UNIVERSITAT AUTÒNOMA DE BARCELONA
Department of Animal and Food Science
Faculty of Veterinary

"Trabajo presentado para la superación de los 15 créditos del Módulo Trabajo Fin de Máster del Máster Oficial en Calidad de Alimentos de Origen Animal"

Application of front-face fluorescence spectroscopy as a tool for evaluating the functional properties of whey

"Aplicación de espectroscopía front-face como herramienta para evaluar las propiedades funcionales del Suero"

Yu Ting Teng

2/Jul/2021

Supervisors:
Manuel Castillo Zambudio
Anna Zamora Viladomiu

Declaro ser la autora de este Trabajo Fin de Máster que se presenta para obtener el grado de Maestría en Calidad de Alimentos de Origen Animal en la Universidad Autónoma de Barcelona, España. Este trabajo no ha sido presentado antes para obtener ningún grado o examen en cualquier otra universidad.

YU TING TENG - DNI Y7245542G

數位簽署者：YU TING TENG - DNI Y7245542G
日期：2021.07.02 14:43:47 +02'00'

Yu Ting Teng

Bellaterra, 2 de julio de 2021.

Los Doctores Manuel Castillo Zambudio y Anna Zamora Viladomiu (Área de Tecnología de los Alimentos del Departamento de Ciencia Animal y de los Alimentos de la Universidad Autónoma de Barcelona)

INFORMAN

Que el trabajo titulado: "Aplicación de espectroscopía front-face como herramienta para evaluar las propiedades funcionales del suero" ha sido realizado bajo nuestra supervisión y tutela por Yu Ting Teng dentro del módulo "Trabajo Fin de Máster" del Máster en Calidad de Alimentos de Origen Animal de la Universidad Autónoma de Barcelona.

Bellaterra (Cerdanyola del Vallés), 2 de julio de 2021.

UAB Universitat Autònoma de Barcelona
Signat digitalment per: Manuel Castillo Zambudio
Sotsdirector del Departament de Ciència Animal i de Facultat de Veterinària
2021.07.02 13:04:38 +02'00'

ANNA ZAMORA VILADOMIU
Firmado digitalmente por ANNA ZAMORA VILADOMIU
Fecha: 2021.07.02 12:27:40 +02'00'

Manuel Castillo Zambudio

Anna Zamora Viladomiu

Acknowledgement

Thank you to my supervisors, Dr. Anna Zamora Viladomiu and Dr. Manuel Castillo Zambudio, for providing guidance and feedback throughout this project, and for the inclusion of my language difficulties.

Thanks to all my colleagues at UAB. Especially, my senior Paulina, for her assistance and accompany, who helped me to gain excellent laboratory techniques and practice.

Last but not the least, I would like to thank my parents for supporting and encouraging me to challenge myself to study abroad.

List of Abbreviation

ANOVA	Analysis of variance
A_0	Initial absorbance
α-La	Alpha-Lactalbumin
β-Lg	Beta-Lactoglobulin
$\beta_{0\text{-}2}$	Regression coefficients
c	Initial protein content
CV	Coefficient of variation
D	Dilution factor
EAI	Emulsifying ability index
ESI	Emulsifying stability index
FFFS	Front-face fluorescence spectroscopy
GEL	Gel-forming index
I_{Trp}	Maximum intensity of tryptophan
n	Number of observations
Ov	Overrun
Ov/TWP	Normalized overrun taking into account the total whey protein content
r	Pearson's correlation coefficient
R^2	Determination coefficient
SEP	Standard error of prediction
t_{Drain}	Drainage time
t_{Drain}/TWP	Normalized drainage time taking into account the total whey protein content
TSI	Turbiscan stability index
TSI/TWP	Normalized TSI taking into account the total whey protein content
TWP	Total protein content of whey
UWP	Undenatured whey protein of milk
WPC	Whey protein concentrate
WPI	Whey protein isolate
W_{Trp}	Emission wavelength corresponding to the maximum intensity of tryptophan
φ	Volume fraction of oil in the emulsion

INDEX

Abstract

This study investigated the feasibility of front-face fluorescence spectroscopy to predict the functional properties of whey. Whey has a commercial interest due to its excellent nutritional value and versatile functional properties, while its attributes depend dramatically on heat treatment, which may alter its suitability for different food applications. Tryptophan front-face fluorescence of the whey and its functional properties, i.e., foaming, gel-forming, and emulsifying properties, were evaluated after milk heat treatment (at 80 °C with holding times of 0, 5, 10, 15, 20, 25 and, 30 min) in order to detect correlations between tryptophan fluorescence and functional parameters and generate predictive models. Whey samples were obtained by isoelectric precipitation of caseins (pH 4.6) from reconstituted skim milk powder enriched with whey protein isolate. As expected, heat provoked a decrease on the undenatured whey proteins of milk, the total concentration of proteins in whey and the intensity of tryptophan fluorescence. Gel-forming and emulsifying properties of whey significantly correlated with the maximum intensity of tryptophan ($P < 0.001$). Concerning foaming properties, only the foam stability index revealed a weak correlation with tryptophan maximum intensity parameters ($P < 0.05$). Merely the predictive models of emulsifying ability possessed a determination coefficient greater than 0.8, albeit most models presented a strong significance ($P < 0.001$). Thus, although significant correlations were observed, the information provided by tryptophan fluorescence was in general not enough to build strong prediction models.

Keywords: milk, whey, front-face fluorescence, heat treatment, functional properties, foam, foaming, gel, gelification, emulsion, emulsification.

Resumen

Este estudio investigó la viabilidad de la espectroscopía de fluorescencia "front-face" para predecir las propiedades funcionales del suero. El suero es de interés comercial por su valor nutricional y versátiles funcionalidades, pero sus atributos dependen drásticamente del tratamiento térmico, que altera sus aplicaciones alimentarias. La fluorescencia "front-face" del triptófano en suero y sus propiedades funcionales, como capacidad de espumante, gelificacante y emulsionante, se evaluaron tras el tratamiento térmico de la leche (80 °C durante 0, 5, 10, 15, 20, 25 y 30 min) con el fin de detectar correlaciones entre la fluorescencia y las propiedades funcionales, y generar modelos predictivos. El suero se obtuvo por precipitación isoeléctrica de caseínas (pH 4,6) en leche desnatada en polvo reconstituida y enriquecida con proteínas séricas deshidratadas. Tal como se esperaba, el tratamiento térmico provocó una disminución de las proteínas séricas nativas en la leche, de la proteína total del suero y de la intensidad de la fluorescencia del triptófano del suero. Las capacidades gelificante y emulsionante se correlacionaron significativamente con la intensidad de fluorescencia máxima del triptófano ($P < 0,001$), que también se correlacionó débilmente con la estabilidad de la espuma ($P < 0,05$). Los modelos predictivos sobre la capacidad emulsionante poseyeron un coeficiente de determinación superior a 0,8, aunque la mayoría de los modelos presentaron una alta significación ($P < 0,001$). En conclusión, aunque se observaron correlaciones significativas, la información proporcionada por la fluorescencia del triptófano fue, en general, insuficiente para generar modelos robustos.

Palabras clave: Leche, suero, fluorescencia "front-face", tratamiento térmico, propiedades funcionales, espuma, gel, gelificación, emulsión, emulsificación.

1. Introduction

Front-face fluorescence spectroscopy (FFFS) could be a process analytical technology suitable for estimating rapid and accurately food properties. It is considered as a potential method that meets the food qualification requirements, since the conventional methods are time-consuming, relatively expensive, labor-intensive, and are not feasible for on-line and in-line monitoring (Babu et al., 2018). However, FFFS can overcome those above inconveniences and provides abundant information with merely one test without destructing or altering the products. Moreover, it can be implemented as a predictive technique for identifying the structural and compositional changes (Taterka, 2016) and quantifying compounds of interest (Ayala et al., 2017; Liu et al., 2018; Alvarado et al., 2019) by detecting the deviation of the emission spectrum of fluorophores, since each of them presents a unique excitation and emission spectrum, resulting from different treatments or storage conditions (Shaikh and O'Donnell, 2017).

This technology is being studied for dairy products since milk possesses several fluorophores naturally or after chemical modification, including tryptophan (Trp), tyrosine and phenylalanine, dityrosine, NADH/FADH, vitamin A, riboflavin, and some Maillard reaction products (Andersen and Mortensen, 2008; Ayala et al., 2020). Among those, Trp is one of the most studied markers to monitor changes in proteins, since it has the greatest quantum yield contributing to stronger intensity of fluorescence emission (James and Patrik, 2001). Thus, it could give relevant information on the thermal treatment of milk, a fundamental step in dairy products manufacturing for improving microbiological quality and extending the shelf life. Several studies have adopted the FFFS to investigate the effect of thermal treatment on the milk by analyzing the fluorescence spectrum of Trp (Dufour and Riaublanc, 1997; Kulmyrzaev et al., 2005; Schamberger and Labuza, 2006; Hougaard et al., 2013; Taterka, 2016; Ayala et al., 2020). However, the degree of heat treatment must be sufficient to induce considerable conformational changes in order to modify the fluorescent response through a shift on the emission wavelength or a change on the maximum fluorescence intensity. For instance, Alvarado (2017) and Ayala et al. (2020) started to observe changes on fluorescence intensity of Trp once the milk underwent 80 °C treatments.

Whey is a fluid by-product produced from the precipitation of proteins in milk (Kilara and Vaghela, 2004) and it is widely used in the food industry due to the excellent nutritional value and versatile functional properties (Xu et al, 2019). Therefore, several studies have been intended to identify the structure and functional properties of whey to improve its utilization (Jiang et al., 2018). Heat treatments are compulsory processes in dairy manufacturing, and the consequent modifications may have not only desirable but also undesirable impact on the techno-functional properties of whey (Nunes and Tavares, 2019), depending on the temperature and the exposure time to the treatment (Nishanthi et al., 2017). In general, whey proteins start to denature at approximately 60 °C, but the substantial conformational changes occur at above 80 °C in that they lose their globular structures exposing the thiol groups and interacting with other whey proteins or casein.

As indicated above, the denaturation of whey proteins resulting from thermal treatment affects the functional properties (Anema, 2020). To be specific, heating is a prerequisite for foaming properties (Devilbiss et al., 1975) because a partial denaturation leads to molecular rearrangements, which are conducive to high viscosity surface for better stability. On the contrary, emulsifying ability decreases by increasing the thermal effect owing to a lesser number of native proteins. For gel-forming properties, heating significantly diminishes the gel strength, first due to the denaturation which provokes less available binding site for molecular interaction, and second, to the aggregation that facilitates the formation of particulate gel, which is weaker than the fine-stranded gel (Chime et al., 2009).

FFFS has been corroborated as a potential method for predicting the concentration of undenatured whey protein of milk (UWP) (Taterka, 2016). Recently, Freire et al. (2020) investigated the synchronous FFF of whey obtained through isoelectric precipitation of caseins (pH 4.6) from milk heated at 70 °C or 90 °C for six holding times (0-30 min). Fluorescence changes were detected in five areas of the synchronous response, of which four were correlated with the concentration of UWP. Among those, one peak intensity with excitation/emission wavelengths of 278 ± 1.9/337 ± 1.3 nm, which was attributed to Trp, showed a strong positive correlation with UWP ($r = 0.85$, $P < 0.001$). As the concentration of UWP decreased, both a decline of intensity and a redshift were observed. However, considering that denaturation of whey proteins influences the functional properties, to the best of our knowledge, no studies have as yet been found whether the potential of FFFS to serve as a tool for predicting the functional properties of whey has been claimed. The current study aimed at evaluating the

potential of FFFS of Trp to determine the functional properties of whey. Specifically, our objectives were to: 1) study the fluorescence response of Trp in whey after milk exposure to a wide range of different heating times, 2) study the effect of heat treatment of milk on whey functionality, and 3) generate models for predicting functional properties based on fluorescence response of Trp in whey.

2. Materials and Methods

2.1 Materials

Low-heat skim milk powder was chosen to minimize previous whey denaturation during the manufacturing process. The standardized skimmed milk powder (low-heat, spray-dried skim milk powder; pH = 6.5, solubility = 99 %, WPNI ≥ 7 mg/g, 800 cfu/g) was supplied by Chr. Hansen SL (Barcelona, Spain). Whey protein isolate (WPI) (protein content > 95 %, HTST-pasteurized, pH = 6.5, 10000 cfu/g) was purchased from Lactalis ingredients (Bourgbarré, France). All other chemicals used were of analytical grade from either PamReac AppliChem (Barcelona, Spain) or Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.2 Sample preparation

2.2.1 Reconstitution

Milk was reconstituted with 12 % (*w/w*) low-heat skim milk powder and 5 % (*w/w*) WPI by dissolving in 40 °C distilled water and stirring with the aid of a stir bar until completely mixed and rapidly cooled to 20 °C. The reconstituted milk was covered with aluminum foil to avoid light-induced oxidation and left at room temperature for 30 min to ensure full hydration.

2.2.2 Heat treatment

The reconstituted milk was heated inside steal sealed tubes in a water bath at 80 °C for seven holding times: 0, 5, 10, 15, 20, 25 and 30 min. The holding times were randomly selected.

2.2.3 Whey separation

After the heat treatment, the pH of the milk was adjusted to 4.5 adding 37 % *w/w* HCl as needed with continuous stirring for casein precipitation and centrifuged at 10,000 ×*g* (Sigma Laboratory Centrifuges 4K15, Osterode am Harz, Germany) for 20 min at 20 °C. The supernatant was filtered through a PRAT DUMAS A125210 filter to obtain whey.

2.3 Front-face florescence spectroscopy of whey

Fluorescence measurements of whey samples were collected using in duplicate a fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, Madrid, Spain) equipped with 15 W lamp "press Xenon lamp" and a "front-face" geometry accessory at 35° (Solid Sample Holder Accessory and Cuvette Kit, Agilent Technologies). The emission spectrum of tryptophan was acquired between 300 and 450 nm with the excitation wavelength set at 290 nm (Ayala et al., 2020). The maximum intensity (I_{Trp}) and the emission wavelength corresponding to the maximum intensity of Trp (W_{Trp}) were collected for detecting the shift and intensity change.

2.4 Undenatured whey protein content of milk

The UWP content of milk was determined following the standard procedure 15.134 documented in Standard Methods for the Examination of Dairy Products (Hooi et al., 2004). Milk samples were diluted with distilled water in a ratio of 1:2. Then, 22 ml of the dilution was mixed with 8 g NaCl in the test tubes and placed in a water bath at 37 °C for 30 min. During the first 15 min, for complete saturation of the diluted milk with NaCl, the test tubes were shaken every 1.5 min outside the heating bath and the next 15 min were kept inside the bath constantly. Without cooling, the solution was filtered through a Whatman 602H filter. Collected 1-ml filtrates were diluted with 10 ml of saturated NaCl solution and mixed slowly inverting 3 times in a test tube. Then, 2 drops of HCl solution were added to generate the turbidity and mixed slowly inverting 3 times again. The mixed solution was left stand 5-10 min and gently inverted twice before being pipetted to the cuvette. The spectra of the samples were collected using a UV–Vis spectrophotometer (Dinko Model UV 4000 spectrophotometer, Barcelona, Spain) with the wavelength set at 420 nm. Then, the percentage of UWP (*w/w*) was calculated with a standard curve. The extraction was performed in duplicates and measurements in triplicates.

2.5 Total protein content of whey

Total protein content of whey (TWP in % *w*/*w*) was analyzed in duplicate by the Dumas combustion method (IDF, 2002). Approximately 0.5 ml of whey solution obtained as described in section 2.2 above was weighted and dried overnight in capsules before analysis with a Leco analyzer (Leco Corporation, St. Joseph, MI). Total protein content of whey was used to normalize functional parameters.

2.6 Functional properties

For the evaluation of the functional properties, whey samples were stored at 4 °C overnight and warmed to 20 °C before the assessments. The emulsifying activity index and the emulsion stability index were measured on the day that whey was obtained.

2.6.1 Foaming properties

Foaming properties, i.e., overrun and foam stability, were evaluated in duplicate with modified methods of Patel and Kilara (1990) and Jambrak et al. (2008). The two 40 ml whey sample aliquots were whipped at room temperature with an electric blender (Silvercrest SHMS 300 A1, Kompernass GMBH, Germany) equipped with a wire whip beater at maximum speed for 5 min. Immediately after, 40 ml of foam was weighted. Foam overrun (%) was calculated by the following equation:

$$Overrun\ (\%) = \frac{\text{wt. of solution} - \text{wt. of foam}}{\text{wt. of foam}} \times 100,$$

where wt. is weight.

Foam stability was quantified by transferring a constant volume of the foam to a funnel with glass wool placed in the top of the stem, which retained the foam but allowed drainage. The time taken for the whole foam mass to drain was taken as a measurement of foam stability.

2.6.2 Gel-forming properties

Gel-forming properties were evaluated in triplicate with modified method of Luck et al. (2013). 10 g of whey protein solution was heated inside the 15 ml centrifuge tubes in the water bath set at 90 °C for 1 h. After gel formation, the sample was centrifuged at 2,000 $\times g$ (Sigma Laboratory Centrifuges 4K15) for 10 min at 20 °C. The weight of the pellet was used as a gel-forming index.

2.6.3 Emulsifying properties

The oil-in-water emulsions were prepared by homogenizing 25 % of sunflower oil (8.3 g) and 75 % of (25 g) whey with ~0.5 ml silicone for preventing the foam formation during emulsification using a homogenizer (Heidolph homogenizer DIAX 900, Merck KGaA, Darmstadt, Germany) at 15,200 rpm for 5 min. An emulsion aliquot of 20 ml was transferred

gently to a turbiscan tube and measured by Turbiscan (Turbiscan Lab Expert, Formulaction, Toulouse, France) for 30 min detecting every 1 min. The slope of turbiscan stability index (TSI) as a function of time from the bottom of the tubes was measured. The whole analysis was performed in duplicate.

For the emulsifying activity index (EAI) and emulsion stability index (ESI), the method of Pearce and Kinsella (1987) was adopted. EAI represents the amount of oil that can be emulsified per unit of protein; ESI is an index of the ability of the emulsion to resist changes through a defined time period (Boye et al., 2010). Both indexes were measured in duplicate. The oil-in-water emulsions were prepared by homogenizing 25 % of sunflower oil (8 g) and 75 % of diluted whey (1:10 dilution, 24 ml) with a homogenizer (Heidolph homogenizer DIAX 900, Merck KGaA) at 15,200 rpm for 5 min. 100 µl of the emulsions were taken immediately (0 min) from the bottom of the emulsion and added into 10 ml of 0.1 % (*w/v*) SDS solution. After mixing the solution by inverting 5 times, the absorbance of the diluted emulsions was measured at 500 nm on a UV–Vis spectrophotometer (Dinko Model UV 4000). The measurement was repeated after 10 min with the same sample and same procedure.
The EAI and ESI values were calculated with the following equation:

$$EAI(m^2/\mathrm{g}) = \frac{2\times 2.303 \times A_0 \times D}{c \times \varphi},$$

where A_0 is the initial absorbance (0 min), D is the dilution factor (0.1), c is the initial protein content (g), and φ is the volume fraction of oil in the emulsion (0.25).

$$ESI(\%) = \frac{A_0}{A_{10}} \times 100,$$

where A_0 is the initial absorbance (0 min), and A_{10} is the absorbance after 10 min.

2.7 Statistical analysis

The complete experiment was replicated four times, except for the EAI and ESI, which were repeated twice. Analysis of variance (ANOVA) and Pearson's correlations were performed with Statgraphics Centurion XVI software (version 16.1.03, 2010, Statgraphics Technologies, Inc., The Plains, Virginia, USA). LSD test was carried out for means comparison and evaluations were based on a significance level of $P < 0.05$. The predictive models were obtained using the maximum R^2 procedure (REG, MAXR) of SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA).

3. Results and Discussions

3.1 Effects of heat on whey proteins

The percentage of UWP of milk as a function of the heat treatment is presented in Table 1. A significant decrease of UWP ($P < 0.05$) was observed as heating time increased; the more intensive the thermal treatment was, the greater the denaturation. Specifically, the concentration of UWP declined dramatically by ~41 % in whey obtained from milk heated for 5 min at 80 °C. In general, heating the whey proteins above 70 °C even for a short period of time, especially α-Lactalbumin (α-La) and *β*- Lactoglobulin (*β*-Lg), two major whey proteins that account for 80 % of total whey nitrogen (Farrell et al., 2004), is sufficient to cause irreversible denaturation (Edwards and Jameson, 2014). Heat induces the exposure of reactive thiol groups of *β*-Lg, which were initially buried within the whey protein structure, and allow them to interact with κ-casein or with other denatured *β*-Lg (Sawyer et al, 1963). As to the α-La, it is believed that there are no direct interactions between α-La and caseins, except for some particular conditions, such as a high pH environment (Doi et al., 1983). Normally, denatured α-La reacts with *β*-Lg first (Baer et al., 1976; Elfagm and Wheelock, 1978), and the formed complex then interacts with κ-casein (Anema, 2020). Subsequently, due to the isoelectric precipitation of casein micelles, some denatured whey proteins precipitated with caseins. As a result, the concentration of TWP decreased as heating time increased as well (Table 1). In fact, according to Pearson's test, a strong positive correlation between the concentrations of UWP and TWP was observed ($r = 0.99$, $P < 0.0001$). Freire (2020) observed no significant differences on UWP of milk heated at 70 °C below 15 min and above 10 min for milk heated at 90 °C. In the present study, 80 °C was applied and the concentration of UWP and TWP differed significantly ($P < 0.05$) among the seven holding times.

Table 1. Effect of heat treatment at 80 °C on the concentration of undenatured whey protein of milk and total protein of whey, and front-face fluorescence response of tryptophan.

Time (min)	UWP (%)	TWP (%)	W_{Trp} (nm)	I_{Trp} (a.u.)
0	2.93 ± 0.13 ^{a}	4.20 ± 0.04 ^{a}	333.91 ± 0.74 ^{b}	74.5 ± 5.83 ^{a}
5	1.74 ± 0.11 ^{b}	2.20 ± 0.11 ^{b}	333.66 ± 0.62 ^{b}	65.3 ± 5.52 ^{b}
10	1.42 ± 0.08 ^{c}	1.83 ± 0.07 ^{c}	333.15 ± 1.65 ^{b}	60.8 ± 2.02 ^{c}
15	1.27 ± 0.09 ^{d}	1.58 ± 0.04 ^{d}	335.28 ± 1.26 ^{a}	59.6 ± 5.07 ^{c}
20	1.11 ± 0.04 ^{e}	1.45 ± 0.03 ^{e}	335.26 ± 1.06 ^{a}	55.7 ± 3.14 ^{d}
25	1.02 ± 0.03 ^{f}	1.33 ± 0.04 ^{f}	336.01 ± 1.46 ^{a}	55.2 ± 2.21 ^{d}
30	0.97 ± 0.05 ^{g}	1.25 ± 0.05 ^{f}	336.53 ± 0.82 ^{a}	52.3 ± 1.57 ^{d}

Mean value ± s.d; n=54 for UWP, n=56 for TWP, I_{Trp} and W_{Trp}; UWP: the concentration of undenaturated whey protein in milk; TWP: total protein content of whey. I_{Trp}: the maximum intensity of tryptophan. W_{Trp}: the emission wavelength corresponding to the maximum intensity of tryptophan. $^{a-g}$: values per column with different letter were significantly different ($P < 0.05$).

3.2 Effect of heat on FFF response of Trp.

Heat induced a redshift and a decrease of Trp fluorescence intensity (Table 1 & Fig. 1). Without heat treatment, the emission spectra of whey exhibited a Trp maximum emission wavelength at around 333.9 nm, whereas for the sample subjected to thermal treatment at 80 °C for 30 min, it increased to approximately 336.5 nm. Significant differences were observed only between two groups, those with a heat treatment ≤10 min and those ≥15 min. Therefore, the emission wavelength of Trp corresponding to the maximum intensity was weakly and negatively correlated with UWP (r = -0.47, $0.01 \leq P < 0.05$) and TWP (r = -0.43, $0.01 \leq P < 0.05$). As to the peak intensity, a significant decline was observed as holding heat time increased, dropping from 74.5 to 52.3 a.u (i.e. a 30 % decrease). The peak intensity of Trp was strongly and positively correlated with the concentration of UWP (r = 0.87, $P < 0.0001$), as well as that of TWP (r = 0.85, $P < 0.0001$). Freire (2020) did not observe statistical differences in the maximum intensity of the area attributed to Trp of whey obtained from milk heat-treated at 70 °C. On the contrary, treating milk at 90 °C provoked a significant decrease of intensity. Moreover, in the same study, Freire (2020) detected a redshift of the whey obtained from milk heated for at least 10 min at 90 °C. Accordingly, Ayala et al. (2020) found that the redshift in milk, followed a quadratic trend with temperatures from 70 to 100 °C as a function of holding time (from 0 to 60 min). The redshift could be due to whey protein denaturation caused by the heat since Trp residues become exposed and move from a nonpolar (folded) to a

polar (unfolded) environment; in contrast, proteins that embed into a micelle may result in a blueshift (Caputo and London, 2003). Different types of whey proteins possess distinct thermal susceptibility. Among whey proteins, α-La is generally regarded as one of the most heat-labile whey proteins, whereas *β*-Lg is one of the most heat-stable whey proteins (Ruegg et al., 1977; Edwards and Jameson, 2020), but some researchers stated that bovine serum albumin and immunoglobulin possess higher heat stability (Mainer et al., 1997; Edwards and Jameson, 2020). However, under the absence of milk fat, all whey proteins denature at 80 °C (de Wit et al, 1983). Moreover, Trp residues are influenced by their proximity to quenching compounds, resulting in a decrease of fluorescence intensity (Taterka, 2016). For instance, *β*-Lg, the most abundant whey protein, which contains two Trp residues, Trp^{19} and Trp^{61}, heat prompts the redshift due to the Trp^{19} exposure from the cavity of *β*-Lg and the decrease of maximum fluorescence intensity is due to the combination of Trp^{61} and disulfide bond or the proximity of Trp^{19} and $Arginine^{124}$ (Manderson et al., 1999).

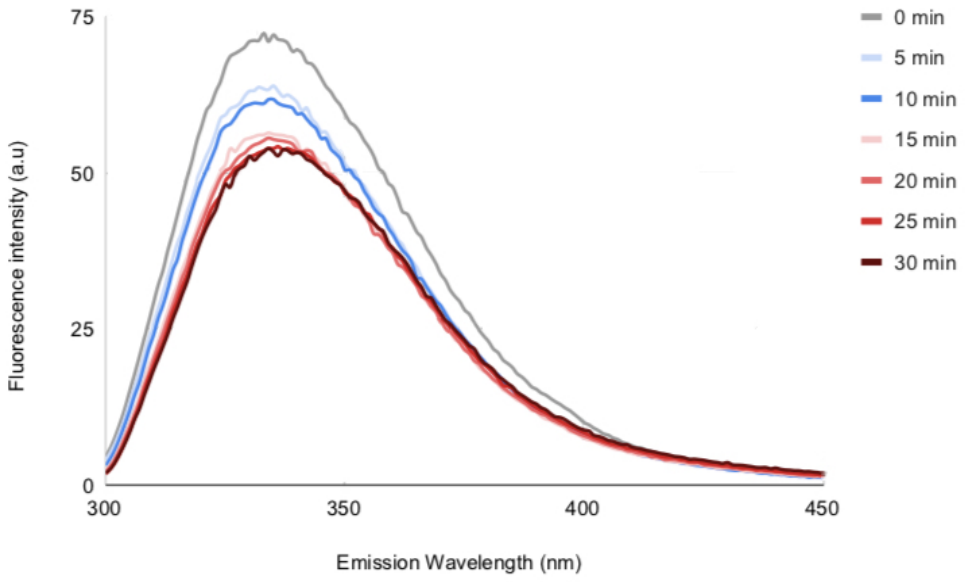

Fig. 1. Fluorescence spectra of tryptophan, corresponding to whey samples obtained from milk heat-treated at 80 °C for different holding times (0-30 min).

3.3 Effects of heat on functional properties

3.3.1 Effects of heat on foaming properties

The effect of heating milk at seven holding times on the foaming behavior of whey was investigated. Table 2 illustrates that foams produced using unheated samples presented the statistically lowest overrun compared with all the whey samples obtained from heated milk, while as the heating time increased no improvement in the foaming capacity was observed and even exhibited a slight decrease of overrun in whey samples acquired from milk heated for 20 and 30 min. However, according to Pearson's test, no correlation of overrun (non-normalized) and the concentration of UWP was observed. The obtained results are in accordance with other literature data (Bals and Kulozik, 2003; Chime et al., 2009) indicating that a partial denaturation favored the foamability. Devilbiss et al. (1975) stated that heating is a prerequisite for foaming. Prolonged heating time posed a detrimental impact on foaming capacity due to further aggregation and polymerization of whey proteins resulting in less available binding sites for protein and air interaction. However, unlike the present study, some studies demonstrated that unheated whey had better foamability (Philips et al., 1990; Davis and Foegeding, 2004) assuming that native protein can absorb to the interface more rapidly, which consequently contributed to the foamability; and Nicorescu et al. (2009) even showed no difference in overrun between native and heated whey.

Foam stability assessed by the time required for the whole foam to drain is shown in Table 2. The worst stability occurred in whey from unheated milk, and as the heating time increased, the tendency of greater foam stability was observed. Although sample collected from milk heated for 20 min demonstrated the best stability, and no statistical difference among heat treatment for 5, 10, and 15 min were observed. Nicorescu et al. (2009) also revealed a similar result; with appropriate thermal treatment the stability was enhanced as well as the excessive heating resulted in an adverse effect. A weak and negative correlation between drainage time and the concentration of UWP (r = -0.44, $0.01 \leq P < 0.05$) was observed, which enhances the idea that the denaturation affects foam stability. The increase of viscosity caused by the heat is one of the major factors that improve foam stability (Davis and Foegeding, 2004), since higher viscosity limits the mobility of the aqueous phase which surrounds the air bubbles, which consequently enhances the ability of foam against drainage (Wang, 2013). Nevertheless, some studies illustrated conversely that the denaturation and aggregation resulted from the heat diminished the foam stability (Bals et al., 2003; Chime et al., 2009).

The inconsistent observations of foaming properties between current and previous studies may be due to differences in the temperature of the treatment that resulted in different denaturation levels, the pH value, and the whey protein content. Philips et al. (1990) pointed out that the pH strongly influences the foaming properties. In our case, the pH was lower than the isoelectric point of whey which resulted in reduced foamability since proteins were more stable to against the unfolding for foam generation. Lee et al. (1992) and Chime et al. (2009) also demonstrated at different pH, the foaming properties differed. As to the whey protein content, in the present study, it decreased as heating time increased (Table 1), unlike other reports that had a constant amount, since the heat treatment was applied to the milk and some denatured whey precipitated with casein during the isoelectric precipitation. In general, higher protein content would have a better foamability, since more whey proteins can interact with air, as well as a better foam stability which is attributed to the increased thickness of the film surrounding air bubbles (Xiong et al., 2020). However, no correlation between TWP concentration and both overrun and drainage time were observed, probably because denaturation increased simultaneously, which compensated the effect of decreasing whey protein amount on foaming properties, since both factors oppositely affected the foaming properties. Therefore, for a better understanding of the effect of denaturation on foaming properties, the overrun and the drainage time had been normalized by the total protein content of whey, and the results became more statistically different among the seven holding times (Table 2). The normalized overrun illustrated a strong and negative correlation with the concentration of UWP ($r = -0.85$, $P < 0.0001$) and TWP ($r = -0.85$, $P < 0.0001$). Similarly, strong and negative correlations between normalized drainage time and the concentration of UWP ($r = -0.65$, $0.001 \leq P < 0.01$) as well as drainage time and TWP ($r = -0.61$, $0.001 \leq P < 0.01$) were demonstrated. Furthermore, better correlation was observed once the whey protein content had been normalized as well.

Table 2. Effect of heat treatment at 80 °C on overrun and drainage time of foam.

Time (min)	Ov (%)	Ov/TWP (% g^{-1})	t_{Drain} (s)	t_{Drain}/TWP (s g^{-1})
0	667 ± 94 ^{b}	15873 ± 2180 ^{e}	461 ± 24.85 ^{c}	10980 ± 657 ^{d}
5	945 ± 232 ^{a}	42684 ± 8737 ^{d}	598 ± 125.86 ^{b,c}	26650 ± 5058 ^{c}
10	892 ± 197 ^{a}	48839 ± 11244 ^{c,d}	586 ± 186.73 ^{b,c}	31493 ± 8912 ^{c}
15	913 ± 85 ^{a}	57590 ± 4446 ^{b,c}	564 ± 159.86 ^{b,c}	35309 ± 10393 ^{c}
20	762 ± 58 ^{a,b}	52565 ± 3750 ^{b,c,d}	1125 ± 605.81 ^{a}	77231 ± 42394 ^{a}
25	969 ± 137 ^{a}	73270 ± 12193 ^{a}	690 ± 46.58 ^{b}	51782 ± 4919 ^{b}
30	819 ± 232 ^{a,b}	65282 ± 16756 ^{a,b}	967 ± 387.40 ^{a}	76835 ± 32591 ^{a}

Mean value ± s.d; n=28 for overrun; n=21 for drainage time. Ov: Overrun of foam; Ov/TWP: Normalized overrun taking into account the total whey protein content; t_{Drain}: Drainage time; t_{Drain}/TWP: Normalized drainage time taking into account the total whey protein content. $^{a-e}$: values per column with different letter were significantly different ($P < 0.05$).

3.3.2 Effects of heat on gel-forming properties

Milk heating significantly deteriorated the gel formation (Table 3). The longer the heating time, the lesser the gel strength, due to greater denaturation of whey, especially *β*-Lg, the most relevant whey protein for gelation (Mulvihill and Kinsella, 1987). Therefore, a positive and statistically significant correlation between gel-forming parameter and the concentration of UWP ($r = 0.87$, $P < 0.0001$) was observed. Besides, a pronounced drop of gel formation took place between samples obtained from unheated milk and milk heated for 5 min, while further heat treatment led to smaller reduction of the gel-forming parameter, which is in agreement with Hongsprabhas and Barbu (1996) and Chime et al. (2009). Extending heating time resulted in further denaturation that in turn increased the protein insolubility (Kilara and Mangino, 1991), which facilitated the formation of particulate gel (Vieth and Reynolds, 2004). The weak gel strength is strongly influenced by the presence of particulate gels, which are the aggregation of whey prior to the gel formation due to the denaturation arising from the heat treatment (Foegeding et al., 1998). The whey protein content plays a critical role in gel strength as well. Lorenzen and Schrader (2006) illustrated that as the whey percentage increased, the gel strength increased significantly, which was also confirmed in the present study with the concentration of TWP data (Table 1). Consistently, Pearson's test showed that the gel-forming properties significantly correlated with the concentration of TWP ($r = 0.87$, $P < 0.0001$).

Table 3. Effect of heat treatment at 80 °C on gel formation and emulsifying properties of whey.

Time (min)	Gel (%)	EAI (m^2 g^{-1})	ESI (%)	TSI (a.u.×100)	TSI/TWP (a.u. g^{-1})
0	51.67 ± 19.76 ^{a}	1.35 ± 0.01 ^{f}	2.74 ± 1.22 ^{b,c}	0.26 ± 0.05 ^{b}	0.06 ± 0.01 ^{e}
5	25.28 ± 7.45 ^{b}	2.05 ± 0.00 ^{e}	2.07 ± 0.48 ^{c,d}	0.40 ± 0.05 ^{a}	0.18 ± 0.03 ^{d}
10	21.01 ± 4.62 ^{c}	2.33 ± 0.15 ^{d}	3.33 ± 1.67 ^{a,b}	0.40 ± 0.03 ^{a}	0.22 ± 0.01 ^{c}
15	16.02 ± 2.22 ^{d}	2.73 ± 0.06 ^{c}	3.82 ± 1.91 ^{a}	0.38 ± 0.04 ^{a}	0.24 ± 0.03 ^{c}
20	14.69 ± 2.15 ^{e}	2.83 ± 0.03 ^{b,c}	1.90 ± 0.31 ^{c,d}	0.42 ± 0.05 ^{a}	0.29 ± 0.04 ^{b}
25	11.21 ± 1.23 ^{f}	3.03 ± 0.17 ^{a,b}	1.77 ± 0.05 ^{d}	0.40 ± 0.02 ^{a}	0.30 ± 0.02 ^{a,b}
30	9.22 ± 2.95 ^{g}	3.10 ± 0.37 ^{a}	2.07 ± 0.87 ^{c,d}	0.39 ± 0.03 ^{a}	0.31 ± 0.01 ^{a}

Mean value ± s.d; n=28 for gel-forming index and TSI, n=14 for EAI and ESI; Gel: gel-forming index; EAI: emulsifying ability index; ESI: emulsifying stability index; TSI: Turbiscan stability index TSI/TWP: Normalized TSI taking into account the total whey protein content. $^{a-g}$: values per column with different letter were significantly different ($P < 0.05$).

3.3.3 Effects of heat on emulsifying properties

The slope of TSI from the bottom part of the tube is displayed in Table 3. The higher value indicates less stability, because this index represents the degree of the particle movement as a function of time. All the whey samples obtained from heated milk had a significantly higher TSI than whey from non-heated milk ($P < 0.05$), but no statistical differences among them were observed. This fact may be attributed to the total protein content of all samples from heated milk which might have been insufficient to cover the oil droplets efficiently. Previous studies investigated the effect of heat on whey directly instead of on milk, which merely altered the denaturation level, but not protein amount (Patel and Kilara, 1990; El-Shibiny et al., 2007; Ghanimah and Ibrahim, 2018). In the present study, although the oil to whey ratio used was the same as other studies, the protein amount reduced significantly (Table 1) due to the thermal treatment since the whey samples applied were obtained after the isoelectric precipitation of caseins (pH 4.6) from the heated milk. Therefore, comparing with other research, the oil to whey protein ratio was not kept constant. However, in general, partial denaturation is conducive to the emulsifying stability, but in the meanwhile, the decrease of the concentration of TWP posed an adverse impact, which had been confirmed by Jiang et al. (2018) demonstrating that WPI had significantly better emulsifying stability than whey protein concentrate (WPC). These above two factors had complementary effects on emulsifying stability and may be another reason that resulted in no statistical difference between whey samples acquired from heated milk. According to Pearson's test, the slope of TSI negatively correlated with the concentration

of UWP (r = -0.72, $P < 0.0001$) and TWP (r = -0.64, $0.0001 \leq P < 0.001$). In order to take only the denaturation into account, TSI had been normalized with the protein content, and a tendency for TSI to increase was observed, in other words, a decrease in emulsifying stability with increasing holding time was found (Table 3). Moreover, a higher correlation coefficient was obtained between the normalized TSI and the concentration of TWP (r = -0.95, $P < 0.0001$) and UWP (r = -0.94, $P < 0.0001$).

The EAI significantly increased by prolonging the heating time (Table 3), and whey obtained from unheated milk possessed the lowest emulsifying capacity. This observation agreed with Dissanayake and Vasiljevic (2009) and Farrag et al. (2016), who also displayed an improvement of EAI once the samples had been heated, since the denaturation made the hidden hydrophobic groups in the globular proteins to expose (Harper, 1992). Therefore, a significant and negative correlation between EAI and the concentration of UWP (r = -0.95, $P < 0.0001$) was found. However, some researchers stated differently (Chime et al., 2009; Jiang et al., 2018), that the denaturation caused by heat impairs the emulsifying ability. In general, heat would lead to a decrease in emulsifying capacity due to irreversible protein denaturation, but a partial protein unfolding may enhance the interfacial properties and accordingly improves the emulsifying ability, which explains the results of the current study (Phillips et al., 1990). Furthermore, the protein content influenced the emulsifying ability as well since whey proteins acted as emulsifiers, that adsorbed at the surface of the newly formed oil droplets (Singh and Ye, 2020). Thus, WPI presented significantly higher EAI than WPC after the heat treatment at 80 °C (Jiang et al., 2018). However, in the present study, according to Pearson's test, EAI negatively correlated with the concentration of TWP (r = -0.94, $P < 0.0001$).

Different from the TSI, the ESI tended to raise as heating time increased first and then dropped with extensive heating time (Table 3). The highest emulsifying stability occurred in whey heated for 15 min. The ability of the protein to stabilize emulsions is associate with the interfacial area that can be coated by proteins (Pearce and Kinsella, 1978). Thus, the exposure of buried hydrophobic groups induced by the heat treatment may have resulted in the enhancement of ESI. In addition, better emulsifying stability with a moderated heating would be explained by a greater zeta potential, which generates a larger electrostatic repulsion to retard the coalescence and creaming (Jiang et al., 2018). However, heating more than 20 min detrimentally influenced the ESI, which is in agreement with Jiang et al. (2018), since further denaturation caused large aggregation of whey proteins, resulting in the inefficiency in covering

the fat droplets (Raiko, 2010). However, in the present study, no correlation was detected between ESI and the concentration of UWP. Moreover, the decreasing whey protein concentration caused by heat may result in less viscosity which diminishes the stability (Hung and Zayas, 1991), but still, no significant correlation between ESI and the concentration of TWP was found.

3.4 Prediction of whey protein concentration and functional properties

A great number of correlations were observed between Trp fluorescence and functional parameters (see Appendix), which indicate that the two Trp fluorescence parameters may have the potential to be used as predictors.

On one hand, the emission wavelength corresponding to maximum fluorescence intensity of Trp correlated with normalized drainage time ($r = 0.54$, $0.01 \leq P < 0.05$), gel formation ($r = -0.44$, $0.01 \leq P < 0.05$), EAI ($r = 0.61$, $0.01 \leq P < 0.05$), and normalized TSI ($r = 0.53$, $0.001 \leq P < 0.01$). On the other, the maximum intensity of Trp correlated with normalized overrun ($r = -0.78$, $P < 0.0001$), drainage ($r = -0.58$, $0.0001 \leq P < 0.001$, and $r = -0.74$, $0.0001 \leq P < 0.001$, for non- and normalized data respectively), gel-forming properties ($r = 0.79$, $P < 0.0001$), and TSI ($r = -0.65$, $0.0001 \leq P < 0.001$, and $r = -0.89$, $P < 0.0001$, for non- and normalized data respectively).

Since Trp fluorescence gave two parameters, only two predictive models were obtained for each variable (Table 4). Except for those models of ESI, all models were strongly significant with $P \leq 0.001$. Those models with the higher R^2 were model II with both W_{Trp} and I_{Trp} as predictors, which were considered as better models. However, only the models of EAI had a R^2 higher than 0.8, which indicates that applying barely one fluorescent marker was not enough to generate a robust predictive model. For instance, Ayala (2018) required four fluorescent compounds in order to predict successfully the concentration of lactulose and furosine in milk, and Babu and Amamcharla (2018) obtained a good R^2 for predicting the solubility of whey protein concentrate with FFF of Trp and Maillard products. Not to mention, in the present study, predicting the functional properties is more challenging since their performances are based on many factors, not only on the concentration of UWP and TWP. Moreover, the R^2 of predictive models of UWP and TWP were merely around 0.67 and 0.65, respectively. As a result, it is

necessary to employ more fluorescent markers to build robust predictive models for not only the functional properties, but also for the concentration of both UWP and TWP.

Table 4. Models for the prediction of undenatured whey protein concentration of milk, total whey protein content and functional properties with FFF responses of Trp.

	Model	β_0	β_1	β_2	R^2	SEP	CV
I^{***}	$UWP = \beta_0 + \beta_1 I_{Trp}$	-2.355^{***}	0.064^{***}	-	0.672	0.372	24.75
II^{***}	$UWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	4.060	0.062^{***}	-0.019	0.674	0.374	24.90
I^{***}	$TWP = \beta_0 + \beta_1 I_{Trp}$	-3.706^{***}	0.094^{***}	-	0.650	0.577	29.16
II^{***}	$TWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	-0.957	0.093^{***}	-0.008	0.651	0.582	29.43
I^{***}	$Ov/TWP = \beta_0 + \beta_1 I_{Trp}$	151872^{***}	-1669^{***}	-	0.402	17039	33.49
II^{***}	$Ov/TWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	-134048	-1588^{***}	839	0.406	17145	33.70
I^{***}	$t_{Drain}/TWP = \beta_0 + \beta_1 I_{Trp}$	198366^{***}	-2543^{***}	-	0.431	23054	52.01
II^{***}	$t_{Drain}/TWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	-444872	-2348^{***}	1886	0.440	23152	52.23
I^{***}	$Gel = \beta_0 + \beta_1 I_{Trp}$	62.460^{***}	1.385^{***}	-	0.557	10.332	48.39
II^{***}	$Gel = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	80.723	1.345^{***}	-0.420	0.559	10.407	48.74
I^{***}	$TSI/TWP = \beta_0 + \beta_1 I_{Trp}$	0.729^{**}	-0.008^{***}	-	0.640	0.052	22.64
II^{***}	$TSI/TWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	-0.966	-0.008^{***}	0.005	0.649	0.052	22.58
I^{***}	$EAI = \beta_0 + \beta_1 I_{Trp}$	7.709^{***}	-0.088^{***}	-	0.848	0.245	8.70
II^{***}	$EAI = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	1.178	-0.086^{***}	1.019	0.850	0.248	8.81
I	$ESI = \beta_0 + \beta_1 I_{Trp}$	-0.409	0.049	-	0.073	1.155	45.71
II	$ESI = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	-27.016	0.058	0.078	0.083	1.172	46.37

n = 56 for UWP, TWP, Ov/TWP, Gel and TSI/TWP; n = 42 for t_{Drain}/TWP; n = 28 for EAI and ESI. ; $\beta_{0\text{-}2}$: regression coefficients; R^2: determination coefficient; SEP: standard error of prediction (units correspond to those of predicted parameters); CV: coefficient of variation (%); I_{Trp}: maximum fluorescence intensity of tryptophan (a.u.); W_{Trp}: emission wavelength corresponding to maximum fluorescence intensity of tryptophan (nm); UWP: undenatured whey protein of milk (%); TWP: total protein content of whey (%); Ov/TWP: normalized overrun taking into account the total whey protein content (% g^{-1}); t_{Drain}/TWP: normalized drainage time taking into account the total whey protein content (s g^{-1}); Gel: gel-forming index; TSI/TWP: normalized turbiscan stability index taking into account the total whey protein content (a.u. g^{-1}); EAI: emulsifying ability index; ESI: emulsifying stability index; Significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

4. Conclusions

Front-face fluorescence spectroscopy was able to detect the distinct fluorescence response of tryptophan after different intensities of thermal treatment. The functional properties were also affected by heat treatment due to denaturation. After taking into account the protein content of the different whey samples, foaming properties and emulsifying ability were improved with increasing holding time. On the contrary, the gel-forming index decreased significantly with prolonging heat treatment. In parallel, significant correlations between the front-face fluorescence response of tryptophan and functional properties of whey were observed. However, albeit the results of functional properties had been normalized, it would be more precise to analyze the sample with merely one dependent variable. Furthermore, although Turbiscan did not provide significant differences among heated samples, probably due to the insufficient protein, the application for stability measurement using this equipment is still recommended owing to the fact that it is a potent and sensitive method.

As to the predictive models, which did not present a sufficient determination coefficient for predicting the functional properties robustly, it is suggested to exploit more fluorescence markers in order to obtain reliable predictive models since front-face fluorescence spectroscopy is a useful, non-invasive, rapid, and relatively cheap technique. Furthermore, owing to the feasibility of measuring turbid samples with front-face fluorescence spectroscopy, it may be interesting to investigate the front-face fluorescence response of the whey powder directly, which would be more convenient for commercial utilization.

Finally, due to the advantages of front-face fluorescence spectroscopy and the increasing popularity of whey application by the industries due to its functional properties, further research studying the prediction of functional properties of whey applying front-face fluorescence technology with more fluorescence markers is valuable.

References

Alvarado, U. (2017). Aplicación de indicadores nativos de fluorescencia para la evaluación rápida de daño térmico en el procesado de leche. Ph.D. dissertation. Universitat Autònoma de Barcelona, Spain.

Alvarado U., Zamora A., Liu J., Saldo J., & Castillo M. (2019). Rapid Quantification of Riboflavin in Milk by Front-Face Fluorescence Spectroscopy: A Preliminary Study. Foods. 20, 9 (1), 6.

Andersen, C.M., & Mortensen, G. (2008). Fluorescence spectroscopy: a rapid tool for analyzing dairy products. J. Agric. Food Chem. 56 (1), 720-729.

Anema S. (2020). The whey proteins in milk: Thermal denaturation, physical interactions, and effects on the functional properties of milk, In Boland M. & Singh H. (eds.) Milk Proteins (3rd ed.) (pp.325-384), London, Academic press.

Ayala, N., Zamora, A., González, C., Saldo, J., & Castillo, M. (2017). Predicting lactulose concentration in heat-treated reconstituted skim milk powder using front-face fluorescence. Food Control, 73, 110-116.

Ayala, N. (2018). Application of native fluorescence tracers for quick evaluation of thermal damage in milk. Ph.D. dissertation. Universitat Autònoma de Barcelona, Spain.

Ayala, N., Zamora, A., Rinnan, A., Saldo, J., & Castillo, M. (2020). The effect of heat treatment on the front-face fluorescence spectrum of tryptophan in skim milk. J. Food Compos. Anal., 92, 103569.

Babu, K.S. & Amamcharla, J.K. (2018). Application of front-face fluorescence spectroscopy as a tool for monitoring changes in milk protein concentrate powders during storage. J. Dairy Sci., 101, 10844-10859.

Babu, K.S., Siliveru, K., Amamcharla, J.K., Vadlani, P.V., & Ambrose R.P.K. (2018). Influence of protein content and storage temperature on the particle morphology and flow ability characteristics of milk protein concentrate powders. J. Dairy Sci., 101, 7013-7026.

Baer, A., Oroz, M. & Blanc, B. (1976). Serological studies on heat-induced interactions of α-lactalbumin and milk-proteins. J. Dairy Res. 43, 419-432.

Bals, A. & Kulozik, U. (2003). Effect of pre-heating on the foaming properties of whey protein isolate using a membrane foaming apparatus. Int. Dairy J., 13 (11), 903-908.

Boye, J.I., Aksay, S., Roufik, S. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. Food Res. Int. 43, 537–546.

Caputo, G. A., & London, E. (2003). Cumulative effects of amino acid substitutions and hydrophobic mismatch upon the transmembrane stability and conformation of hydrophobic alpha-helices. Biochemistry, 42(11), 3275-85.

Chime, J.J., Konrad G., Kleinschmidt, Th., & Gorzki, L. (2009). Thermal modification of functional properties of whey proteins. Milchwissenschaft. 64, 400-404.

Davis, J. P., & Foegeding, E. A. (2004). Foaming and interfacial properties of polymerized whey protein isolate. Food Chem. and Toxicol., 69, 404-410.

Devilbiss, E.D., Holsinger, V.H., Posti, L.P., & Pallansch, M.J. (1975). Properties of whey protein concentrate foams. Food Technol. 28, 40-48.

de Wit, J.N., Klarenbeek, G. & Honlelez-Backx, E. (1983). Evaluation of functional properties of whey protein concentrates and whey protein isolates. 1. Isolation and characterization. Neth. Milk Dairy J., 3737-3749.

Dissanayake, M. & Vasiljevic, T. (2009). Functional properties of whey proteins affected by heat treatment and hydrodynamic high-pressure shearing**.** J. Dairy Sci., 92, 1387-1397.

Dufour, E., & Riaublanc, A. (1997). Potentiality of spectroscopic methods for the characterisation of dairy products. I. Front-face fluorescence study of raw, heated and homogenised milks. Le Lait, 77(6), 657-670.

Edwards, P. & Jameson, G. (2020). Structure and Stability of Whey Proteins. In Boland M. & Singh H. (eds.) Milk Proteins (3rd ed.) (pp. 251-291), London, Academic press.

Elfagm, A.A. & Wheelock, J.V. (1978). Heat interaction between α-lactalbumin, β-lactoglobulin and casein in bovine milk. J. Dairy Sci. 61, 159-163.

El-Shibiny, S., Farrag, A.F., El-Garawany, G. & Assem, F.M. (2007). Rheological and Functional Properties of Whey Protein Concentrate and β-Lactoglobulin and α-Lactalbumin Rich Fractions. Int. J. of Dairy Sci., 2(3), 196-206.

Farrag, A., El-Sayed, M.M., El-Sheikh, M.M., Shendy, A. & Soliman, T. (2016). Effect of heat treatments of whey proteins supplemented with sodium caseinate on the emulsions properties. Res. J. Pharm., Biol. Chem. 7, 2613-2621.

Farrell Jr., H.M., Jimenez-Flores, R., Bleck, G.T., Brown, E.M., Butler, J.E., Creamer, L.K., Hicks, C.L., Hollar, C.M., Ng- Kwai-Hang, K.F. & Swaisgood, H.E. (2004). Nomenclature of the proteins of cows' milk—sixth revision. J. Dairy Sci. 87 (6), 1641-1674.

Ferry J.D. (1948). Protein gels. Adv. Protein Chem., 4, 1-78.

Foegeding, E. A., Gwartney, E. A. & Errington, A. D. (1998). Chapter 9: Functional Properties of Whey Proteins in Forming Networks. In Whitaker, J. R., Shahidi F., Munguia A. L., Yada, R. Y., Fuller, G. Functional Properties of Proteins and Lipids (pp, 145-157), Washington, American Chemical Society.

Freire, P., Zamora A. & Castillo M. (2020). Effect of Milk Heat Treatment on Acid Whey: Front-Face Fluorescence Response and Undenatured Whey Proteins. 6th International Conference on Food Chemistry & Technology (FCT-2020)- A Virtual Conference held on October 05-06.

Ghanimah, M. & Ibrahim, E. (2018). Effect of pH, carbohydrates, and NaCl on functional properties of whey proteins. J. Sustain. Agri. Sci., 44(2), 93-99.

Harper, W.J., (1992). Functional properties of whey protein concentrate and their relationship to ultrafiltration. In Boer, R., Jelen, P. & Puchan, Z. (eds.) New applications of membrane processes (pp. 77-108), Belgium, International Dairy Federation.

Hongsprabhas, P. & Barbut, S. (1996). Ca^{2+} induced gelation of whey protein isolate: Effects of pre-heating. Food Res. Int., 29 (2), 135-139.

Hooi, R., Barbano, D.M., Bradley, R.L., Budde, D., Bulthaus, M., Chettiar M., Lynch, J. & Reddy, R. (2012). Chapter 15. Chemical and Physical Methods. In Michael Wehr, H. & Frank, J. F. (eds.) Standard methods for the examination of dairy products (17^{th} ed.) (pp. 498-501), Washington, American Public Health Association.

Hougaard, A. B., Lawaetz, A. J., & Ipsen, R. H. (2013). Front face fluorescence spectroscopy and multi-way data analysis for characterization of milk pasteurized using instant infusion. LWT, 53(1), 331-337.

Hung, S. & Zayas, J. (1991). Emulsifying Capacity and Emulsion Stability of Milk Proteins and Corn Germ Protein Flour. J. Food Sc., 56, 1216-1218.

IDF. (2002). Milk and milk products – Determination of nitrogen content. IDF Standard 185:2002/ISO 14891. Belgium, International Dairy Federation.

Jambrak, A.R., Mason, T.J., Lelas, V., Herceg, Z. & Herceg I.L. (2008). Effect of ultrasound treatment on solubility and foaming properties of whey protein suspensions. J. Food Eng., 86 (2), 281-287.

James, T. V., & Patrik, R. C. (2001). Mechanisms of Tryptophan Fluorescence Shifts in Proteins. Biophys. J., 80(5), 2093-2109.

Jiang, S., Altaf Hussain, M., Cheng, J., Jiang, Z., Geng, H., Sun, Y., Sun, C., & Hou, J. (2018). Effect of heat treatment on physicochemical and emulsifying properties of polymerized whey protein concentrate and polymerized whey protein isolate. LWT, 98, 134-140.

Kilara, A. & Mangino, M.E. (1991). Relationship of Solubility of Whey Protein Concentrates to Thermal Properties Determined by Differential Scanning Calorimetry. J.Food Sci., 56, 1448-1449.

Kilara, A., & Vaghela, M. N. (2004). Whey proteins. In Yada R.Y. (eds.) Proteins in Food Processing (2^{nd} ed.) (pp. 72-99), Duxford, Woodhead publishing.

Kulmyrzaev, A. A., Levieux, D., & Dufour, E. (2005). Front-face fluorescence spectroscopy allows the characterization of mild heat treatments applied to milk. Relations with the denaturation of milk proteins. J. Agr. Food Chem., 53(3), 502-507.

Lee, S.Y., Morr, C.V. & HA, E.Y. (1992), Structural and Functional Properties of Caseinate and Whey Protein Isolate as Affected by Temperature and pH. J. Food Sci., 57. 1210-1229.

Liu, J., Zamora, A., Castillo, M. & Saldo, J. (2018). Using front-face fluorescence spectroscopy for prediction of retinol loss in milk during thermal processing. LWT, 87, 151-157.
Lorenzen, P. C. & Schrader, K. (2006). A comparative study of the gelation properties of whey protein concentrate and whey protein isolate. Le Lait, 86 (4),259-271.

Luck, P., Vardhanabhuti, B. Yong, Y., Laundon, T., Barbano, D. & Foegeding E. (2013). Comparison of functional properties of 34% and 80% whey protein and milk serum protein concentrates. J. Dairy Sci., 96, 5522-5531.

Mainer, G., Sanchez, L., Ena, J.M. & Calvo, M. (1997). Kinetic and thermodynamic parameters for heat denaturation of bovine milk IgG, IgA and IgM. J. Food Sci. 62 (5), 1034-1038.

Manderson, G. A., Hardman, M. J., & Creamer, L. K. (1999). Effect of heat treatment on bovine beta-lactoglobulin A, B, and C explored using thiol availability and fluorescence. J. Agr. Food Chem., 47(9), 3617-3627.

Mulvihill, D., & Donovan, M. (1987). Whey Proteins and Their Thermal Denaturation - A Review. Ir. J. Food Sci. Technol., 11(1), 43-75.

Nicorescu, I., Loisel, C., Vial, C., Riaublanc, A., Djelveh, G., Cuvelier, G., & Legrand J. (2009). Effect of dynamic heat treatment on the physical properties of whey protein foams. Food Hydrocoll., 23, 1209-1219.

Nishanthi, M., Chandrapala, J., & Vasiljevic, T. (2017). Compositional & structural properties of whey proteins of sweet, acid and salty whey concentrates and their respective spray dried powders. Int. Dairy J., 49-56.

Nunes, L. & Tavares G.M. (2019). Thermal treatments and emerging technologies: Impacts on the structure and techno-functional properties of milk proteins. Trends Food Sc. Technol., 90, 88-99.

Ooi, H., Tokuyama, T" Kuo, F.H., Ibuki. F.H. & Kanamori, M. (I983). Heal-induced complex formation between κ-casein and α-lactalbumin. Agric. BioI. Chem. 47, 2817-2824.

Patel, M.T. & Kilara A. (1990). Studies on whey protein concentrates. 2. Foaming and emulsifying properties and their relationship with physicochemical properties**.** J. Dairy Sci., 73, 2731-2740.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a turbidimetric technique. J. Agric. Food Chem. 1978, 26, 3, 716-723.

Phillips, L., Schulman, W. and Kinsella, J. (1990). pH and heat treatment effects on foaming of whey protein isolate. J. Food Sci., 55: 1116-1119.

Raikos, V. (2010). Effect of heat treatment on milk protein functionality at emulsion interfaces. A review. Food Hydrocoll., 24 (4), 259-265.

Ruegg, M., Moor, U., & Blanc, B. (1977). A calorimetric study of thermal denaturation of whey proteins in simulated milk ultra-filtrate. J. Dairy Res. 44, 509-520. Sawyer, W.H. (1969). Complex between β-lactoglobulin and κ-casein. A review. J. Dairy Sci. 52, 1347-1355.

Schamberger, G. P., & Labuza, T. P. (2006). Evaluation of Front-face Fluorescence for Assessing Thermal Processing of Milk. J. Food Sci., 71(2), 69-74.

Shaikh, S., & O'Donnell C. (2017). Applications of fluorescence spectroscopy in dairy processing: A review. Curr. Opin. Food Sci., 17, 16-24.

Singh, H. & Ye, A. (2020). Interactions and functionality of milk proteins in food emulsions. In Boland M. & Singh H. (eds.) Milk Proteins (3rd ed.) (pp. 467-497), London, Academic press.

Taterka, H. (2016). Optical prediction models of whey protein denaturation in thermally treated milk for the development of an inline sensor. Ph.D. dissertation. Universitat Autònoma de Barcelona, Spain.

Veith, P.D. & Reynolds, E.C. (2004). Production of a high gel strength whey protein concentrate from cheese whey. J. Dairy Sci., 87, 831-840.

Wang, Z. (2013). Foaming properties of whey protein isolate and λ-carrageenan mixed systems. Master dissertation. University of Missouri, MO, USA.

Xiong, X., Ho, M.T., Bhandari, B., & Bansal, N. (2020). Foaming properties of milk protein dispersions at different protein content and casein to whey protein ratios. Int. Dairy J., 109, 104758.

Xu, H., Zhang, T., Lu, Y., Lin, X., Hu, X., Liu, L., & Wu, X. (2019). Effect of chlorogenic acid covalent conjugation on the allergenicity, digestibility & functional properties of whey protein. Food Chem., 298, 125024.

Appendix– correlation table

	W_{Trp}	I_{Trp}	UWP	TWP
UWP	r = -0.47 n = 28 p = 0.0116 *	r = 0.8673 n = 28 p = 0.0000 ***		r = 0.9919 n = 28 p = 0.0000 ***
TWP	r = -0.4316 n = 28 p = 0.0218 *	r = 0.8544 n = 28 p = 0.0000 ***	r = 0.9919 n = 28 p = 0.0000 ***	
Ov	r = 0.0405 n = 28 p = 0.8380	r = -0.2842 n = 28 p = 0.1427	r = -0.2996 n = 28 p = 0.1214	r=-0.3466 n = 28 p = 0.0708
Ov/TWP	r = 0.3590 n = 21 p = 0.1100	r = -0.7829 n = 21 p = 0.0000 ***	r = -0.8508 n = 21 p = 0.0000 ***	r=-0.8516 n = 21 p = 0.0000 ***
t_{Drain}	r = 0.4014 n = 21 p=0.0713	r = -0.5783 n = 21 p = 0.0060 **	r = -0.4443 n = 21 p = 0.0436 *	r=-04110 n = 28 p = 0.0642
t_{Drain}/TWP	r = 0.5453 n = 21 p = 0.0106 *	r = -0.7414 n = 21 p = 0.0001 ***	r = -0.6465 n = 21 p = 0.0015 **	r=-0.6134 n = 21 p = 0.0031 **
Gel	r = -0.4358 n = 28 p = 0.0204 *	r = 0.7939 n = 28 p = 0.0000 ***	r = 0.8696 n = 28 p = 0.0000 ***	r=0.8703 n = 28 p = 0.0000 ***
EAI	r = 0.6082 n = 14 p = 0.0210 *	r = -0.9490 n = 14 p = 0.0000 ***	r = -0.9482 n = 14 p = 0.0000 ***	r=-0.9358 n = 14 p = 0.0000 ***
ESI	r = 0.0526 n = 14 p = 0.8582	r = 0.3077 n = 14 v0.2845	r = 0.1315 n = 14 p = 0.6450	r=0.1151 n = 14 p = 0.6953
TSI	r = 0.1572 n = 28 p = 0.4244	r = -0.6531 n = 28 p = 0.0002 ***	r = -0.7247 n = 28 p = 0.0000 ***	r=-0.6394 n = 28 p = 0.0002 ***
TSI/TWP	r = 0.5326 n = 28 p = 0.0035 **	r = -0.8882 n = 28 p = 0.0000 ***	r = -0.9476 n = 28 p = 0.0000 ***	r=-0.9417 n = 28 p = 0.0000 ***

r: Pearson's correlation coefficient; n: number of observations. Significance: *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$. UWP: undenatured whey protein of milk (%); TWP: total protein content of whey (%); W_{Trp}: emission wavelength corresponding to maximum fluorescence intensity of tryptophan (nm); I_{Trp}: maximum fluorescence intensity of tryptophan (a.u.); Ov: Overrun; Ov/TWP: normalized overrun (%) taking into account the total whey protein content (% g^{-1}); t_{Drain}: drainage time (s); t_{Drain}/TWP: normalized drainage time (s) taking into account the total whey protein content (s g^{-1}); Gel: gel-forming index; EAI: emulsifying ability index; ESI: emulsifying stability index; TSI: turbiscan stability index; TSI/TWP: normalized turbiscan stability index taking into account the total whey protein content (a.u. g^{-1}).