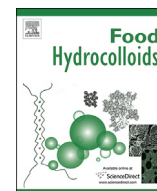


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Influence of moderate electric fields on gelation of whey protein isolate



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ABSTRACT

Proteins are one of the food constituents most affected by heating, and some of the changes involve their unfolding, denaturation and gelation. Ohmic heating has often been claimed to improve the quality of foodstuffs due to its uniform heating and (putative) presence of a moderate electric field (MEF). However, this is still subject to discussion, so it is important to determine the effect of ohmic heating and of its MEF upon food constituents. Hence, the aim of this work was to evaluate the effects of MEF on denaturation, aggregation and viscoelastic properties of whey protein isolate (WPI), and compare them with those obtained via conventional heating under identical treatment conditions (up to 30 min at 85 °C). Results have shown that MEF interferes with whey protein unfolding and aggregation pathways at relatively high temperatures. MEF treatments have resulted in WPI solutions possessing more 8 and 10% of native β -Lactoglobulin and α -Lactalbumin, respectively, after 30 s of heating at 85 °C, when compared with a conventional heating method. Protein aggregates from MEF-treated WPI solutions presented a maximum increase in size of 78 nm, whereas conventional heating produced an increase of 86 nm. Unlike in conventional heating, aggregation of whey proteins during MEF was not sufficiently strong to form a true elastic gel network, since decreases in both storage and loss modulus were observed following MEF treatment. Our results suggest that MEF may provide a novel method for production of a whey protein matrix with distinctive gel-forming properties.

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1. Introduction

Whey proteins have increasingly been used as nutritional and a functional ingredients in several food formulations, particularly in the form of dry whey, such as whey protein concentrate (WPC) and whey protein isolate (WPI). The functionality of whey ingredients is determined by changes in their physical and chemical properties during manufacture that commonly include such thermal processing as pre-heating, pasteurization or sterilization. β -Lactoglobulin (β -lg) is the most abundant globular protein found in whey, thus overriding functional (e.g. gelation and emulsification) and nutritional properties of whey derivatives (Lefevre, Subirade, & Pezolet, 2005). The effects of heat on denaturation of pure and enriched fractions of β -lg has been extensively reported (Nicorescu

et al., 2008). It is known that heating at high temperatures (>60 °C) produces thermal denaturation of globular whey proteins.

The process of denaturation of globular whey proteins is assumed to consist of at least two steps: a partial unfolding of the native protein, and a subsequent aggregation of unfolded molecules (Nielsen, Singh, & Latham, 1996). In particular, unfolding of the native conformation of globular whey proteins at neutral pH leads to exposure of free sulfhydryl groups (SH) and hydrophobic amino acid side-chains, normally occluded within bovine serum albumin (BSA) and β -lg (Kazmierski & Corredig, 2003; Schmitt et al., 2009; Shimada & Cheftel, 1989). With further heat treatment, free SH may rapidly interchange with existing disulfide bonds to generate new inter- and intramolecular disulfide bonds that will engage toward protein aggregation (Fairley, Monahan, German, & Krochta, 1996; Schokker, Singh, Pinder, & Creamer, 2000). The formation of intermolecular disulfide bonds by sulfhydryl–disulfide interchange is considered one of the major mechanisms of protein aggregation, and is mainly governed by formation

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of β -lg oligomers that combine into large aggregates (Havea, Singh, & Creamer, 2002).

However, the mechanism of protein unfolding and aggregation is complex and may be influenced by many factors, such as electrostatic and hydrophobic interactions, hydrogen bonding and disulfide cross-linking (Havea et al., 2002; Kazmierski & Corredig, 2003; Mulvihill, Rector, & Kinsella, 1990). Several studies have shown that the processes of protein unfolding and aggregation occur to different extents depending on the conditions prevailing in the aqueous solution (e.g. pH, ionic strength, ionic calcium, fat content, presence of lactose and protein composition/concentration), and heating treatments (Anema & Li, 2003; Dalglish & Banks, 1991; Law & Leaver, 2000; Nicorescu et al., 2008; Verheul, Roefs, & de Kruif, 1998).

Ohmic heating (OH) has been receiving increased attention due to its uniform transmission of thermal energy and extremely rapid heating rates that enable high temperatures to be applied without inducing coagulation or excessive denaturation of the constituent proteins (Parrot, 1992). In this sense, this technology provides processed products of higher quality (i.e. where such thermal labile compounds as proteins and vitamins can be preserved) than those obtained by conventional heating technologies (Castro, Teixeira, Salengke, Sastry, & Vicente, 2003; Machado, Pereira, Martins, Teixeira, & Vicente, 2010; Parrot, 1992). During OH treatment, electric current passes through food that behaves as a resistor in an electrical circuit, and heat is internally generated according to Joule's law (De Alwis & Fryer, 1990). OH technology can be distinguished from other electrical heating methods: by a) presence of electrodes contacting the foods; b) frequency range applied (from 50 to 25,000 Hz); and c) unrestricted, and typically sinusoidal waveform. OH is also known by the name of moderate electric fields (MEF) due to the application of an electric field of relatively low intensity (arbitrarily defined between 1 and 1000 V cm⁻¹) aiming at controlling permeabilization of membranes and other non-thermal effects (Machado et al., 2010). However, only a few scientific and technical works have focused on the effects of MEF upon enriched fractions of β -lg (which is the whey protein most susceptible to heat treatments). Recently, it was shown that MEF may lead to protein conformational disturbances during heat-induced denaturation of WPI (Pereira, Souza, Cerqueira, Teixeira, & Vicente, 2010; Pereira, Teixeira, & Vicente, 2011). Hence, is expected that MEF will influence protein aggregation kinetics and gelation, and accordingly affect WPI functional properties. Knowledge of WPI aggregation and gelation behavior upon MEF treatment would be of great significance to the food industry once this whey product is more often used than pure fractions of proteins essentially due to low cost and high availability. Despite WPC being considered the most widespread ingredient, the study of heat-induced aggregation of WPC is usually hindered by the presence of high quantities of fat, lactose and other impurities, which can modify the aggregation behavior and the role of the primary functional proteins (Kazmierski & Corredig, 2003). The objective of this work was therefore to evaluate the formation of soluble whey protein aggregates from WPI, under the presence and absence of MEF at almost neutral pH conditions. Fine-stranded WPI gels were also produced under acidic conditions, and characterized through small amplitude oscillatory dynamic measurements – in an attempt to provide new insights about MEF effects upon non-covalent protein interactions.

2. Material and methods

2.1. Whey protein isolate

WPI powder (Lacprodan DI-9212) used was kindly supplied by Arla Foods Ingredients (Viby, Denmark), and was essentially free of

lactose (max 0.5%) and fat (max 0.2%), with a protein content of 91% (of dry weight). Protein composition of WPI powder was determined by reverse-phase high-performance liquid chromatography (RP-HPLC): α -lac 22.8%, BSA 1.7%, β -lgA 44%, β -lgB 30.7% and immunoglobulins (IG) 1.1% on a protein basis. The amount of proteins considered to be in their "native" state (i.e. soluble at pH 4.6) was 85% of the total protein content (Pereira et al., 2011).

2.2. Whey protein solutions

Protein stock solutions of 3 and 10% (w/w) were prepared by dissolving the WPI powder in 50 and 20 mM sodium phosphate buffer (pH = 6), respectively. Both WPI solutions were then stirred continuously overnight at refrigeration temperature (5 °C), to ensure full rehydration. For studies of protein unfolding, denaturation and production of soluble protein aggregates, WPI solution at 3% (w/w) was prepared with final pH adjusted to 6.8 with 1 M of NaOH (Merck, Germany). It has been shown that, when WPI solutions of low protein concentration (1–3%) heated under neutral pH, small amounts of added salts, lead to the formation of soluble protein aggregates via both disulfide and hydrophobic interactions (Purwanti et al., 2011; Ryan et al., 2012; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007). For the development of protein gel, was used the WPI solution at 10% (w/w) and final pH was adjusted to 3.0 with 1 M of HCl (Merck, Germany). Heating WPI with high protein concentration (8–11%) and under acidic conditions can yield a gel stabilized by non-covalent bonding (Aymard, Nicolai, Durand, & Clark, 1999; Otte, Zakora, & Qvist, 2000). Electrical conductivity of the prepared WPI solutions ranged approximately from ca. 1000 to 1500 μ S cm⁻¹, which allowed OH effect to be observed.

2.3. Heating units

2.3.1. Conventional heating (COV)

Experiments were performed in a double-walled water-jacketed reactor vessel (3 mm of internal diameter and 100 mm height), as reported previously (Pereira et al., 2010). Treatment temperature was controlled by circulating thermo-stabilized water from a bath (set at the same temperature as that selected for the treatment) in order to better control temperature. A magnetic stirrer was introduced inside the reactor vessel to homogenize the solution and improve heat transfer during the heating cycle. Temperature evolution was measured with a type-K thermocouple (1 °C, Omega, 709, U.S.A.), placed at the geometric center of the sample volume and connected to a data logger (National Instruments, USB-9161, U.S.A.) – working with Lab View 7 Express software (National Instruments, NI Data logger).

2.3.2. Moderate electric fields (MEF)

MEF treatments were performed in a cylindrical glass reactor (30 cm total length and an inner diameter of 2.3 cm), with two stainless steel electrodes placed at each edge isolated by polytetrafluoroethylene (PTFE) caps, as described elsewhere (Pereira et al., 2010, 2011). A gap of 10 cm between the electrodes (the treatment chamber) was used for the experiments. The supplied voltage, and consequently temperature were controlled through the use of a function generator (Agilent 33,220 A, Bayan Lepas, Malaysia; 1 Hz–25 MHz and 1–10 V) connected to an amplifier system (Peavey CS3000, Meridian, MS, USA; 0.3 V–170 V). During heating phase and holding treatment, the MEF applied varied from 15 to 22 V cm⁻¹ and 4 to 8 V cm⁻¹, respectively, whereas electrical frequency was of 25 kHz. At this high frequency, (25 kHz), electrochemical reactions at the electrode interface are reduced, thus minimizing potential corrosion and leakage of metals to the

medium (Pataro et al., 2014; Pinto et al., 2013). Temperature measurement was performed as previously described for conventional heating. A data logger was employed to record continuously and simultaneously voltage, and current intensity across the samples during heating.

2.3.3. Heat treatments

WPI dispersions were heated through COV and MEF at a holding temperature of 85 °C. Both heating treatments were performed with heating rate of 19.1 ± 0.2 °C min⁻¹ and held constant at 85 °C for 30 min. At different holding time intervals (5, 10, 15, 20 and 30 min), samples were removed and cooled immediately in ice water at 4 °C for 10 min. A close similarity of the temperature profiles was obtained during both COV and MEF treatments (see Appendix A, Fig. A.1). This close pairing of heating rate and holding temperature is always a necessary condition to evaluate the non-thermal effects of MEF heating (Pereira et al., 2011).

2.4. Measurements

2.4.1. Reactivity of free sulfhydryl (SH) groups

The reactivity of free sulfhydryl (SH) groups on WPI solution was determined immediately after heat treatments using Ellman's 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) method, as described previously (Pereira et al., 2010). DTNB reacts with thiol compounds from whey proteins to produce 1 mol of *p*-nitrothiophenol anion/mol of thiol (Ellman, 1959), thus giving rise to a yellow color. During heating treatments, WPI samples of 100 µL were taken at different holding times (5–30 min) and diluted to 1 ml with phosphate buffer (5 mM, pH 8.0). To these solutions, was added 12 µL of 5 mM of DTNB (Sigma–Aldrich, Spain), prepared in pH 8.0 phosphate buffer. Color development was allowed to proceed for 40 min, and absorbance of samples was read on a microplate reader (Biotech Synergy HT) at 412 nm. Concentration of free SH groups was calculated using a standard curve of L-cysteine (Sigma–Aldrich, Spain) at pH 8.0, in the range of 12–95 µM.

2.4.2. Reversed-phase high-performance liquid chromatography (RP-HPLC)

The level of soluble native whey protein in the unheated and heated WPI solutions was evaluated by RP-HPLC. Denatured whey proteins were removed from the WPI solutions by adjusting the pH to 4.6, as described before (Pereira et al., 2011). The samples were held for 30 min, and then centrifuged at 15,558 g for 5 min in a Mikro 120 Microfuge centrifuge and filtered through filter paper (S & S Number 605). The filtrate containing the soluble (undenatured) whey proteins was injected in a reverse phase column C18 Symmetry 300, Waters USA (5 mm, 300 Å, 250 × 4.2 mm² i.d.), installed in a liquid chromatography system formed by an intelligent HPLC pump PU-2080 Plus, a 3-Line Degasser DG-2080-53, and a UV-970 detector (Jasco, Japan). A Symmetry guard-column (Waters, USA) was used as pre-column. Gradient elution was carried out with a mixture of two solvents (Ferreira, Mendes, & Ferreira, 2001): solvent A was 0.1% trifluoroacetic acid (TFA) in water, and solvent B was 0.1% TFA in a mixture of 95% aqueous acetonitrile and 5% water. The column was used at room temperature, the effluent was monitored at 280 nm and the total run time was 20 min. Standard calibration curves were produced by the external standard method with solutions that contained pure bovine β-IgA, β-IgB and α-lac in the range of 0.20–7.0 mg ml⁻¹, 0.30–9.4 mg ml⁻¹ and 0.03–3.3 mg ml⁻¹, respectively. At these concentration ranges, a linear relationship between the concentrations of β-Ig and α-lac was observed (results not shown). The identification of protein peaks was made by comparison of the retention times with those of the corresponding standards, which in order of increasing time

were 8.6, 9.7 and 10.0 min for α-lac, β-IgA and β-IgB (see Appendix A, Fig. A.2).

2.4.3. Native polyacrylamide gel electrophoresis (Native-PAGE)

In order to evaluate the heat treatment effects on denaturation and aggregation patterns of whey protein subunits, unheated and heated WPI samples were analyzed using Native-PAGE or “non-denaturing” gel electrophoresis. Native-PAGE analyses were carried out using the Mini-Protean II dual slab cell system equipped with a PAC 300 power supply (Bio-Rad Laboratories, Hercules, CA, USA). The resolving and stacking gel contained 10 and 3.7% of polyacrylamide, respectively. Whey protein samples were mixed with twice their volume of a non-reducing loading buffer of TRIS 0.5 mol L⁻¹ at (pH 6.8, 50% glycerol and 0.02% bromophenol blue). The gels were maintained overnight in 50% ethanol and 10% of acetic acid solution, stained with Coomassie Brilliant Blue (R250) solution and destained with a 5% ethanol and 7.5% acetic acid solution. The integrated intensities of whey protein bands were determined using the Bio-Rad software “Bio-Rad Quantity One”, associated with a GS-800 Densitometer (Bio-Rad Laboratories, Hercules, CA, USA). The quantity of each protein was determined as a percentage of that in the unheated samples (Anema & Li, 2003).

2.4.4. Particle size analyses

Particle size measurements were made by dynamic light scattering (DLS) using a Zetasizer Nano (ZEN 3600, Malvern Instruments Ltd., Malvern, U.K.), equipped with a He–Ne laser of 632.8 nm and 4 mW. Measurements of the dynamics of scattered light were collected applying backscatter detection NIBS (Non-Invasive Back-Scatter) at 173°, which reduces multiple scattering and allows higher concentrations to be measured. Samples of 1 ml of unheated (control) and heated (by COV and MEF) WPI solutions were poured into disposable sizing cuvettes and measurements were carried out, at least, in triplicate. The temperature of the cell was maintained at 25 ± 0.5 °C during measurement. Average diffusion coefficients were determined by the method of cumulants fit and were translated into average particle diameters (*Z*-value) using Stokes–Einstein relationship (Anema & Li, 2003). Polydispersity index (PDI) derived from cumulants analysis of the DLS measurements was also evaluated and describes the width or the relative variance of the particle size distribution. On the basis of previous works (Pereira et al., 2010, 2011), the variable parameters chosen for the formation of soluble whey protein aggregates (i.e. protein concentration, pH, buffer capacity and heating conditions) were selected to avoid coagulation or gel formation during heating, as this would impair size measurements.

2.4.5. Dynamic rheological measurements

Acidic WPI solutions at 10% of protein were heat-treated in order to induce formation of fine-stranded translucent gels (Errington & Foegeding, 1998). Gels were cooled in an ice water bath for 30 min, and held in the tubes overnight at 4 °C prior to rheological measurements. Small amplitude oscillatory dynamic rheological measurements were then performed on WPI gels. Measurements of elastic (*G'*) and viscous (*G''*) moduli were recorded in the oscillatory mode as a function of time, at intervals of 30 s. The oscillation stress amplitude was set at 0.1% and the frequency at 1 Hz. Stress sweeps were applied to check that data were acquired within the linear viscoelastic region.

2.4.6. Phase-contrast light microscopy

Microscopy was used to observe the physical structure of gels as described. One drop of WPI gel treated by COV or MEF was placed on acetone-cleaned microscope slides, and covered with a cover slip. Analyses were performed using an inverted microscope (Carl Zeiss,

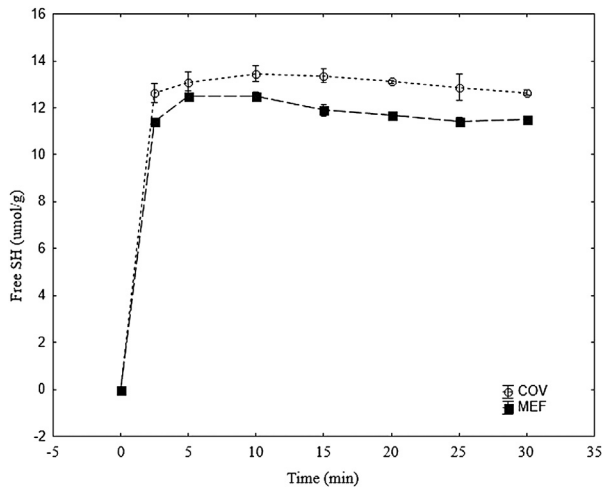


Fig. 1. Concentration of free sulfhydryl groups (SH) as a function of holding time during conventional (COV) and moderate electric fields (MEF) thermal treatments, at a holding temperature of 85 °C.

Axiovert 40, Ontario, Canada), equipped with a digital camera (Nikon D60) and using the phase contrast field at 40× magnification.

2.5. Statistical analysis

All statistical analyses involving experimental data were performed using Statistica software version 10.0.228.8 (StatSoft Inc.). Statistical significance was determined by Student's *t* and Turkey's tests, using 0.05 as preselected level of significance. Unless otherwise stated, all the following experiments were run at least in triplicate, and all measured parameters are means of four experimental points.

3. Results and discussion

3.1. Unfolding of whey proteins

β-Lactoglobulin contributes with 90–95% of the SH groups found in skim milk, and milk-derived protein ingredients (Owusu-Apenten & Chee, 2004). Thermal processing at denaturation temperatures (above 60 °C) involves changes in protein conformation that convert masked SH groups to free SH ones, thus becoming available for the formation of covalent disulfide bonds. The reactivity of free SH groups, measured at 85 °C under COV and MEF treatments as a function of time, is shown in Fig. 1. Activation and reactivity of SH groups in WPI solutions were observed upon both treatments (COV and MEF), and essentially reached a plateau value after early stages of heating (first 5 min). By 10 min of heating, the differences between both treatments became significant ($*p < 0.05$), and it was observed that COV samples presented more

1.2 μmol of SH per g of WPI than samples treated by MEF ($*p < 0.05$). These results do somehow agree with those previously reported, where COV heating led to higher reactivity of SH groups (more 2.2 μmol of SH per g of WPI) than samples treated by MEF ($*p < 0.05$), under the same heating temperature and time conditions (85 °C for 30 min) (Pereira et al., 2010). These results suggest that structural transitions and unfolding of β-Ig were less favored during MEF treatment, possibly due to less aggressive thermal effects associated with the presence of an alternating electric field. Denaturation kinetics are primarily driven by covalent bonding of unfolded proteins, which in turn is closely related to the availability and reactivity of the free SH groups of unfolded proteins (Kroslak, Sefcik, & Morbidelli, 2007). Therefore, the lower reactivity of free SH observed during MEF may contribute to low whey denaturation rates at early stages of the process and change the role of whey protein aggregation. Overall, calculated SH levels were slightly higher than the published values (Aboshama & Hansen, 1977; Cosio, Mannino, & Buratti, 2000; Owusu-Apenten, 2005), probably due to the use of WPI instead of skim milk fractions, and to different analytical and quantification techniques being employed. Nevertheless, the results obtained indicate unmasking and activation of SH groups in whey proteins upon extended heat treatment, as observed and reported in previous studies at temperatures above 70 °C (Koka, Mikolajcik, & Gould, 1968; Owusu-Apenten, 2005; Owusu-Apenten & Chee, 2004).

3.2. Whey protein denaturation

In order to study the influence of MEF and COV treatment upon extent of protein denaturation, samples from the same batch of WPI solution were analyzed before and during the heating process through RP-HPLC. The mean results and corresponding standard errors expressing heat-induced denaturation of whey proteins as a function of heating treatment are presented in Table 1. As expected, concentration of soluble native whey proteins decreased with an increase in holding time, and the degrees of denaturation were higher than 90% after 5 min of heating at 85 °C. The denaturation levels observed were slightly higher (10–15%) than those observed elsewhere (Anema & Li, 2003; Dannenberg & Kessler, 1988; Oldfield, Singh, Taylor, & Pearce, 1998). This can be explained by the fact that, during processing to obtain WPI powder, partial unfolding of the major whey proteins may occur that results in a more rapid denaturation on the subsequent heating treatments (Anema & McKenna, 1996). Moreover, these authors studied the disappearance of native whey proteins in skim raw milk fractions, and suggested that some of the constituents may have influenced the rate of denaturation.

When COV and MEF treatments were compared, significant differences ($*p < 0.05$) were found between the native total contents of whey immediately after 30 s of heating at 85 °C. In fact, after a holding time of 30 s, WPI solutions presented more 8 and 10% of soluble native β-IgA and α-lac, respectively, than WPI

Table 1
Reversed-phase high-performance liquid chromatography (RP-HPLC) quantification of β-Lactoglobulin (β-Ig) A and B and α-Lactalbumin (α-Lac) contents present in unheated and heated WPI solutions treated through moderate electric fields (MEF) and conventional (COV) heating, at a holding temperature of 85 °C and holding times ranging from 0.5 to 10 min.

Holding (min)	α-lac (mg ml ⁻¹)		β-IgA (mg ml ⁻¹)		β-IgB (mg ml ⁻¹)	
	COV	MEF	COV	MEF	COV	MEF
0	2.23 ± 0.12		7.53 ± 0.29		6.45 ± 0.15	
0.5	0.42 ± 0.02 ^a	0.64 ± 0.00 ^b	3.00 ± 0.11 ^a	3.58 ± 0.09 ^b	1.44 ± 0.12 ^a	1.75 ± 0.05 ^a
5	0.06 ± 0.00 ^c	0.06 ± 0.00 ^c	0.89 ± 0.04 ^c	0.93 ± 0.05 ^c	0.41 ± 0.00 ^b	0.43 ± 0.00 ^b
10	0.04 ± 0.00 ^d	0.04 ± 0.00 ^d	0.46 ± 0.01 ^d	0.45 ± 0.01 ^d	0.33 ± 0.00 ^c	0.34 ± 0.00 ^c

For each whey protein, means with different superscripts are significantly different ($*p < 0.05$).

solutions treated by COV under identical thermal profiles ($*p < 0.05$). These variations were dissipated for extended holding times (5 and 10 min), where no significant differences were observed between these treatments ($*p > 0.05$). The results outlined in Table 1 also indicate that the disappearance of native β -lg was faster for β -lgB than for β -lgA, which is in agreement with previous works (Croguennec, O’Kennedy, & Mehra, 2004; Nielsen et al., 1996).

Isolation and quantification of native whey proteins through RP-HPLC involves precipitation at low pH that can induce protein denaturation or produce low yields (Lozano, Giraldo, & Romero, 2008). Therefore, the influence of MEF on the extent of whey protein denaturation was also assessed by Native-PAGE. This method is considered fast and versatile for the quantification of whey proteins, and reflects the exact amounts of native proteins in samples submitted to different thermal treatments (Lin, Sun, Cao, Cao, & Jiang, 2010), by separating proteins in their native form based on charge, hydrodynamic size and shape of the molecule. Fig. 2 shows that, through Native-PAGE under non-reducing conditions, at least four major bands appear: β -lgA, β -lgB, α -lac and BSA. The whey protein bands on the resultant native gels were identified by concurrent electrophoresis of β -lg and α -lac samples. Some other protein bands (such as BSA) were identified by comparing the results with reported patterns (Glibowski, Mleko, & Wesolowska-Trojanowska, 2006; Havea, Singh, Creamer, & Campanella, 1998).

The results of Native-PAGE were quantified with gel densitometry, in attempts to measure the loss of the major native whey proteins. Since the amount of bound dye is proportional to the protein content, changes in the amount of protein fractions can be detected through the integrated intensities of whey protein bands (Anema & Li, 2003; Pásztor-Huszár, 2008; Schokker, Singh, Pinder, Norris, & Creamer, 1999). Table 2 displays the contents, in percentage of native whey proteins, determined by Native-PAGE during heating treatments. In general, both COV and MEF treatments have reduced considerably the contents of native whey proteins, and the denaturation levels observed were in good agreement with published literature (Anema & Li, 2003). In both heat-treated WPI solutions, the loss of BSA was faster than that of β -lg or α -lac, whereas the loss of α -lac was faster than that of β -lg thus agreeing with previous studies performed on whey protein (Havea et al., 2002). In particular, BSA was completely denaturated during the early stages of heating, since the corresponding band was lost after the first 5 min of heating at 85 °C. The decline in native β -lg was

considerably slower than that of α -lac; after 15 min of heating at 85 °C, the protein bands associated with native β -lgA (~14%) and β -lgB (~6%) remained in the gel. Conversely, native α -lac was completely lost after 10 min of heating. Isolated α -lac is considered one of the least heat-stable of the milk proteins; however, denaturation at relatively high temperatures can be substantially reversible, thus accounting for its apparently high thermal stability observed in bovine milk (Anema & McKenna, 1996; Larson & Roller, 1955; Lin et al., 2010). However, there are also studies showing that dimeric β -lg possesses a high stabilization free energy at neutral pH in phosphate buffer, which can explain the higher heat stability of β -lg against α -lac (Apenten, Khokhar, & Galani, 2002). Results also have shown that β -lgB was irreversibly denatured to a greater extent than β -lgA variant – in agreement with previous results from RP-HPLC. In fact, after 20 min of holding at 85 °C, only a small amount of native β -lgA was still present in the gel. This is in agreement with studies showing that B variant is less heat stable than A variant, in the range of temperatures studied; and with realization that, to achieve the same degree of denaturation of β -lgA, the holding time had to be increased by 30–40% as compared to that necessary for the corresponding denaturation of β -lgB (Anema & McKenna, 1996; Dannenberg & Kessler, 1988).

In agreement with the previous results obtained through RP-HPLC, whey protein denaturation was lower for MEF than COV treatment during the early stages of heating, under identical thermal profiles. MEF treatment determined higher amounts of native whey proteins (i.e. more 8 and 10% of soluble native β -lgA and α -lac, respectively), than COV, being this difference statistically significant for holding times of 5 min ($*p < 0.05$). This result does not agree with the one obtained through RP-HPLC, where no significant differences were noticed for holding times of 5 min between MEF and COV.

Moreover, denaturation levels observed via RP-HPLC were much higher than those observed by Native-PAGE. Samples heated for 5 min at 85 °C and analyzed by RP-HPLC showed a rather low percentage of native β -lg (~8%), whereas a considerable amount of protein with the same electrophoretic mobility as native β -lg (~25–35%) was still observed through Native-PAGE after the same heat treatment. This could simply be due to a greater sensitivity of PAGE technique, or else indicate that some of the irreversibly altered whey proteins had the same electrophoretic mobility as their native counterparts (Schokker et al., 1999).

Native-PAGE revealed that heating at 85 °C by COV and MEF, resulted in the disappearance of native β -lg bands, and the

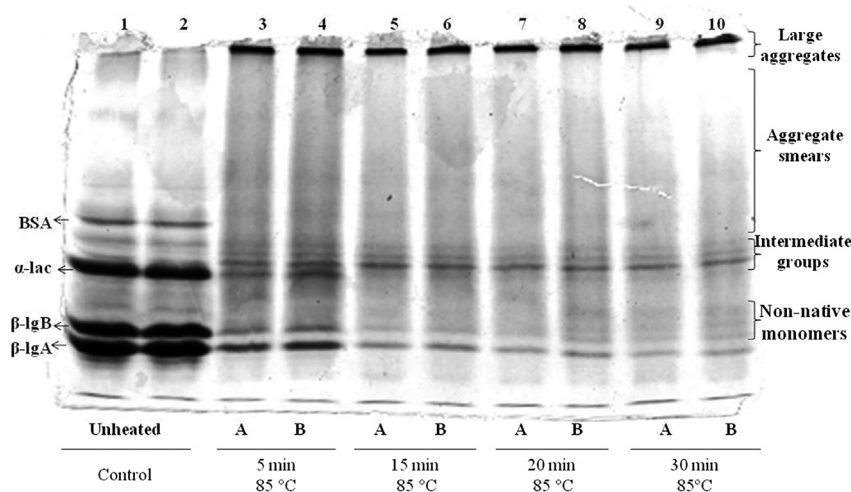


Fig. 2. Example of native polyacrylamide gel electrophoresis (Native-PAGE) gel bands of WPI solutions treated by moderate electric fields (MEF) and conventional (COV) heating, at a holding temperature of 85 °C and holding times ranging from 5 to 30 min.

Table 2
Native polyacrylamide gel electrophoresis (Native-PAGE) quantification of β -Lactoglobulin (β -lg) and α -Lactalbumin (α -lac) contents present in unheated and heated WPI solutions treated via moderate electric fields (MEF) and conventional (COV) heating, at holding temperature of 85 °C and holding times ranging from 5 to 30 min.

Holding (min)	α -lac (%)		β -lgA (%)		β -lgB (%)	
	COV	MEF	COV	MEF	COV	MEF
5	6.6 ± 0.4 ^a	9.0 ± 0.7 ^b	34.5 ± 1.0 ^a	42.8 ± 2.1 ^b	21.0 ± 4.6 ^a	27.2 ± 1.5 ^a
15	nd	nd	12.5 ± 0.5 ^c	13.9 ± 1.9 ^c	6.0 ± 1.6 ^b	5.4 ± 0.1 ^b
20	nd	nd	9.9 ± 1.2 ^{c,d}	10.6 ± 1.0 ^{c,d}	3.5 ± 1.2 ^c	2.8 ± 0.3 ^c
30	nd	nd	5.4 ± 0.1 ^d	6.7 ± 0.5 ^d	1.8 ± 0.1 ^d	2.5 ± 0.1 ^d

For each whey protein, means with different superscripts are significantly different (* $p < 0.05$). nd, not detected.

formation of protein species with lower electrophoretic mobility. Faded bands appeared between native β -lg and α -lac bands, which were identified by several authors as non-native monomers (Manderson, Hardman, & Creamer, 1998; Schokker et al., 2000). Faint bands in the region between BSA and β -lg have become distinct after the first 5 min of heating, and are identified as intermediate protein groups, such as β -lg dimers and trimers, that were formed during heating (Cho, Singh, & Creamer, 2003; Havea et al., 2002). Heating at 85 °C resulted in a sharp decrease in “native-like” β -lg and α -lac bands, and a concomitant appearance of both aggregate smears and high molecular mass protein aggregate bands (Fig. 2); this indicates extensive protein aggregation, and consequently aggregation of β -lg and α -lac (Glibowski et al., 2006; Schokker et al., 2000). Heating α -lac is responsible for producing non-native monomeric groups of α -lac dimers and larger aggregates. Previous studies showed that the irreversible transformation of α -lac into disulphide-bonded dimers, trimers and non-native forms is faster because of the presence of β -lg. Formation of β -lg: α -lac disulphide-bonded dimers is also caused by the heat effect (Havea et al., 1998; Hong & Creamer, 2002).

Protein concentration is an important factor concerning its denaturation by heat because of their tendency to aggregate when at high concentrations. (Glibowski et al., 2006; Schokker et al., 2000; Wehbi et al., 2005). In fact, protein large aggregates were at the top of the resolving gel – characterized by a molecular mass of ca. 250 kDa. Molecules with molecular mass above 1000 kDa remained at the top of the wells, and only the molecules up to 250 kDa could enter the resolving gel (Glibowski et al., 2006). The appearance of the large aggregate smear region changed with heating time for both MEF and COV treatments. In general, a maximum increase in the dye intensity of aggregate smear region was observed at 5 min of heating time (Fig. 2, lanes 3 and 4); the samples heated for 30 min showed a less intense aggregate smear band (Fig. 2, lanes 9 and 10). This may be a clue to an increase in the overall size (and molecular weight) of the heat-induced protein aggregates and complexes, which probably become trapped on the top of the stacking wells (Cho et al., 2003; Glibowski et al., 2006).

Both results from RP-HPLC and Native-PAGE indicate that MEF can preserve higher contents of native proteins during early stages of heating than COV (see Tables 1 and 2), which is in agreement with previous results regarding denaturation kinetics of whey proteins (Pereira et al., 2011). This lower denaturation observed during MEF is also supported by results from reactivity of free SH measurements. Moreover, it was possible to confirm the formation of soluble aggregates of whey proteins upon MEF treatment of WPI solutions through Native-PAGE (Fig. 2).

3.3. Whey protein aggregation

It is known that denaturation of β -lg under neutral pH and low ionic strength in the temperature range of 60–70 °C forms transparent dispersions, containing relatively small polymeric protein

particles with sizes < 100 nm (Hoffmann, Roefs, Verheul, VanMil, & DeKruif, 1996; Roefs & De Kruif, 1994). Native-PAGE provided evidence for the formation of high molecular mass aggregates of whey proteins after heating treatments of WPI solutions at 85 °C (Fig. 2). However, it was not possible to observe the effect of COV and MEF upon aggregation extent using electrophoresis, because large aggregates were formed and did not penetrate into the resolving gel after 5 min of heating (Fig. 2). Through DLS analysis, it was possible to confirm formation of soluble whey protein aggregates upon heating at 85 °C. The fundamental size distribution generated by DLS is an intensity distribution, which can be converted using Mie theory to a volume distribution – or can be used through the cumulant method to find the mean average (Z_{avg}), i.e. the size of a particle that corresponds to the mean of the intensity distribution. Z_{avg} is beneficial when citing a single average value for the purpose of comparison (Gordon & Pilosof, 2010).

Fig. 3 shows the size distribution curves obtained for unheated WPI dispersions, initially prepared at 3% (w/v), and the size distributions for the same dispersion after heating at 85 °C. In terms of intensity, the size distribution curves (sensitive to particles of large sizes) showed a first population with a maximum located between 2 and 10 nm (peak 1), a second population with a maximum between 16 and 78 nm (peak 2), and a final population at 122–712 nm (peak 3). This polymodal distribution of unheated WPI dispersions agrees with published literature. In particular, non-aggregated whey proteins, such as β -lg and α -lac monomers and dimers, remain below 7 nm (Gimel, Durand, & Nicolai, 1994; Nicorescu

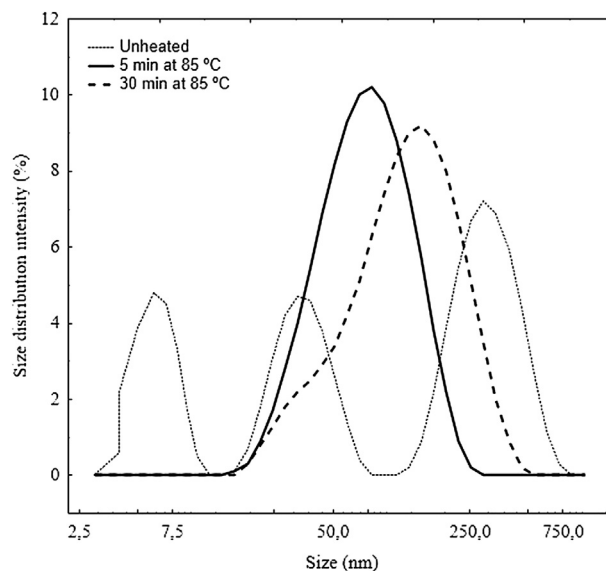


Fig. 3. Examples of particle size distribution intensity curves of whey protein isolate (WPI) solutions obtained via dynamic light scattering measurement, after heating at a holding temperature of 85 °C for 5 and 30 min.

et al., 2008; Roefs & De Kruif, 1994); this corresponds to peak 1 distribution observed in unheated WPI. Peak 2 and 3 correspond to native whey aggregates, which are commonly observed in industrial hydrated WPI (Grácia-Juliá et al., 2008; Nicorescu et al., 2008).

When WPI solutions were heated at 85 °C, the intensity of the size distribution curves unfolded disappearance of peak 1 (observed after 5 min of heating), with a concomitant increase of peak 2 and partial disappearance of peak 3 (see Fig. 3). Peak 3 may correspond to “clumps” of whey proteins that became disaggregated upon heating. Peak 2 distribution was progressively shifted towards larger sizes, ranging from 8 to 130 nm and 9 to 230 nm, for 5 and 30 min of heating, respectively. These results are consistent with those by Sharma et al. (Sharma, Haque, & Wilson, 1996) who observed a sharp decrease in small size particles (1–9 nm) with a concomitant increase in aggregates of larger sizes (100–500 nm) upon heating 5% (w/v) β -lg (pH 7.0) at 70 °C for 5 min. Grácia-Juliá et al. (2008) also observed that the maximum of peak 2 was progressively shifted towards larger sizes.

Fig. 4 indicates that slight oscillations in the values of electrical conductivity (σ) contributed to the different patterns of aggregation observed. These oscillations (ranging from 1000 to 1500 $\mu\text{S cm}^{-1}$) can be justified by very small variations on WPI powder weighting, preparation of buffer solution, pH adjustments or protein solubilization. However, irrespective of the electrical conductivity value, when the size of whey protein aggregates from COV- and MEF-treated solutions were compared, it was possible to observe that MEF produced always smaller structural changes ($*p < 0.05$), particularly after 30 min of heating. Unheated WPI presented a high

PDI (see Appendix A, Fig. A.3), as a consequence of the polymodal distribution of native WPI solutions. Upon heating, large aggregates have disappeared (corresponding to peak 3); hence, the size distribution became monomodal, and a drastic decrease in PDI was observed. With longer heating, PDI increased slightly to an identical plateau of approximately 0.270 in both heating treatments ($*p > 0.05$) – thus indicating that, within instrumental limits, the distribution was not broadening upon heating.

According to Native-PAGE results, heat-induced whey aggregates started to appear after 5 min of heating, and became larger with increasing heat time. However, the size increase observed during the first 5 min represented about 60–70% of the total size increase found after 30 min of heating (Fig. 3). The aggregation mechanism of WPI at neutral pH is close to that of pure β -lg (Mahmoudi, Mehalebi, Nicolai, Durand, & Riaublanc, 2007). Therefore, this aggregation behavior is considered to be the result of unfolding, and subsequent aggregation of β -lg, which occur through: disulphide exchange reactions (propagation) (Kazmiński & Corredig, 2003); reactions between reactive, unfolded intermediates and native β -lg molecules; and reactions by thiol group-thiol group (termination) of reactive intermediates (Hoffmann et al., 1996; Roefs & De Kruif, 1994). During thermal exposure at pH values above the isoelectric point, the unfolding of the protein's native globular structure exposes the hydrophobic region of β -lg, containing disulphide bonds and the free SH group (Laligant, Dumay, Valencia, Cuq, & Cheftel, 1991). The reactivity of the SH group for further reactions, such as disulphide exchange, is enhanced at high temperatures such as 85 °C; and, when combined

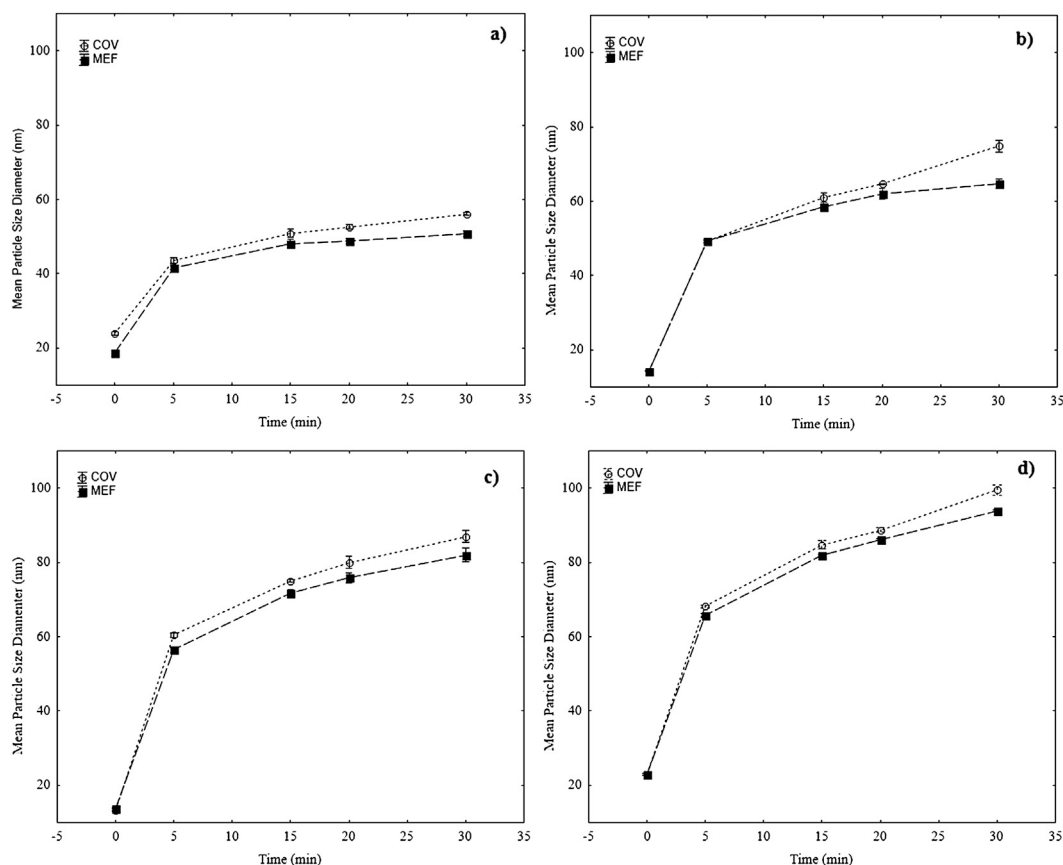


Fig. 4. Hydrodynamic size diameter (Z_{avg}) of whey protein aggregates as a function of heating holding time during heating of WPI solutions, at a holding temperature of 85 °C and different electrical conductivities: a) 1180 $\mu\text{S cm}^{-1}$; b) 1443 $\mu\text{S cm}^{-1}$; c) 1476 $\mu\text{S cm}^{-1}$; and d) 1533 $\mu\text{S cm}^{-1}$.

with long heating times, an increase in the probability of collisions leading to increased formation of WPI aggregates occurs (Gracia-Juliá et al., 2008; Kazmierski & Corredig, 2003).

It is also known that irreversible denaturation of whey proteins is characterized by an unfolding (first order-step) followed by aggregation (second-order step) (Tolkach & Kulozik, 2005). Theoretically speaking, if unfolding of β -lg is enhanced and if heat-treated β -lg contains a higher amount of free SH groups, the aggregation step will be favored (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). These observations are consistent with results obtained in this study, as they clearly show that MEF leads to less extensive whey protein aggregation –, in addition to the lower denaturation rates and reactivity to SH, when compared with COV, under identical thermal conditions. In this study, thermal histories of both MEF and COV treatments were made equal, including heating and holding time. Absence of very hot surfaces ($T \leq 85$ °C) and continuous stirring also assured a maximum similarity between heating methods (see Appendix A, Fig. A.1). Therefore, the different patterns of aggregation observed during MEF can be attributed to factors other than heating kinetics and efficiency. Apparently, MEF effects dictated marked differences in whey protein denaturation ($*p < 0.05$), when compared to those from COV under equivalent heating rates and holding times. As a consequence of this kinetic behavior, MEF lead also to less pronounced aggregation.

3.4. Whey protein gelation

The network structure in a heat-induced globular protein gel is strongly dependent on the balance between attractive and repulsive forces among denatured protein molecules during aggregation (Ikeda & Morris, 2002). Under neutral pH and low ionic strength, there is an increased electrostatic repulsion and formation of covalent disulfide bonds that reduce protein aggregate size (Schmitt et al., 2007). Whey protein aggregates formed under these conditions require higher levels of salts to screen the charge and form gels (Vardhanabhuti & Foegeding, 1999; Vardhanabhuti, Foegeding, McGuffey, Daubert, & Swaisgood, 2001). Moreover, the presence of an electric field may induce, together with changes in the reactivity of SH, disturbances on non-covalent interactions between proteins. Therefore, gel formation under conditions that induce protein interactions through non-covalent bonding were promoted in this study.

At acidic pH (far from the protein isoelectric point, which is ~ 5.2) and low ionic strength, a gel network form that is composed of fine strands in the order of nanometer in size (Ikeda & Morris, 2002; Kavanagh, Clark, & Ross-Murphy, 2000), where intermolecular covalent disulfide bonding is unlikely to occur (Aymard et al., 1999; Otte et al., 2000). WPI fine-stranded and transparent gels were produced at pH 3 after heating at 85 °C for 30 min.

Fig. 5 shows the frequency sweep of storage (G') and loss (G'') moduli of WPI gels formed by COV and MEF treatments. For both treatments, G' and G'' exhibited a very weak frequency dependence. COV gels presented always a G' larger than G'' over the entire frequency range; such features are characteristic of a true gel (Eissa, 2005). On the other hand, MEF originated a weaker gel structure than COV treatment, since decreases in both G' and G'' occurred. In addition, MEF resulted in nearly identical values for G' and G'' , or alternatively higher G'' than G' , thereby demonstrating two important points: 1) the number of disulfide linkages formed during the polymerization step is small, which is consistent with results concerning aggregation and reactivity to free SH; and 2) said disulfide linkages have a negligible effect on gel rheology. Since G' is related to the degree of cross-linking, this suggests the presence of additional cross-links in the COV treated sample (Eissa, 2005), when compared with MEF sample.

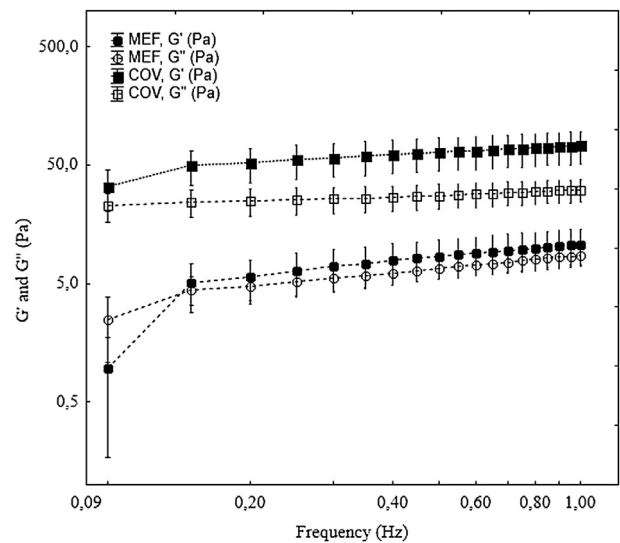


Fig. 5. Viscoelastic behavior of WPI 10% (w/w) gels obtained through conventional (COV) and moderate electric fields (MEF) heating, at a holding temperature and a holding time of 85 °C and 30 min, respectively. Closed symbols correspond to the storage modulus (G') and open symbols to the loss modulus (G''). Squares correspond to COV gels, and circles to MEF gels. The oscillation stress amplitude was set at 0.1% and the frequency at 1 Hz. Each frequency sweep represents the mean average of triplicated experiments.

During the initial stages of heating gelation, a transition from the protein native to a “progel” state occurs, and this is associated with unfolding and denaturation of the protein. The progel state is usually a viscous liquid state in which some degree of protein

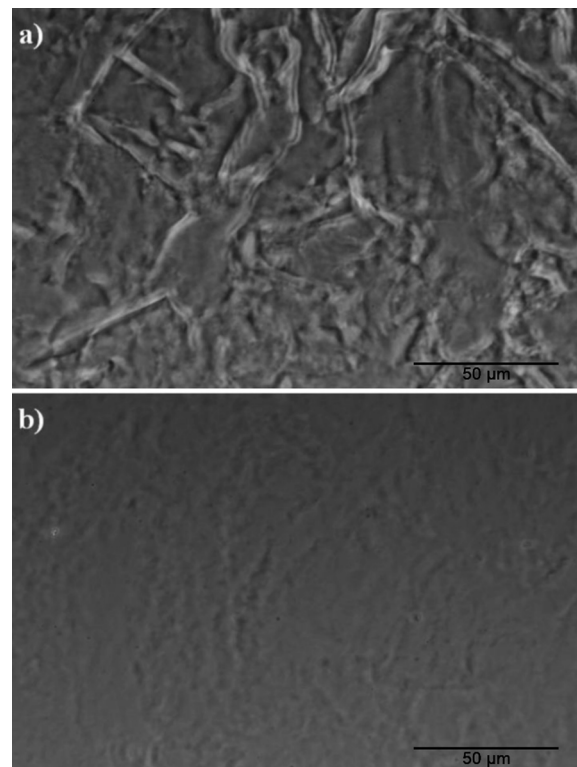


Fig. 6. Phase contrast micrograph at 40 \times magnification of WPI gels obtained through: a) conventional (COV) and b) moderate electric fields (MEF) heating, at a holding temperature and time of 85 °C and 30 min, respectively. Scale bar corresponds to 50 μ m.

polymerization has already occurred (Geara, 1999). Therefore, it can be suggested that a progel state was achieved at the end of MEF treatment. This progel or liquid-like state was made apparent by the fact that G'' was not dominated by G' during small amplitude oscillatory dynamic measurements at 25 °C (see Fig. 5).

Our results show that slight differences at nano-scale on the patterns of denaturation/aggregation have a great impact on physical properties of protein network structures. Aggregation and cross-linking patterns of whey proteins during MEF were not sufficiently high to form a true elastic gel network. Weaker gels with different dynamic viscoelastic behavior could be obtained under MEF. Furthermore, it was shown that lower levels of α -lac denaturation can be obtained during the early stages of MEF treatments. This may influence the gelation and the viscoelastic behavior of the MEF gels.

It has been reported that increasing the proportion of α -lac in protein solutions results in gels characterized by an open microstructure and reduced elastic and viscous moduli (Rabiey & Britten, 2009). These observations were also supported by phase-contrast light microscopy analyses. Fig. 6 shows the microstructure of the WPI gels produced. MEF gels exhibited a smooth and homogeneous appearance; whereas COV gels presented a well visible heterogeneous appearance, probably due to the presence of a more irregular and compact protein network structures.

3.5. Non-thermal effects of MEF

It is important to emphasize the lack of information pertaining to non-thermal effects of MEF on whey protein aggregation mechanisms. The presence of MEF, coupled with temperature may affect molecular flexibility or stability of whey proteins, and therefore aggregation –, likely in the same way of other extrinsic factors, e.g. pH and ionic strength (Sava et al., 2005). MEF may imply conformational disturbances on tertiary protein structure due to rearrangement of hydrogen bonds, hydrophobic interactions and ionic bonds.

During aggregation, application of an alternating electric field may also interfere with electrostatic interactions that play a major role in folding, conformational stability and protein–protein interactions (Neves-Petersen & Petersen, 2003). Non-covalent interactions may be impaired in the reorientation of hydrophobic clusters occurring in protein structure during application of MEF, thus affecting physical aggregation and gelation. This hypothesis is also supported by the differences found in SH crosslink between treatments (see Fig. 1), which were not as significant as the COV and MEF gels structures suggest (see Fig. 5). This indicates that MEF treatment may affect other kinds of interactions. Application of electric fields may also affect ionic movement in the medium, and modify the molecular environment due to the increased number of ions and their different distributions around the protein molecules (Castro, Macedo, Teixeira, & Vicente, 2004). Alternatively, the combined effects of MEF and sinusoidal frequency may promote splitting of large aggregates induced by thermal processing, thus enhancing the formation of small particles.

4. Conclusions

Heat-induced whey protein denaturation and aggregation is an important phenomenon that has been linked to milk fouling of heat exchangers, among other examples. There is still much to understand on the influence of heat upon the functional properties of whey proteins, especially because these depend on how the heat treatment is applied. Experimental data showed that MEF at 85 °C (at low ionic strength and neutral pH) produced a less extended denaturation of β -IgA during early stages of heating, when

compared with indirect heating. It is thus possible to conclude that not only unfolding and denaturation is reduced by MEF, but also size of the aggregates is smaller and remains always below that of the corresponding COV treatments. In addition, these lower rates of denaturation and aggregation may impose significant changes in the gelation mechanisms of whey proteins.

Together with other factors such as pH, ionic strength, protein concentration and the type of heating method (direct or indirect) used for protein denaturation should not be underestimated, once they may influence the viscoelastic dynamic behavior of gels. Non-thermal effects due to the presence of an electrical field must be taken into account during aggregation step of whey proteins; the alternating movement of electrical charges seems to disturb non-covalent interactions between proteins, thus leading to formation of gels with distinct viscoelastic behavior. However, a more complete study is in order for a better understanding of how the electric field interacts, at a molecular level, with individual whey proteins, so as to clarify the events occurring during unfolding, aggregation and gelation steps.

Heating combined with application of MEF may affect quality, as well as functional and technological properties of whey-derived products. Moreover, application of MEF during heating treatment may offer a great potential for development of GRAS (generally recognized as safe) whey protein hydrogels – with diverse mechanical and microstructural features, and thus improved textural properties.

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Appendix A

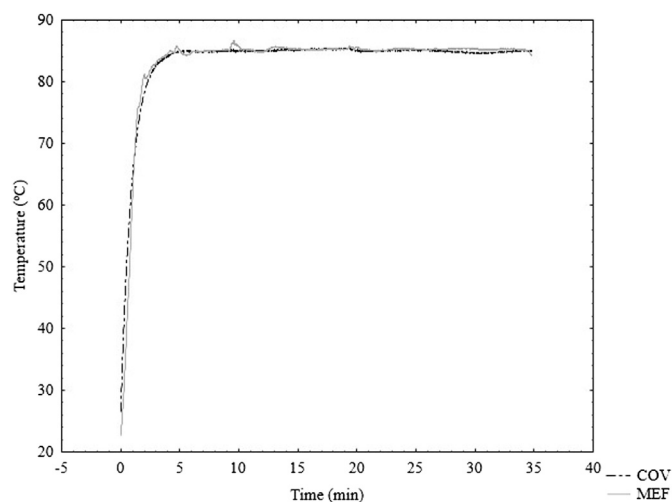


Fig. A.1. Example of heating rate and holding time of thermal treatments applied at 85 °C, for conventional (COV) and moderate electric fields (MEF) heating.

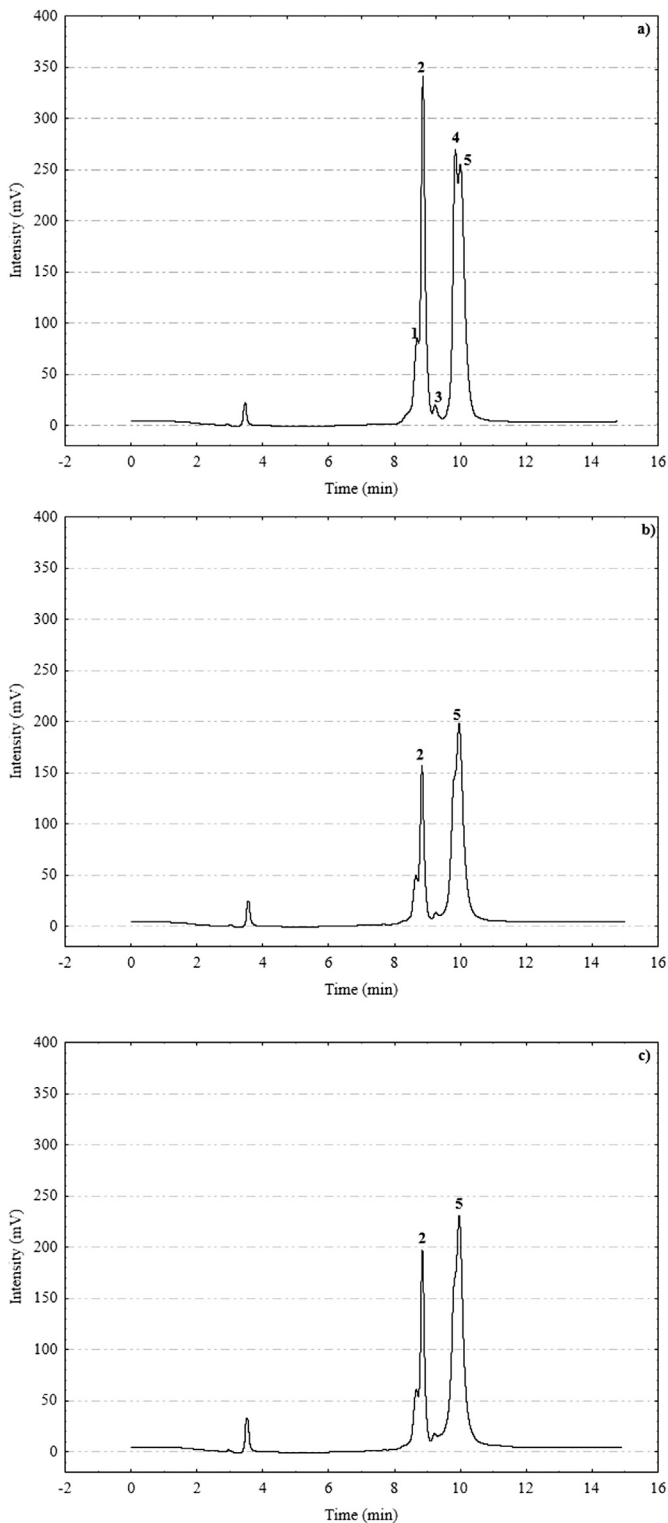


Fig. A.2. Example of chromatograms obtained for unheated (a), and heated WPI samples at 85 °C through conventional (COV) (b) and moderate electric fields (MEF) (c) heating, for a holding time of 30 s: (1) BSA; (2) α -lac; (3) immunoglobulins (IG); (4) β -lgB; and (5) β -lgA.

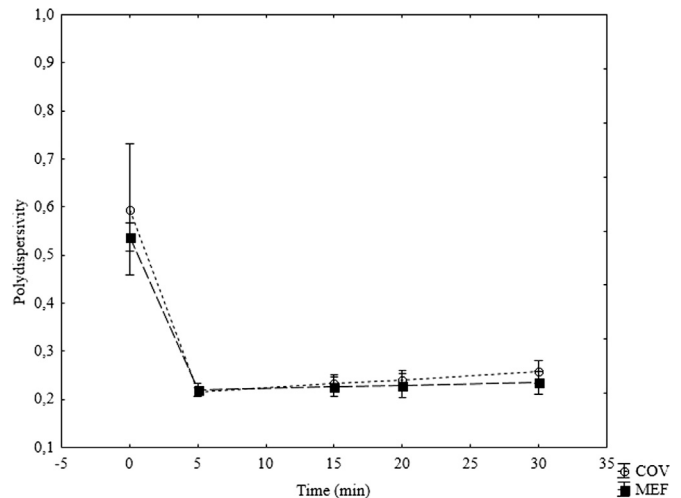


Fig. A.3. Polydispersity index (PDI) of whey protein aggregates as a function of heating holding time, during conventional (COV) and moderate electric fields (MEF) heating, at a holding temperature of 85 °C.

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