Short communication: Annatto in Cheddar cheese-derived whey protein concentrate is primarily associated with milk fat globule membrane

D. Zhu and S. Damodaran¹

Department of Food Science, University of Wisconsin at Madison, 1605 Linden Drive, Madison 53706

ABSTRACT

The yellow color of Cheddar cheese whey arises from a residual amount of annatto that partitions into the whey during Cheddar cheese manufacture. Bleaching of the color using hydrogen peroxide or benzoyl peroxide is often a prerequisite to produce an acceptable neutral-colored whey protein concentrate and isolate. However, the use of these strong oxidizing agents often generates off-flavors as a result of lipid oxidation and results in loss of nutritive value due to protein oxidation. The objective of this study was to determine the extent of partitioning of annatto between protein, milk fat globule membrane (MFGM), and aqueous (serum) phases of cheese whey so that a simple method can be developed to remove annatto from cheese whey. The MFGM was separated from Cheddar cheese whey using a recently developed novel method. Quantitative analysis of the distribution of annatto in the fat-free whey protein isolate (WPI), the MFGM fractions, and the serum phase revealed that annatto was not bound to the protein fraction but was mostly distributed between the serum phase and the MFGM fraction. The results showed that a colorless WPI or whey protein concentrate could be produced from Cheddar cheese whey by separation of MFGM from the whey, followed by diafiltration. This approach will negate the need for using bleaching agents.

Key words: annatto color, whey protein concentrate, milk fat globule membrane

Short Communication

Total cheese production in the United States in 2009 was about 10.1 billion pounds, of which about 31.7% was Cheddar cheese (USDA, 2010). Thus, Cheddar cheese whey is a major starting material for the manufacture of whey protein concentrates (WPC) and isolates (WPI). A significant amount of Cheddar cheese produced is yellow because annatto (norbixin) is used

as the major colorant. About 20% of the annatto added to milk during Cheddar cheese manufacture partitions into the cheese whey (Chapman et al., 1980). When whey is subsequently converted to WPC and WPI, the residual annatto imparts an undesirable yellow color to these whey protein products (Kang et al., 2010). To eliminate the yellow color, whey processors often bleach Cheddar cheese whey by treating it with hydrogen peroxide or benzoyl peroxide before spray drying (Kang et al., 2010). However, it has been shown that bleaching causes oxidation of whey lipids, which results in liberation of volatile off-flavors, such as hexanal, heptanal, and octanal, in WPC (Croissant et al., 2009). In addition, it is likely that the above oxidants may cause oxidation of several amino acid residues, such as methionine, lysine, tyrosine, histidine, and tryptophan (Shacter, 2000), resulting in decreased nutritive value of whey proteins.

The dairy industry predominantly uses norbixin, instead of bixin, as a cheese colorant because it is water-soluble and dispersible (Giuliano et al., 2003). Because norbixin is water-soluble and dispersible, it is often assumed that norbixin in whey is present mainly in the serum phase. If this indeed is the case, then it should be possible to completely remove norbixin from the retentate during the manufacture of WPC80 (WPC with 80% protein) and WPI, which involves a series of ultrafiltration and diafiltration steps to remove lactose and other low molecular weight substances. However, this is not the case; most norbixin remains in the retentate. This has led some to suggest that norbixin in whey might be tightly bound to whey proteins in the same way that it binds to caseins in Cheddar cheese curd (Govindarajan and Morris, 1973; Hammond et al., 1975). Presumably, this suggestion was made because β -LG is a retinol-binding protein (Cho et al., 1994).

Norbixin is a diapocarotenoid with carboxyl groups at both ends of a 24-carbon isoprenoid chain with conjugated double bonds. The hydrophobicity and hydrophilicity characteristics of the molecule might suggest that in solution norbixin is in the form of micelles dispersed in the aqueous medium, rather than in a “soluble” state. If this conjecture is correct, then the micellar norbixin would more likely to be associated

Received August 22, 2011.

Accepted October 23, 2011.

¹Corresponding author: sdamodar@wisc.edu

with the milk fat globule membrane (**MFGM**) particles than with globular proteins in Cheddar cheese whey. Recently, it has been shown that the MFGM particles separated from cheese whey are predominantly in the form of bilayer lamellar sheets (Zhu and Damodaran, 2011). Association of norbixin with MFGM particles may occur via adsorption to the lamellar surface or via incorporation into the bilayer structure of the MFGM.

The literature is ambiguous regarding the state of annatto in Cheddar cheese whey. In the present study, we investigated the distribution of norbixin in the serum, protein, and MFGM phases of Cheddar cheese whey to clear this ambiguity so that a better alternative to the use of peroxides can be developed to decolorize Cheddar cheese whey.

Clarified and pasteurized Cheddar cheese whey was obtained from the dairy plant in the Department of Food Science at the University of Wisconsin-Madison. The MFGM was isolated from cheese whey using a novel method described elsewhere (Damodaran, 2011). Briefly, Cheddar cheese whey (3 L) was first ultrafiltered 3-fold (3 $\times$) using a bench-top Millipore ProFlux M12 Tangential Flow Filtration System using a spiral-wound 10-kDa molecular weight cut-off polysulfone membrane (Millipore Corp., Billerica, MA). Two hundred milliliters of this original retentate (**OR**) was removed and freeze-dried. The remaining retentate (0.8 L) was then diafiltered 3-fold using 2.4 L of distilled water. The conductivity of the final retentate was less than 500 μ S/cm. The final diafiltered retentate (**DR**) was recovered (0.8 L) and divided into two 375-mL portions. One portion was freeze-dried and the other portion was treated as follows. The pH of the solution was adjusted to 4.2 using 2 M HCl and the solution was incubated for 30 min at 35°C. The MFGM fragments in DR flocculated during this incubation period. The solution was then centrifuged at 3,000 $\times g$ for 10 min. The supernatant, which contained almost all whey proteins (Damodaran, 2011), and the MFGM pellet were separated. The pellet was resuspended in 100 mL of distilled water. The pH of the supernatant was adjusted to 6.7. Both the supernatant and the resuspended MFGM pellet were freeze-dried.

The annatto content of the 4 freeze-dried samples [i.e., OR, DR, MFGM isolate, and freeze-dried supernatant (WPI)] was determined by the ammonia:ethanol extraction procedure as described elsewhere (Hammond et al., 1975). The absorption spectrum of norbixin in 1:5 ammonia solution (30%):ethanol solvent is shown in Figure 1. The spectrum exhibited 3 peaks, at 427, 449, and 479 nm. Based on this, a standard curve was constructed by plotting absorbance at 479 nm against norbixin concentration in 1:5 ammonia:ethanol solvent. From the slope of the standard curve, the extinction co-

efficient of 1% norbixin in the ammonia:ethanol solvent was determined to be 2,515 at 479 nm. This value was very similar to the extinction coefficient value of 2,503 at 282 nm in 0.1 N KOH solvent reported by Scotter et al. (1994). This extinction coefficient was used to determine the concentration of norbixin in the freeze-dried whey fractions. Briefly, about 0.1 to 0.5 g of the sample was accurately weighed and placed in a glass tube. Two milliliters of 30% ammonia solution was added and the mixture was vigorously vortexed for 1 min. To this was added 10 mL of anhydrous ethanol and the mixture was vortexed again for 1 min and centrifuged at 16,000 $\times g$ for 10 min at 5°C. The supernatant was decanted, 2 drops of a phosphate solution (containing 1.24 M NaH_2PO_4 and 0.76 M Na_2HPO_4) was added, and the tube was vortexed for 30 s, left in the dark for 30 min, and then centrifuged at 16,000 $\times g$ for 10 min at 5°C. The absorbance of the supernatant was measured at 479 nm against ammonia:ethanol solvent as the blank. The concentration of norbixin in the whey samples was determined using the extinction coefficient value of 2,515 for 1% at 479 nm. Triplicate determinations were done on each sample.

The appearance of OR, DR, and the MFGM-free supernatant before and after freeze-drying is shown in Figure 2; the OR and DR were bright yellow. However, when the MFGM particles were removed from the DR by selective precipitation at pH 4.2, the resulting supernatant was colorless. Upon freeze-drying, the OR, DR,

Figure 1. Absorption spectrum of norbixin in 1:5 ammonia:ethanol solvent.

Figure 2. Visual appearances of various fractions and phases in Cheddar cheese whey. OR = original retentate; DR = diafiltered retentate; MFGM = milk fat globule membrane; WPI = whey protein isolate.

and the MFGM powders were bright yellow, whereas the WPI was very white. These results indicate that the annatto in the DR sample was primarily associated with the MFGM phase.

Quantitative analysis of the distribution of annatto levels in OR, DR, WPI, and MFGM powders resulting from 375 mL of the 3-fold concentrated OR is shown in Table 1. The total annatto content in 375 mL of OR was 18.09 μg . Upon 3-fold diafiltration, the annatto content decreased to 13.16 μg , indicating that 4.93 μg of annatto was removed in the filtrate during diafiltration. Apparently, this fraction was in the soluble or micellar form in the serum phase of OR. When the MFGM was precipitated from DR, the MFGM fraction

contained 10.76 μg of annatto, and the WPI (supernatant) fraction contained 1.87 μg of annatto. When the WPI fraction was dialyzed against water for 24 h, the resulting WPI contained no detectable annatto, suggesting that the residual amount of annatto (1.87 μg) that was present before dialysis was not tightly bound to the protein. It is likely that this residual amount of annatto in WPI could be the remnant of the serum phase annatto in the OR that was not completely removed during the 3-fold diafiltration step.

Contrary to the common view that annatto in whey is mostly bound to whey proteins, the results presented here clearly indicate that about 60% of annatto in whey exists in the MFGM phase and the remainder is in

Table 1. Distribution of annatto among various phases in 375 mL of 3-fold-concentrated Cheddar cheese whey

Sample	Dry solid (g)	Annatto content ($\mu\text{g/g}$; $\pm$ SD ¹)	Total amount of annatto (μg ; $\pm$ SD ¹)
3 $\times$ whey retentate (OR)	27.73	0.652 ± 0.005	18.09 ± 0.14
3-fold diafiltered 3 $\times$ whey retentate (DR)	8.50	1.548 ± 0.02	13.16 ± 0.11
Milk fat globule membrane pellet (freeze-dried)	2.2	4.76 ± 0.08	10.76 ± 0.21
Lyophilized supernatant (whey protein isolate)	6.16	0.30 ± 0.01	1.87 ± 0.06
Dialyzed and lyophilized supernatant (whey protein isolate)	6.16	Not detected	~ 0

¹The standard deviation was based on triplicate measurements ($n = 3$).

freely dispersed form in the serum phase, which could be removed by diafiltration.

Based on the results presented here, we argue that the use of bleaching agents, such as hydrogen peroxide and benzoyl peroxide (which causes oxidation of essential amino acids and lipids, and loss of nutritive value) to eliminate the yellow color problem in Cheddar cheese whey is not ideal. Decolorization of Cheddar cheese whey can be achieved simply by removing the MFGM fragments using the process outlined previously (Damodaran, 2011). This process can be easily integrated into the existing commercial whey processing operations, which has the additional benefit of manufacturing a fat-free WPI or WPC.

REFERENCES

- Chapman, H. R., S. Y. Thompson, and H. M. Slade. 1980. The use of carotenoid preparations for colouring Cheddar cheese. *J. Soc. Dairy Technol.* 33:162–164.
- Cho, Y., C. A. Batt, and L. Sawyer. 1994. Probing the retinol-binding site of bovine β -lactoglobulin. *J. Biol. Chem.* 269:11102–11107.
- Croissant, A. E., E. J. Kang, R. E. Campbell, E. Bastian, and M. A. Drake. 2009. The effect of bleaching agent on the flavor of liquid whey and whey protein concentrate. *J. Dairy Sci.* 92:5917–5927.
- Damodaran, S. 2011. A straightforward process for removal of milk fat globule membranes and production of fat-free whey protein concentrate from cheese whey. *J. Agric. Food Chem.* 59:10271–10276.
- Giuliano, G., C. Rosati, and P. M. Bramley. 2003. To dye or not to dye: Biochemistry of annatto unveiled. *Trends Biotechnol.* 21:513–516.
- Govindarajan, S., and H. A. Morris. 1973. Pink discoloration in Cheddar cheese. *J. Food Sci.* 38:675–678.
- Hammond, E. G., J. Chang, and G. W. Reinbold. 1975. Colorimetric method for residual annatto in dry whey. *J. Dairy Sci.* 58:1365–1366.
- Kang, E. J., R. E. Campbell, E. Batian, and M. A. Drake. 2010. Annatto usage and bleaching in dairy foods. *J. Dairy Sci.* 93:3891–3901.
- Scotter, M. J., S. A. Thorpe, S. L. Reynolds, L. A. Wilson, and P. R. Strutt. 1994. Characterization of annatto food coloring components using high performance liquid chromatography with photodiode-array detection. *Food Addit. Contam.* 11:301–315.
- Shacter, E. 2000. Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* 32:307–326.
- USDA. 2010. National Agricultural Statistics Service. Accessed April 2010. USDA, Washington, DC.
- Zhu, D., and S. Damodaran. 2011. Composition, thermotropic properties, and oxidative stability of freeze-dried and spray-dried milk fat globule membrane isolated from cheese whey. *J. Agric. Food Chem.* 59:8931–8938.