and time point in triplicate during separate sessions. Three samples were selected from previous work by the authors (WPC00, WPC05, and WPC20) (Bull et al., 2017) to represent a range of sensory attributes associated with drying and physical characteristics.

The study was given a favourable ethical opinion for conduct by the University of Reading, School of Chemistry, Food and Pharmacy (study number 27/15). Previous studies have indicated that 5 subjects

are sufficient for such *in vivo* studies where difference in retention due to sample differences were greater than inter-individual differences (Cook et al., 2018).

2.2.1 ***Saliva collection***

During each session, each volunteer was presented with one type of sample (WPC00, WPC05 or WPC20) to avoid crossover effects. A blank sample of unstimulated saliva was collected from each participant before consuming any sample. The volunteer was presented with 5 mL of the sample and instructed to swill it around their mouth for 10 s before swallowing, after which a countdown timer was started. The timer was set to count down from 5, 10, 20, 30, 45, 60, 120, 180, 240 or 300 s, with these times presented in a balanced order across samples and volunteers (using Williams Design Latin squares). At the end of the timer, the volunteer was prompted to spit their saliva into a collection tube for analysis. Volunteers rinsed with warm water during a 2 min enforced break to clear mouth of any residual sample before testing the subsequent sample-time combination. Only one sample type was presented per session (one session per day), with one aliquot (5 mL) per time point. Triplicate sessions for each WPC sample gave a total of 30 saliva samples per volunteer per sample. Volunteers recorded number of natural swallows of saliva accumulation in the mouth between swallowing the sample and collection of saliva ($\leq$1 min^{-1}), to ensure saliva was naturally removed from the mouth at similar rates. As this was the case, no intervention was needed to prevent volunteers from excessively swallowing saliva between the initial swallowing of the sample. Collection tubes were weighed before and after spitting to monitor saliva weight for each volunteer.

2.2.2 ***Protein quantification***

Protein concentration was determined using the Bradford microplate assay (Bradford, 1976; Zor & Seliger, 1996) in triplicate on each of the triplicate saliva collections, giving a total of 9 readings per volunteer per time point for each of three samples (Epoch, Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA).

The total amount of whey protein for each sample was calculated as the sample weight (assuming saliva density at 1 g mL^{-1} (Kubala et al., 2018)) multiplied by the protein concentration determined by the

Bradford assay, subtracting each volunteer's baseline. The baseline was calculated as the average protein concentration in the volunteer's blank saliva sample (from their 9 sessions) multiplied by the weight of the sample.

2.3 Free ionic calcium measurement

Free ionic calcium in WPC samples was measured using a calcium selective ion electrode (Sentek, Essex, UK) and pH meter as described by Lin, Lewis, and Grandison (2006). Measurements were performed in triplicate on each of three processing replicates at ambient temperature (20.3 ± 0.1 °C). Calibration of the electrode was performed using 0.5, 1, 2.5 and 5 mM solutions of $CaCl_2$ in a dilution standard comprising 13.5 mM imidazole and 67.5 mM KCl.

2.4 Accessible thiol group measurement

Accessible thiol content of WPC samples was measured using an adaptation of Ellman's assay (Bravo-Osuna, Teutonico, Arpicco, Vauthier, & Ponchel, 2007; Withers, Cook, et al., 2013). 10% WPC samples were diluted using a phosphate buffer (0.1 M, pH 8) to reach a final concentration of 2 mg mL^{-1}. DTNB was dissolved in phosphate buffer (0.3 mg mL^{-1}) and added to the dilute samples in a ratio of 1:1. The treated samples were left to incubate in the dark for 2 h before absorbance at 412 nm was measured (Epoch, Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA). Cysteine hydrochloride standards (25 – 750 μM in phosphate buffer) were used to establish a standard curve, from which the thiol content of the samples was calculated. Measurements were performed in triplicate on each of three processing replicates.

2.5 Circular dichroism

CD spectra were recorded using a Chirascan CD Spectrophotometer (Applied Photophysics Ltd., Leatherhead, UK) in both near and far-UV ranges. Measurements were performed in triplicate on three processing replicates for each sample; the spectrum of the solvent was subtracted from the average of the triplicate scans of samples.

Near-UV spectra were recorded over a range of 450 down to 260 nm using a 1 mm pathlength cuvette with a step size of 2 nm. For the near-UV spectra, WPC samples were diluted to obtain a total concentration of 1% (w/v). Far-UV spectra were recorded over a range of 280 to 185 nm using a 0.1 mm pathlength cuvette with a step size of 1 nm. For this wavelength range, WPC samples were diluted to obtain a total concentration of 0.1% (w/v).

2.6 Nuclear magnetic resonance spectroscopy

Samples of WPC00, WPC05, WPC10, and WPC20 were prepared at concentrations of 10 mg mL^{-1} in a mixture of deuterium oxide and water (10% D_2O, 90% H_2O; Sigma-Aldrich). ^1H NMR spectra were recorded for all samples. 2D NOESY spectra were collected for all samples to confirm the appearance of peaks in overlapped ^1H spectra of WPC samples. A standard pre-saturation sequence was used to suppress water signals. All spectra were recorded on a 700 MHz Bruker Avance III spectrometer (Bruker, Billerica, MA, USA).

2.7 Statistical analysis

IBM SPSS Statistics (version 21) was used to carry out three-way repeated measures analysis of variance (RM-ANOVA) on the *in-vivo* retention data using sample (n = 3), assessors (n = 5) and time (n = 10) as explanatory variables. Analytical data were analysed by one-way ANOVA using IBM SPSS Statistics (version 21). Multiple pairwise comparisons were also carried out using IBM SPSS Statistics (version 21) using Tukey's HSD test (p = 0.05).

3 Results and discussion

3.1 In vivo protein retention method

All collected saliva samples showed a general trend of initially increasing in protein concentration over time, followed by a gradual decrease, with a plateau reached at approximately 3 min post swallow (Figure 1, 180 s). WPC20 was significantly higher in protein in the collected saliva samples overall compared to WPC00 (p = 0.007), tending to give higher protein weights over the first 60 s. While WPC20 peaked at 30 s; WPC05 peaked at 10 s before gradually declining, although there were no

significant differences in protein collected due to sample at any specific time point. Average saliva weights collected for each time point are shown in Figure 2; there was no significant effect of sample or time on saliva weight.

It is known that thermally treated whey protein gives an increased perception of mouthdrying (Bull et al., 2017), which builds up over repeated consumption and is consistent with mechanisms of mucoadhesion (Vardhanabhuti & Foegeding, 2010; Withers, Cook, et al., 2013). We hypothesise the build-up of a drying sensation to be caused by an accumulation of WPC in the oral cavity. The *in vivo* oral retention results obtained here showed retention of WPC in the mouth for up to 1 min after just one 5 mL sip. As previous work has shown repeated consumption leading to prolonged drying sensation (Bull et al., 2017) we anticipate that such repeated intake would lead to a greater building up of protein in the oral cavity. The higher protein weights observed in saliva for WPC20 indicate the presence of more protein in the mouth than for WPC00; this correlates with a higher sensory score for drying, mouthcoating, and chalky in WPC20, alongside larger particle sizes, as shown in previous work by the authors (Bull et al., 2017). WPC05 was not found to be significantly different to either WPC00 or WPC20. The protein weight in collected saliva increases initially, presumably due to the combination of a release of adhered whey protein from the mucosal surfaces into the saliva, and an increased production of protein in stimulated saliva. After the maximum, the protein levels in the saliva decrease as the WPC is removed from the oral mucosa and swallowed. We have established in a further paper that using a non-protein liquid bolus, formulated using whey permeate rather than WPC, does not lead to protein accumulation in the oral cavity post swallowing (Norton et al., 2020). This therefore concludes that the main cause of increase in oral retention of protein post consumption results from whey protein rather than salivary proteins. In addition, having demonstrated above that there was no difference in saliva weight post consumption of WPC20 versus WPC00, this strongly indicates that the WPC20 did not stimulate saliva flow and hence lead to an increase in salivary protein versus the WPC00. In summary, the data strongly supports that the increase in oral protein post consumption of the WPC20 sample is due to protein from the whey remaining in the mouth rather than in increase in salivary protein.

Andrewes et al. (2011) found similar results when studying whey protein isolate (WPI) and whole saliva *in vitro*. They measured turbidity, with maximum turbidity measured 60 s after simulating swallowing 5 mL of acidic WPI solution. Due to the acidic nature of the WPI samples used, the clear samples became turbid upon the increased pH caused by the constant addition of saliva. The WPC samples used in the current study had a neutral pH (6.5 – 6.7); therefore, changes observed are not related to pH-driven aggregation.

Average saliva weights were consistent across time points, with no significant difference found across the samples, showing that the effects observed are not due to an increased amount of stimulated saliva; however, a possible change in saliva composition must be considered. Salivary proteins have been found to increase in concentration when an astringent compound was present in the mouth (Dinnella, Recchia, Vincenzi, Tuorila, & Monteleone, 2010), contributing to the higher protein weights after consuming WPC; however, this has only been shown using polyphenol astringents, which have a different mechanism of action to the mouthdrying caused by dairy proteins.

The volunteers used in this study were healthy young adults (aged 25 – 30). Older adults (aged 65 and older) have been shown to have saliva with higher levels of protein, K^+, Cl^-, amylase, lysozyme, albumin and secretory immunoglobulin (Nagler & Hershkovich, 2005b). Older adults also have a reduced salivary flow rate in comparison to younger adults (Nagler & Hershkovich, 2005a). These factors affect mucoadhesion of WPC in many ways: a reduced salivary flow would expose more mucosal tissue to the WPC, leaving it available to adhere; a decreased flow rate would also reduce the rate of clearing in the mouth; an increased proportion of salivary proteins would allow more interactions to occur, increasing mucoadhesion; different salivary proteins may interact more strongly, and therefore alter the degree and mechanism of mucoadhesion. This supports the proposed hypothesis that an increase in denaturation would lead to an increase in mucoadhesive strength.

3.2 Free ionic calcium and accessible thiol concentration

No significant difference was observed in free ionic calcium between the samples, and therefore it is unlikely that calcium will have an effect on the mucoadhesive strength of the WPC samples. Free thiol groups can increase as a result of protein denaturation (Zeiler & Bolhuis, 2015), and indeed the free thiol concentration measured did increase significantly with heating time of the WPC (Table 1).

Table 1: Free ionic calcium and accessible thiol concentrations in 10% w/v WPC samples. Mean values ± 2 standard deviations. Superscript letters in a column indicate significantly different groupings (p = 0.05).

Sample	Ca^{2+} concentration (mM)	Accessible thiol concentration (mM)
WPC00	3.45 ± 0.53 [a]	4.03 ± 0.44[a]
WPC05	3.41 ± 0.32 [a]	5.50 ± 0.66[b]
WPC10	3.38 ± 0.26 [a]	5.57 ± 0.95[b]
WPC20	3.08 ± 0.57 [a]	6.87 ± 0.79[c]

As no significant difference was observed in free ionic calcium concentrations between WPC samples, this is an unlikely cause for the increased retention in WPC20; however, it does not discount that calcium binding to mucin could contribute to the drying mechanism in all WPC samples equally. The trend that free ionic calcium decreased with heating time might indicate that less free ionic calcium is available to interact with mucin, and instead the calcium is bound to the whey proteins due to structural changes; however, this non-significant effect requires further proof.

There was an increase in accessible thiol groups with longer heating times, which would increase mucoadhesive strength due to formation of disulfide bridges with cysteine groups in mucins (Bernkop-Schnürch, 2005). This increase in thiol may be due to conformational changes upon denaturation as observed by CD and NMR. Sava, Van der Plancken, Claeys, and Hendrick (2005) found that surface thiol groups of β-LG increased with heating time at 70 °C at neutral pH, consistent with the findings in the present study with WPC. This increase in accessible thiols and therefore mucoadhesive potential appears to be an underlying cause of the increase in oral retention observed for WPC20.

3.3 Circular dichroism

Far-UV CD spectra were collected on more dilute samples than near-UV spectra to reduce light scattering and improve signal. Spectra for all samples show a broad peak at 208 nm with an inflection around 220 nm; although the intensity of the 208 nm peak increased with heating WPC from 0 to 5 and 10 min, after 20 min of heating the peak was the same as for the native sample (Figure 3).

Higher concentrations were used for near-UV CD spectra to allow observation of characteristics in this wavelength region, as excessive light scattering was not an issue. A change in structure was observed upon heating in the aromatic region (260 – 310 nm); with a reduction in the peak size for samples with higher heating times (

).

In order to compare samples with unknown concentrations of specific proteins, the CD curve shape must be compared, rather than peak intensities as these relate to concentration: spectra were normalised by the area between 0 and the CD curve. These normalised spectra were each subtracted from WPC00 spectra in order to compare the differences observed upon heating of the samples for different times. Spectra in display error bars around zero as a measure of significant differences between samples.

Far-UV CD spectra found an increase in signal around 180 – 210 nm, with larger differences seen for WPC10, and fewer significant differences between WPC20 and WPC00. An increase in negative signal was observed for WPC20 between 250 and 280 nm in comparison to WPC00 and WPC10. Near-UV CD spectra showed significant differences moving further towards lower wavelengths with increasing heating time, with larger differences observed from the WPC00 spectra for WPC10 and WPC20 ().

The far-UV CD spectra for WPC samples in this study were similar to those observed previously for WPI (Liu & Zhong, 2013; Tomczynska-Mleko et al., 2014) with a peak around 208 nm, corresponding to an α-helix, and a broad peak around 220 nm, characteristic of β-sheets (Greenfield, 2006). The decrease in peak intensity for WPC20, compared to that of WPC00 could be due to a confounding factor, such as an increase in turbidity caused by aggregation, which would increase absorbance and

interfere with the signal; however, all samples were quality checked for absorbance during CD analysis (absorbance < 2).

The near-UV CD spectra showed differences in the structure of WPC heated at different time points, with a small decrease in the near-UV peak (260 – 300 nm) corresponding to changes in tertiary structure. Such changes lead to the exposure of thiol groups, hydrophobic regions, or functional groups able to form hydrogen bonds, which increases mucoadhesive strength.

To the authors' knowledge, no literature exists showing near-UV CD spectra for WPC or WPI; however, near-UV spectra exist for β-LG, α-LA (Mercade-Prieto, Paterson, & Wilson, 2007; Moro, Baez, Busti, Ballerini, & Delorenzi, 2011; Rodiles-Lopez et al., 2010; Wijesinha-Bettoni et al., 2007). Moro et al. (2011) showed a reduction in peaks (285 and 292 nm) of β-LG upon heating at 85 °C due to a tryptophan residue absorbance (Trp19) reflecting structural changes of the β-barrel within the protein where the residue sits (Matsuura & Manning, 1994). The near-UV CD spectrum for α-LA, reported by Wijesinha-Bettoni et al. (2007) contained a peak at 270 nm, which disappears upon the unfolding of the protein after heat treatment. The results in this study are consistent with these findings as the broad negative peak in near-UV spectra of WPC samples occurs around 260 – 310 nm, with minima appearing at around 270, 285 and 290 nm. This negative peak decreases in size with heating time, indicating a structural change within the β-barrel of β-LG and a change in tertiary structure.

3.4 Nuclear magnetic resonance

Full ^{1}H NMR spectra are given in Supplementary data S1; two regions (8.8 – 8.0 ppm; 3.2 – 1.8 ppm) are enlarged in Figure 5. Full assignments of β-LG and α-LA have been previously reported (Uhrinova et al., 1998; Alexandrescu et al., 1992), and hence are not reported here. The yellow bands highlight regions where spectral differences exist between the four samples. Structural differences between WPC00 and the three heated samples were inferred from ^{1}H NMR, using 2D NOESY spectra to confirm.

Three signals were present only in WPC00 (δ 8.35, 2.31, 1.82), corresponding to the amide proton of Ser^{27}, the H_{γ} of Gln^{68}, and the H_{β} of Leu^{95} respectively of β-LG (Belloque et al., 1998). These residues

lie within the β-barrel strands of β-LG, and the disappearance of these peaks indicates the loss of structure in these regions caused by thermal denaturation. This change in structure increases exposure of hydrophobic regions, which increases mucoadhesive strength (Celebioglu et al., 2017).

One further signal was more pronounced in WPC00 than the heated samples (δ 3.16), likely to be from a H_{β}, R_2NH, or R_2NCH, although it was not possible to determine the specific residue. One signal was more pronounced in the heated samples than WPC00 (δ 3.10), corresponding to $Lys_{100\ of}$ β-LG, located in the short loop between two β-strands (Belloque et al., 1998), also linked to the loss of structure of the β-barrel affecting the structure of the connecting loops.

A trend of increasing signal intensity with heating time was observed in three areas (δ 2.61, 2.59, 2.41). It is suggested that these arise from the release of small sulfur-containing compounds from heating of the whey protein such as dimethyl disulfide and methionine (Jo et al., 2019; White et al., 2013).

Differences between multiplets were seen, with similarities between WPC05 and WPC10 (δ 8.89 – 8.50, 8.17 – 8.29). The differences seen in multiplets across 8.17-8.29 ppm, correspond to amide protons in the α-helix of β-LG, with a decrease in signal demonstrating a decrease of structure upon heating. This corresponds to work carried out by Belloque et al., who found these signals entirely disappeared after heating β-LG at 75 °C for 1 h (1998). The unfolding of this α-helix exposes Cys^{121} alongside a hydrophobic region of the protein (Qi et al., 1995; Iametti et al., 1996), these have the potential to form both covalent and hydrophobic interactions with the oral mucosa, leading to increased oral retention.

It is worth noting, that the structures of β-LG and α-LG can form dimers via thiol-disulfide exchange reactions, where the structures will be different than those observed in individual spectra (Havea et al., 2001).

4 Conclusions

Whey protein samples were heated for varying times (0, 5 and 20 min; 70 °C) and the retention of protein in the mouth was measured using an *in vivo* technique. Higher protein levels were found in

saliva collected after consuming WPC20 than WPC00, indicating that protein in WPC20 is retained longer in the mouth, which can be explained by mucoadhesion. Previous findings of a higher sensory score in drying, mouthcoating and chalky for WPC20 (Bull et al., 2017) correlate to the increase of protein in the oral cavity over 60 s post consumption. An increase in accessible thiol concentration with heating time is consistent with the proposed mechanism of mucoadhesion as the source of whey protein derived mouthdrying. This is supported by the adhesion of whey protein to the oral mucosa, the denaturation of whey protein increasing mucoadhesive strength, and by the conformational changes inferred by the CD and NMR spectra found on heating WPC in this study. Conformational changes showed loss of secondary and tertiary structure with heating time which correlate to the unfolding of protein leading to changes within the β-barrel and the α-helix of β-LG, both of which expose buried hydrophobic regions. In addition to the increase in hydrophobicity, these conformational changes expose and release sulfur-containing compounds; all factors that lead to increases in mucoadhesive properties. Future studies should account for differences of saliva characteristics associated with aging, in order to study the conditions which may affect the oral retention of whey protein and perception of drying by older adults.

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Conflict of interests

Declaration of interest: none

References

Alexandrescu, A. T., Broadhurst, R. W., Wormald, C., Chyan, C. L., Baum, J., Dobson, C. M. (1992). H-1-NMR assignments and local environments of aromatic residues in bovine, human and guinea-pig variants of alpha-lactalbumin. *European Journal of Biochemistry, 210*(3), 699-709

Andrewes, P., Kelly, M., Vardhanabhuti, B., & Foegeding, E. A. (2011). Dynamic modelling of whey protein-saliva interactions in the mouth and relation to astringency in acidic beverages. *International Dairy Journal, 21*(8), 523-530

Belloque, J., Smith, G. M. (1998). Thermal denaturation of beta-lactoglobulin. A H-1 NMR study. *Journal of Agricultural and Food Chemistry 46*(5), 1805-1813

Bernkop-Schnürch, A. (2005). Thiomers: A new generation of mucoadhesive polymers. *Advanced Drug Delivery Reviews, 57*(11), 1569-1582

Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2), 248-254

Bravo-Osuna, I., Teutonico, D., Arpicco, S., Vauthier, C., & Ponchel, G. (2007). Characterization of chitosan thiolation and application to thiol quantification onto nanoparticle surface. *International Journal of Pharmaceutics, 340*(1-2), 173-181

Bull, S. P., Hong, Y., Khutoryanskiy, V. V., Parker, J. K., Faka, M., & Methven, L. (2017). Whey protein mouth drying influenced by thermal denaturation. *Food Quality and Preference, 56*, 233-240

Cave, R. A., Cook, J. P., Connon, C. J., & Khutoryanskiy, V. V. (2012). A flow system for the on-line quantitative measurement of the retention of dosage forms on biological surfaces using spectroscopy and image analysis. *International Journal of Pharmaceutics, 428*(1-2), 96-102

Celebioglu, H. Y., Gudjonsdottir, M., Meier, S., Duus, J. O., Lee, S., & Chronakis, I. S. (2015). Spectroscopic studies of the interactions between beta-lactoglobulin and bovine submaxillary mucin. *Food Hydrocolloids, 50*, 203-210

Celebioglu, H. Y., Kmiecik-Palczewska, J., Lee, S. & Chronakis, I. S. (2017). Interfacial shear rheology of beta-lactoglobulin-Bovine submaxillary mucin layers adsorbed at air/water interface. *International Journal of Biological Macromolecules 102*, 857-867

Chandrapala, J., Zisu, B., Kentish, S., & Ashokkumar, M. (2012). The effects of high-intensity ultrasound on the structural and functional properties of alpha-Lactalbumin, beta-Lactoglobulin and their mixtures. *Food Research International, 48*(2), 940-943

Chandrapala, J., Zisu, B., Palmer, M., Kentish, S., & Ashokkumar, M. (2011). Effects of ultrasound on the thermal and structural characteristics of proteins in reconstituted whey protein concentrate. *Ultrasonics Sonochemistry, 18*(5), 951-957

Cook, S. L., Bull, S. P., Methven, L., Parker, J. K., & Khutoryanskiy, V. V. (2017). Mucoadhesion: A food perspective. *Food Hydrocolloids, 72*, 281-296

Cook, S. L., Woods, S., Methven, L., Parker, J. K., & Khutoryanskiy, V. V. (2018). Mucoadhesive polysaccharides modulate sodium retention, release and taste perception. *Food Chemistry, 240*, 482-489

Dewit, J. N., & Swinkels, G. A. M. (1980). A differential scanning calorimetric study of the thermal-denaturation of bovine beta-lactoglobulin - thermal-behavior at temperatures up to 100 degrees C. *Biochimica Et Biophysica Acta, 624(1),* 40-50

Dinnella, C., Recchia, A., Vincenzi, S., Tuorila, H., & Monteleone, E. (2010). Temporary modification of salivary protein profile and individual responses to repeated phenolic astringent stimuli. *Chemical Senses, 35*(1), 75-85

Dresselhuis, D. M., de Hoog, E. H. A., Stuart, M. A. C., & van Aken, G. A. (2008). Application of oral tissue in tribological measurements in an emulsion perception context. *Food Hydrocolloids, 22*(2), 323-335

Etzel, M. R. (2004). Manufacture and use of dairy protein fractions. *Journal of Nutrition, 134(4),* 996S-1002S

Gosney, M. (2003). Are we wasting our money on food supplements in elder care wards? *Journal of Advanced Nursing, 43*(3), 275-280

Greenfield, N. J. (2006). Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols, 1*(6), 2876-2890

Havea, P., Singh, H., & Creamer, L. K. (2001). Characterization of heat-induced aggregates of beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin in a whey protein concentrate environment. *Journal of Dairy Research, 68*(3), 483-497

Havea, P., Singh, H., & Creamer, L. K. (2002). Heat-induced aggregation of whey proteins: Comparison of cheese WPC with acid WPC and relevance of mineral composition. *Journal of Agricultural and Food Chemistry, 50*(16), 4674-4681

Iametti, S., et al. (1996). Modifications occur at different structural levels during the heat denaturation of beta-lactoglobulin. *European Journal of Biochemistry, 237*(1), 106-112

Indrawati, L., Stroshine, R. L., Narsimhan, G. (2007), Low-field NMR: A tool for studying protein aggregation. *Journal of the Science of Food and Agriculture, 87*(12), 2207-2216

Jo, Y., Carter, B. G., Barbano, D. M. & Drake, M. A. (2019). Identification of the source of volatile sulfur compounds produced in milk during thermal processing. *Journal of Dairy Science 102*(10), 8658-8669Kubala, E., Strzelecka, P., Grzegocka, M., Lietz-Kijak, D., Gronwald, H., Skomro, P., & Kijak, E. (2018). A Review of Selected Studies That Determine the Physical and Chemical Properties of Saliva in the Field of Dental Treatment. *BioMed Research International, 2018*, 13

Lin, M. J., Lewis, M. J., & Grandison, A. S. (2006). Measurement of ionic calcium in milk. *International Journal of Dairy Technology, 59*(3), 192-199

Liu, G., & Zhong, Q. (2013). Thermal aggregation properties of whey protein glycated with various saccharides. *Food Hydrocolloids, 32*(1), 87-96

Matsuura, J. E., & Manning, M. C. (1994). Heat-induced gel formation of beta-lactoglobulin - A study on the secondary and tertiary structure as followed by circular-dichroism spectroscopy. *Journal of Agricultural and Food Chemistry, 42*(8), 1650-1656

Mercade-Prieto, R., Paterson, W. R., & Wilson, D. I. (2007). The pH threshold in the dissolution of beta-lactoglobulin gels and aggregates in alkali. *Biomacromolecules, 8*(4), 1162-1170

Moro, A., Baez, G. D., Busti, P. A., Ballerini, G. A., & Delorenzi, N. J. (2011). Effects of heat-treated beta-lactoglobulin and its aggregates on foaming properties. *Food Hydrocolloids, 25*(5), 1009-1015

Nagler, R. M., & Hershkovich, O. (2005a). Age-related changes in unstimulated salivary function and comvosition and its relations to medications and oral sensorial complaints. *Aging Clinical and Experimental Research, 17*(5), 358-366

Nagler, R. M., & Hershkovich, O. (2005b). Relationships between age, drugs, oral sensorial complaints and salivary profile. *Archives of Oral Biology, 50*(1), 7-16

Norton, V.; Lignou, S.; Bull, S.P.; Gosney, M.A.; Methven, L. (2020). An Investigation of the Influence of Age and Saliva Flow on the Oral Retention of Whey Protein and Its Potential Effect on the Perception and Acceptance of Whey Protein Beverages. *Nutrients*, *12*(9), 2506

Qi, X. L., et al. (1995). Thermal-denaturation of beta-lactoglobulin - Effect of protein-concentration at pH-6.75 and pH-8.05. *Biochimica Et Biophysica Acta, 1248*(1), 43-49

Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2015). Conformational changes of beta-lactoglobulin induced by shear, heat, and pH-Effects on antigenicity. *Journal of Dairy Science, 98*(7), 4255-4265

Riou, E., Havea, P., McCarthy, O., Watkinson, P., & Singh, H. (2011). Behavior of protein in the presence of calcium during heating of whey protein concentrate solutions. *Journal of Agricultural and Food Chemistry, 59*(24), 13156-13164

Rodiles-Lopez, J. O., Arroyo-Maya, I. J., Jaramillo-Flores, M. E., Gutierrez-Lopez, G. F., Hernandez-Arana, A., Barbosa-Canovas, G. V., Niranjan, K., & Hernandez-Sanchez, H. (2010). Effects of high hydrostatic pressure on the structure of bovine alpha-lactalbumin. *Journal of Dairy Science, 93*(4), 1420-1428

Sava, N., Van der Plancken, I., Claeys, W., & Hendrick, M. (2005). The kinetics of heat-induced structural changes of beta-lactoglobulin. *Journal of Dairy Science, 88*(5), 1646-1653

Thirawong, N., Kennedy, R. A., & Sriamornsak, P. (2008). Viscometric study of pectin-mucin interaction and its mucoadhesive bond strength. *Carbohydrate Polymers, 71*(2), 170-179

Tomczynska-Mleko, M., Kamysz, E., Sikorska, E., Puchalski, C., Mleko, S., Ozimek, L., Kowaluk, G., Gustaw, W., & Wesolowska-Trojanowska, M. (2014). Changes of secondary structure and surface tension of whey protein isolate dispersions upon pH and temperature. *Czech Journal of Food Sciences, 32*(1), 82-89

Uhrinova, S., Uhrin, D., Denton, H., Smith, M., Sawyer, L., Barlow, N. (1998) Complete assignment of H-1, C-13 and N-15 chemical shifts for bovine beta-lactoglobulin: Secondary structure and topology of the native state is retained in a partially unfolded form. *Journal of Biomolecular NMR, 12*(1), 89-107

Vardhanabhuti, B., & Foegeding, E. A. (2010). Evidence of interactions between whey proteins and mucin their implication on the astringency mechanism of whey proteins at low pH. *Gums and Stabilisers for the Food Industry 15*, 137-146

Wada, R., Fujita, Y., & Kitabatake, N. (2006). Effects of heating at neutral and acid pH on the structure of beta-lactoglobulin A revealed by differential scanning calorimetry and circular dichroism spectroscopy. *Biochimica Et Biophysica Acta-General Subjects, 1760*(6), 841-847

White, S. S., Fox, K. M., Jervis, S. M. & Drake, M. A. (2013). Influence of heating and acidification on the flavor of whey protein isolate. *Journal of Dairy Science*, *96*(3), 1366-1379

Wijesinha-Bettoni, R., Gao, C., Jenkins, J. A., Mackie, A. R., Wilde, P. J., Mills, E. N. C., & Smith, L. J. (2007). Heat treatment of bovine alpha-lactalbumin results in partially folded, disulfide bond shuffled states with enhanced surface activity. *Biochemistry, 46*(34), 9774-9784

Withers, C. A., Cook, M. T., Methven, L., Gosney, M. A., & Khutoryanskiy, V. V. (2013). Investigation of milk proteins binding to the oral mucosa. *Food & Function, 4*, 1668–1674

Withers, C. A., Gosney, M. A., & Methven, L. (2013). Perception of thickness, mouth coating and mouth drying of dairy beverages by younger and older volunteers. *Journal of Sensory Studies, 28*(3), 230-237

Ye, A., Streicher, C., & Singh, H. (2011). Interactions between whey proteins and salivary proteins as related to astringency of whey protein beverages at low pH. *Journal of Dairy Science*, *94*, 5842–5850

Ye, A., Zheng, T., Ye, J. Z., & Singh, H. (2012). Potential role of the binding of whey proteins to human buccal cells on the perception of astringency in whey protein beverages. *Physiology and Behaviour, 106*, 645–650

Zeiler, R. N. W., & Bolhuis, P. G. (2015). Exposure of thiol groups in the heat-induced denaturation of beta-lactoglobulin. *Molecular Simulation, 41*(10-12), 1006-1014

Zor, T., & Seliger, Z. (1996). Linearization of the Bradford protein assay increases its sensitivity: Theoretical and experimental studies. *Analytical Biochemistry, 236*(2), 302-308

Figure Captions

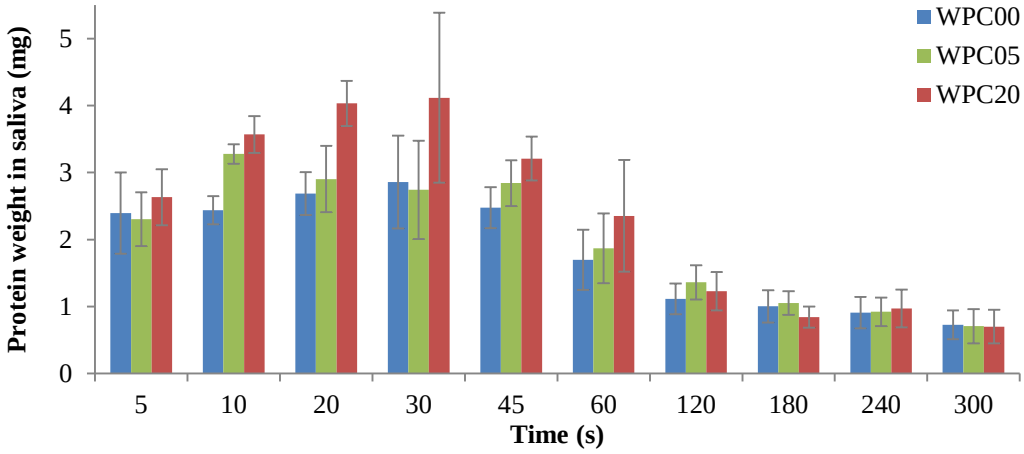
Figure 1: Average protein weight in collected saliva after consumption of 5 mL of sample over 5 min (all time points collected were independent). Average baseline saliva protein weight is subtracted for each volunteer. Saliva is assumed to have a density of 1 g mL^{-1}. Error bars represent ± 2 standard error of the mean.

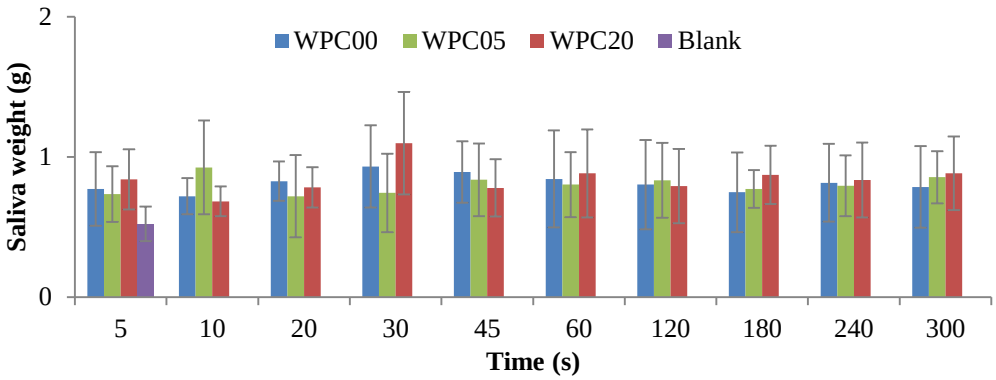
Figure 2: Average saliva weights of all volunteers over 5 min of collection.Error bars represent ± 2 standard error of the mean.

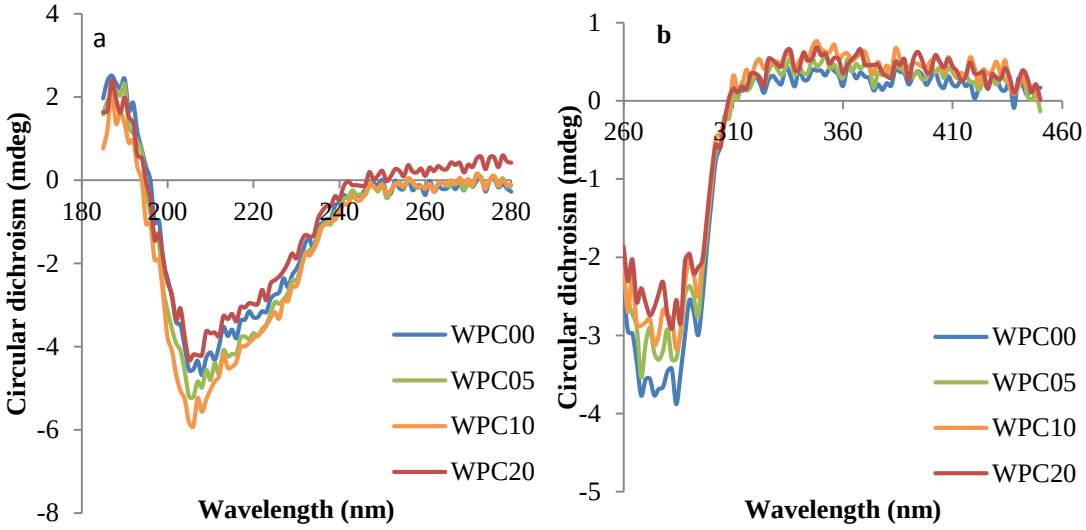
Figure 3: a) Far-UV and b) near-UV CD spectra comparing WPC00, WPC05, WPC10 and WPC20.

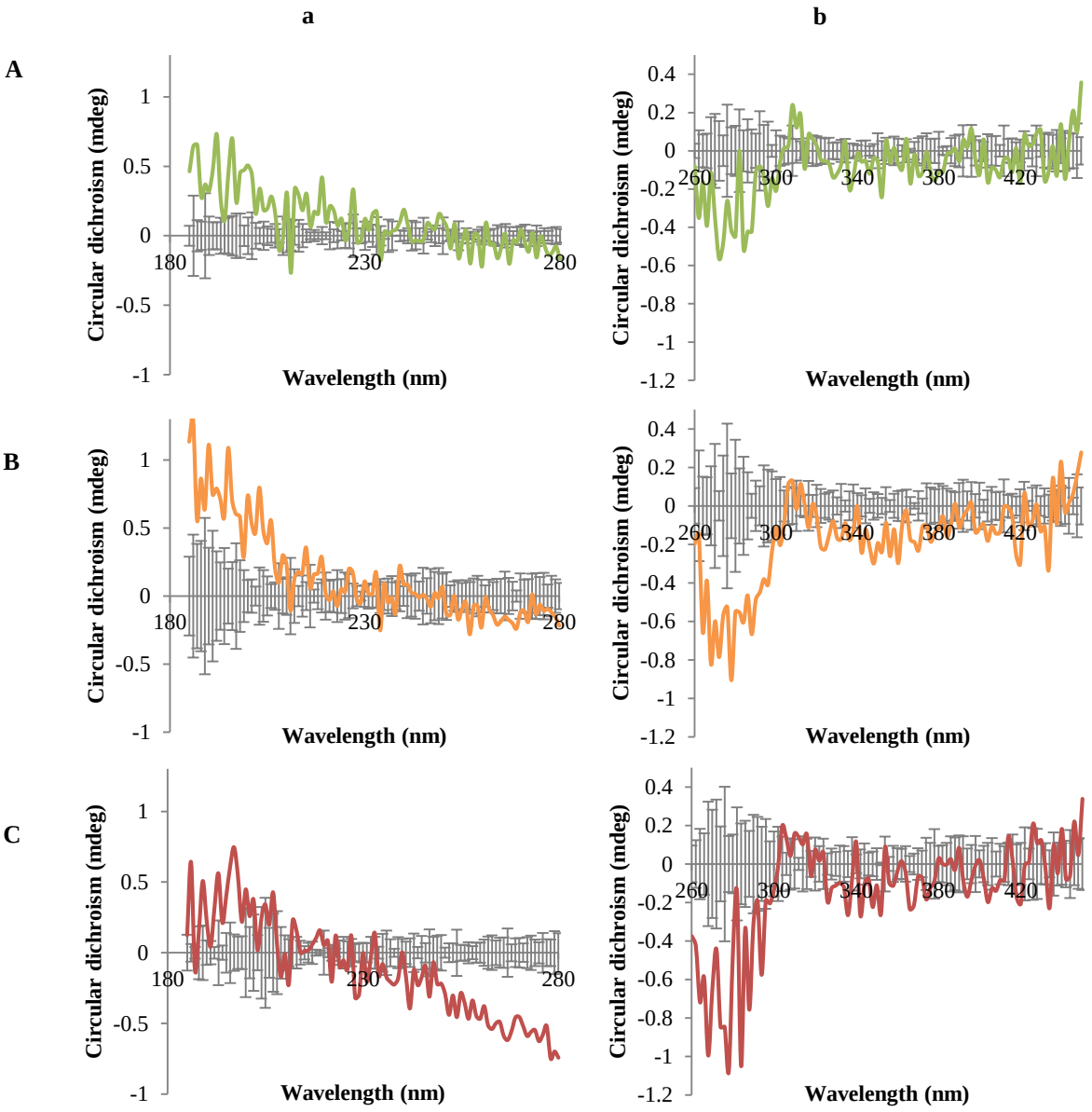
Figure 4: Column a) far-UV and column b) near-UV CD difference spectra of normalised WPC00 spectrum minus other WPC sample spectra. Row A: WPC05; row B: WPC10; row C: WPC20. Error bars represent ± 2 standard deviations and give an indication of significant difference between the samples.

Figure 5: ^{1}H NMR spectra of WPC samples (A: 8.8 – 8.1 ppm; B: 3.2 – 1.8 ppm); WPC00 (blue), WPC05 (green), WPC10 (orange) and WPC20 (red). Differing peaks highlighted for clarity.








a
b
A
B
C
Circular dichroism (mdeg)
Wavelength (nm)
1
0.5
0
-0.5
-1
180
230
280
0.4
0.2
-0.2
-0.4
-0.6
-0.8
-1.2
260
300
340
380
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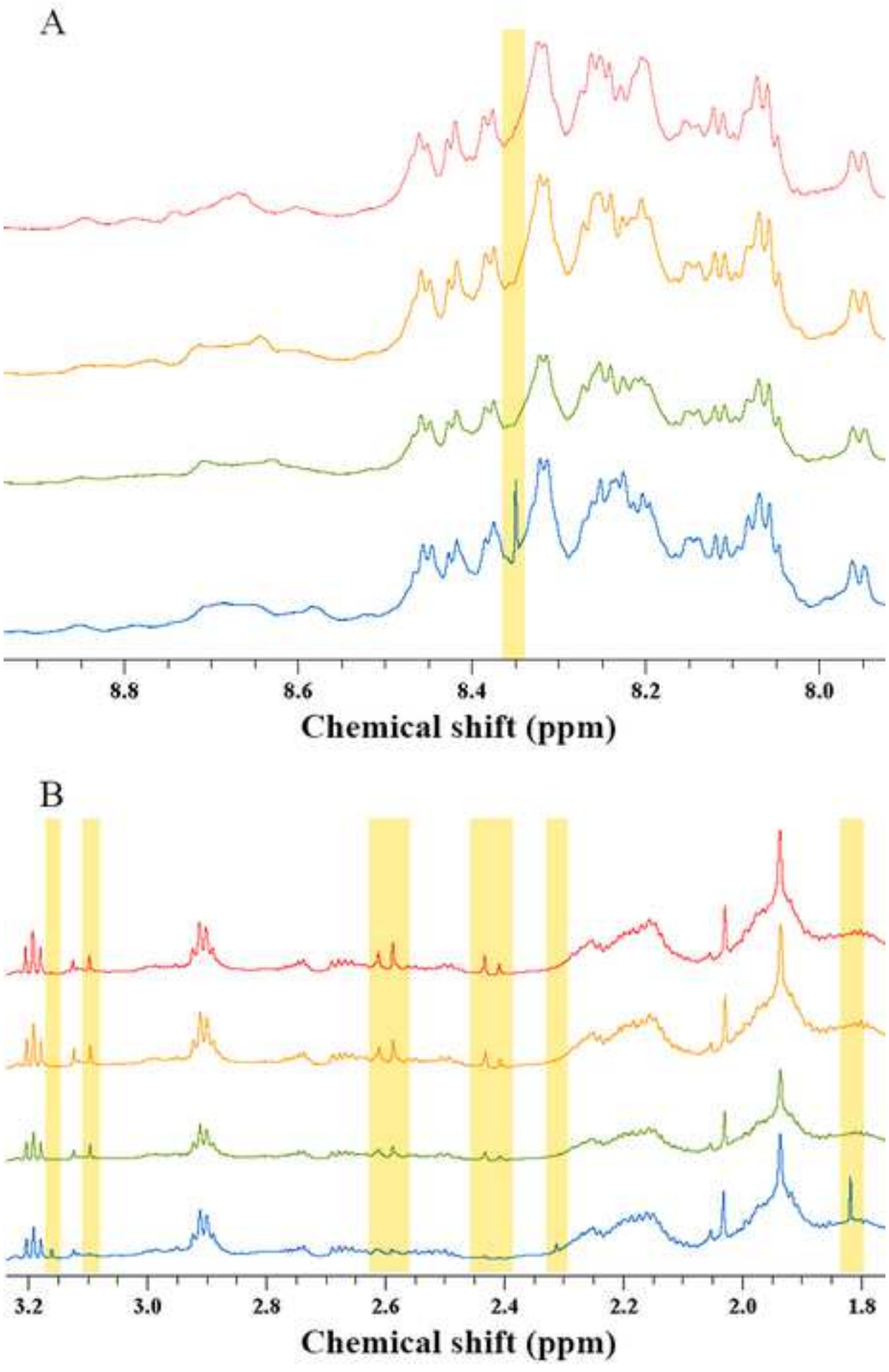

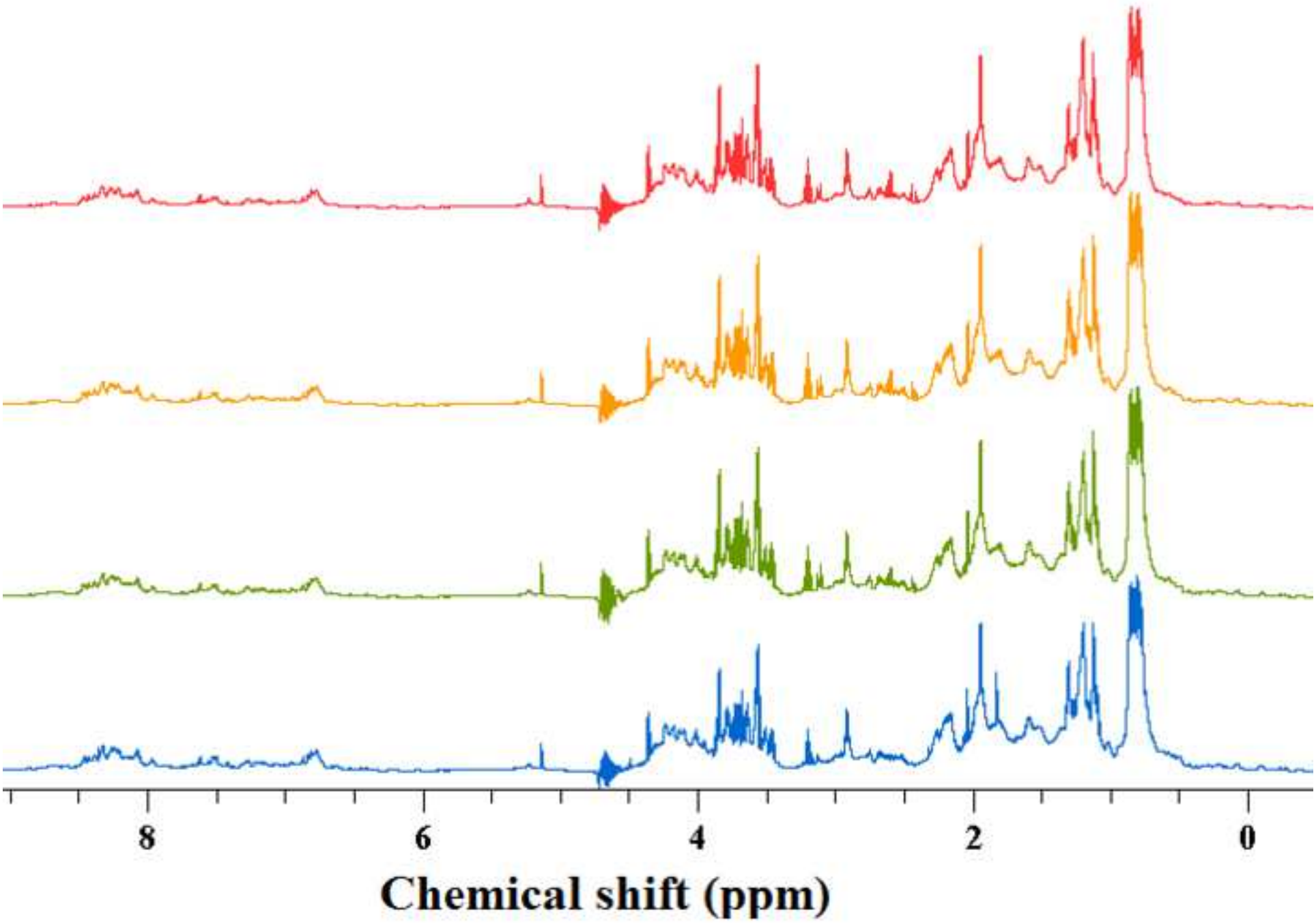

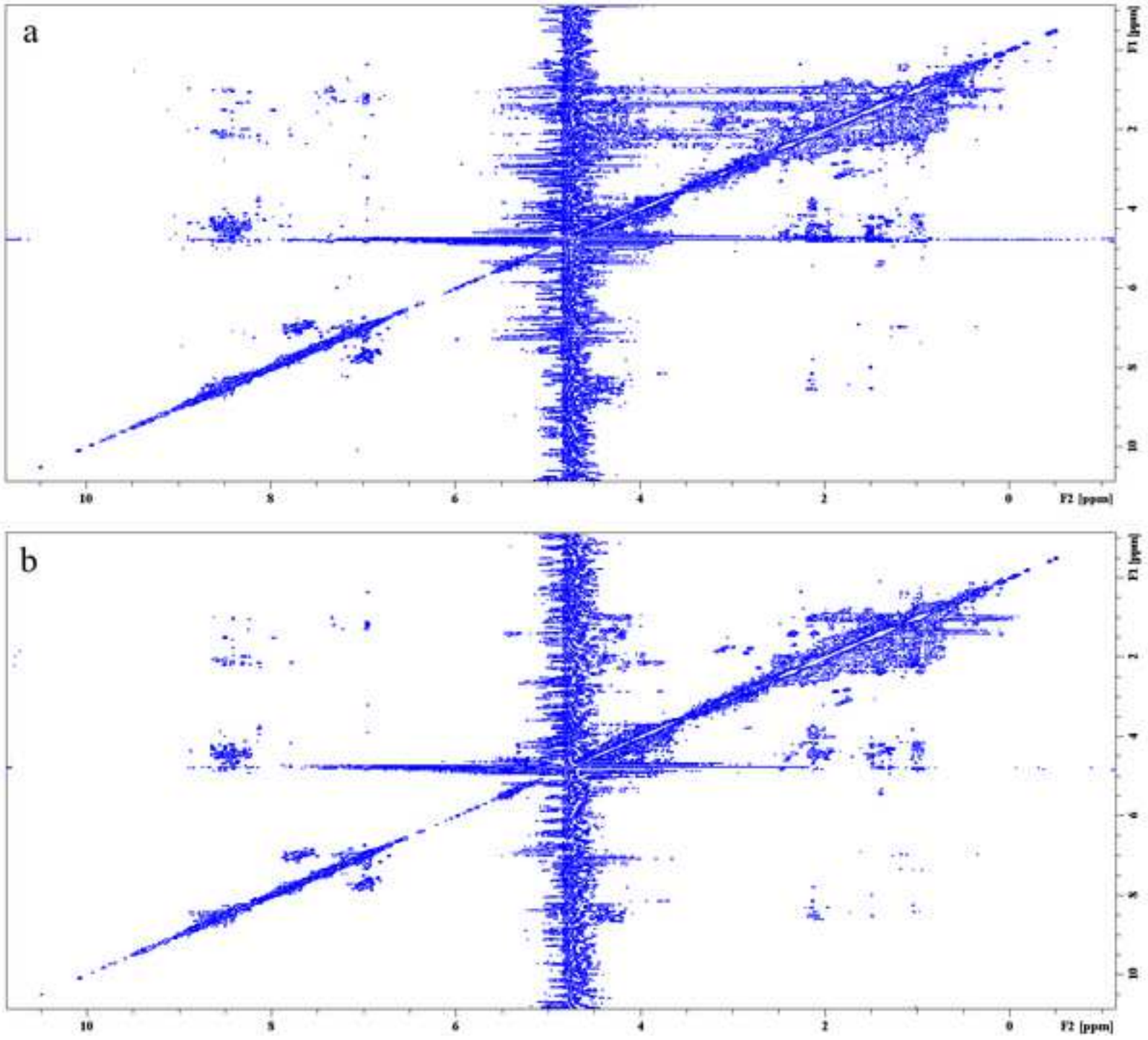

Supplementary data

Supplementary Data S1: Full ^{1}H NMR spectra of WPC samples; WPC00 (blue), WPC05 (green), WPC10 (orange) and WPC20 (red).

Supplementary Data S2: 2D NOESY NMR spectra of WPC samples; a) WPC00, and b) WPC20.

Credit Author Statement

The following authors have contributed to the article: Stephanie Bull, Vitaliy Khutoryanskiy, Jane Parker, Marianthi Faka and Lisa Methven. The authors' responsibilities were as follows: SB, VK, JP, MF and LM designed the research; SB conducted the research; SB analysed the data and wrote the manuscript; SB, JP, MF, VK and LM all contributed to the manuscript and SB had primary responsibility for the final content. All authors listed have made substantial contributions to the manuscript and they all have given final approval of the final version of the manuscript.

*Declaration of Interest Statement

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: