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Structure of whey protein hydrolysate used as emulsifier in wet and dried oil delivery systems: Effect of pH and drying processing

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ABSTRACT

The secondary structure of whey protein concentrate hydrolysate (WPCH), used as an emulsifier in oil delivery systems, was investigated using Synchrotron Radiation Circular Dichroism (SRCD). The effect of pH on the conformation of peptides in solution and adsorbed at the oil/water interface, as well as the thermal stability of the systems was studied. Furthermore, oil-loaded microcapsules were produced by spray-drying or electrospraying to investigate the influence of encapsulating agents (glucose syrup, maltodextrin) and drying technique on the secondary structure of WPCH at the oil/water interface. Enzymatic hydrolysis resulted in peptides with a highly unordered structure (~60% turns and unordered regions) in solution. However, WPCH adsorption onto the oil/water interface increased the α-helical content resulting in an improved thermal stability. The encapsulating agents and spray-drying process did not modify the conformation of WPCH at the oil/water interface. Nonetheless, electrospraying affected the SRCD spectra obtained for WPCH adsorbed at the oil/water interface.

1. Introduction

Bioactive oils (e.g., oils rich in omega-3 polyunsaturated fatty acids) present numerous health effects and thus the food industry is highly interested in the development of fortified food products rich in these bioactive lipids (Punia et al., 2019). However, the extremely low oxidative stability, together with other inherent drawbacks of bioactive oils (e.g., low solubility), still represent a challenge to the food industry when it comes to their inclusion in complex food matrices. Hence, the design and development of efficient oil delivery systems, such as oil-in-water emulsions or oil-loaded micro/nanocapsules, is of utmost importance. To produce oil delivery systems, it is necessary to emulsify the oil in the continuous phase, which is normally water-based, and then drying is carried out when dried encapsulates are required (Rahmani-Manglano et al., 2020a).

Proteins are amphiphilic molecules that have been extensively used in the food industry as emulsifiers to stabilize oil-in-water emulsions used as: i) a liquid delivery system itself (Horn et al., 2012; Nielsen & Jacobsen, 2013), or ii) employed as feed emulsion to the dryer to produce dry micro/nanocapsules (Miguel et al., 2019; Takeungwongtrakul et al., 2015). Moreover, protein hydrolysates have shown enhanced functional properties (e.g., emulsifying and antioxidant) with respect to their parent proteins (Liceaga & Hall, 2018). Enzymatic hydrolysis of proteins to a low degree of hydrolysis (DH; DH $\leq$ 10%) results in the release of surface-active peptides, which diffuse, adsorb, unfold and reorient faster at the oil/water (O/W) interface when compared to the parent protein (Peighambardoust et al., 2021). Furthermore, the conformational changes induced during hydrolysis also favour the exposure of hydrophobic groups, originally buried inside the protein structure, that also contribute to the improved emulsifying activity of protein hydrolysates (Liceaga & Hall, 2018). In addition, and depending on the proteolysis conditions (e.g., type of enzyme, temperature, pH, time), protein hydrolysates might also exhibit higher antioxidant activity than the native proteins as a result of an increased exposure of amino acid residues capable of inhibiting lipid oxidation through different mechanisms, e.g., radical scavenging and/or metal chelation (Elias et al., 2008; Peighambardoust et al., 2021). The latter is of special relevance since protein hydrolysates exhibiting both emulsifying and antioxidant activities allow the location of antioxidants at the oil/aqueous phase (in emulsions) or at the oil/shell phase (in dried encapsulates), which is the place where lipid oxidation is initiated in heterogeneous systems (García-Moreno et al., 2021; Padial-Domínguez et al.,

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2020a; Tamm et al., 2015). In this line, whey protein concentrate hydrolysates (WPCHs) have been reported to exhibit high emulsifying (Padial-Domínguez et al., 2020b) and antioxidant activities (e.g., radical scavenging activity, metal chelating and reducing power) (Padial-Domínguez et al., 2020a) and have been used as emulsifier/antioxidant to stabilize either oil-in-water emulsions (Padial-Domínguez et al., 2020a) or oil-loaded microcapsules (Rahmani-Manglano et al., 2020b) showing high oxidative stability.

Some research has already studied the structure of pure proteins contained in whey (e.g., α-lactalbumin, β-lactoglobulin and bovine serum albumin; BSA) (Day et al., 2014; Zhai et al., 2010, 2011, 2012). However, to the best of our knowledge, no studies have yet been reported on the structure of peptides present in hydrolysates when adsorbed at the O/W interface. Structural organization and interaction of emulsifier peptides at the O/W interface affect the mechanical properties of the interfacial peptides film (e.g., viscoelasticity) (García-Moreno et al., 2021). The latter strongly affects the physical and oxidative stability of O/W emulsions, as well as the encapsulation efficiency (EE) and oxidative stability of dried micro/nanocapsules loaded with bioactive oils. Moreover, the effect of pH on the interfacial structure of peptides needs further investigation since above or below the isoelectric point (pI) the peptides will be negatively or positively charged, respectively. This is extremely important since it determines the adsorption of peptides onto the O/W interface as well as the viscoelasticity of the peptide interfacial layer (Ruiz-Álvarez et al., 2022). Furthermore, to the best of the authors' knowledge, the influence of the encapsulating agent (e.g., carbohydrates), and of the drying method used to produce oil-loaded micro/nanocapsules (e.g., spray-drying or electrospraying) on the structure of the peptides at the O/W interface remains to be investigated.

Synchrotron radiation circular dichroism (SRCD) is an advanced technique used to study the secondary structure of proteins and peptides in scattering and/or absorbing media such as O/W emulsions (Wallace & Janes, 2001). The high flux of synchrotron radiation light sources allows spectra collection at the vacuum ultraviolet (VUV) wavelength region (<190 nm) with higher signal-to-noise ratios than conventional circular dichroism (CD), allowing better and more accurate determination of secondary structure (Wallace & Janes, 2001; Zhai et al., 2013). Indeed, SRCD has been recently employed to determine the secondary structure of proteins (Day et al., 2014; Zhai et al., 2010, 2011, 2012) and synthetic peptides (García-Moreno et al., 2021) both in solutions and at the O/W interface. Nevertheless, the use of SRCD to determine the predominating structure of peptides present in protein hydrolysates and their thermal stability both in solution and at the O/W interface have not been yet studied.

Therefore, this work aimed at: i) quantitatively evaluating by SRCD the secondary structure of WPCH in solution or adsorbed at the O/W interface at different pH (pH 2 or pH 8), ii) investigating the thermal stability of WPCH in solution or adsorbed at the O/W interface at different pH (pH 2 or pH 8), and iii) studying the changes in the secondary structure of WPCH used as an emulsifier to produce oil-loaded micro/nanocapsules using different encapsulating agents (e.g., glucose syrup or maltodextrin) and obtained by different drying techniques (e.g., spray-drying or electrospraying). Thus, this research will advance our knowledge on the interfacial structure of emulsifier peptides present in whey protein hydrolysate, which will open up its use for the development of more efficient liquid and dried oil delivery systems.

2. Materials and methods

2.1. Materials

Whey protein concentrate (WPC: 35 wt% protein, 52 wt% lactose, 2.8 wt% fat, 2.5 wt% moisture and 7.7 wt% ash), lactose and maltodextrin (MD; DE21) were kindly donated by Abbott Laboratories (Granada, Spain). Glucose syrup (GS; DE38, C*Dry 1934) and pullulan (PUL) were provided by Cargill Germany GmbH (Krefeld, Germany) and Hayashibara Co., Ltd. (Okayama, Japan), respectively. Tricaprylin oil and sodium phosphate (monobasic anhydrous and dibasic dihydrate) were purchased from Sigma-Aldrich (St. Louis, MO). Ortho-Phosphoric acid (85%) was supplied by Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was provided by Panreac Quimica S.A. (Barcelona, Spain).

2.2. Production of whey protein concentrate hydrolysate (WPCH)

WPCH was produced with Alcalase® (Novozymes, Bagsværd, Denmark) to a degree of hydrolysis of 10% (DH 10%), as described by Rahmani-Manglano et al. (2020b). In brief, WPC was diluted in distilled water to a final protein content of 40 g/L. The reaction was carried out at 50 °C and pH 8 and the DH was determined using the pH-stat method by employing an automatic titrator 718 Stat Titrino (Metrohm AG, Herisau, Switzerland) (Rahmani-Manglano et al., 2020b). The protein hydrolysate solution was then heated to 100 °C for 5 min to deactivate the enzyme, before being freeze-dried in a Labconco freeze-drying system (Kansas City, MO, USA) and stored at 4 °C until further use. The protein content of the resulting WPCH was ca. 33% (Padial-Domínguez et al., 2020a). A heat treatment without enzyme addition (WPC + heat) was carried out at the hydrolysis conditions mentioned (50 °C and pH 8) to evaluate the effect of only heat on the structure of WPC. The protein solution obtained was also heated to 100 °C for 5 min and then freeze-dried and stored at 4 °C.

2.3. Preparation of solutions and emulsions

Stock solutions were prepared dissolving ca. 6 wt% of WPC or WPCH in phosphate buffer 10 mM (pH 8 or pH 2), stirring overnight (500 rpm) at 4 °C. From the stock solutions, protein solutions and emulsions were produced either by addition of phosphate buffer or emulsification of tricaprylin oil. Oil-in-water (O/W) emulsions were produced by dispersing 5 wt% of tricaprylin oil in the aqueous phase containing WPC or WPCH. The protein content of the emulsions was fixed at 2 wt%. First, a coarse emulsion was produced using an Ultraturrax T-25 homogenizer (IKA, Staufen, Germany) at 15,000 rpm for 2 min. The oil was added during the first minute, and the coarse emulsion was then homogenized using a two-valve homogenizer (PandaPLUS 2000; GEANiro Soavi, Lübeck, Germany) at a pressure range of 450/75 bar, applying 3 passes. An SDS-stabilized O/W emulsion was also prepared by homogenizing 5 wt% tricaprylin oil with 95 wt% aqueous solution (2 wt% SDS in 10 mM phosphate buffer, pH 8) under the same conditions as described above. This emulsion and dilutions from it were used for SRCD baseline correction.

2.4. Production of spray-dried capsules

The emulsions fed to the spray-dryer consisted of 5 wt% tricaprylin oil-in-water emulsions stabilized with WPCH (6 wt%, which is equivalent to 2 wt% protein), containing GS or MD as encapsulating agent (28 wt%). The aqueous phase (10 mM phosphate buffer, pH 8) and the emulsions were produced as described in Section 2.3 by also dissolving the encapsulating agents in the aqueous phase. The oil load of the resulting spray-dried capsules was ca. 13 wt%. The drying process was carried out in a lab-scale spray-drier (Büchi B-190; Büchi Labortechnik, Flawill, Switzerland) at 180/90 °C as the inlet/outlet temperature, respectively. The drying air flow was fixed to 25 Nm3/h.

2.5. Production of electrosprayed capsules

For electrosprayed emulsions, the aqueous phase was produced dissolving WPCH (3.5 wt%), PUL (1.5 wt%) and GS or MD (15 wt%) in phosphate buffer 10 mM at pH 8. Again, the aqueous phase and the emulsions were produced as described in Section 2.3. The final

tricaprylin oil load of the emulsion was 3 wt%. Pullulan was added as thickening agent in order to increase the stability of the Taylor cone during the electrospraying process. It should be noted that despite the differences in the formulation when compared to spray-drying, the protein/oil ratio (P/O = 0.4) and the oil load (ca. 13%) of the resulting capsules was the same irrespective of the drying method. The electrospraying process was carried out using SpinBox equipment (Bioinicia-Fluidnatek S.L.U., Valencia, Spain) consisting of a syringe pump, a drying chamber equipped with a variable high voltage power supply and a 15 × 15 cm collector plate made of stainless steel. The flow rate was fixed to 1 mL/h. The voltage applied was 17 kV. A 16G needle (Proto Advantage, Canada) was coupled to the monoaxial injector and the needle tip was 15 cm from the collector plate. The electrospraying process was carried out at room temperature (relative humidity, RH: 35–43%) in batches of 1 h.

2.6. SRCD measurements

SRCD measurements were carried out on the AU-CD beamline at the ASTRID2 synchrotron radiation source, (ISA, Department of Physics & Astronomy, Aarhus University in Denmark) with slight differences from our previous work (García-Moreno et al., 2021). As usual the operation of the SRCD spectrometer was confirmed daily using camphorsulfonic acid for optical rotation magnitude and wavelength (Miles et al., 2004). A 0.01 cm path length quartz Suprasil cell (Hellma GmbH & Co., Germany) was used for far-UV SRCD measurements at 25 °C. The far-UV SRCD spectra were recorded in triplicate from 270 to 170 nm in 1 nm steps, with a dwell time of 2.1 s per point.

For SRCD measurements, the solutions of WPC or WPCH were diluted in phosphate buffer at pH 8 or pH 2 (1:19, v/v) to reduce absorbance. All emulsion samples were measured untreated and after centrifugation (Eppendorf Minispin®, Eppendorf Nordic, Denmark). Centrifugation was conducted at 13,400 rpm for 15 min to separate the resulting bottom phase (aqueous phase) from the top phase (oil phase). The top phase was later re-suspended. This was carried out to separate the excess WPC or WPCH in the aqueous phase, which allowed the CD signal to be obtained from only the peptides located at the O/W interface. The re-suspended emulsions were further diluted in phosphate buffer at pH 8 or pH 2 (1:3, v/v) to adjust the maximum absorbance measured.

Temperature scans were carried out for WPCH in solution and at the O/W interface both at pH 8 and pH 2, with SRCD measurements taken at temperatures from 5.7 °C to 84.5 °C in steps of 5 °C, recorded in triplicate at each step.

2.7. SRCD data analysis and calculation of peptides secondary structure

The samples of WPC and WPCH naturally contain lactose, which itself has a CD spectrum starting below 193 nm (Fig S6.1). Therefore, in order to remove this contribution from the solution spectra, a spectrum of lactose at the appropriate concentration was subtracted from each spectrum. This same treatment was applied to the aqueous portion of the emulsion samples, where it is assumed that the majority of the lactose is free in solution and therefore largely removed from the re-suspended oil-droplet portion of the emulsions. The spectra were converted to delta epsilon units using the peptide concentration obtained from absorbance at 205 nm (Anthis & Clore, 2013) and corrected for scattering where necessary (Nordén et al., 2010). There are several factors which may affect the accuracy of the concentration determined in this way, most particularly for the emulsion samples. When light is elastically scattered from particles with a size similar to or smaller than the wavelength, it may not be detected by the detector. Where scattering occurs, it is evident in the absorbance spectrum which is measured simultaneously with the CD spectrum. A long, non-zero sloping tail of the absorbance curve can be fitted using an equation based on Rayleigh scattering to simulate the effect of scattering over the full spectrum (Nordén et al., 2010), allowing for a corrected absorbance at 205 nm to be obtained. Another factor involved, is that the emulsions are being separated into aqueous and oil-droplet portions, leaving unknown quantities of oil remaining in each portion. The latter means that it is difficult to match an underlying baseline for absorbance due to the oil, which results in an incorrect absorbance calculation for the peptide. A best estimate of both the underlying scattering and absorbance from the oil was carried out through combining baselines of different oil concentrations, at or less than the dilution factor from the original emulsion concentration. Therefore, if a re-suspended emulsion was diluted by a factor of 4 for measurement, baselines of the SDS stabilized emulsion at this same dilution factor were combined with a higher dilution factor (e.g., 1/8), until absorbance matched the sample at long wavelength. The amino acid composition of WPCH was used to determine the molar extinction coefficient and mean residual weight (Anthis & Clore, 2013) for all samples.

Another effect of light scattering from the emulsions is a change in the underlying CD baseline, due to the very different paths of light through the system compared to normal solution measurements. The SDS stabilized emulsion, produced in the same way as for the WPCH, enables the light scattering effects to be reasonably-well reproduced and therefore allowing a baseline correction of the peptide stabilized emulsions. Before analysis, all spectra were zeroed at long wavelength (270–280 nm), where no CD signal from the sample occurs.

The proportions of each secondary structure components were determined using a web-based calculation server DICHROWEB that incorporates various methods and a wide range of protein spectral databases (Whitmore & Wallace, 2007). The calculation method used in this study was CDSSTR with the reference set SMP180 (Abdul-Gader et al., 2011; Sreerama & Woody, 2000). These data were supported through additional fitting using SELCON3 analysis and the SMP180 reference set. Further details can be found in Section S3 of the Supplementary Information. Due to the nature of the emulsions, in particular due to the scattering which occurs from the droplets, the accuracy of peptide concentration determination is lower than for those in solution. In view of this, although the CDSSTR and SELCON3 routines report both regular and distorted α-helix and β-sheet structures, we report only the total helix and sheet structure to avoid over interpretation of the details of the structure content calculations. However, despite these higher uncertainties for the emulsions, which may affect the absolute values obtained through the secondary structure fitting, relative changes in the structure determination are still credible and the changes in CD spectral shapes from solution to emulsion samples reflecting significant folding changes are clearly observed.

2.8. Statistical analysis

The secondary structure fitting data were subjected to analysis of variance (ANOVA) by using Statgraphics version 5.1 (Statistical Graphics Corp., Rockville, MD, USA). Tukeýs multiple range test was used to determine significant differences between mean values. Differences between mean values were considered significant at a level of confidence of 95% ($p < 0.05$).

3. Results and discussion

3.1. Secondary structure of WPCH in solution and at the O/W interface

The far-UV SRCD spectra and the secondary structure composition (% α-helix, β-strands, turns and unordered) of WPC and WPCH in solution and at the tricaprylin oil–water interface (pH 2 or pH 8) are shown in Figs. 1 and 2 (with full data sets shown in Fig. S1.1).

Irrespective of the pH, the SRCD spectra of WPC in solution (Fig. 1a, b) showed a minimum at ca. 210 nm, a zero crossing at ca. 200 nm and a maximum at 190 nm, which is characteristic of proteins with a β-sheet-rich structure (Zhai et al., 2011). Although a high content of α-helical

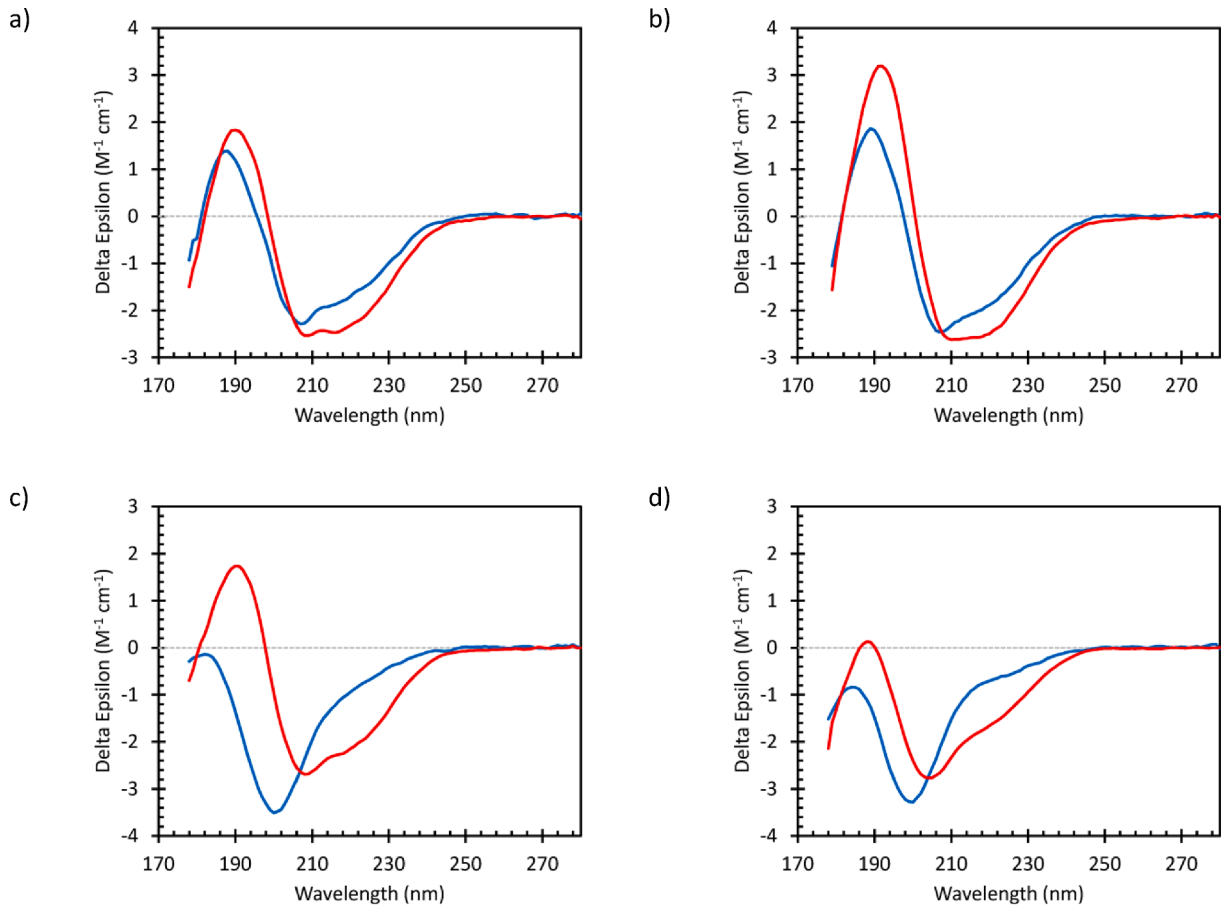

Fig. 1. Far-UV SRCD spectra in solution (blue line) or at the tricaprylin oil–water interface (red line) of a) WPC at pH 8, b) WPC at pH 2, c) WPCH at pH 8, and d) WPCH at pH 2. Light scattering of oil droplets in the emulsions was corrected using sodium dodecyl sulfate (SDS)-stabilised emulsions. WPC: whey protein concentrate; WPCH: whey protein concentrate hydrolysate. The data presented here are averaged over several replicates for each sample. Fig S1.1 includes the spectra of all replicates, plotted with the averaged data. Secondary structure analysis was carried out on the averaged curves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structure in solution has been found for other proteins contained in whey (e.g., α-lactalbumin and BSA) (Day et al., 2014; Zhai et al., 2012), β-lactoglobulin (β-LG) has been reported to exhibit a β-sheet-dominant conformation (Zhai et al., 2010, 2011; Zhang & Keiderling, 2006). The latter could explain our results since β-LG constitutes ca. 60% of whey proteins (Khan et al., 2019) and its high content may mask the contribution of other proteins present in lower quantity in terms of the secondary structure (e.g., ~15% of α-lactalbumin and ~ 8% BSA). The calculated ordered secondary structure composition of WPC is in agreement with previous studies of β-LG secondary structure in solution (16 and 33% of α-helix and β-sheet, respectively) (Zhai et al., 2011), with disordered structures (turns and unordered regions) of WPC in solution accounting for ~ 54% (Fig. 2a).

On the contrary, the far-UV SRCD spectra of WPCH showed a maximum at ca.185 nm and only a minimum at 200 nm (Fig. 1c,d), indicating a predominantly unordered structure in aqueous solution (Hoffmann et al., 2016), both at pH 2 and pH 8 (around 60% is made up of turns and unordered regions) (Fig. 2a). The ordered structure is predominantly β-sheet, with<10% α-helix structure (Fig. 2a). These changes in the conformation of WPCH in solution with respect to the WPC are a consequence of protein hydrolysis, since similar heat treatment using the hydrolysis conditions, but without enzyme (WPC + heat), did not significantly modify the secondary structure of WPC (Fig. S2.1 and S2.2). Hence, it can be concluded that enzymatic hydrolysis resulted in peptides with lower α-helix structure and higher unordered regions in solution than the parent protein, regardless of the pH (Fig. 2a).

In order to study the conformational changes induced by the adsorption of the native protein (WPC) or peptides (WPCH) at the O/W interface, the excess of un-adsorbed protein/peptides in the continuous phase was removed by centrifuging the parent emulsions (Section 2.6). The far-UV SRCD spectrum of an emulsion is comprised of signals from both protein/peptides present in the aqueous phase and at the O/W interface. Thus, to study solely the protein/peptides conformation at the O/W interface, there should be a minimum of un-adsorbed protein in the system (Zhai et al., 2010, 2012). As shown in Fig. S4.1, the emulsions had a very high content of un-adsorbed protein/peptides in the aqueous phase, since the CD spectra of the untreated emulsions were practically the same that the ones obtained for the solutions. Indeed, the spectra shown for the aqueous phase separated from the emulsions were almost identical to those of untreated emulsions and solutions (Fig. S4.1), which highlights the importance of the centrifugation step for studying protein/peptide conformation at the interface. Fig. 1 shows that the adsorption of WPC and WPCH at the O/W interface induced changes in their secondary structure when compared to the structure in solution. The CD spectra of WPC and WPCH adsorbed at the O/W interface are characteristic of a more α-helix-rich secondary structure showing two double minima at 209 nm and 222 nm and a maximum at ca. 190 nm (Hoffmann et al., 2016). In the case of WPC, the α-helical content increased by around 10% going from the solution to the tricaprylin O/W interface, which was accompanied by a slight decrease in both the β-sheet content and disordered structures. These structural changes are due to the reorientation of hydrophobic residues of proteins in WPC towards the oil phase in the emulsion, thus enhancing the interaction

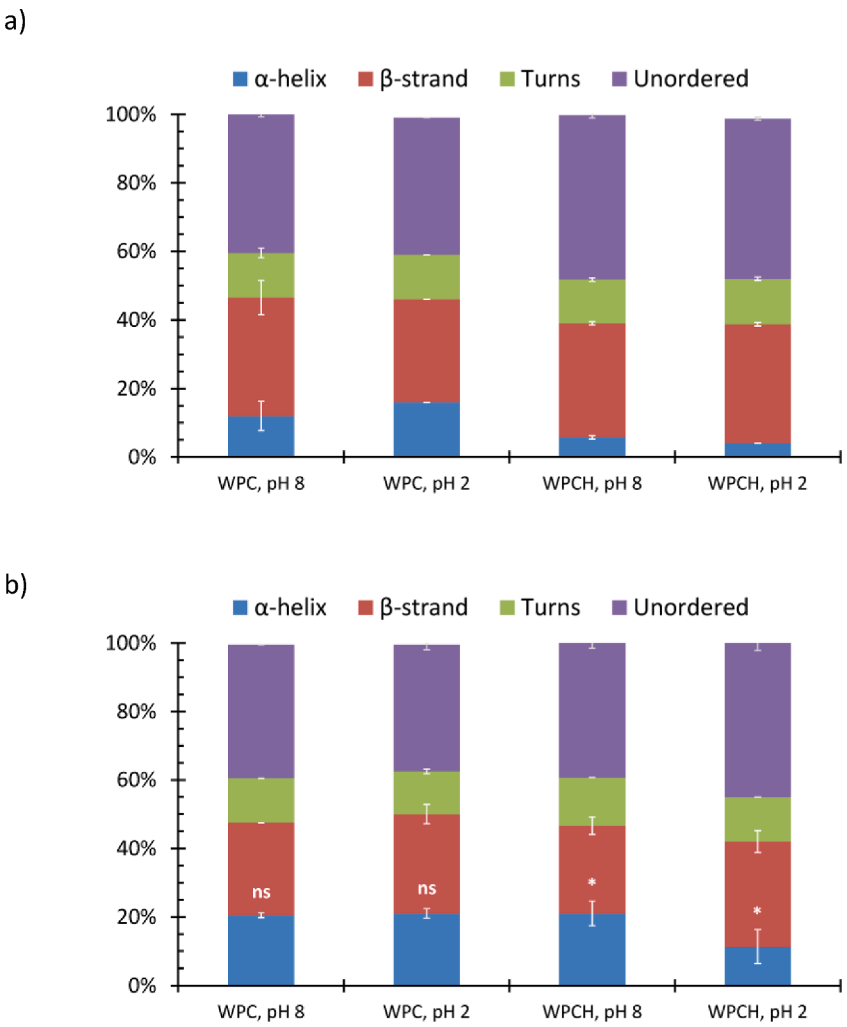

Fig. 2. Secondary structure composition (% α-helix, β-strand, turns and unordered) of whey protein concentrate (WPC) or whey protein concentrate hydrolysate (WPCH) in a) solution, and b) adsorbed at the tricaprylin oil–water interface at pH 8 or pH 2. The results derived from deconvolution of the respective SRCD spectra. For each emulsifier at fixed pH, ns: denotes not significant differences ($p > 0.05$) in % α-helix between solution and at the tricaprylin oil–water interface, whereas *: denotes significant differences ($p < 0.05$).

protein-oil (Zhai et al., 2011). However, although these results are consistent with previous studies on the secondary structure of β-LG upon adsorption at the O/W interface (Zhai et al., 2010, 2011), the increase of α-helical structure, compared to that in solution, was not statistically significant ($p > 0.05$) in the case of WPC (Fig. 2b). On the contrary, a significant ($p < 0.05$) increase in α-helical structure was found for WPCH at the O/W interface when compared to solution (Fig. 2b). Although protein hydrolysis of WPC gave rise to peptides with a highly unordered structure in aqueous solution (~60%) and high β-sheet content, adsorption of peptides present in WCPH at the O/W interface resulted in a slight lipid-induced β-to-α conformational transition. The non-native α-helical content increased by>10% going from aqueous solution to the O/W interface, with a concomitant β-sheet reduction. Furthermore, and similarly to WPC, the emulsion pH (pH 2 or pH 8) had a minor effect on the secondary structure of protein/peptides upon adsorption at the O/W interface. However, it should be noted that the pH 2 emulsions had a lower physical stability than those at pH 8, noticeable at time of measurement as inhomogeneity of the sample, resulting in a more significant variation in resulting spectra of peptide at the O/W interface (Fig S1.1). These results contrast with previous studies on the role of electrostatic interactions on whey proteins (β-LG)-lipids associations (Zhang & Keiderling, 2006). The authors reported that these associations were strongly affected by the protein net charge since at pH values under the protein isoelectric point (pI = 5.5) the lipid-induced non-native α-helical structure could be further increased at the expense of both β-sheet and unordered structures, contrary to what occurred at pH values above the pI. However, our results suggest that hydrophobic interactions played a major role (over electrostatic interactions) on conformational changes of protein/peptides when adsorbed at the O/W interface. It has been previously reported that structural changes of protein/peptides upon adsorption to the interface in emulsions are largely driven by the hydrophobic effect due to reorientation and solvation of the hydrophobic region of proteins/peptides towards the oil phase (Bañuelos & Muga, 1996).

Furthermore, our results show that hydrophobic interactions were enhanced in the case of WPCH over WPC, since the content of α-helical structure significantly increased at the O/W interface compared to that in solution (Fig. 2b). This is explained on the basis that protein hydrolysis resulted in smaller and more flexible peptides, thus favouring the exposure and interaction of the hydrophobic residues with the oil phase (O'Regan and Mulvihill, 2010; Rahali et al., 2000).

3.2. *Thermal stability of WPCH in solution and at the O/W interface*

The thermal stability of WPCH in aqueous solution and adsorbed at the tricaprylin O/W interface (pH 2 and pH 8) was investigated by recording the far-UV SRCD spectra of the samples at different temperatures (from ca. 6 °C to 85 °C) (Figs. 3 and 4). These measurements were carried out on two replicates of each solution sample. After heating, the samples were cooled back to 25 °C. In addition, at the spectra minima wavelengths (WLs), the CD signal evolution with temperature was extracted and fit to a sigmoidal curve from which the melting (transition) temperature (Tm) of the system was determined (Section S5 in the Supplementary Information).

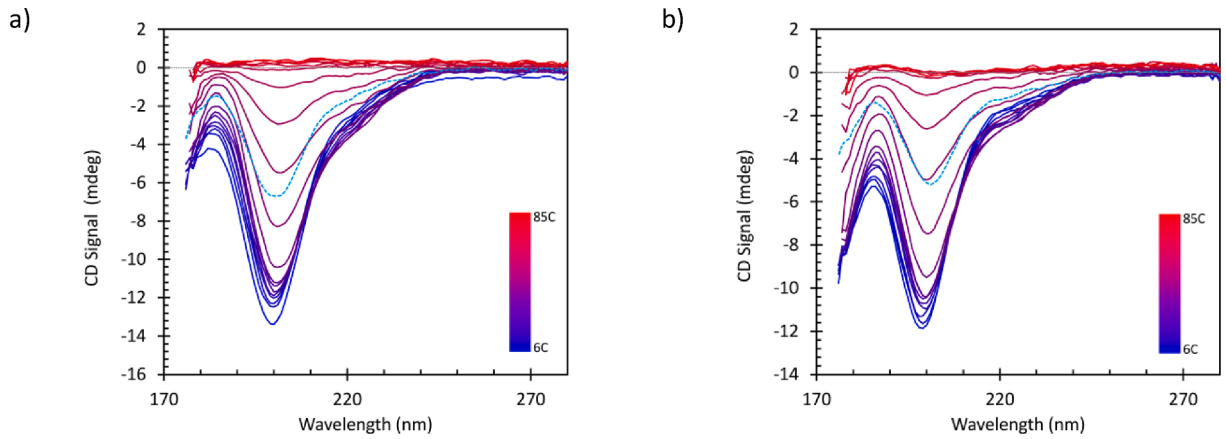

Fig. 3. Effect of temperature on the conformation of whey protein hydrolysate WPCH in solution at pH 8 (a) and at pH 2 (b). The data have been treated using 10 mM sodium phosphate buffer baselines and zeroed at long wavelengths (270–280 nm). The spectra also contain a small contribution from lactose at wavelengths below 193 nm. The light blue dotted curves are the spectra measured after the system was re-cooled to 25 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

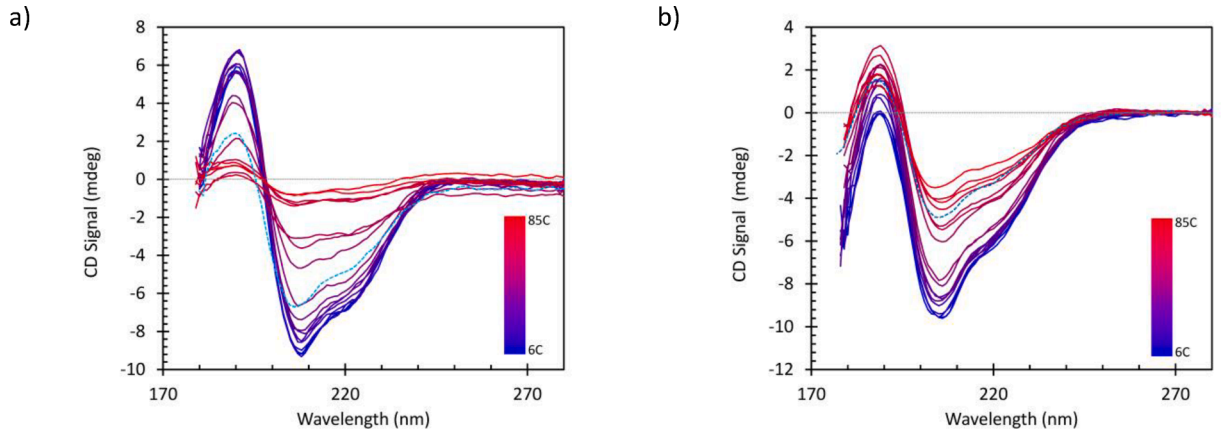

Fig. 4. Effect of temperature on the conformation of whey protein hydrolysate (WPCH) at the tricaprylin oil/water interface at pH 8 (a) and pH 2 (b). The data have been treated using an SDS stabilised emulsion measured at each temperature and zeroed at long wavelengths (270–280 nm). See S5 in the SI for more details.

In solution, the CD spectra of WPCH remained relatively unaltered up to ca. 38 °C regardless of the pH (Fig. 3). However, from 43 °C onward, the intensity of the spectra decreased gradually with the shape changing slightly, and was then completely lost at temperatures above 66 °C (flat spectra). These changes in the CD spectra of the peptides in solution are representative of a heat-induced denaturation of the secondary structure and the stability of the sample in the cell, yet this was partially reversible since some of the structure was recovered after re-cooling to 25 °C. The Tm of WPCH in solution was not affected by the pH, with similar values at pH 2 or pH 8 of $\sim 52 \pm 1$ °C (determined from the sigmoidal fits, Section S5 of the Supplementary Information, with the CD signal data recorded at 200 nm for all sample replicates).

There is a higher degree of uncertainty interpreting the temperature measurements on the emulsion samples (Fig. 4). Due to the scattering nature of the system and the change in scattering occurring as the temperature is increased, matching an underlying baseline for each temperature step is difficult. Fig. S5.2.1 shows the scans of an SDS stabilised emulsion taken with the same scanning parameters as for the samples. It clearly shows the changing nature of the scattering from the emulsion and therefore the underlying CD baseline of the system which will occur in these samples. However, there is no guarantee that an SDS stabilised emulsion will change in the same way as one containing the WPCH peptide. Therefore, in order to determine how much the baseline may affect the outcome, temperature scans were also treated using a single SDS emulsion baseline taken at room temperature for all temperatures. A full description and all data produced in this way are presented in Section S5 of the Supplementary Information. Another factor which may affect these emulsion temperature scans is the stability (due to the age) of an emulsion sample. Measurements of WPCH emulsions at both pH 8 and pH 2 were repeated on separately prepared samples, though prepared at the same time, and more significant differences in behaviour were seen compared to the solution samples.

The data presented in Fig. 4 have been treated with baselines measured on an SDS emulsion taken at the same temperature as the sample. Despite these additional difficulties, the behaviour of WPCH at the O/W interface system with increasing temperature is apparently different to that in solution. Although the CD signal for the solution samples at higher temperature becomes zero from temperatures around 70 °C (Fig. 3 and Figs in Section S5.1 of the Supplementary Information), the emulsion systems appear to show a higher level of stability, with a significant CD signal from the peptide remaining even at the highest temperature (Fig. 4), particularly at pH 2. These results suggest that the α-helical structure of WPCH at the O/W interface was retained to a higher extent at pH 2, so the peptides adsorbed at the interface are less prone to unfolding and aggregation upon heating. Furthermore, the range of temperatures over which the CD signal changed along the sigmoidal (data recorded at 209 nm and 222 nm) was broader at pH 2 than at pH 8 (Fig. S5.2.2–5.2.9). These results contrast with the calculated secondary structure of the peptides in WPCH adsorbed at the O/W interface (Fig. 2b) since slightly higher α-helical content was found for

WPCH adsorbed at the O/W interface at pH 8 than at pH 2, which does not explain the observed slightly lower thermal stability of WPCH at the O/W at pH 8 when compared to pH 2.

The errors associated with the repeated measurements of the samples (Section S5.3 of the Supplementary Information) make it difficult to be certain of a change in stability of the peptides at the O/W interface based on the thermal stability alone. For samples at pH 8 there is no apparent difference in the transition temperature, and for pH 2, the difference in transition temperature between the solutions and O/W interface is marginally outside one standard deviation of the error. However, combined with the CD spectra, which indicate that a signal from the peptide could still be observed at the highest temperatures for those in the O/W interface samples, this suggests an improvement of stability of the peptide at the O/W interface. These results are consistent with other studies on the thermal stability of whey proteins at the O/W interface (e.g., α-lactalbumin, β-LG, BSA), which reported that protein adsorption at the O/W interface resulted in an enhanced resistance to thermal denaturation compared to proteins in solution (Day et al., 2014; Zhai et al., 2010, 2011, 2012; Zhang & Keiderling, 2006). The latter might be attributed to an enhanced hydrophobic accessible surface area while these peptides are in contact with oil at the oil/water interface.

Although WPCH adsorption to the O/W interface only slightly improved the thermal stability of peptides at pH 8 in the present study, it has been reported that at these conditions their emulsifying properties are optimal (Padial-Domínguez et al., 2020b). These authors studied the influence of pH on the emulsifying activity index (EAI) and the emulsifying stability index (ESI) of WPCH and found that these were maximized at pH 8. Furthermore, a recent study on the interfacial properties of WPCH reported that WPCH at pH 8 showed higher interfacial adsorption and led to an interface with higher dilatational modulus than at pH 2. These enhanced interfacial properties of WPCH at pH 8 favoured the formation of smaller oil droplets in emulsions and a more resistant interfacial peptide layer leading to higher physical stability of emulsions stabilized with WPCH at pH 8 compared to pH 2 (Ruiz-Álvarez et al., 2022). Indeed, it is worth mentioning that differences in the physical appearance of emulsions was observed before measuring SRCD, with the emulsion stabilized with WPCH at pH 2 exhibiting creaming which was not observed for the emulsion stabilized at pH 8. Thus, the creaming in emulsion at pH 2 could affect the interaction of peptides at the interface influencing their thermal stability, although further research is required to confirm this.

WPCH has been used as emulsifier to produce fish oil-in-water emulsions (Padial-Domínguez et al., 2020a) and fish oil-loaded microcapsules (Rahmani-Manglano et al., 2020b), both at pH 8, showing high physical and oxidative stability. Hence, in the following section we aim to study the influence of the encapsulating agent and the drying method on the secondary structure of WPCH at the O/W interface at pH 8.

3.3. Effect of the encapsulating agent and the drying method on the secondary structure of WPCH at the O/W interface

Before studying the influence of the drying method, e.g., spray-drying or electrospraying, on the secondary structure of WPCH used as emulsifier to produce tricaprylin-loaded micro/nanocapsules, the influence of the encapsulating agents used for that purpose (e.g., GS, MD, PUL) on the secondary structure of WPCH in solution and at the O/W interface (pH 8) was investigated (Fig. 5). The spectra have been treated using the appropriate concentration of encapsulating agent included in the baseline scans as GS, MD and PUL have significant CD signals at low wavelengths starting below 200 nm, in order to remove any contribution from them in the final spectra (Section S6 of the Supplementary Information).

In aqueous solution, the delta epsilon plots of all the different samples overlapped, showing the same shape with the positive and negative peaks at ca. 185 nm and 200 nm, respectively (Fig. 5a). The similarities of the plots indicate that the addition of the encapsulating agents (e.g., GS, MD and/or PUL) did not modify the secondary structure of the peptides present in WPCH in solution. This indicates that there is not a significant interaction between these carbohydrates and WPCH. For samples of WPCH adsorbed at the O/W interface (pH 8), these were treated in the same way as the other emulsions samples, centrifuging the emulsion to remove the majority of the aqueous portion and re-suspending the oil portion in the buffer. However, while any minimal remaining contribution from lactose in previous samples could be ignored due to the very low starting concentration and CD signal (Section S6 of the Supplementary Information), these samples contain significant amounts of encapsulating agents with large CD signals. These may not be completely removed in the process of separating the oil portion from the emulsion, leaving an unknown quantity of encapsulating agents in the measured O/W interface samples. As it is not known how much remains, the spectra have not been treated to remove them thus the resulting spectra may have contributions from these encapsulating agents below 200 nm. Despite this, the addition of the encapsulating agents does not appear to significantly change the CD signal intensities of the spectra and, as has occurred with WPCH in solution, the shape and peaks of the spectra remained similar among the samples (Fig. 5b). Hence, the results found for the peptides' CD spectra in emulsion in the presence of encapsulating agents (Fig. 5b) are consistent with those of the peptides' CD spectra in solution (Fig. 5a) such that the same conclusion was drawn: the addition of encapsulating agents did not affect the secondary structure of WPCH at pH 8 in solution or at the

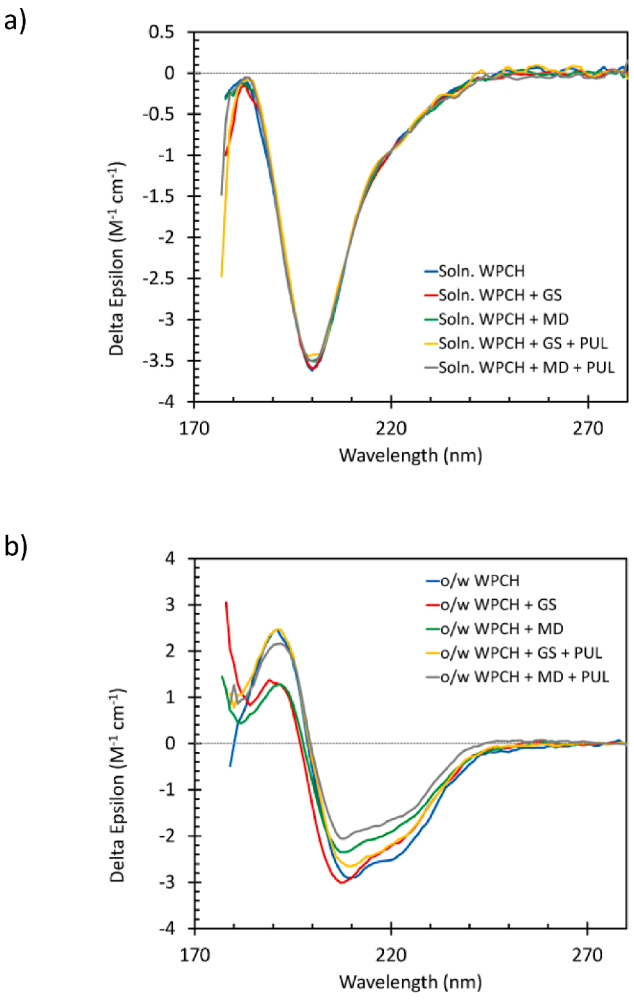

Fig. 5. Effect of the encapsulating agent on the conformation of whey protein hydrolysate (WPCH) in a) solution, and b) at the tricaprylin oil/water interface. GS: glucose syrup. MD: maltodextrin. PUL: pullulan.

O/W interface in any significant way.

Fig. 6 shows the far-UV SRCD spectra of the parent emulsions dried to produce the micro/nanocapsules and the reconstituted emulsions, i.e., those produced after re-dissolving the obtained micro/nanocapsules in buffer at pH 8. It should be noted that both parent and reconstituted emulsions were centrifuged as described in Section 2.6 in order to remove the excess of WPCH in the aqueous phase. After spray-drying for both encapsulating agents, GS or MD, it was found that the structure of WPCH adsorbed at the O/W interface remained practically unaltered since the shape of the CD spectra of both the parent and reconstituted emulsions were the same (Fig. 6a,b). After drying, it could be observed that the positive and negative peaks were found at practically the same WLs (190, 209, and 222 nm, respectively). Spray-drying might denature proteins due to thermal stress, shear stress, air–liquid interfacial stress and/or dehydration stress (Kusonwiriyawong et al., 2009). Nonetheless, our results show that these stresses were not sufficient to significantly change the conformational structure of the peptides present in WPCH and adsorbed at the O/W interface during spray-drying. In this line, Anandharamakrishnan et al. (2007) reported that moderate outlet gas temperatures (60–80 °C) could prevent excessive denaturation of whey protein isolate (WPI) during spray-drying, which was studied by differential scanning calorimetry (DSC). Likewise, Haque et al. (2015) spray-dried WPI at 180/80 °C (inlet/outlet air temperature, respectively) without significant protein loss due to denaturation and/or aggregation, although the secondary structure composition was different from that of unprocessed WPI (slight decrease in α-helix and β-sheet content). Furthermore, Kusonwiriyawong et al. (2009) studied the secondary structure of untreated BSA and BSA (5 or 10 %w/w) after spray-drying in the presence of chitosan (1 %w/v) at 100/63 °C (inlet/outlet air temperature) and found that the respective CD spectra, represented as molar ellipticity, overlapped. The latter is line with our results, where no conformational changes of WPCH at the O/W interface were observed between the parent and reconstituted emulsions when spray-drying the parent emulsions (180/90 °C inlet/outlet air temperature). Despite the use of high drying temperatures (100–200 °C), spray-drying (in the co-current operational mode) has been reported as a suitable method to produce microcapsules loaded with heat-sensitive bioactive compounds (Rahmani-Manglano et al., 2020a). Atomization of the infeed emulsion/dispersion results in fine droplets which assures that the internal mass transfer rate is high enough to evaporate most of the solvent at the inlet air wet bulb temperature (Twb) (constant- rate drying period). Subsequently, in the falling-rate drying period, the external mass transfer (from the surface of the droplet to the air) is also high allowing a reduced contact time, therefore very short residence time of the microcapsules in the drying chamber (5–30 s). Furthermore, in the co-current operational mode, the temperature of the dried product is 15–20 °C lower than that of the outlet air temperature, thus limiting thermal degradation (Anandharamakrishnan et al., 2007; Kusonwiriyawong et al., 2009).

On the other hand, the CD spectra of WPCH adsorbed at the O/W interface prior to and after electrospraying were different for both of the main encapsulating agents assayed, GS or MD (Fig. 6c,d), suggesting

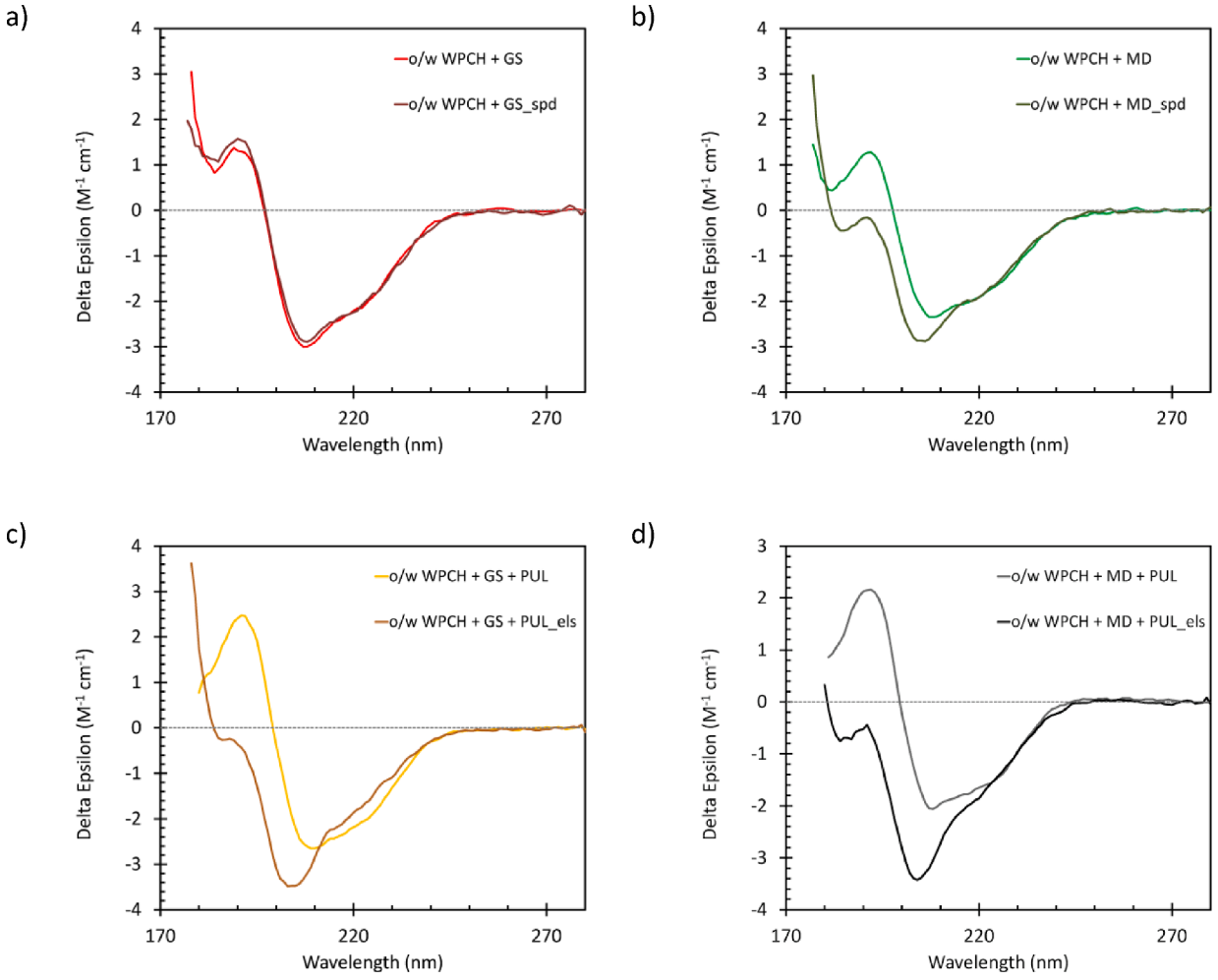

Fig. 6. Effect of the drying method on the conformation of whey protein hydrolysate (WPCH) at the tricaprylin oil/water interface at pH 8: a) and b) capsules obtained by spray-drying (spd), c) and d) capsules obtained by electrospraying (els). GS: glucose syrup. MD: maltodextrin. PUL: pullulan.

that the secondary structure of WPCH at the O/W interface changed during processing by electrospraying. The shape of the CD spectra after electrospraying for both emulsions containing GS and MD are representative of proteins/peptides with a high content of irregular structure showing a sharp minimum at ca. 200 nm (Fig. 6c,d) (Hoffmann et al., 2016). Electrospraying is based on applying a high-voltage electrostatic field to charge the surface of a solution that is ejected from the tip of the injector in the form of fine droplets due to Coulombic repulsion forces (Jacobsen et al., 2018). The process is carried out at room temperature, thus thermal degradation is avoided. Therefore, this technique has gained attention as an alternative to conventional spray-drying for the production of micro/nanocapsules loaded with thermo-sensitive compounds (e.g., vitamins, probiotics, antioxidants, and omega-3 oils) (Jacobsen et al., 2018). However, the dispersion/emulsion to be dried is subjected to electrostatic stresses, which could also cause some degradation of the components during processing. In this regard, Xiang (2008) studied the effect of pulsed electric fields (PEFs) on the structural modification of WPI, by tryptophan emission fluorescence spectroscopy. This technique gives information on the hydrophobicity or hydrophilicity of the environment to which tryptophan residues (Trp), originally buried in the hydrophobic core of globular proteins, are exposed. At an excitation WL of ca. 295 nm, Trp buried inside the globular protein core emits at WLs with a maximum at ca. 335 nm. However, when Trp are exposed to a hydrophilic environment (e.g., aqueous solvent due to protein denaturation) a red shift to higher WLs occurs, with a maximum at ca. 355 nm (Zhai et al., 2013). This technique has been widely used to study conformational changes of proteins contained in whey (e.g., α-lactalbumin, β-LG or BSA) either in solution or adsorbed at the O/W interface (Casterlain & Genot, 1994; Zhai et al., 2011, 2012). In the study by Xiang (2008), the authors reported that increasing the electric field intensity (from 20 kV/cm to 30 kV/cm) and the number of pulses (from 10 to 20 pulses) resulted in an increased fluorescence intensity of WPI, related to protein conformational changes which was further confirmed by DSC. The results showed that WPI subjected to an electric field of 20 kV/cm and 30 pulses denatured ~ 44% of the protein. Correspondingly, Perez & Pilosof (2004) found that β-LG was denatured by PEF (an electric field intensity of 12.5 kV/cm denatured 40% of protein after 10 pulses). The authors explained protein denaturation on the basis that, under an electric field, β-LG underwent conformational changes that would have exposed hydrophobic residues and the sulfhydryl group (SH), promoting hydrophobic and SH/S—S interactions, and therefore leading to protein aggregation. In the same line as the aforementioned studies, our results point to the electrostatic field applied between the injector and the collector of the electrospraying setup while the parent emulsions are sprayed/dried causing the secondary structural changes of the peptides present in WPCH and adsorbed at the O/W interface. Moreover, it is worth mentioning that electrosprayed capsules (80% of the capsules were below 1 μm) had a considerably lower size when compared to spray-dried capsules (90% of the capsules were below 20 μm) (results not shown). The reduced size of the electrosprayed capsules, when compared to spray-dried capsules, could also affect the observed changes in interfacial structure. In any case, further research is required to investigate how the electrospraying processing conditions (e.g., voltage, flow-rate, and distance from injector to collector) and particle size influence protein/peptide conformation when adsorbed at the O/W interface.

4. Conclusions

Protein hydrolysis of WPC to a low DH (10%) resulted in peptides (WPCH) with a lower α-helical structure in solution than the parent protein, and with a more unordered structure (<10% α-helix structure and ca. 60% turns and unordered regions). However, WPC and WPCH adsorption to the O/W interface led to an increase in the α-helical content (>10% increase in the case of WPCH), accompanied by a concomitant slight reduction of β-sheet and unordered regions. This lipid-induced conformational transition was more pronounced in the case of WPCH. Moreover, our results showed that pH (pH 2 and pH 8) had little effect on the WPC or WPCH conformation in solution and at the O/W interface. Although protein/peptides-lipid interactions are governed by electrostatic and hydrophobic interactions, our results show that the latter was the major driving force since pH, and thus the protein/peptides net charge, did not modify WPC nor WPCH secondary structure either in solution or at the O/W interface. In addition, it was found that WPCH adsorbed at the O/W interface slightly enhanced its thermal stability, particularly at pH 2. The presence of the encapsulating agents (e.g., glucose syrup or maltodextrin) did not affect the secondary structure of WPCH both in solution and at the O/W interface. Finally, it was found that the stresses exerted by spray-drying (e.g., thermal stress, shear and interfacial stress) were not sufficient to denature the peptides present in WPCH when adsorbed at the O/W interface. However, although electrospraying was carried out at room temperature, electrostatic stresses derived of applying high voltage to the emulsion caused structural changes in the peptides present in WPCH when adsorbed at the O/W interface.

CRediT authorship contribution statement

Nor E. Rahmani-Manglano: Methodology, Investigation, Formal analysis, Writing – original draft. **Nykola C. Jones:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Søren V. Hoffmann:** Methodology, Formal analysis, Writing – review & editing. **Emilia M. Guadix:** Supervision, Writing – review & editing, Funding acquisition. **Raúl Pérez-Gálvez:** Writing – review & editing. **Antonio Guadix:** Writing – review & editing. **Pedro J. García-Moreno:** Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133169.

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