Effect of annatto addition and bleaching treatments on ultrafiltration flux during production of 80% whey protein concentrate and 80% serum protein concentrate¹

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ABSTRACT

The goals of this study were to determine if adding annatto color to milk or applying a bleaching process to whey or microfiltration (MF) permeate influenced ultrafiltration (UF) flux, diafiltration (DF) flux, or membrane fouling during production of 80% whey protein concentrate (WPC80) or 80% serum protein concentrate (SPC80). Separated Cheddar cheese whey (18 vats using 900 kg of whole milk each) and MF permeate of skim milk (18 processing runs using 800 kg of skim milk each) were produced to make WPC80 and SPC80, respectively. The 6 treatments, replicated 3 times each, that constituted the 18 processing runs within either whey or MF permeate UF were as follows: (1) no annatto; (2) no annatto + benzoyl peroxide (BPO); (3) no annatto + hydrogen peroxide (H₂O₂); (4) annatto; (5) annatto + BPO; and (6) annatto + H₂O₂. Approximately 700 kg of whey or 530 kg of MF permeate from each treatment were heated to 50°C and processed in 2 stages (UF and DF) with the UF system in batch recirculation mode using a polyethersulfone spiral-wound UF membrane with a molecular weight cutoff of 10,000 Da. Addition of annatto color had no effect on UF or DF flux. The processes of bleaching whey or MF permeate with or without added color improved flux during processing. Bleaching with H₂O₂ usually produced higher flux than bleaching with BPO. Bleaching with BPO increased WPC80 flux to a greater extent than it did SPC80 flux. Though no differences in mean flux were observed for a common bleaching treatment between the WPC80 and SPC80 production processes during the UF stage, mean flux during WPC80 DF was higher than mean flux during SPC80 DF for each

bleaching treatment. Water flux values before and after processing were used to calculate a fouling coefficient that demonstrated differences in fouling which were consistent with flux differences among treatments. In both processes, bleaching with H₂O₂ led to the largest reduction in fouling. No effect of annatto on fouling was observed. The reasons for flux enhancement associated with bleaching treatments are unclear.

Key words: ultrafiltration, flux, bleaching, annatto

INTRODUCTION

Flux decline and fouling during UF of whey have been studied extensively (Tong et al., 1989; Heng and Glatz, 1991; Rao, 2002) because they limit processing efficiency. Sweet and acid wheys are usually treated before UF to remove or inactivate potential foulant material that may reduce process flux (Heng and Glatz, 1991; Pouliot, 1996). These pretreatments may involve upstream unit operations such as microfiltration (MF) or centrifugal separation to remove foulant, chemical adjustments such as pH modification, mineral chelation, or preheating to inactivate foulant, or a combination of the 2 (Pouliot, 1996). Because proteins, minerals, and lipids are generally considered the most prevalent foulants, pretreatments are usually intended to increase protein solubility, limit calcium phosphate precipitation and calcium bridging during UF, or remove lipids from the whey (Pouliot, 1996). As such, the chemistry and composition of the process feed stream is expected to influence UF fouling and flux decline. Though studies regarding flux decline during UF of MF permeate of skim milk have not been as common, data by Britten and Pouliot (1996) and Nelson and Barbano (2005) indicate that the composition and pH of MF permeate are more similar to those of sweet whey than acid whey.

Whey protein concentrates (WPC) and serum protein concentrates are created by ultrafiltering cheese whey or 0.1 µm MF permeate of skim milk, respectively, to concentrate serum proteins (SP) and remove lactose and minerals (Nelson and Barbano, 2005). The

Received July 31, 2012.

Accepted December 23, 2012.

¹Use of names, names of ingredients, and identification of specific models of equipment is for scientific clarity and does not constitute any endorsement of product by authors, Cornell University, or the Northeast Dairy Foods Research Center.

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UF retentate can be concentrated by evaporation and spray dried to make a shelf-stable powder. Because SP in these ingredients are highly sought after, producers may further reduce the lactose and mineral contents of WPC and serum protein concentrates below what a single UF step would accomplish by diafiltering (**DF**) the UF retentate before evaporation and drying. Diafiltration involves diluting the UF retentate with water and then repeating the UF process. This step washes out the nonprotein-soluble milk solids that pass through the UF membrane and increases the protein content of the powder. Although serum protein concentrates and 80% serum protein concentrate (**SPC80**) solutions have been shown to exhibit improved sensory characteristics under certain conditions and greater clarities than their WPC counterparts (Evans et al., 2009, 2010), they are not widely available in the dairy industry. Conversely, WPC and 80% whey protein concentrate (**WPC80**) are by-products of cheese manufacture that are widely available. Over 175 million kg of WPC were produced in the United States in 2008 (IDFA, 2009).

In the United States, the majority of WPC and WPC80 are produced from Cheddar and mozzarella whey. Cheddar is often colored using annatto, a yellow to orange food colorant derived from the *Bixa orellana* shrub (Kang, et al., 2010), to maintain cheese color consistency throughout the year. The principal color molecules in annatto are the carotenoids bixin and norbixin (Kang, et al., 2010). Unfortunately, not all of the bixin and norbixin remain in the cheese; some of these colorants pass into the Cheddar whey. Because whey products made from Cheddar cheese whey with added annatto color may contribute an undesirable yellow hue to a food product in which it is subsequently used, manufacturers of WPC and WPC80 often bleach the whey before spray drying to whiten the final protein concentrate. Currently, 2 bleaching agents, benzoyl peroxide (**BPO**) and hydrogen peroxide (H_2O_2), are approved and deemed generally recognized as safe for bleaching whey in the United States (US FDA, 2011a,b). Benzoyl peroxide completely degrades to benzoic acid during the bleaching process and this residue in whey products may not be allowed in some countries. When H_2O_2 is used for bleaching, residual H_2O_2 must be broken down into molecular oxygen and water with catalase enzyme.

When examining the functional properties of WPC80 that had been bleached with BPO or H_2O_2 , Jervis et al. (2012) noted that protein solubility increased after bleaching with H_2O_2 . Because increased protein solubility has been linked with a more sustainable UF flux (Heng and Glatz, 1991; de la Casa, 2007), it stands to reason that the bleaching treatments described above may improve membrane flux. Currently, BPO may only be added to whey for the purposes of bleaching and

H_2O_2 may be used for bleaching or as an antimicrobial during electrodialysis (US FDA, 2011a,b), but neither may be used exclusively for enhancing membrane flux. However, if a processor were to treat colored whey with BPO or H_2O_2 before UF with the intent of bleaching, any flux-enhancing benefits could also be realized. No study has quantified the effects of bleaching whey or MF permeate on UF flux. Moreover, even though annatto addition is not expected to affect UF flux, no study has verified this hypothesis. The objectives of this study were to measure the effects of bleaching treatments and annatto coloring of whey and MF permeate on UF and DF flux during the production of WPC80 and SPC80 and to examine these treatments' effects on a polyethersulfone spiral-wound membrane's tendency to foul during WPC80 and SPC80 processing.

MATERIALS AND METHODS

Experimental Design

For both WPC80 and SPC80 manufacture, a 3×2 full factorial design was employed with 3 levels of bleaching (no bleaching treatment applied, 50 mg/kg BPO treatment, and 500 mg/kg H_2O_2 treatment) and 2 levels of coloring (no annatto and 0.066 mL of annatto/kg of milk). The experiments were replicated 3 times, resulting in 18 total processing runs for WPC80 manufacture and 18 total processing runs for SPC80 manufacture. Each individual processing run was conducted over 3 consecutive days in a week.

WPC80 Production

Cheddar Cheese Whey Manufacture. On the first day of processing, raw whole milk (about 900 kg) for Cheddar cheese production was pasteurized with a plate heat exchanger (model 080-S, AGC Engineering, Manassas, VA) at 72°C for 16 s, cooled to 4°C, and held overnight. The following day, the pasteurized milk was manufactured into Cheddar cheese and Cheddar cheese whey as described by Evans et al. (2009). For the treatments with added annatto, the colorant (Annatto cheese color - 2X, P/N 70741, Chr. Hansen Inc., Milwaukee, WI) was added to the milk (0.066 mL/kg of milk) before ripening. The curds and whey were continuously stirred at 38°C until the target whey draining pH of 6.45 was attained. The whey was drained through a sieve to remove cheese fines and immediately pasteurized using a plate heat exchanger equipped with regeneration, heating, and cooling sections (model 080-S, AGC Engineering) at 72°C for 16 s. The whey was cooled to 50°C at the exit of the pasteurizer and immediately processed with a cream separator (model 619,

DeLaval Inc., Kansas, MO) to reduce the fat content. The fat contents of the whey before and after separation were $0.21\% \pm 0.02$ and $0.042\% \pm 0.004$, respectively. The whey was mixed and sampled directly before and after cream separation. After separation, if the whey was not going to be bleached, it was cooled to 4°C with a plate heat exchanger and held overnight at $\leq 4^{\circ}\text{C}$.

Bleaching of Whey. If the whey was going to be bleached, it was recirculated through a plate heat exchanger in a large stainless steel tank to heat it to 66°C . Two different bleaches were used, BPO at 50 mg/kg (Oxylite Type XX benzoyl peroxide 32% by weight, Nelson Jameson, Marshfield, WI) or H_2O_2 at 500 mg/kg (35% H_2O_2 , FCC grade, Columbus Chemical Industries Inc., Columbus, WI). When bleaching with BPO, the powdered bleach was mixed with about 30 kg of whey using a high-shear mixer then added to the remainder of the whey. The whey was held for 30 min at 66°C with agitation then cooled with a plate heat exchanger and held overnight at $\leq 4^{\circ}\text{C}$. When bleaching with H_2O_2 , the liquid bleach was added to about 30 kg of whey, mixed with the remainder of the separated whey, and agitated for 30 min at 66°C . The H_2O_2 concentration in the liquid bleach was diluted from 35 to 10% concentration. The 10% concentration was verified with a 10% H_2O_2 test strip (Indigo Instruments, Niagara Falls, NY). If the H_2O_2 concentration was lower than 10%, then the actual concentration was calculated and the amount of H_2O_2 was adjusted to achieve an added level of 500 mg/kg in the cheese whey. After 30 min at 66°C , the whey was cooled with a plate heat exchanger to 50°C , liquid catalase enzyme derived from *Aspergillus niger* (FoodPro CAT, PD 216626-2.0EN, Danisco, Madison, WI) was added at 20 mg/kg, and the whey was mixed for 10 min. The whey was then cooled with a plate heat exchanger and held overnight at $\leq 4^{\circ}\text{C}$.

UF of Whey. The following day, approximately 700 kg of separated whey was weighed into a vat, heated to 50°C using a plate heat exchanger, and processed with a UF system in batch recirculation mode using a polyethersulfone spiral-wound UF membrane (model 3838, GEA NIRO Inc., Hudson, WI; nominal molecular weight cutoff: 10,000 Da, surface area: 13.6 m^2). Before processing, the UF membrane was short cleaned using the procedure described by Evans et al. (2009). The membrane was then flushed with 50°C reverse osmosis (RO) water to neutral pH and the clean water flux was determined. The following standard conditions were applied when determining UF water flux measurements: an inlet pressure of 172 kPa was applied using only the feed pump, 50°C RO water was fed to the system, and water flux was measured by collecting permeate in a tared, 19-L bucket over 30 s, weighing it, and dividing

the derived mass flow rate (kg/h) by the membrane surface area (m^2). A volumetric flux was derived from the mass flux, assuming 1 L of permeate = 1 kg at 50°C . The initial clean water flux was about 54 L/m^2 per h. During processing, the system was operated in a constant pressure mode with 276 kPa of retentate inlet pressure, 103 kPa of retentate outlet pressure, and no backpressure on the permeate side of the membrane. Whey was ultrafiltered for about 120 min. Every 15 min during processing, flux was measured and samples of the permeate and retentate were taken for compositional analysis using an infrared spectrophotometer (IR; Lactoscope FTIR, Delta Instruments, Drachten, the Netherlands) to monitor the process. The IR was calibrated using modified milk samples as described by Kaylegian et al. (2006). Ultrafiltration was continued until the protein content of the retentate was 41% protein as a percentage of lactose plus fat plus protein, as determined using IR. The corresponding concentration factor (CF) was about $5\times$. After UF, the retentate was diluted with pasteurized RO water at 50°C to bring the weight back to the original weight of the starting whey for DF. The membrane was not cleaned before proceeding to the DF stage. The mixture was recirculated through the membrane for 5 min to ensure complete mixing and then the DF process was started. The diluted UF retentate was diafiltered for about 135 min. Diafiltration continued until the protein content of the retentate measured by IR was 91.2 to 91.6% protein as a percentage of lactose plus fat plus protein in the retentate. The corresponding CF was about $11.3\times$. After producing the liquid WPC80, the UF system was cleaned using the procedure described by Evans et al. (2009). The fouled water flux before cleaning was, on average, 39% of the initial clean water flux (21 vs. 54 L/m^2 per hour), and the clean water flux after cleaning was similar to the clean water flux before processing (about 51 L/m^2 per hour).

SPC80 Production

MF Permeate Production. On the first day of processing, raw whole milk (about 1150 kg) was pasteurized with a plate heat exchanger (model 080-S, AGC Engineering) at 72°C for 16 s, cooled to 50°C , and separated with a centrifugal cream separator (model 619, DeLaval Inc.). After separation, the skim milk (about 1,060 kg) was kept at 50°C and processed with a UF system in batch recirculation mode using a polyethersulfone spiral-wound membrane (model 3838, GEA NIRO Inc.) with a molecular weight cutoff of 10,000 Da. This was a different membrane than the one used for both WPC80 and SPC80 productions. Before

processing, the UF membrane was cleaned following the procedure described by Evans et al. (2009). The skim milk was ultrafiltered for about 4 h to achieve a CF of approximately $2.2\times$. After UF, RO water was used to dilute the retentate back to the original true protein (TP) content of the skim milk, as determined by IR. For the treatments with added annatto, the colorant (Annatto cheese color - 2X, P/N 70741, Chr. Hansen Inc.) was added (0.066 mL/kg of milk) to the diluted UF retentate. The diluted UF retentate was then cooled using a plate heat exchanger and stored overnight at $\leq 4^{\circ}\text{C}$. The UF system was then cleaned as described by Evans et al. (2009). This UF step was conducted to reduce the lactose content of the MF feed so that the MF retentate produced in this study could be used in another study. In practice, SPC80 could be produced without ultrafiltering the skim milk before MF. In such a case, SPC80 UF and DF processing parameters (i.e., CF and IR targets) would be similar to those used during the production of WPC80 described above.

The next day, the diluted UF retentate was microfiltered in a continuous feed-and-bleed $3\times$ process using a pilot-scale system equipped with ceramic Membralox graded permeability membranes (Pall Corp., Cortland, NY; nominal pore diameter = $0.1\text{ }\mu\text{m}$, surface area = 1.7 m^2). The day before processing, the MF system was cleaned as described by Zulewska et al. (2009). The system setup was similar to the one described by Zulewska et al. (2009), but with the following additions: the retentate recirculation pump was equipped with a variable frequency drive (MC Series, model M12100C, Lenze AC Tech, Uxbridge, MA) and a magnetic flow transmitter (Foxboro I/A series IMF25, Invensys Process Systems, Plano, TX) so that the cross-flow velocity could be controlled and monitored, respectively.

The MF system was started on 50°C RO water and a transition from water to milk occurred with both the feed and recirculation pumps running. Retentate and permeate removal rates were set to 60 and 120 L/h, respectively. These removal rates resulted in a $3\times$ CF and a flux of 72 L/m^2 per hour, which were maintained throughout the process. The retentate recirculation rate was approximately 712 L/min, which corresponded to a cross-flow velocity of 7.1 m/s. Typical retentate and permeate inlet pressures were 455 and 240 kPa, respectively, and typical retentate and permeate outlet pressures were 225 and 255 kPa, respectively. To begin the process, 150 kg of the diluted UF retentate was used to flush the 50°C water out of the system. About 46 kg of retentate and 92 kg of permeate were collected in standard 38 L milk cans, the weights were recorded using a high capacity scale (Champ, Ohaus Corp., Florham Park, NJ), and both were discarded. While flushing

the water out of the system, samples of retentate and permeate were collected every 5 to 15 min and analyzed for composition by IR to ensure that all of the water had been removed from the system. After flushing the water from the system, the remaining skim milk (about 800 kg) was added to the system and retentate and permeate were collected continuously. Once the process was completed, the system was cleaned as described by Zulewska et al. (2009). If the permeate was not going to be bleached, it was cooled to 4°C with a plate heat exchanger and held overnight at $\leq 4^{\circ}\text{C}$.

Bleaching of MF Permeate. If the MF permeate was going to be bleached, it was subjected to the same bleaching treatments described above for whey, then cooled to 4°C with a plate heat exchanger and held overnight at $\leq 4^{\circ}\text{C}$.

UF of MF Permeate. The following day, approximately 530 kg of MF permeate was weighed into a vat, heated to 50°C using a plate heat exchanger, and processed using the same UF system used for WPC80 manufacture. Before processing, the UF membrane was short cleaned following the procedure described by Evans et al. (2009). The initial clean water flux was about 53 L/m^2 per hour. During processing, the system was operated in a constant pressure mode with 276 kPa of retentate inlet pressure, 103 kPa of retentate outlet pressure, and no backpressure on the permeate side of the membrane. The MF permeate was ultrafiltered for about 90 min. Every 15 min during processing, flux was measured and samples of permeate and retentate were taken for composition analysis using IR to monitor the process. Ultrafiltration was continued until the protein content of the retentate was 51% protein as a percentage of lactose plus fat plus protein, as determined by IR. The corresponding CF was about $3.8\times$. After UF, the retentate was diluted with pasteurized RO water at 50°C to bring the weight back to the original total weight of the starting MF permeate for DF. The membrane was not cleaned before proceeding to the DF stage. The mixture was recirculated through the membrane for 5 min to ensure complete mixing, then the DF process was started. Diluted UF retentate was DF for about 120 min. Diafiltration was continued until the protein content of the retentate measured by IR was 92% protein as a percentage of lactose plus fat plus protein in the retentate. The corresponding CF was about $11.2\times$. After producing the liquid SPC80, the UF system was cleaned as described by Evans et al. (2009). The fouled water flux before cleaning was, on average, 36% of the initial clean water flux (19 vs. 53 L/m^2 per hour), and the clean water flux after cleaning was similar to the clean water flux before processing (about 53 L/m^2 per hour).

Fouling Coefficient Calculations

Water flux before and after manufacturing WPC80 and SPC80 were used to estimate membrane fouling after processing. A fouling coefficient (**FC**) as proposed by Rao (2002) was calculated for each processing run using the following equation: $FC = 1 - (\text{fouled water flux} / \text{clean water flux})$. Greater FC values indicated a greater loss of water permeability (i.e., more membrane fouling). Mean values were calculated from the 3 replicates of each treatment and reported. In one WPC80 run, a fouled water flux was not recorded. To account for this missing fouled water flux value, an average of the 2 remaining fouled water flux values for the treatment was used with the run's clean water flux value to calculate the run's FC.

Chemical Analyses

The MF permeate was analyzed for TS, fat, total nitrogen (**TN**), NPN, and noncasein nitrogen (**NCN**) content using forced air oven drying (AOAC International, 2000; method 990.20; 33.2.44), ether extraction (AOAC International, 2000; method 989.05; 33.2.26), Kjeldahl (AOAC International, 2000; method 991.20; 33.2.11), Kjeldahl (AOAC International, 2000; method 991.21; 33.2.12), and Kjeldahl (AOAC International, 2000; method 998.05; 33.2.64) methods, respectively. Crude protein was calculated by multiplying TN by 6.38, TP was calculated by subtracting NPN from TN and multiplying by 6.38, CN was calculated by subtracting NCN from TN and multiplying by 6.38, and serum protein content was calculated by subtracting NPN from NCN and multiplying by 6.38. The separated Cheddar cheese whey was measured for TS, fat, and CP using the same methods described above. The pH of the Cheddar whey and MF permeate were measured with a solid polymer electrode (HA405-DXK-S8/120, Mettler-Toledo, Columbus, OH) and an Accumet 915 pH meter (Fisher Scientific, Pittsburgh, PA) that was calibrated at 50°C using standard pH 4 and 7 buffer solutions (Fisher Scientific).

Statistical Analysis

All data were analyzed by ANOVA using the Proc GLM procedure of SAS (SAS version 8.02, SAS Institute Inc., Cary, NC). To determine if significant differences ($P < 0.05$) had occurred in composition or pH among color and bleaching treatments of the separated Cheddar cheese whey or MF permeate, the GLM was dependent variable = bleach + color + replicate + bleach $\times$ color + error. To determine if significant differences ($P < 0.05$) had occurred in TS, fat, CP,

and pH between separated Cheddar cheese whey and MF permeate regardless of color or bleaching treatments, the GLM was dependent variable = feed type + replicate (feed type) + error. The ANOVA models comparing whey and MF permeate TS, fat, CP, and pH independent of the color and bleaching treatments were significant overall ($P < 0.05$); therefore, the LSM of the whey and MF permeate were compared using the LSMEANS statement.

To determine if significant differences ($P < 0.05$) had occurred in UF or DF flux among bleaching or color treatments, a full split-plot ANOVA model was constructed for each product (WPC80 or SPC80) during each processing stage (UF or DF) using the following main effects and all 2-way and 3-way interactions: bleach, color, replicate, and processing time. Additionally, due to the nonlinear decay of flux over time, predictors were constructed from the squared time term and all possible 2-way and 3-way interactions. Bleach, color, bleach $\times$ color, bleach $\times$ replicate, and color $\times$ replicate were treated as whole-plot variables in the split-plot ANOVA models. Consequently, the error term for the whole-plot variables would be bleach $\times$ color $\times$ replicate if the bleach, color, and replicate terms were all included in the model. Time was treated as a continuous subplot variable in the split-plot ANOVA models. Distortion of the ANOVA by multicollinearity in the model was minimized by transforming the processing time (Glantz and Slinker, 2001). Time was transformed as follows: transformed time = processing time – [(last processing time – first processing time)/2]. This transformation made the data set orthogonal with respect to time. Predictors within each model were examined for significance ($P < 0.05$) using the output from the Type III sums of squares table. A backward elimination procedure was used to remove nonsignificant terms ($P > 0.05$) from each model to create reduced models. Nonsignificant terms that took part in significant higher order interactions were allowed to remain in the reduced models. An *F*-test was carried out on each reduced model to verify that its predictive ability was not significantly different ($P > 0.05$) from that of its respective full model (Ott and Longnecker, 2004). This criterion was satisfied for all 4 models. Because the effect of color was determined to be nonsignificant ($P > 0.05$), the whole-plot error term for each final model was bleach $\times$ replicate. All reduced ANOVA models (Table 1, Table 2) were significant overall ($P < 0.05$); therefore, LSM for the bleaching treatments were compared using the LSMEANS statement with a Tukey-Kramer adjustment for multiple comparisons. To emphasize differences in the average flux of a treatment as opposed to initial or final flux values, data were truncated before analysis

Table 1. Analysis of variance split-plot design with degrees of freedom, type III sums of squares (SS), and *P*-values for predictive variables of ultrafiltration (after first 15 min of processing) and diafiltration (before last 15 min of processing) flux during the production of 80% whey protein concentrate

Predictive variable	Ultrafiltration			Diafiltration		
	df	SS	<i>P</i> -value	df	SS	<i>P</i> -value
Whole plot						
Bleach	2	846.55	0.0003	2	717.56	0.0005
Color	1	NS ¹	NS	1	NS	NS
Replicate	2	105.54	0.0138	2	41.56	0.0844
Bleach × color	2	NS	NS	2	NS	NS
Bleach × replicate ²	4	14.03	<0.0001	4	17.02	<0.0001
Color × replicate	2	NS	NS	2	NS	NS
Bleach × color × replicate	4	NS	NS	4	NS	NS
Sub plot						
Time	1	594.83	<0.0001	1	77.52	<0.0001
Time × bleach	2	20.64	<0.0001	2	4.90	0.0030
Time × color	1	NS	NS	1	NS	NS
Time × replicate	2	4.28	0.0036	2	NS	NS
Time × bleach × color	2	NS	NS	2	NS	NS
Time × bleach × replicate	4	NS	NS	4	NS	NS
Time × color × replicate	2	NS	NS	2	NS	NS
Time × time	1	33.44	<0.0001	1	14.66	<0.0001
Time × time × bleach	2	NS	NS	2	NS	NS
Time × time × color	1	NS	NS	1	NS	NS
Time × time × replicate	2	NS	NS	2	NS	NS
Time × time × bleach × color	2	NS	NS	2	NS	NS
Time × time × bleach × replicate	4	NS	NS	4	NS	NS
Time × time × color × replicate	2	NS	NS	2	NS	NS
Reduced model df	14			12		
Reduced error df	142			158		
Total df	156			170		
R ²	0.97			0.94		

¹The predictive variable was not significant ($P > 0.05$) and not included in the reduced model.

²Used as whole-plot error term for bleach and replicate in final model.

by removing the first time point during UF (i.e., UF flux at 0 min) and the last time point during DF (i.e., DF flux at end of processing). Mean flux values (i.e., the average flux over the entire processing time less the initial and final measurements) for bleaching treatments among the WPC80 and SPC80 processes were also compared. The GLM for each processing stage (UF or DF) was flux = bleach + feed + bleach × feed + rep (feed) + error. Both processing stage ANOVA models were significant overall ($P < 0.05$), therefore the LSM for the bleach × feed interaction were compared using the LSMEANS statement with a Tukey-Kramer adjustment for multiple comparisons.

To determine if significant differences ($P < 0.05$) had occurred in membrane FC due to bleaching or color treatments, an ANOVA model was constructed for each product (WPC80 or SPC80) using the following main effects and all 2-way interactions: bleach, color, and replicate. After the previously described model-reduction procedure was applied, the final GLM for each product was FC = bleach + replicate + error. All reduced ANOVA models were significant overall ($P < 0.05$), therefore the LSM for the bleaching treatments

were compared using the LSMEANS statement with a Tukey-Kramer adjustment for multiple comparisons.

RESULTS AND DISCUSSION

UF Stage Feed Compositions

No composition or pH differences were detected ($P > 0.05$) among bleaching or color treatments within a given UF feed material (i.e., separated Cheddar whey or MF permeate; Table 3). Therefore, even though 18 different cheese manufactures and 18 different MF processing runs of skim milk took place, the composition of the separated wheys and MF permeates were very consistent from processing run to run. Separated Cheddar whey TS, fat, and CP concentrations were all higher ($P < 0.0001$) than those of the MF permeate (Table 3). Whey TS were higher because lactose, soluble minerals, and NPN were removed from the skim milk used to produce the MF permeate during the UF step before MF. The resulting UF retentate was subsequently diluted back to the skim milk's original mass with RO water, thereby further reducing the lactose, soluble mineral,

Table 2. Analysis of variance split-plot design with degrees of freedom, type III sums of squares (SS), and *P*-values for predictive variables of ultrafiltration (after first 15 min of processing) and diafiltration (before last 15 min of processing) flux during the production of 80% serum protein concentrate

Predictive variable	Ultrafiltration			Diafiltration		
	df	SS	<i>P</i> -value	df	SS	<i>P</i> -value
Whole plot						
Bleach	2	156.90	0.0347	2	749.07	0.0005
Color	1	NS ¹	NS	1	NS	NS
Replicate	2	17.83	0.4468	2	16.53	0.2628
Bleach × color	2	NS	NS	2	NS	NS
Bleach × replicate ²	4	35.93	<0.0001	4	17.39	<0.0001
Color × replicate	2	NS	NS	2	NS	NS
Bleach × color × replicate	4	NS	NS	4	NS	NS
Subplot						
Time	1	129.72	<0.0001	1	22.58	<0.0001
Time × bleach	2	NS	NS	2	4.78	0.0005
Time × color	1	NS	NS	1	NS	NS
Time × replicate	2	NS	NS	2	NS	NS
Time × bleach × color	2	NS	NS	2	NS	NS
Time × bleach × replicate	4	NS	NS	4	NS	NS
Time × color × replicate	2	NS	NS	2	NS	NS
Time × time	1	7.05	0.0071	1	NS	NS
Time × time × bleach	2	NS	NS	2	NS	NS
Time × time × color	1	NS	NS	1	NS	NS
Time × time × replicate	2	NS	NS	2	NS	NS
Time × time × bleach × color	2	NS	NS	2	NS	NS
Time × time × bleach × replicate	4	NS	NS	4	NS	NS
Time × time × color × replicate	2	NS	NS	2	NS	NS
Reduced model df	10			11		
Reduced error df	86			129		
Total df	96			140		
R ²	0.82			0.96		

¹The predictive variable was not significant ($P > 0.05$) and not included in the reduced model.

²Used as whole-plot error term for bleach and replicate in final model.

and NPN concentrations in the serum phase. The whey also likely contained more solubilized colloidal calcium phosphate due to the mineral complex's migration from the CN micelles into the whey during the cheese making process's acidification step. Whey fat content was greater than that of the MF permeate because the 0.10 μm MF membrane used to produce the MF permeate retained the majority of the few fat globules present in the MF feed material (i.e., diluted UF retentate of skim milk). Whey CP concentration was higher than MF permeate CP because some NPN was washed out of the skim milk used to make the MF permeate in the UF and dilution steps before MF. Also, MF permeate lacked the proteolysis products created during the cheese-making process (such as glycomacropeptide). Whey pH was lower ($P < 0.0001$) than MF permeate pH (Table 3) due to the fermentation of lactose to lactic acid during the cheese-making process.

Flux During 80% Protein Concentrate Production

WPC80. The addition of annatto did not affect ($P > 0.05$) UF or DF flux during the production of WPC80 (Table 1). However, bleaching treatments increased (P

< 0.05) flux when compared with those without bleach (Tables 1 and 4). During whey UF (Figure 1; Table 4), treatments without bleach exhibited lower flux than BPO treatments, which in turn exhibited lower flux than H_2O_2 treatments ($P < 0.05$). The same pattern occurred during whey DF (Figure 2; Table 4); treatments without bleach exhibited lower flux than BPO treatments, which in turn exhibited lower flux than H_2O_2 treatments ($P < 0.05$).

SPC80. The addition of annatto did not affect ($P > 0.05$) UF or DF flux during the production of SPC80 (Table 2). However, bleaching treatments increased ($P < 0.05$) flux in most instances (Tables 2 and 5). During MF permeate UF (Figure 3; Table 5), H_2O_2 treatment flux was higher ($P < 0.05$) than flux in treatments without bleach. No differences ($P > 0.05$) in UF flux between treatments without bleach and treatments bleached with BPO or between the 2 bleaching treatments were detected. During MF permeate DF (Figure 4; Table 5), treatments without bleach exhibited lower flux than BPO treatments, which in turn exhibited lower flux than the H_2O_2 treatments ($P < 0.05$).

Comparison of WPC80 and SPC80. Because the addition of annatto was not found to influence flux

Table 3. Mean ($n = 3$) compositional¹ and pH² data for separated Cheddar cheese whey and 0.1- μ m microfiltration (MF) permeate of skim milk used to feed the ultrafiltration unit during production of 80% whey protein concentrate and 80% serum protein concentrate, respectively

Item	Treatment ³						SEM	R ²	Mean
	NA-NB	NA-BPO	NA-H ₂ O ₂	A-NB	A-BPO	A-H ₂ O ₂			
Separated whey ^a									
TS	6.710	6.703	6.728	6.706	6.727	6.710	0.021	0.68	6.714
Fat	0.043	0.037	0.044	0.042	0.043	0.040	0.003	0.61	0.043
CP	0.909	0.896	0.916	0.918	0.917	0.912	0.015	0.66	0.911
pH	6.37	6.35	6.39	6.36	6.36	6.36	0.030	0.29	6.37
MF permeate ^a									
TS	3.266	3.170	3.223	3.245	3.309	3.328	0.084	0.71	3.257
Fat	0.001	0.004	0.003	0.004	0.005	0.007	0.003	0.48	0.004
CP	0.725	0.723	0.701	0.719	0.723	0.735	0.029	0.49	0.721
NPN	0.098	0.105	0.095	0.102	0.100	0.104	0.004	0.68	0.100
NCN	0.677	0.666	0.607	0.664	0.621	0.645	0.043	0.51	0.647
TP	0.627	0.618	0.606	0.618	0.623	0.632	0.027	0.45	0.621
CN	0.048	0.056	0.093	0.055	0.102	0.091	0.036	0.49	0.074
SP	0.579	0.562	0.513	0.563	0.521	0.541	0.041	0.50	0.546
pH	6.71	6.66	6.71	6.72	6.71	6.73	0.036	0.57	6.71

^aMeans within the same row are not different among treatments ($P > 0.05$).

¹CP = total nitrogen $\times$ 6.38; NPN = nonprotein nitrogen $\times$ 6.38; NCN = noncasein nitrogen $\times$ 6.38; TP = true protein = CP minus NPN; CN = CP minus NCN; SP = serum proteins = NCN - NPN.

²pH determined at 50°C.

³No annatto and no bleach (NA-NB), annatto and no bleach (A-NB), no annatto and 50 mg/kg benzoyl peroxide (NA-BPO), annatto and 50 mg/kg benzoyl peroxide (A-BPO), no annatto and 500 mg/kg hydrogen peroxide (NA-H₂O₂), and annatto and 500 mg/kg hydrogen peroxide (A-H₂O₂).

in any of the above instances, mean flux values of the 3 bleaching treatments and 2 production processes (i.e., WPC80 and SPC80) were compared without considering the color treatment (Table 6). No differences ($P > 0.05$) were observed in mean UF flux during WPC80 and SPC80 production within any of the common bleaching treatments. However, SPC80 mean DF flux was lower ($P < 0.0001$) than the observed flux during WPC80 processing for each bleaching treatment. This difference was surprising, given that the SPC80 feed material for the DF stage only contained about 65, 9, and 79% of the TS, fat, and CP, respectively, when compared with that of the WPC80 DF feed (Table 3). Relative to that of whey, the pH of the MF permeate was higher; this may have caused the lower flux observed during SPC80 DF. A higher pH, which increased further as a result of the dilution before DF (about 6.63 for WPC80 DF

feed and about 6.82 for SPC80 DF feed), would favor the formation of apatite calcium structures, which are known to promote fouling (Hayes et al., 1974; Hickey et al., 1980). Though the calcium content of the MF permeate was likely lower than that of the separated whey, the form of the calcium in the MF permeate could be a more aggressive foulant. The implication of these results is that a manufacturer choosing to process its milk solids into SPC80 instead of WPC80 may experience a slightly lower flux during DF regardless of the bleaching treatment chosen.

Membrane Fouling During 80% Protein Concentrate Production

WPC80. No differences in membrane fouling, as measured by FC (Figure 5), were observed during

Table 4. Mean ($n = 3$) ultrafiltration (UF) and diafiltration (DF) flux (L/m² per hour) at 50°C during the production of 80% whey protein concentrate from separated Cheddar cheese whey under different treatments

Item	Treatment ¹						SEM	R ²
	NA-NB	NA-BPO	NA-H ₂ O ₂	A-NB	A-BPO	A-H ₂ O ₂		
UF	14.9 ^c	18.5 ^b	20.0 ^a	14.7 ^c	18.6 ^b	20.1 ^a	0.60	0.97
DF	16.8 ^c	20.0 ^b	21.6 ^a	16.0 ^c	19.6 ^b	22.1 ^a	0.64	0.94

^{a-c}Means in the same row not sharing a common superscript are different ($P < 0.05$).

¹Treatments: no annatto and no bleach (NA-NB), annatto and no bleach (A-NB), no annatto and 50 mg/kg benzoyl peroxide (NA-BPO), annatto and 50 mg/kg benzoyl peroxide (A-BPO), no annatto and 500 mg/kg hydrogen peroxide (NA-H₂O₂), and annatto and 500 mg/kg hydrogen peroxide (A-H₂O₂).

Figure 1. Mean ($n = 3$) flux at 50°C during the ultrafiltration of separated Cheddar cheese whey treated with no annatto and no bleach ($\diamond$), annatto and no bleach ($\blacklozenge$), no annatto and 50 mg/kg benzoyl peroxide ($\circ$), annatto and 50 mg/kg benzoyl peroxide ($\bullet$), no annatto and 500 mg/kg hydrogen peroxide ($\square$), and annatto and 500 mg/kg hydrogen peroxide ($\blacksquare$) before processing.

Figure 2. Mean ($n = 3$) flux at 50°C during the diafiltration of separated Cheddar cheese whey treated with no annatto and no bleach ($\diamond$), annatto and no bleach ($\blacklozenge$), no annatto and 50 mg/kg benzoyl peroxide ($\circ$), annatto and 50 mg/kg benzoyl peroxide ($\bullet$), no annatto and 500 mg/kg hydrogen peroxide ($\square$), and annatto and 500 mg/kg hydrogen peroxide ($\blacksquare$) before processing.

WPC80 processing due to annatto coloring of the whey ($P > 0.05$). Fouling coefficients were lower ($P < 0.01$) for WPC80 production when bleaching treatments were applied to whey (Figure 5). Furthermore, processes in which the wheys were bleached with BPO exhibited higher FC than processes in which wheys were bleached with H_2O_2 ($P < 0.01$). A strong negative correlation ($R^2 = 0.93$) was noted between the average of the UF and DF flux values and FC for each treatment (Figure 6), indicating that flux differences were associated with differences in membrane fouling.

SPC80. No differences in membrane fouling, as measured by FC (Figure 7), were observed during SPC80 processing due to annatto coloring of the MF permeate ($P > 0.05$). Processes that did not include a bleaching step and processes in which the MF permeates were bleached with BPO resulted in higher FC ($P < 0.001$; i.e., more fouling) than processes in which MF permeates were bleached with H_2O_2 (Figure 6). No differ-

ences in FC were detected between treatments without bleaching steps and those that included BPO bleaching steps ($P > 0.05$). A strong negative correlation ($R^2 = 0.82$) was noted between the average of the UF and DF flux values and fouling coefficient for each treatment (Figure 6), indicating that flux differences were associated with differences in membrane fouling.

Possible Reasons for the Observed Effects of Bleaching Treatments

Because this study sought to explore the effect of industrial bleaching treatments on UF flux, the effects of the bleaching agents were confounded with other inherent aspects of the bleaching processes. Specifically, the 66°C, 30-min heat-and-hold step used during bleaching was not applied to the unbleached treatments because this heating step would not be required if a processor chose not to bleach. Consequently, the aforementioned

Table 5. Mean ($n = 3$) ultrafiltration (UF) and diafiltration (DF) flux (L/m^2 per hour) at 50°C during the production of 80% serum protein concentrate from 0.1- μm microfiltration permeate of skim milk under different treatments

Item	Treatment ¹						SEM	R ²
	NA-NB	NA-BPO	NA-H ₂ O ₂	A-NB	A-BPO	A-H ₂ O ₂		
UF	16.7 ^b	16.5 ^{ab}	19.5 ^a	15.9 ^b	17.4 ^{ab}	18.9 ^a	0.96	0.82
DF	14.3 ^c	15.6 ^b	19.9 ^a	13.9 ^c	16.2 ^b	20.0 ^a	0.54	0.96

^{a-c}Means in the same row not sharing a common superscript are different ($P < 0.05$).

¹Treatments: no annatto and no bleach (NA-NB), annatto and no bleach (A-NB), no annatto and 50 mg/kg benzoyl peroxide (NA-BPO), annatto and 50 mg/kg benzoyl peroxide (A-BPO), no annatto and 500 mg/kg hydrogen peroxide (NA-H₂O₂), and annatto and 500 mg/kg hydrogen peroxide (A-H₂O₂).

Figure 3. Mean ($n = 3$) flux at 50°C during the ultrafiltration of 0.1- μm microfiltration permeate of skim milk treated with no annatto and no bleach ($\diamond$), annatto and no bleach ($\blacklozenge$), no annatto and 50 mg/kg benzoyl peroxide ($\circ$), annatto and 50 mg/kg benzoyl peroxide ($\bullet$), no annatto and 500 mg/kg hydrogen peroxide ($\square$), and annatto and 500 mg/kg hydrogen peroxide ($\blacksquare$) before processing.

Figure 4. Mean ($n = 3$) flux at 50°C during the diafiltration of 0.1- μm microfiltration permeate of skim milk treated with no annatto and no bleach ($\diamond$), annatto and no bleach ($\blacklozenge$), no annatto and 50 mg/kg benzoyl peroxide ($\circ$), annatto and 50 mg/kg benzoyl peroxide ($\bullet$), no annatto and 500 mg/kg hydrogen peroxide ($\square$), and annatto and 500 mg/kg hydrogen peroxide ($\blacksquare$) before processing.

findings should be considered results of the total treatment applied, not simply the bleaching agent used. With this in mind, we will address factors specific to the bleaching treatments that may have contributed to our results.

The BPO and H_2O_2 treatments affected flux values to varying degrees. This suggests that these bleaching treatments may reduce membrane fouling through different mechanisms. A key difference between the bleaching agents was the form in which they were used. Whereas H_2O_2 was added as a liquid, BPO was added as a powder. Approximately 68% of this powder was composed of cornstarch filler to facilitate the delivery of the BPO. This equated to a concentration of about 0.01% starch being incorporated into the feed material. Though sugars such as lactose are not generally regarded to be potential foulants (Rice et al., 2009), high molecular weight carbohydrates such as amylose (a component of cornstarch) have been shown to foul polymeric membranes during cross-flow UF at concentrations as low as 0.01 g/L (about 0.001%; Susanto

and Widiassa, 2009). Consequently, the starch carrier in the BPO may have offset some of the treatment's flux-enhancing effects by contributing to fouling.

The fact that the H_2O_2 treatment required the use of catalase enzyme and the BPO treatment did not should also be considered. It is doubtful that this source of variation contributed to the H_2O_2 treatment's observed flux-enhancing properties. According to its specification sheet, the catalase enzyme preparation contained the following ingredients: protein (enzymes), water, sodium chloride, sodium citrate, and sodium biphosphate. The only enzyme present in this product is catalase, so the potential for proteolytic activity from contaminating enzymes can be ruled out. Sodium citrate and sodium biphosphate are excellent chelating agents; they are capable of binding calcium ions which would otherwise contribute to calcium bridging, thereby promoting membrane fouling. However, the maximum concentration of these salts combined would be less than 0.002% in the whey or MF permeate based on the 20 mg/kg catalase delivery rate. This concentration would be be-

Table 6. Mean ($n = 6$) ultrafiltration (UF) and diafiltration (DF) flux (L/m^2 per hour) at 50°C during the production of 80% whey protein concentrates (WPC) and 80% serum protein concentrates (SPC) made from separated Cheddar cheese whey or 0.1- μm microfiltration permeate of skim milk, respectively, treated with no bleach (NB), 50 mg/kg benzoyl peroxide (BPO), or 500 mg/kg hydrogen peroxide (H_2O_2) before processing

Item	WPC-NB	WPC-BPO	WPC- H_2O_2	SPC-NB	SPC-BPO	SPC- H_2O_2	SEM	R^2
UF	14.8 ^d	18.6 ^{ab}	20.1 ^a	16.3 ^{cd}	17.0 ^{bc}	19.2 ^a	0.90	0.86
DF	16.4 ^c	19.8 ^b	21.8 ^a	14.1 ^d	15.9 ^c	20.0 ^b	0.73	0.95

^{a-d}Means in the same row not sharing a common superscript are different ($P < 0.05$).

Figure 5. Effect of annatto colorant and bleaching treatments (NB = no bleach; BPO = 50 mg/kg benzoyl peroxide; H₂O₂ = 500 mg/kg hydrogen peroxide) of separated Cheddar cheese whey on fouling coefficients [1 – (fouled water flux/clean water flux)] of polyethersulfone membranes after the production of 80% whey protein concentrate. Higher values indicate more fouling. Different letters (a–c) indicate significant differences ($P < 0.05$) among treatments; SE = 0.02; $R^2 = 0.91$.

Figure 7. Effect of annatto colorant and bleaching treatments (NB = no bleach; BPO = 50 mg/kg benzoyl peroxide; H₂O₂ = 500 mg/kg hydrogen peroxide) of 0.1- μ m microfiltration permeate of skim milk on fouling coefficients [1 – (fouled water flux/clean water flux)] of polyethersulfone membranes after the production of 80% serum protein concentrate. Higher values indicate more fouling. Different letters (a,b) indicate significant differences ($P < 0.05$) among treatments; SE = 0.03; $R^2 = 0.86$.

low the useful addition of sodium citrate (0.2%) quoted by Maubois (1980) for whey flux improvement and far below the approximate 0.4%, which was found to be effective by Taddei et al., (1988).

Bleaching with H₂O₂ improved both WPC80 and SPC80 flux in all instances. Past studies that have examined the effects of H₂O₂ on SP in whey (Cooney and

Morr, 1972) have used peroxide concentrations far in excess of that used in the present study (0.05% wt/wt). When treating dialyzed whey with H₂O₂ concentrations between 1 and 2%, Cooney and Morr (1972) observed considerable SP denaturation and aggregation. However, they noted that no SP denaturation or aggregation was observed in whey treated with 0.5% H₂O₂ at 25°C. Recent work by Jervis et al. (2012), done in conjunction with this study, focused on the functional properties of the WPC80 produced by each treatment. They observed that when heating 10% (wt/wt) solutions of treated WPC80 at 90°C at pH 7, only the concentrates bleached with H₂O₂ failed to gel after 10 min. Upon adjustment of the pH to 4.6 and subsequent heating, only the H₂O₂ treatments still exhibited some soluble protein (i.e., protein that did not gel). By heating 10% (wt/wt) solutions of the SPC80 produced in the present study to 90°C at pH 4.6, Campbell et al. (2013) also determined that only those SPC80 which had been subjected the H₂O₂ treatment did not gel after 10 min. Because the solutions prepared from WPC80 and SPC80 that were bleached with BPO did gel under these conditions, it can be surmised that these bleaching treatments affected the SP differently. The apparent increase in protein solubility with the H₂O₂-treated WPC80 samples observed by Jervis et al. (2012) was attributed to SP proteolysis incurred during the bleaching treatment. Because the 90°C heat treatments used by Jervis et al. (2012) and Campbell et al. (2013) were greater than

Figure 6. Linear correlations between average of ultrafiltration and diafiltration flux values and fouling coefficients [1 – (fouled water flux/clean water flux)] from treatments within 80% whey protein concentrate (WPC80; ■) and 80% serum protein concentrate (SPC80; ○) ultrafiltration production processes.

the UF processing temperature (50°C) and the denaturation temperature of SP (about 70°C), it cannot be concluded that a similar advantage in protein solubility would be observed during UF processing. However, it is well established that protein solutions will exhibit flux minima during UF at the protein's isoelectric point due to absorption caused by minimum protein solubility (Swaminathan et al., 1981; Hanemaaijer et al., 1989; de la Casa et al., 2007). Heng and Glatz (1991) determined that flux during UF of acid whey was reduced when SP were precipitated (i.e., solubility was reduced) using carboxymethyl cellulose before processing. Therefore, if the H₂O₂ treatment proteolyzed the SP, thus creating more soluble peptide fractions, membrane flux would be expected to increase, as was observed. Consequently, the mechanism which improves membrane flux when bleaching with H₂O₂ may be protein-driven. It is possible that the BPO bleaching process affected flux by altering the SP structure as well, but this may have occurred to a much lesser extent based on the protein solubility data presented by Jervis et al. (2012) and Campbell et al. (2013).

Tong et al. (1989) observed that the proteinaceous foulant responsible for flux decline during sweet whey UF was composed of CN proteolysis products and α -LA. The former was only present when the cheese had been coagulated with calf rennet as opposed to a protease derived from *Mucor pusillus*. These proteolysis products were absent from the UF foulant when whey produced with calf rennet was treated with the *M. pusillus* protease before UF. Presumably, the microbial coagulant proteolyzed the CN peptides even further, causing roughly a 40% increase in UF flux. Casein proteolysis products were absent from the MF permeate used to produce SPC80 in the present study. If the flux differences observed between the unbleached and BPO treatments during WPC80 production were due to the breakdown of CN proteolysis products, one would not expect to observe differences in flux or fouling between the unbleached and BPO treatments during SPC80 production. This was the case, as the BPO treatment flux during SPC80 production was not different ($P > 0.05$) from that of the unbleached treatment flux during SPC80 production (Table 6). Furthermore, BPO flux during SPC80 production was not different ($P > 0.05$) from that of the BPO treatment flux during WPC80 production (Table 6). Consequently, the mechanism by which BPO enhances flux may be due to an effect on CN proteolysis products. The same logic would follow if the BPO acted on the fat to cause changes in flux, as the fat content of the MF permeate was almost negligible compared with that of the separated Cheddar whey (0.004% vs. 0.04%; Table 3). Therefore, the

mechanism by which BPO enhances flux may also be due to an effect on fat.

CONCLUSIONS

Ultrafiltration and DF flux from WPC80 and SPC80 production processes were examined after each process's feed material had been subjected to annatto coloring and bleaching treatments. Addition of annatto color had no effect on UF or DF flux. Bleaching whey or MF permeate with or without added color usually improved flux during processing. Bleaching with H₂O₂ produced a higher flux than bleaching with BPO. Bleaching with BPO increased WPC80 flux to a greater extent than it did SPC80 flux. Though no differences in mean flux were observed for a common bleaching treatment between the WPC80 and SPC80 production processes during the UF stage, mean flux during WPC80 DF was higher than mean flux during SPC80 DF for each bleaching treatment. Water flux values before and after processing were used to calculate an FC that demonstrated differences in fouling which were consistent with flux differences resulting from the bleaching treatments. In both processes, bleaching with H₂O₂ led to the largest reduction in fouling. No effect of annatto on fouling was observed. The reasons for flux enhancement associated with bleaching treatments are unclear.

ACKNOWLEDGMENTS

The authors thank the New York State Milk Promotion Board (Albany, NY) and Northeast Dairy Foods Research Center (Ithaca, NY) for partial funding of this research. The technical assistance of Irma Amelia, Tim Barnard, Steve Beckman, Michelle Bilotta, Maureen Chapman, Chassidy Coon, Aneela Hameed, Emily Hurt, and Mark Newbold from the Department of Food Science at Cornell University (Ithaca, NY) was greatly appreciated.

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