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Tryptophan front-face fluorescence and functional properties of whey: A preliminary study

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ABSTRACT

This study investigated the potential 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. However, its attributes depend dramatically on heat treatment, which may alter its suitability for different food applications. Tryptophan front-face fluorescence of whey and its functional properties, i.e., foaming, gel-forming, and emulsifying properties, were evaluated after milk heat treatment (at 80 °C with seven holding times) to detect correlations between tryptophan fluorescence and functional parameters and generate predictive models. Whey samples were obtained by isoelectric precipitation of caseins from reconstituted skim milk powder enriched with whey protein isolate. As expected, heat treatments induced an increase in whey protein denaturation, as well as a decrease in total whey protein concentration and tryptophan fluorescence intensity. 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 ($P < 0.05$). Consequently, models presented strong significances ($P < 0.001$). Specifically, using only tryptophan fluorescence allowed successful prediction of emulsifying properties but was not enough for foaming and gel forming properties.

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 cannot be used for on-line or in-line monitoring (Babu et al., 2018). However, FFFS can overcome these inconveniences and provides abundant information with only 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 (Alvarado et al., 2019; Ayala et al., 2017; Liu et al., 2018) 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 & 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 & Mortensen, 2008; Ayala et al., 2020). Among those, Trp is one of the most studied markers to monitor changes in proteins since it has a considerable quantum yield contributing to higher intensity of fluorescence emission (James & Patrik, 2001). Thus, it could give relevant information on the thermal treatment of milk, a fundamental step in manufacturing dairy products to improve microbiological quality and extend 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 & Riaublanc, 1997; Ayala et al., 2020; Hougaard et al., 2013; Kulmyrzaev et al., 2005; Schamberger & Labuza, 2006; Taterka, 2016). Specifically, FFFS at an excitation wavelength of 290 nm has been corroborated as a potential method for predicting the concentration of undenatured whey protein (UWP) of milk by quantifying the intensity of the peak at 340 nm (Taterka, 2016). However, the degree of heat treatment must be sufficient to induce

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considerable conformational changes to modify the fluorescent response through a shift on the emission wavelength or a change in the maximum fluorescence intensity. For instance, Ayala et al. (2020) started to observe changes in 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 & Vaghela, 2004). Whey proteins are widely used in the food industry due to their 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 impacts on the techno-functional properties of whey (Nunes & 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. However, 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 and a concomitant high viscosity surface for better stability. On the contrary, emulsifying ability decreases by increasing the thermal effect due to lower content of native proteins. For gel-forming properties, heating significantly diminishes the gel strength, first due to the denaturation, which provokes less available binding sites 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).

As mentioned before, there are several studies of the fluorescence of Trp in milk, however there are very few that applied fluorescence spectroscopy directly in whey. MurilloPulgarín, Alañón, and Alañón (2005) presented the fluorescence characteristic of several wheys, where milk was subjected to different heat treatments, showing the full spectrum (with excitation and emission ranges of about 220–320 nm and 260–435 nm, respectively) and remarking that tyrosine and Trp are responsible for the main intrinsic fluorescence of whey in that area. Trp fluorescence on whey was also used by Liu et al. (2017) as a detection index to evaluate the adulteration of milk with milk powder.

However, to the best of our knowledge, no studies linking both FFFS and functional properties of whey have been found in literature. Therefore, the present 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, 10,000 cfu/g) was purchased from Lactalis ingredients (Bourgbarré, France). All other chemicals used were of analytical grade from either PanReac AppliChem (Barcelona, Spain) or Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.2. Sample preparation

2.2.1. Heat treatment

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 entirely mixed and rapidly cooled to 20 °C. Then, it was covered with aluminum foil to avoid light-induced oxidation and left at room temperature for 30 min to ensure full hydration. 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.

2.2.2. Whey separation

Whey separation was performed following a method based on those of Nishanthi et al. (2017) and Bertrand-Harb et al. (2002). 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 fluorescence 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 to detect the shift and intensity change.

2.4. 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.

2.5. 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 thrice in a test tube. Then, two drops of HCl solution were added to generate the turbidity and mixed slowly inverting thrice 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.

2.6. 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). 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.

Both overrun and drainage time were normalized dividing the obtained data by the total protein content of whey.

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 2000 ×*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. TSI values were normalized dividing the obtained data by the total protein content of whey.

For the emulsifying activity index (EAI), the method of Pearce and Kinsella (1978) was adopted. EAI represents the amount of oil that can be emulsified per unit of protein (Boye et al., 2010). This index was 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 EAI values were calculated with the following equation:

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

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).

2.7. Statistical analysis

The complete experiment was replicated four times, except for the EAI, which was repeated twice. Analysis of variance (ANOVA), taking as factors heat treatment time, replica and their interaction, 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. Indeed, Lamb et al. (2013) observed that UWP followed a first-order response to heating time at 80 °C detected by differential scanning calorimetry. Specifically, in the present study, 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; Edwards & Jameson, 2020), is sufficient to cause irreversible denaturation (Edwards & Jameson, 2020; Anema, 2020). 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 (Anema, 2020). 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, 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 the present study, 80 °C was applied and the concentration of UWP and TWP differed significantly ($P < 0.05$) among the seven holding times, and according to Pearson's test, a strong positive correlation between the concentrations of UWP and TWP was observed ($r = 0.99$, $P < 0.0001$).

3.2. Effect of heat on FFFS response of Trp

Heat induced a redshift and a decrease of Trp fluorescence intensity

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

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

Mean value ± s.d.; n = 54 for UWP, n = 56 for TWP, n = 55 for W_{Trp} and I_{Trp}; TWP: total protein content of whey; UWP: the concentration of undenaturated whey protein in milk; 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 ($P < 0.05$).

(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. 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). 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 & 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 (Edwards & Jameson, 2020), but some researchers stated that bovine serum albumin and immunoglobulin possess higher heat stability (Edwards & Jameson, 2020; Mainer et al., 1997). 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).

Concerning Pearson's correlations, 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 ($r = -0.43$, $0.01 \leq P < 0.05$). Redshift at 80 °C occurs only above 15 min and presents values of about 2–3 nm provoking the weak correlation with both UWP and TWP. At higher temperatures, i.e., 90 and 100 °C, the emission wavelength moves up to 14 nm (Ayala et al., 2020). In addition, 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 ($r = 0.85$, $P < 0.0001$).

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 improvement in the foaming capacity was observed. The obtained results are in accordance with other literature data (Bals & 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 (Phillips et al., 1990; Davis & Foegeding, 2004) assuming that native protein can absorb to the interface more rapidly, which consequently contribute 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

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}	$15{,}873 \pm 2{,}180^{e}$	461 ± 25^{c}	$10{,}980 \pm 657^{d}$
5	945 ± 232^{a}	$42{,}684 \pm 8{,}737^{d}$	$598 \pm 126^{b,c}$	$26{,}650 \pm 5{,}058^{c}$
10	892 ± 197^{a}	$48{,}839 \pm 11{,}244^{c,d}$	$586 \pm 187^{b,c}$	$31{,}493 \pm 8{,}912^{c}$
15	913 ± 85^{a}	$57{,}590 \pm 4{,}446^{b,c}$	$564 \pm 160^{b,c}$	$35{,}309 \pm 10{,}393^{c}$
20	$762 \pm 58^{a,b}$	$52{,}565 \pm 3{,}750^{b,c,d}$	$1{,}125 \pm 606^{a}$	$77{,}231 \pm 42{,}394^{a}$
25	969 ± 137^{a}	$73{,}270 \pm 12{,}193^{a}$	690 ± 47^{b}	$51{,}782 \pm 4{,}919^{b}$
30	$819 \pm 232^{a,b}$	$65{,}282 \pm 16{,}756^{a,b}$	967 ± 387^{a}	$76{,}835 \pm 32{,}591^{a}$

Mean value ± s.d.; n = 56 for overrun, n = 42 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. $^{a\text{-}e}$: values per column with different letter were significantly different ($P < 0.05$).

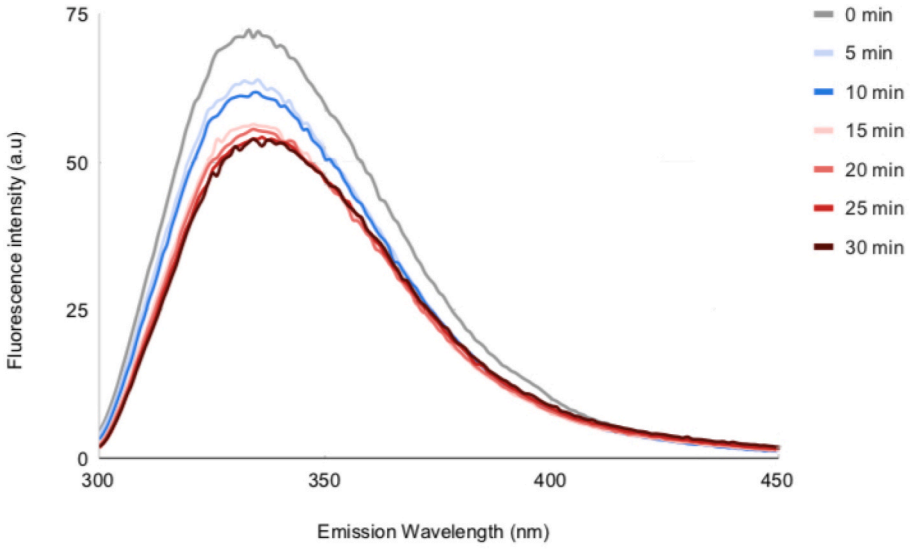

Fig. 1. Fluorescence spectra of tryptophan, corresponding to whey samples obtained from milk heat-treated at 80 °C for different holding times (0–30 min).

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 was observed. Richert (1979) also revealed a similar result; with appropriate thermal treatment the foam stability was enhanced, but the excessive heating (80 °C, 30 min, pH 7) resulted in an adverse effect. The increase of viscosity caused by a previous heat-treatment is one of the major factors that improve foam stability (Davis & 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. Phillips et al. (1990) pointed out that the pH strongly influences the foaming properties. In our case, the pH was lower than 4.6, since separation of whey was achieved through precipitation of caseins by lowering the pH down to their isoelectric point, 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).

According to Pearson's test, non-normalized overrun showed no significant correlation ($P \geq 0.05$) neither with UWP nor with TWP. Concerning drainage time, it showed a weak and negative correlation with UWP ($r = -0.44$, $0.01 \leq P < 0.05$), but no correlation ($P \geq 0.05$) with TWP. Denaturation increased simultaneously with decreasing whey protein amount, 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 outcomes became more statistically different among the seven holding times (Table 2). The normalized overrun illustrated a strong and negative correlation with 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 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.

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 & Donovan, 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 Barbut (1996) and Chime et al. (2009). Extending heating time resulted in further denaturation that in turn increased the protein insolubility (Kilara & Mangino, 1991), which facilitated the formation of particulate gel (Veith & 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 (%)	TSI (a.u. × 100)	TSI/TWP (a.u. g^{-1})	EAI ($m^2\ g^{-1}$)
0	51.67 ± 19.76^{a}	0.26 ± 0.05^{b}	0.06 ± 0.01^{e}	1.35 ± 0.01^{f}
5	25.28 ± 7.45^{b}	0.40 ± 0.05^{a}	0.18 ± 0.03^{d}	2.05 ± 0.00^{e}
10	21.01 ± 4.62^{c}	0.40 ± 0.03^{a}	0.22 ± 0.01^{c}	2.33 ± 0.15^{d}
15	16.02 ± 2.22^{d}	0.38 ± 0.04^{a}	0.24 ± 0.03^{c}	2.73 ± 0.06^{c}
20	14.69 ± 2.15^{e}	0.42 ± 0.05^{a}	0.29 ± 0.04^{b}	2.83 ± 0.03^{b,c}
25	11.21 ± 1.23^{f}	0.40 ± 0.02^{a}	0.30 ± 0.02^{a,b}	3.03 ± 0.17^{a,b}
30	9.22 ± 2.95^{g}	0.39 ± 0.03^{a}	0.31 ± 0.01^{a}	3.10 ± 0.37^{a}

Mean value ± s.d.; n = 83 for gel-forming index; n = 56 for TSI; n = 28 for EAI. Gel: gel-forming index; EAI: emulsifying activity 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 Turbiscan stability index (TSI) from the bottom part of the tube is displayed in Table 3. The higher value indicates less stability, because this index represents the phase separation 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 (El-Shibiny et al., 2007; Ghanimah & Ibrahim, 2018; Patel & Kilara, 1990). 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).

According to Pearson's test, the slope of TSI negatively correlated with both 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. After normalization, higher correlation coefficients were obtained ($r = -0.95$ with $P < 0.0001$ for TWP and $r = -0.94$ with $P < 0.0001$ for UWP). However, this observation differed from Jiang et al. (2018), who found better emulsifying stability after heating due to a greater zeta potential, that generated a larger electrostatic repulsion to retard the coalescence and

creaming, while Raikos (2010) stated that further denaturation caused large aggregation of whey proteins, resulting in the inefficiency in covering the fat droplets, which explained the decreasing of emulsion stability in the current study.

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). 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 & Ye, 2020). Thus, WPI presented significantly higher EAI than WPC after the heat treatment at 80 °C (Jiang et al., 2018).

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 indicated 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, two predictive models were obtained for each variable (Table 4). All models were strongly significant with $P \leq 0.001$, and 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 for TSI and EAI had a R^2 higher than 0.8 and 0.9, respectively, which indicates that applying merely 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 FFFS 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.

4. Conclusions

Since whey functional properties were distinctively affected by different thermal treatments and front-face fluorescence spectroscopy detected differences in tryptophan response, significant correlations between tryptophan fluorescence and functional properties of whey were observed.

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.

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Table 4
Models for the prediction of undenatured whey protein concentration of milk, total whey protein content and functional properties with front-face fluorescence response of tryptophan.

	Model	β_0	β_1	β_2	R^2	SEP	CV
I***	$UWP = \beta_0 + \beta_1 I_{Trp}$	−2.82***	0.07***		0.75	0.33	21.92
II***	$UWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	7.13	0.07***	−0.05	0.76	0.33	22.18
I***	$TWP = \beta_0 + \beta_1 I_{Trp}$	−4.40***	0.11***		0.73	0.52	26.12
II***	$TWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	1.66	0.10***	−0.02	0.73	0.53	26.60
I***	$Ov/TWP = \beta_0 + \beta_1 I_{Trp}$	166,121***	−1,904***		0.60	12,572	24.71
II***	$Ov/TWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	−489,586	−1,721***	1,925	0.62	12,522	24.62
I***	$t_{Drain}/TWP = \beta_0 + \beta_1 I_{Trp}$	228,952***	−3,047***		0.55	20,534	46.33
II***	$t_{Drain}/TWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	−1,446,857	−2,565***	4,918	0.59	20,042	45.22
I***	$Gel = \beta_0 + \beta_1 I_{Trp}$	−72.96***	1.56***		0.63	9.61	45.01
II***	$Gel = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	168.98	1.49***	−0.71	0.63	9.75	45.65
I***	$TSI/TWP = \beta_0 + \beta_1 I_{Trp}$	0.81***	−0.01***		0.79	0.04	17.29
II***	$TSI/TWP = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	−1.64	−0.010***	0.01	0.80	0.04	17.07
I***	$EAI = \beta_0 + \beta_1 I_{Trp}$	7.83***	−0.09***		0.90	0.20	7.17
II***	$EAI = \beta_0 + \beta_1 I_{Trp} + \beta_2 W_{Trp}$	−2.95	−0.09***	0.03	0.90	0.21	7.39

n = 28 for UWP, TWP, Ov/TWP, Gel and TSI/TWP; n = 21 for t_{Drain}/TWP; n = 14 for EAI. $\beta_{0\text{-}2}$: 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 activity index (m^2 g^{-1}). Significance: *** $P \leq 0.001$.

CRediT authorship contribution statement

Yu Ting Teng: Methodology, Investigation, Writing – original draft, preparation. **Paulina Freire:** Methodology, Investigation, Writing – review & editing. **Anna Zamora:** Conceptualization, Methodology, Data curation, Supervision, Writing – review & editing. **Manuel Castillo:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113589.

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