ABSTRACT

This study evaluated the role of protein concentration and milk protein ingredient [serum protein isolate (SPI), micellar casein concentrate (MCC), or milk protein concentrate (MPC)] on sensory properties of vanilla ready-to-drink (RTD) protein beverages. The RTD beverages were manufactured from 5 different liquid milk protein blends: 100% MCC, 100% MPC, 18:82 SPI:MCC, 50:50 SPI:MCC, and 50:50 SPI:MPC, at 2 different protein concentrations: 6.3% and 10.5% (wt/wt) protein (15 or 25 g of protein per 237 mL) with 0.5% (wt/wt) fat and 0.7% (wt/wt) lactose. Dipotassium phosphate, carrageenan, cellulose gum, sucralose, and vanilla flavor were included. Blended beverages were preheated to 60°C, homogenized (20.7 MPa), and cooled to 8°C. The beverages were then preheated to 90°C and ultrapasteurized (141°C, 3 s) by direct steam injection followed by vacuum cooling to 86°C and homogenized again (17.2 MPa first stage, 3.5 MPa second stage). Beverages were cooled to 8°C, filled into sanitized bottles, and stored at 4°C. Initial testing of RTD beverages included proximate analyses and aerobic plate count and coliform count. Volatile sulfur compounds and sensory properties were evaluated through 8-wk storage at 4°C. Astringency and sensory viscosity were higher and vanillin flavor was lower in beverages containing 10.5% protein compared with 6.3% protein, and sulfur/eggy flavor, astringency, and viscosity were higher, and sweet aromatic/vanillin flavor was lower in beverages with higher serum protein as a percentage of true protein within each protein content. Volatile compound analysis of headspace vanillin and sulfur volatiles was consistent with sensory results: beverages with 50% serum protein as a percentage of true protein and 10.5% protein had the highest concentrations of sulfur volatiles and lower vanillin compared with other beverages. Sulfur volatiles and vanillin, as well as sulfur/eggy and sweet aromatic/vanillin flavors, decreased in all beverages with storage time. These results will enable manufacturers to select or optimize protein blends to better formulate RTD beverages to provide consumers with a protein beverage with high protein content and desired flavor and functional properties. Key words: protein beverage, flavor, micellar casein, milk protein

INTRODUCTION

Consumers continue to seek out products that offer benefits for their health and wellness. A recent survey indicated that 76% of shoppers ages 18 to 80 yr in the United States made small changes in their diet throughout the year to achieve an overall healthier diet (International Food Information Council, 2016). From the survey results, researchers concluded that taste continues to have the greatest influence (83%) on the decision to buy foods and beverages, followed by price (73%), followed by healthfulness, convenience, and sustainability, in that order. In that same survey, protein was the most sought-after nutrient, with 64% of respondents indicating that they were trying to consume more protein in their diet. The convenience and variety of ready-to-drink (RTD) protein beverages attracts new consumers who are seeking ways to increase protein intake. Dairy proteins, specifically milk and whey proteins, are the most desirable types of protein in RTD beverages, and ≥15 g of protein per serving are favored (Oltman et al., 2015; Harwood and Drake, 2019). Producers of high-protein milk-based beverages have a wide range of dried milk protein-based ingredients to select from for beverage formulation, including nonfat dry milk, milk protein concentrates (MPC) and milk protein isolates, and whey protein concentrates and isolates, with new milk protein ingredients becoming available, such as micellar casein concentrate (MCC) and milk serum protein isolate (SPI); i.e., milk-derived...
The RTD beverages currently on the market have a broad range of protein content from 10 to 40 g of protein per serving. However, the reported variability of the amount of protein per serving can partially be attributed to inconsistent sizing, which range from 236 to 591 mL per serving. Manufacturers are increasing concentrations of protein in beverages in response to consumer demand, and this change presents technical challenges (i.e., thermal and flavor stability) to formulate and manufacture these products that are affected by higher concentrations of protein.

Two main categories of RTD beverages exist: neutral pH beverages and acidified beverages. Neutral pH and low-acid beverages generally have a pH between 4.6 and 7.5, whereas acidified beverages ideally have a pH between 2.5 and 3.0 (Etzel, 2004; Rittmanic and Burginton, 2006). The pH range of the beverages dictates the thermal treatment the beverages will require, as an H <4.6 lowers the heat resistance of bacteria (Palop and Martínez, 2005). Generally speaking, acidic beverages are traditionally processed by “hot fill,” where the beverage is heated to at least 83°C (target temperature 90 to 95°C), then filled into a container, and inverted while still hot (Frederick et al., 2010; Rushing and Foegeding, 2010). Shelf-stable neutral pH beverages are required to undergo either retort processing (115–125°C for about 10–40 min), or UHT treatment (135–150°C with a hold time of at least 2–6 s), followed by cooling and filling aseptically into previously sterilized containers. These beverages can also be processed by ultrapasteurization (UP; 138°C for at least 2 s), filled, and refrigerated, and are designated extended shelf life. The pH and thermal treatments determine the specific protein or proteins that can be used in RTD beverages. In acidic beverages, casein-containing milk protein ingredients cannot be used, as they precipitate at acidic pH (pH <4.6), whereas whey proteins are soluble at low pH. At neutral pH, whey proteins are not heat stable. Caseins, however, are very heat stable, with some casein ingredients such as sodium caseinate being able to withstand heating at 140°C for hours at pH 7 without visible changes (O’Mahony and Fox, 2014).

The physicochemical properties of casein and whey protein ingredients influence the desired nutritional profile, functionality, and sensory properties of RTD protein beverages (Carter et al., 2021). Many studies have investigated the heat stability of individual dairy proteins at various concentrations and matrix effects such as pH, ionic strength, and addition of various complexes (Vardhanabhuti and Foegeding, 2008; Yong and Foegeding, 2008, 2010; de Wit, 2009; Donato and Guyomarc’h, 2009). Recently, Kelleher et al. (2018) compared physical properties of whey protein isolate solutions with 4, 6, and 8% (wt/wt) protein and a pH of 6.8, processed by direct or indirect heating at 121°C and 135°C for 2 s. However, to our knowledge, previous studies have not evaluated the roles of different milk protein blends and protein concentration on sensory properties of RTD protein beverages. The goal of this study was to evaluate the role of protein concentration and milk protein ingredient (i.e., SPI, MCC, or MPC) on sensory properties and volatile compounds of model vanilla-flavored RTD protein beverages.

MATERIALS AND METHODS

Experimental Design

Beverage formulations were based on a factorial complete block design (protein concentration and protein composition) with repeated measures across time. The beverage treatments (n = 10 total) were combinations of true protein (TP) content (6.3 or 10.5%, wt/wt) and 5 compositions of dairy protein ingredients (100% MCC, 100% MPC, 18:82 SPI:MCC, 50:50 SPI:MCC, or 50:50 SPI:MPC). The protein concentrations were selected to represent 15 or 25 g of protein per 237-mL serving (Harwood and Drake, 2019). The protein blends represented various milk protein sources available to beverage processors. Liquid proteins were manufactured on d 1, and then beverages were blended and processed on d 2 and stored at 4°C. Proximate analysis, volatile compound analysis, descriptive sensory analysis, aerobic plate count, and coliform count were performed on the beverages. All analyses, excluding proximate analysis, were performed every 2 wk throughout an 8-wk period. The entire experiment was replicated twice.

Milk Protein Processing and Other Beverage Ingredients

Raw bovine skim milk (0.09% ± 0.04 fat, SSC <300,000) was secured from the North Carolina State University Dairy Enterprise System (Raleigh, NC) on the morning of each processing run. Raw skim milk was collected into a glycol-chilled, jacketed storage tank, which was cleaned and sanitized immediately before milk receipt, and then held at 4°C until use (~2 h). Skim milk was HTST pasteurized (11.34 kg/min) using a plate heat exchanger (model T4 RGS-16/2, SPX Flow Technology) at 72°C with a hold time of 16 s. The pasteurized skim milk was cooled to 4°C, prefiltered by a Nexis T Filter (NXT 10-30U-M7S, Pall Corporation), and stored at 4°C in a 1,200-kg jacketed stainless-steel tank (type A & G, Chester-Jensen Company).
**Micellar Casein Concentrate**

The 95% serum protein–reduced liquid MCC was manufactured as described by Cheng et al. (2018) targeting the stage-3 microfiltered retentate for a TP concentration between 12.8 and 13.6%, confirmed by a mid-infrared spectrophotometer (Lactoscope FTA, Delta Instruments). After the third stage, all of the retentate was added back to the feed tank, and the retentate exit was further concentrated from 8.6% to between 12.8 and 13.6% TP. Once target protein was achieved, the retentate was collected directly into sanitized milk cans and cooled to <10°C using a glycol water bath. Following the initial cooling, the cans were stored at 4°C in a walk-in cooler overnight.

**Milk Protein Concentrate**

The MPC was processed using the UF protocol described by Carter et al. (2018). The liquid MPC was collected directly into sanitized milk cans and cooled to <10°C using a glycol water bath. Following the initial cooling, the cans were stored at 4°C in a walk-in cooler overnight.

**Serum Protein Isolate**

Frozen SPI was purchased from South Dakota State University (Brookings, SD) and stored at −20°C. The SPI was produced by ceramic microfiltration of skim milk, followed by 2 stages of UF with diafiltration to remove lactose and concentrate the protein. Frozen SPI was thawed at 4°C in a walk-in cooler for 3 d. The SPI contained 22.3% protein and 24.73% total solids.

**Cream, Lactose, and Water**

High-temperature short-time pasteurized cream containing about 41% fat (wt/vol) was obtained from the North Carolina State University Dairy and stored at 4°C before use. Composition of the cream was verified by mid-infrared spectrophotometry (Lactoscope FTA, Delta Instruments) before formulation. Lactose monohydrate (Hilmar 5120 Refined Edible Lactose 200 mesh, 25 kg) was donated by Hilmar Ingredients. Potable water was processed to produce deionized water (MarCor, unit number 1933-2).

**Additional Ingredients**

All beverages were also formulated to include 0.015% (wt/wt) carrageenan (Ticaloid 780, TIC Gums), 0.4% (wt/wt) cellulose gel (Ticaloid Pro HC 988, TIC Gums), 0.03% (wt/wt) sucralose (100% Sucralose Powder, Sweet Solutions LLC), 0.15% (wt/wt) dipotassium phosphate (Consolidated Chemical), and 0.5% (wt/wt) vanilla flavoring (Glanbia Nutritionals).

**Beverage Formulation**

Mean values of the liquid protein ingredients are provided in Table 1. Formulations were calculated using concentrations of fat (0.5% wt/wt), TP, casein as a percent of true protein (CN%TP), serum protein, and lactose (0.7% wt/wt; Misawa et al., 2016). The additional ingredients previously noted were constant across all beverage formulations. Target percent lactose and fat were also constant across beverage formulations. These values were entered into an Excel XP (Microsoft Corp.) spreadsheet designed to use the Generalized Reduced Gradient (GRG) optimization solver function in Excel, with the multistart option selected. Desired final target composition [fat, TP, CN%TP, serum protein, lactose, and final weight (35.0 kg)] of each batch was also entered into the spreadsheet. The output of the optimization function for each formulation was saved into the final formula table used for processing.

**Processing**

Liquid ingredients (deionized water, cream, and protein ingredients) were weighed into a sanitized stainless steel milk can (38-kg capacity) and mixed. Dry ingredients were weighed out and combined. The powder was slowly added to the wet ingredients with constant mixing. The final mix for each beverage was allowed to continue to mix for an additional 5 min before being preheated to 60°C using a Microthermics UHT/HTST Lab-25 EH VH pasteurization unit and homogenized at 20.7 MPa (17.2 MPa first stage, 3.4 MPa second stage) using a 2-stage homogenizer (NS2006H, GEA Niro Soavi) followed by cooling to 8°C using the tube-in-tube cooling section of the Microthermics unit, before being placed into sanitized milk cans and stored at 4°C until all samples were homogenized, and then the Microthermics EH VH system was reconfigured and heat sterilized (approximately 3 h total).

### Table 1. Mean (n = 2) composition of liquid protein ingredients (serum protein isolate, SPI; milk protein concentrate, MPC; and micellar casein concentrate, MCC) used in beverage formulations; means provided on a wet weight basis as percent by weight

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Total solids</th>
<th>Fat</th>
<th>Protein</th>
<th>Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI</td>
<td>24.73</td>
<td>0.01</td>
<td>22.48</td>
<td>0.20</td>
</tr>
<tr>
<td>MPC</td>
<td>16.99</td>
<td>0.29</td>
<td>14.81</td>
<td>0.56</td>
</tr>
<tr>
<td>MCC</td>
<td>15.58</td>
<td>0.31</td>
<td>13.38</td>
<td>0.53</td>
</tr>
</tbody>
</table>
The Microthermics EH VH was then reconfigured for final heating with direct steam injection and filling with an ultraclean-fill hood/sterile product outlet (Microthermics). The time to purge the flow system was previously determined by flushing water out of the system and measuring the freezing point of the milk (Cryoscope, model no. 4250, Advanced Instruments Inc.) and the fat, protein, and lactose concentrations (Lactoscope FTA, Delta Instruments).

The entire system was heat sterilized before processing by heating until the product outlet reached at least 121°C and held for at least 20 min per filling side. Following heat sterilization, the beverages were introduced into the system and preheated to 90°C. Beverages were heated to a final temperature of 141°C using direct culinary steam injection (model LG-30, Electro-Steam Generator Corp.) via a Microthermics Steam Injection Module. The beverages were held for 3 s at 141°C, vacuum cooled to 86°C, and homogenized again at 20.7 MPa (17.2 MPa first stage, 3.4 MPa second stage). The beverages were cooled to 8°C using the tube-in-tube cooling section of the Microthermics unit and then filled into sanitized bottles [473 mL (16 oz), item ID B252SS, Container and Packaging Supply Inc.] using the clean fill hood. Samples were stored at 4°C until testing.

**Analysis Methods**

**Proximate and Microbial Analysis.** Milk-based ingredients were analyzed in triplicate using the following analytical methods: total solids by direct forced-air method (AOAC International, 2012; method number 990.20), fat by ether extraction, (AOAC International, 2012; method number 989.05), lactose by spectrophotometric enzymatic method (AOAC International, 2012; method number 2006.06), total nitrogen (AOAC International, 2012; method number 991.21), NPN (AOAC International, 2012; method number 991.20), and noncasein nitrogen (NCN; AOAC International, 2012; method number 998.05). True protein was calculated as total nitrogen minus NPN multiplied by 6.38; casein was calculated as total nitrogen minus noncasein nitrogen multiplied by 6.38; and serum protein content was calculated by subtracting NPN from noncasein nitrogen and multiplying by 6.38. Beverages were tested after processing and at each time point for aerobic plate count and coliform count (Petrifilm Aerobic Count Plate and Petrifilm Coliform Count Plate, 3M Food Safety) to evaluate microbial quality.

**Descriptive Sensory Analysis.** Sensory analysis was conducted in accordance with the North Carolina State University Institutional Review Board for the Protection of Human Subjects in Research regulations. Sensory properties of the beverages were evaluated by 7 trained panelists at each time point (3 females, 4 males, ages 21 to 55 yr). Each panelist had a minimum of 80 h of previous experience evaluating flavor and texture attributes of dairy products using the Spectrum method (Meilgaard et al., 2007) and at least 40 h of experience with evaluation of fluid milk sensory attributes using an established sensory language (Croissant et al., 2007; Lee et al., 2017; Jo et al., 2018). Attributes evaluated included aroma intensity, sweet aromatic/ vanilla, cooked milky, sulfur/eggy, sweet taste, astringency, metallic mouthfeel, and viscosity. Beverages (30 mL) were dispensed into 59-mL soufflé cups labeled with random 3-digit blinding codes and sealed with lids (Dart Container Corp.). The beverages were prepped and stored under reduced light to prevent the formation of light oxidized flavors and were evaluated at 15°C. Each panelist evaluated each treatment at each time point in duplicate.

**Headspace-SPME-GC-MS of Vanillin.** Headspace vanillin content was determined using an Agilent 7820 GC with 5975 MSD (Agilent Technologies Inc.) and a ZB-5 MS column (30-m length × 0.25-mm internal diameter × 0.25-μm film thickness, Phenomenex). Sample introduction was accomplished using a CombiPal Autosampler (CTC Analytics). Analytical conditions for GC-MS were adapted from Vazquez-Landa-veralde et al., (2005). At each time point, 5 mL of milk along with 20 μL of internal standard (benzyl benzoate in methanol at 200 mg/L) was added to 20-mL solid-phase microextraction (SPME) autosampler vials with steel screw tops, containing silicone septa faced in Teflon (Microliter Analytical), in triplicate. Vials were equilibrated for 25 min at 40°C with 4 s of pulsated agitation at 250 rpm. A single 50/30-μm divinylbenzene/ carboxen/polydimethylsiloxane (DVB/CAR/PDMS, Supelco) 1-cm fiber was used for all analysis. The SPME fiber was exposed to the beverages for 40 min at a depth of 31 mm. The fiber was retracted and injected at 50 mm in the GC inlet for 5 min. The GC oven was initially held at 40°C for 3 min with a gradual increase of 10°C/min to 150°C and held for 1 min, then raised at a rate of 20°C/min to 250°C and maintained for 5 min. The SPME fiber was introduced into the inlet using splitless mode at 250°C (0.75-min valve delay). All analyses were performed at a constant flow rate of 1 mL/min with helium. Scanning parameters were set from 30 to 275 m/z to identify compounds of interest. The MS transfer line was maintained at 250°C with the quadrupole at 150°C and source at 230°C. Vanillin was quantified using the extracted ion of 152 m/z.

**Headspace-SPME-GC-MS/MS.** Selected sulfur compounds (Jo et al., 2018), hydrogen sulfide (H2S) and carbon disulfide (CS2), were measured using an
Agilent 7890B GC applied to an Agilent 7000C triple-quadrupole mass spectrometer (GC-MS/MS) equipped with a sulfur selective flame photometric detector (Agilent Technologies Inc.) equipped with a ZB-5 MS column (30-m length × 0.25-mm internal diameter × 0.25-μm film thickness, Phenomenex). Sample introduction was accomplished using a CombiPal Autosampler (CTC Analytics). Five milliliters of sample along with 20 μL of internal standard (ethyl methyl sulfide in diethyl ether at 1.65 mg/L, Sigma Aldrich) was added to 20-mL SPME autosampler vials with steel screw tops, containing silicone septa faced in Teflon (Microliter Analytical). The analytical conditions and the multiple reaction monitoring transition for selected sulfur compounds were followed as described by Jo et al. (2018). Dwell times were set to ensure 3 to 3.1 cycles over a peak. The experiments were performed in triplicate at each time point. MassHunter Qualitative and Quantitative Analysis software (version B.08.00, Agilent Technologies Inc.) were used for data analysis. The relative concentration of each compound was calculated based on the response ratio of the quantifier ion to that of internal standard.

Statistics

A general linear mixed model was fit using the GLIMMIX procedure in SAS (version 9.4, SAS Institute Inc.). Fixed effects included protein blend, protein amount, time, and all interactions. A random effect was included to account for replication effects as well as repeated measures taken across time. Least squares means were used to determine significant interactions and main effects. The $F$-values calculated from the type III sum of squares for the $F$-values of fixed and interactions effects were summed, and each individual $F$-value for each effect was divided by the sum of all the $F$-values, to report a relative percentage of variation explained by each fixed and interaction effect. Additionally, for time 0, the general linear model (GLM) procedure of SAS was used to determine the effects of protein source and protein amount and their interaction. Least squares means were used to investigate significant interactions and main effects.

RESULTS AND DISCUSSION

The results of the proximate analysis following processing are shown in Table 2. In general, the protein and casein contents of the beverages were as expected. Small amounts of variability are due to differences in compositional measurement of the liquid dairy ingredients by mid-infrared spectrophotometry during formulation, and the final beverage compositions were

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Low, 6.3%</th>
<th>High, 10.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Fat</td>
<td>Total nitrogen</td>
</tr>
<tr>
<td>100 MCC</td>
<td>0.38 ± 0.01</td>
<td>7.16 ± 0.02</td>
</tr>
<tr>
<td>100 MPC</td>
<td>0.51 ± 0.01</td>
<td>6.22 ± 0.02</td>
</tr>
<tr>
<td>18:82 SPI:</td>
<td>0.39 ± 0.01</td>
<td>7.09 ± 0.02</td>
</tr>
<tr>
<td>MCC</td>
<td>0.50 ± 0.01</td>
<td>6.39 ± 0.10</td>
</tr>
<tr>
<td>50:50 SPI:</td>
<td>0.43 ± 0.01</td>
<td>6.82 ± 0.10</td>
</tr>
<tr>
<td>MCC</td>
<td>0.49 ± 0.01</td>
<td>6.39 ± 0.11</td>
</tr>
<tr>
<td>50:50 SPI:</td>
<td>0.51 ± 0.01</td>
<td>6.47 ± 0.17</td>
</tr>
</tbody>
</table>

MCC = micellar casein concentrate, MPC = milk protein concentrate, and SPI = milk serum protein isolate. Total nitrogen, non-casein nitrogen, NPN, true protein, and casein contents are expressed on a protein basis by multiplying Kjeldahl nitrogen by 6.38.
confirmed by Kjeldahl nitrogen and Mojonnier fat extraction. Our beverage formulation goal was to achieve a wide range of CN%TP within both the low and high protein concentrations by using different combination of milk protein ingredients. This was achieved with the 100 MCC, 100 MPC, 18/82 SPI/MCC, 50/50 SPI/MCC, and 50/50 SPI/MPC in low protein concentration beverages having a CN%TP of 91.4, 82.7, 78.6, 53.9, and 46.6%, respectively, and the high protein concentration beverages having a CN%TP of 92.7, 83.6, 81.1, 56.1, and 47.6%, respectively. No coliforms were detected in beverages at any time point. The aerobic plate counts for the beverages were also <10^2 cfu/mL throughout 8 wk at 4°C storage.

### Sensory Analysis

The overall orthonasal aroma scores of the beverages was affected by time and protein amount \((P < 0.05; \text{Table } 3)\). Overall aroma scores decreased from time 0 through 4 wk at 4°C. Overall aroma scores were

<table>
<thead>
<tr>
<th>Factor</th>
<th>Overall aroma</th>
<th>Sweet aromatic/vanillin</th>
<th>Cooked/milky</th>
<th>Sulfur/eggy</th>
<th>Astringency</th>
<th>Viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trt</td>
<td>37.1*</td>
<td>54.5*</td>
<td>65.7*</td>
<td>48.8*</td>
<td>37.5*</td>
<td>27.0*</td>
</tr>
<tr>
<td>Prot</td>
<td>27.1*</td>
<td>32.0*</td>
<td>13.8*</td>
<td>0.5</td>
<td>60.1*</td>
<td>64.1*</td>
</tr>
<tr>
<td>Trt × prot</td>
<td>0.3</td>
<td>0.2</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
<td>2.9*</td>
</tr>
<tr>
<td>Time</td>
<td>22.0*</td>
<td>11.7*</td>
<td>16.2*</td>
<td>46.3*</td>
<td>1.9*</td>
<td>4.1*</td>
</tr>
<tr>
<td>Trt × time</td>
<td>2.1</td>
<td>0.2</td>
<td>1.2</td>
<td>3.4*</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Time × prot</td>
<td>10.8*</td>
<td>1.3*</td>
<td>2.1*</td>
<td>0.5</td>
<td>0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Trt × time × prot</td>
<td>0.6</td>
<td>0.1</td>
<td>0.5</td>
<td>0.3</td>
<td>0.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*1Metallic and sweet taste were not included, as these mean attributes did not change by more than ±0.2 across treatment, protein amount, or storage time.

* \(P < 0.05\).
higher for 6.3% protein beverages compared with 10.5% protein beverages across 8-wk storage at 4°C (results not shown). The main effects of protein content and protein blend accounted for about 64% of the variance in the beverages (Table 3). At time 0, the lower-protein beverages and beverages with lower amounts of serum protein as the percentage of true protein (i.e., 100% MCC) had higher \((P < 0.05)\) sweet aromatic/vanillin scores compared with those with higher amounts of protein or higher amounts of serum protein as a percentage of true protein (i.e., 50/50 SPI/MPC; Figure 1). Sweet aromatic/vanillin flavor decreased \((P < 0.05)\) in all beverages with storage time at 4°C (Table 3), with larger decreases in the lower-protein beverages (protein concentration by time interaction) compared with the higher-protein beverages.

The main effects of protein blend, followed by protein amount and time, explained 65.7, 13.8, and 16.2% of the variance, respectively, in cooked/milky flavor intensity in the beverages \((P < 0.05; \text{Table 3})\). Beverages with a higher amount of serum protein as a percentage of true protein had higher intensities of cooked/milky flavor (Figure 2). In contrast, sulfur/eggy flavor was not affected by protein amount \((P > 0.05; \text{Table 3})\). Protein blend and storage time were the 2 main factors which affected sulfur/eggy flavor intensity \((P < 0.05)\), explaining about 49 and 48% of variation in sulfur/eggy flavor intensity, respectively (Table 3). Beverages with higher amounts of serum protein as a percentage of true protein had higher sulfur/eggy flavor intensities at time 0 (Figure 3). Sulfur/eggy flavor intensities decreased with storage time \((P < 0.05; \text{Figure 4})\) and were not influenced \((P > 0.05)\) by protein concentration (Table 3). Sulfur/eggy flavor intensity average for both protein concentrations corresponded in rank order with the CN%TP in the formulations, with the beverage with the lowest CN%TP having the highest sulfur/eggy flavor intensity. Virtually no sulfur/eggy flavor was detectable in the 100% MCC-based beverage, which had the lowest milk serum protein content. The MCC purity was about 92% on a CN%TP basis, measured before thermal treatment. It has been well documented that sulfur/eggy flavors in UHT and ultrapasteurized milk decrease with storage time (Mehta, 1980; Rerkrai et al., 1987; Steely, 1994; Lee et al., 2017; Jo et al., 2018), and this is consistent with the time-dependent decrease in sulfur/eggy flavor in the current study (Figure 4). Sulfur/eggy flavor has been sourced to whey protein (Jo et al., 2019).

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Figure 3. Least squares means of sulfur/eggy intensity of vanilla-flavored protein beverages from 5 different protein blends (trt; Table 1) at time 0. The figure is the average intensity of each treatment, as there was no effect of protein concentration (prot) at time 0 \((P > 0.05)\). Sensory attributes were scaled on a 0- to 15-point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Lee et al., 2017; Jo et al., 2018). Figures are graphed with a maximum axis value of 10 to demonstrate differences among beverages. Different letters indicate differences among the beverages \((P < 0.05)\). The main effect (trt) was the only significant effect \((P < 0.05)\) at time 0; prot and prot \(\times\) trt were not significant \((P > 0.05)\). Error bars above each bar indicate standard error of 0.116. MCC = micellar casein concentrate; MPC = milk protein concentrate; SPI = serum protein isolate.

Figure 4. Least squares means of trained panel sensory perception of sulfur/eggy flavor of vanilla-flavored protein beverages from 5 different protein blends (trt; Table 1) across 8 wk of storage at 4°C (time). Sensory attributes were scaled on a 0- to 15-point intensity scale consistent with the Spectrum method (Meilgaard et al., 2007). Most dairy flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Lee et al., 2017; Jo et al., 2018). Graphed with a maximum axis value of 5 to demonstrate differences among beverages. The interaction of time \(\times\) trt was significant \((P < 0.05)\). Standard error 0.116. MCC = micellar casein concentrate; MPC = milk protein concentrate; SPI = serum protein isolate.
Beverage astringency was primarily affected by protein amount and protein blend, with those 2 parameters explaining about 97.6% of the variation in beverages astringency (Table 3). Beverages with higher protein and higher amounts of serum protein as a percentage of true protein had higher astringency (Figure 5). We found no significant interactions between any main effects for astringency (P > 0.05). Cheng et al. (2019) documented that astringency increased with true protein concentration in milk beverages across 3 to 5% protein. The amount of serum protein as a portion of true protein had no influence on astringency in the study by Cheng et al. (2019), but those beverages were high-temperature short-time pasteurized (versus UP with direct steam injection in the present study), and the amount of protein was lower than that in the current study. Higher-heat treatments such as UP have been shown to increase the astringency of beverages (Lee et al., 2017; Li et al., 2018). This may be due to the increased denaturation of the whey proteins under more intense heat treatments leading to different interactions of these proteins with various parts of the oral mucosa and increasing the friction within the mouth. We detected a statistically significant effect of time on the astringency of the beverages (P < 0.05; Table 3); however, the increase observed was from a mean astringency intensity of 2.7 at wk 0 to a mean astringency intensity of 2.9 at wk 8. Although statistically significant, this increase would not be practically significant. However, increases in astringency over time could be attributable to further protein network formation and aggregation during storage, as previously seen.

Table 4. Relative percentage of ANOVA F-values (from type III sum of squares) with the F-value for each dependent variable divided by the sum of F-values for all dependent variables multiplied by 100, for volatile compound analysis of vanillin by solid-phase microextraction GC-MS of vanilla-flavored protein beverages from 5 different protein blends (trt) and 2 protein concentrations (prot) across 8-wk storage at 4°C (time)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Relative percentage of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trt</td>
<td>23.0*</td>
</tr>
<tr>
<td>Prot</td>
<td>19.0*</td>
</tr>
<tr>
<td>Trt × prot</td>
<td>6.9*</td>
</tr>
<tr>
<td>Time</td>
<td>42.4*</td>
</tr>
<tr>
<td>Trt × time</td>
<td>2.1*</td>
</tr>
<tr>
<td>Time × prot</td>
<td>5.3*</td>
</tr>
<tr>
<td>Trt × time × prot</td>
<td>1.3*</td>
</tr>
</tbody>
</table>

*P < 0.05.
by Li et al. (2018). Sensory panelists also noted chalkiness and graininess in the 10.5% protein 50:50 SPI: MPC beverages at wk 6 and 8, which is also consistent with protein aggregation. Larger particles can indicate protein aggregation following their denaturation by high-heat treatment (Dalgleish, 1990, 1992; Anema and Li, 2003). The denaturation of the proteins, especially the serum proteins, may account for higher astringency in the beverages with about 50% of serum protein as a percentage of true protein. Astringency perception and astringency of dairy proteins is complex and has been attributed to more than one parameter and more than one mechanism (Carter et al., 2020). Additional research would be needed to elucidate whether an increased particle size was documented in beverages with increased astringency or whether other factors are involved.

Protein concentration explained 64.1% of the differences in the sensory perception of beverage viscosity, followed by protein blend (27%) and time (4.1%; Table 2). Higher-protein beverages and beverages with higher amounts of serum protein had greater viscosity than lower-protein beverages or beverages with...
lower amounts of serum protein as a percentage of true protein (Figure 6). High-protein beverages made from 100% MCC did show thermoreversible gelling, which has also been previously documented in 18% protein liquid MCC (Amelia and Barbano, 2013). This increase in beverage viscosity with higher protein content is also in agreement with the results of several previous studies (Lutz et al., 2009; Wagoner and Foegeding, 2017; Cheng et al., 2019).

Sensory perception of viscosity increased over time ($P < 0.05$; Table 2), which was expected as secondary aggregation and protein networks formed, but increases were small, similar to those observed with astringency.

Table 5. Relative percentage of ANOVA $F$-values (from type III sum of squares) with the $F$-value for each dependent variable divided by the sum of $F$-values for all dependent variables multiplied by 100 for volatile compound analysis of hydrogen sulfide and carbon disulfide by solid-phase microextraction GC-MS/MS from 5 different protein blends (trt) and 2 protein concentrations (prot) across 8-wk storage at 4°C (time)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Hydrogen sulfide</th>
<th>Carbon disulfide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trt</td>
<td>43.3*</td>
<td>32.0*</td>
</tr>
<tr>
<td>Prot</td>
<td>1.9*</td>
<td>1.7*</td>
</tr>
<tr>
<td>Trt × prot</td>
<td>0.1*</td>
<td>0.0</td>
</tr>
<tr>
<td>Time</td>
<td>49.7*</td>
<td>60.2*</td>
</tr>
<tr>
<td>Trt × time</td>
<td>4.8*</td>
<td>5.8*</td>
</tr>
<tr>
<td>Time × prot</td>
<td>0.2*</td>
<td>0.3*</td>
</tr>
<tr>
<td>Trt × time × prot</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* $P < 0.05$.

Figure 9. Least squares means of volatile compound analysis of hydrogen sulfide by solid-phase microextraction GC-MS/MS of vanilla-flavored protein beverages from 5 different protein blends and 2 protein concentrations (6.3 and 10.5%; Table 1) at time 0. Different letters indicate differences among the beverages ($P < 0.05$). Error bars above each bar indicate a standard error of 3.311. MCC = micellar casein concentrate; MPC = milk protein concentrate; SPI = serum protein isolate.

For low-protein beverages, sensory viscosity averaged across treatments increased only slightly, from 2.3 at time 0 to 2.4 following 8-wk storage at 4°C, whereas high-protein beverages increased from 2.7 to 3.0, respectively ($P < 0.05$; results not shown). An increase in instrumental viscosity during storage has been observed in previous studies involving UHT milk products (Datta and Deeth, 2001; Elliott et al., 2005; Deeth and Lewis, 2016; Anema, 2017). Additional work with protein beverages, focused on rheology and rheological changes and their relationship to sensory perception, is needed.

**Headspace Vanillin**

Storage time and protein blend, followed by protein amount, explained the majority of the variation (42.4, 23, and 19%, respectively) in headspace vanillin concentration of the beverages (Table 4). These results were generally consistent with the sensory perception of sweet aromatic/vanillin flavor, with time of storage explaining less variation in sensory perception than the effect of storage time on headspace vanillin. The low-protein (6.3% wt/wt) beverages had higher amounts of headspace vanillin than their equivalent high-protein (10.5% wt/wt) treatments (Figure 7). Beverages with lower amounts of serum protein as a percentage of true protein had higher amounts of headspace vanillin than...
beverages made with higher amounts of serum protein as a percentage of true protein. With increased true protein or increased serum protein as a percentage of true protein, the decrease in headspace vanillin and sensory perception of sweet aromatic/vanillin flavor suggests that higher protein decreases instrumental analysis and sensory perception of vanillin, and that these effects are increased by serum protein more than by casein. These results are consistent with previous work, which demonstrated that trained panel sensory perception of vanillin flavor intensity was lower in whey protein solutions than in casein solutions (McNeill and Schmidt, 1993; Hansen and Booker, 1996). Our results also support the conclusions of Hansen and Heinis (1991), who showed that increasing the concentration of whey protein in solution (0.125 to 0.5% wt/wt) caused a reduction in perceived sensory vanillin flavor intensity. Li et al. (2000) reported that caseinate in an aqueous solution (2% wt/vol) had a higher amount of free vanillin, determined by HPLC, than whey protein isolate, showing instrumentally that whey proteins have a higher affinity for binding vanillin than casein. Another study using HPLC to explore interactions of vanillin in model systems found that more free vanillin was present in 3% solutions of sodium caseinate than in a 3% solution of bovine serum albumin, and also that 6% concentrations of the proteins decreased the levels of free vanillin more than 3% solutions (Chobpattana et al., 2002). Storage time also decreased vanillin content of beverages by instrumental and sensory perception (Tables 3 and 4; Figure 8). Decreases of vanillin with storage may be due to absorption or transfer of vanillin through the packaging, as well as by additional binding of vanillin by proteins.

**Headspace Sulfur Compounds**

The main effects of protein blend and time, followed by protein blend by time interaction, accounted for the majority of the variation in hydrogen sulfide (43.3, 49.7, and 4.8%, respectively) and carbon disulfide (32.0, 60.2, and 5.8%, respectively) and in the beverages (Table 5). These results were consistent with the sensory perception of sulfur/eggy flavors, except that protein amount did not have an effect ($P > 0.05$) on the sensory perception of sulfur/eggy flavors. Jo et al.
(2019) recently demonstrated that hydrogen sulfide and carbon disulfide were responsible for sulfur/eggy and cooked/burnt flavors in UP milk and that the serum protein fraction of milk protein was the source of these volatile sulfur compounds. The hydrogen sulfide and carbon disulfide content of the beverages at wk 0 are shown in Figures 9 and 10, respectively. Low-protein (6.3%) beverages had lower amounts of volatile sulfur compounds than their equivalent high-protein beverages. The biggest influence in initial headspace hydrogen sulfide and carbon disulfide was the protein blend (Table 4), consistent with the results of Jo et al. (2019). Low-protein (6.3%) beverages had lower amounts of volatile sulfur compounds than their equivalent high-protein beverages. The biggest influence in initial headspace hydrogen sulfide and carbon disulfide was the protein blend (Table 4), consistent with the results of Jo et al. (2019). Beverages with higher amounts of serum protein as a percentage of true protein had higher amounts of hydrogen sulfide and carbon disulfide. Volatile sulfur compounds decreased in beverages with storage time (Figures 11, 12). Previous work has also demonstrated that these compounds decreased in UP milk across 14 d of storage at 4°C (Steely, 1994; Jo et al., 2018). The specific type of packaging will also affect the rate of dissipation of cooked and sulfur flavors, depending on the permeability of gases through the package (Dunkley and Stevenson, 1987; Rysstad et al., 1998). Jo et al. (2019) further demonstrated that serum proteins were the primary source of these volatile compounds in fluid milk and milk beverages (3.3% protein). Many of these sulfur compounds are formed during Maillard reactions with sulfur-containing amino acids during the UHT heat process, so proteins with higher amounts of sulfur-containing amino acid residues, such as serum proteins, will produce more volatile sulfur compounds during heating (Mehta, 1980; Al-Attabi et al., 2008).

**CONCLUSIONS**

The amount and type of protein used when formulating RTD beverages has multiple effects on flavor. Higher protein increased beverage astringency and sensory viscosity. Beverages with higher proportions of serum protein as a percentage of true protein had lower levels of vanillin and higher levels of sulfur compounds (hydrogen sulfide and carbon disulfide), concurrent with lower intensities of sweet aromatic/vanilla flavor and higher intensities of sulfur/eggy flavor by sensory analysis. Vanillin and sensory perception of vanilla/vanillin flavor were higher and remained higher through 8-wk storage.
storage in beverages made with MCC compared with beverages made with other protein blends. This study establishes some of the key factors that contribute to the final sensory properties of RTD protein beverages.

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REFERENCES


ORICDS

D. M. Barbano © https://orcid.org/0000-0002-0206-7028
M. A. Drake © https://orcid.org/0000-0002-4744-2493