Identification of the source of volatile sulfur compounds produced in milk during thermal processing

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ABSTRACT

Volatile sulfur compounds in ultra-pasteurized (UP) milk are the major contributors to sulfur/burnt and eggy flavors, and these flavors are disliked by consumers. Previous research has established distinct differences in flavor profiles of fluid milk processed by high temperature, short time pasteurization (HTST) and UP by direct steam injection (DSI-UP) or indirect heat (IND-UP). An understanding of the contribution of the individual milk proteins to sulfur off-flavors would clarify the source of sulfur flavors in UP milks. The objective of this study was to determine the source of volatile sulfur compounds in fluid milk with a specific focus on the comparison of heat treatment effects on milks by HTST and UP. Formulated skim milks (FSM) were manufactured by blending micellar casein concentrate and serum protein isolate (SPI). Three different caseins as a percentage of true protein (FSM95, FSM80, and FSM60) were formulated to determine the source of sulfur/burnt and eggy flavors. Freshly processed micellar casein concentrate or SPI were blended to achieve a true protein content of about 3.2%. Raw skim milk served as a control. Skim milk and FSM were pasteurized at 78°C for 15 s (HTST) or 140°C for 2.3 s by IND-UP or DSI-UP. The experiment was replicated twice. Sensory properties of milks and FSM were documented by descriptive sensory analysis. Volatile sulfur compounds in milks and FSM were evaluated using solid-phase microextraction followed by gas chromatography-triple quadrupole mass spectrometry combined with a sulfur selective flame photometric detector. The FSM with higher SPI as a percent of true protein had higher sensory sulfur/burnt and eggy flavors along with elevated concentrations of hydrogen sulfide and carbon disulfide compared with skim milk or

FSM with lower proportions of SPI. Sulfur compounds including dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, dimethyl sulfoxide, and methional were not associated with sulfur/burnt and eggy flavors, which suggests that these compounds may not specifically contribute to the sulfur/burnt and eggy off-flavors of UP milks. Sensory panelists found higher overall aroma impact, and cooked, sulfur/burnt, and eggy flavors for DSI-UP, followed by IND-UP and HTST. The combination of sensory and instrumental methods used in the current study effectively identified that milk serum proteins are the source of sulfur compounds in milk, and further confirmed the contribution of hydrogen sulfide and carbon disulfide to eggy and sulfur/burnt flavors, respectively.

Key words: skim milk, volatile sulfur compound, ultrapasteurization

INTRODUCTION

Thermal processing of milk has facilitated product safety and a longer shelf life. In the United States, milk processing involves HTST pasteurization (minimum of 72°C for 15 s), providing approximately 3 wk of shelf life, and ultra-pasteurization (UP) or UHT, or both, for extended shelf life of milk (Boor, 2001). The thermal processing conditions for UP milk are defined as at or above 138°C (280°F) for at least 2 s (21 CFR 131.3, FDA, 2017), and this product generally has an extended shelf life under refrigerated conditions, up to 75 to 80 d. Ultrahigh-temperature milk has the same thermal process as for UP milk, but the milk is aseptically packaged to be shelf stable (FDA, 2015).

Despite the advantage of a longer shelf life compared with conventional HTST milk, UP and UHT milk have stronger overall flavor intensities, including undesirable cooked and sulfur/eggy off-flavors, which can create a possible barrier to UP/UHT milk acceptance by consumers (Perkins and Deeth, 2001; Lee et al., 2017). Recently, Jo et al. (2018) demonstrated that sulfur compounds, including hydrogen sulfide (H₂S), carbon disulfide (CS₂), dimethyl sulfide (DMS), dimethyl

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disulfide (**DMDS**), and dimethyl trisulfide (**DMTS**), distinguished UP milks from HTST milks. These compounds were associated with the higher cooked flavor and distinct sulfur/eggy flavor in UP milks. The study further suggested that H_2S and CS_2 were the key responsible compounds for sulfur/eggy flavor. Jo et al. (2018) also established that the sulfur compound profile was different between the 2 heat treatment methods for UP milk, indirect heating (**IND**) and direct steam injection (**DSI**). Those key compounds included H_2S , **DMTS**, and methional for **DSI-UP** and **DMS** for **IND-UP**, indicating that differences in flavor formation by Maillard reactions and protein denaturation during thermal processing affected the flavor quality of UP milks and resulted in distinct flavor differences between **DSI-UP** and **IND-UP** milks (Jo et al., 2018).

The primary source of volatile sulfur compounds in milk has been suggested to be due to the denaturation of serum (whey) protein and sulfur-containing AA in serum protein (Hutton and Patton, 1952), as well as the Maillard reaction, a thermally induced reaction between sulfur-containing AA and reducing sugars (McGorrrin, 2011). Sulfur compounds can be also be derived from proteins in the milk fat globule membrane during thermal treatment (Mehta, 1980; Calvo and de la Hoz, 1992). The amount of sulfur-containing AA (cystine/cysteine, methionine) in milk is variable based on the milk protein fraction, casein, and serum. Methionine is present in casein and serum protein at equivalent levels (Belitz et al., 2009), which possibly can make both casein and serum proteins the primary source of **DMS**, **DMDS**, and **DMTS** via Strecker degradation. Cystine and cysteine are higher in serum protein than casein, which can be key precursors of H_2S and CS_2 during heat treatment of milk (Belitz et al., 2009). Constituents of serum protein including β -LG, α -LA, BSA, and immunoglobulin contain sulfur AA in different ratios. While BSA (17 disulfide bonds and 1 free sulfhydryl group) and immunoglobulin (12 intrachain and 4 interchain disulfide bonds) are in low concentration, they have a much larger number of intramolecular disulfide bonds and content of sulfur-containing AA per monomeric protein than the high abundance milk serum proteins. While β -LG has a higher amount of methionine, α -LA contains a higher amount of cystine/cysteine (Heine et al., 1991). Changes in properties of these milk proteins are affected by heat treatment (temperature), leading to denaturation, which involves unfolding and subsequent aggregation of unfolded protein molecules and flavor formation (Kühn et al., 2006). As serum proteins are more heat labile than casein (Brodkorb et al., 2013), the effect of heat treatment on serum protein can be more significant than casein in relation to distinct sulfur/cooked flavors.

There are established characteristic properties of serum protein in response to the heat treatment of milk. Those include the content of sulfhydryl groups (Hutton and Patton, 1952; Pofahl and Vakaleris, 1968) and the denaturation of serum proteins (Hutton and Patton, 1952; Larson and Roller, 1955; Lyster, 1970; Manji and Kakuda, 1986; Elliott et al., 2005; Lee et al., 2017). Although studies have suggested that these properties are the source(s) of the formation of sulfur compounds and sulfur and cooked flavors (Hutton and Patton, 1952; Mehta, 1980; Calvo and de la Hoz, 1992), previous studies have not elucidated the protein fractions and the compounds responsible for the distinct sulfur flavor in milk in conjunction with different heat treatments. A greater understanding of the source(s) of cooked and sulfur flavors in UP milk can be obtained if flavor properties of milk protein fractions generated during milk heat treatment are elucidated.

Membrane technology has been applied in milk and dairy products since the 1970s and includes several unit operations that involve water or bacteria removal, and milk fat or protein separation (Pouliot, 2008). Membrane separation of milk proteins has expanded the versatility of dairy ingredients and facilitated the development of new concepts of dairy products, such as extended-shelf-life milks, and high protein beverages (Pouliot, 2008; Smithers, 2008). The application of microfiltration (**MF**) to skim milk followed by UF can be applied to generate serum protein isolate (**SPI**) and 95% serum protein reduced micellar casein concentrate (**MCC**; Nelson and Barbano, 2005; Zulewska et al., 2009; Beckman et al., 2010). The formation of sulfur compounds can be dependent on these distinct protein fractions and their physical changes caused by heat treatment. More importantly, identification of the source of sulfur compounds from milk proteins during thermal processing of milk can help to indicate sulfur off-flavors in UP milk and further predict consumer acceptability. No previous studies have compared protein source and effects of heat treatment on milk and milk protein beverages with different proportions of casein and serum proteins processed by HTST or UP. The objective of this study was to determine the source of volatile sulfur compounds in skim milk and formulated skim milk (**FSM**) with a range of proportions of casein and serum proteins and to measure the effect of thermal process on volatile sulfur flavor compound formation.

MATERIALS AND METHODS

Skim Milk, MCC, and SPI Production

Source of Skim Milk. Raw bovine skim milk ($0.087\% \pm 0.003$ fat, SCC <300,000) was obtained from

the North Carolina State University Dairy Enterprise System (Raleigh, NC). The raw skim milk was divided into 2 portions: one for skim milk, and one for MCC and SPI production. This process was replicated twice on 2 different days.

MCC Manufacture. Raw skim milk (530 kg) was HTST pasteurized at 72°C for 15 s with a plate heat exchanger (model T4 RGS-16/2, SPX Flow Technology, Greensboro, NC) and cooled to 4°C. The pasteurized skim milk was prefiltered at 4°C using a Nexis T filter (NXT 10–30U-M7S, Pall Corp., Port Washington, NY). The milk was then heated to 50°C with a plate heat exchanger (serial no. G201400849, Plate ID: SR1, SPX Flow Technology). The pasteurized and prefiltered skim milk was microfiltered using a ceramic MF system (Tetra Alcross M7, TetraPak Filtration Systems, Aarhus, Denmark), equipped with 0.1 µm nominal pore diameter graded permeability membrane (model EP1940GL0.1 u, AGP1020, alumina, Pall Corp.). A 3-stage and a continuous 3 × MF process were used to produce a 95% serum protein reduced MCC with true protein concentration between 8.4 to 8.6% as described by Zulewska and Barbano (2014) and Cheng et al. (2018). Before the first stage, 160 kg of milk was taken into the MF system to flush water and both the retentate and permeate collected from this time were discarded. The first stage started when the flush ended. Then, 370 kg of milk was added as the feed for the first stage. In the second and third stages, the MF retentate was diluted with deionized water (2 kg of deionized water for every 1 kg of retentate), heated to 50°C, and diafiltered with the ceramic MF system to produce 3 × retentate. This diafiltration was repeated to complete a 3-stage process and the MF system was run continuously and not stopped and restarted from stage to stage. At the end of stage 3, all retentate (liquid MCC) was mixed, cooled to 4°C, transferred into light shielded high-density polyethylene (**HDPE**) half-gallon containers (Upstate Niagara Cooperative, Buffalo, NY), and stored at 4°C and then used for FSM production the following day.

SPI Manufacture. Several batches of SPI were produced and combined to obtain enough liquid SPI for the study (Cheng et al., 2018). Raw skim milk was

HTST pasteurized at 72°C for 15 s and prefiltered as previously described above. The MF permeate collected from the first stage of MCC production was placed in the UF feed tank and heated to 50°C with a plate heat exchanger. The UF feed was processed by a 2-stage UF and the UF retentate was used to produce liquid SPI as described by Cheng et al. (2018). The UF process was continued until the protein concentration in the UF retentate reached between 27 to 28% protein measured by a Fourier-transform mid-infrared milk analyzer (LactoScope FTIR, Delta Instruments BC, Drachten, the Netherlands). The final UF retentate (liquid SPI) from the second stage was transferred into light-shielded HDPE half-gallon containers (Upstate Niagara Cooperative) and stored at −18°C until FSM production.

FSM Production. The FSM was prepared by blending 3 different ratios of MCC (95, 80, and 60%) and SPI (5, 20, and 40%) as a percentage of the final true protein content (3.2%) to produce 3 formulated skim milks (Table 1; **FSM95**, **FSM80**, and **FSM60**, respectively). Final protein and lactose content of each FSM was adjusted to the equivalent percentage as skim milk (3.2 and 4.7%, respectively) with each protein blend and with UF permeate from SPI production and lactose (Hilmar 5120 200 mesh, Hilmar Ingredients, Hilmar, CA).

Thermal Processing

Raw skim milk and blended FSM were processed by HTST, DSI-UP, or IND-UP as described by Lee et al. (2017). A microthermics EHVH pasteurization unit (Microthermics, Raleigh, NC) with a Microthermics Steam Injection Module and a 2-stage homogenizer (model NS2006H, GEA Niro Soavi, Parma, Italy) was used for milk processing. For the HTST treatment, raw skim milk and blended FSM was preheated to 60°C, pasteurized at 78°C for 15 s, and cooled to 10°C. The DSI-UP products were preheated to 90°C, pasteurized at 140°C for 2.3 s under 330 kPa pressure by direct culinary steam injection (model LG-30, Electro-Steam Generator Corp., Alexandria, VA) using a Microthermics Steam Injection Module with Cub 5 software

Table 1. Mean fat, true protein, and anhydrous lactose for 2 replicates of skim milk, and formulated skim milks with casein as a percent of true protein of 95, 80, and 60% (FSM95, FSM80, and FSM60, respectively)¹

Component (%)	Skim milk	FSM95	FSM80	FSM60
Fat	0.07	0.05	0.04	0.04
True protein	3.24	3.15	3.19	3.18
Anhydrous lactose	4.68	4.42	4.67	4.67

¹FSM = formulated skim milks that were made as blends of micellar casein concentrate and serum protein isolate.

(version 3.1), then cooled to 85°C by vacuum cooling under a 1,040-mmHg vacuum. The DSI-UP milk was then homogenized and cooled to 10°C. For the IND-UP, products were preheated to 90°C, then UP at 140°C for 2.3 s by the indirect heater. The milk was cooled to 85°C before homogenization and then cooled by a second cooler to 10°C. All milks were homogenized at 20.7 MPa total pressure with 3.4 MPa on the second stage. All milks were packaged in light-shielded, half-gallon HDPE containers (Upstate Niagara Cooperative), stored at 4°C, and sampled at d 1 postprocessing for analyses. Sampling was conducted at d 1 postprocessing since it is established that volatile sulfur compounds and sulfur and eggy flavors diminish during refrigerated storage of milk (Jo et al., 2018) and that these flavors are disliked by US consumers (Lee et al., 2017). Our goal in the current study was to elucidate the source of these off flavors and as such, we evaluated milks when volatile sulfur compounds and sulfur flavors were at their highest intensities.

Chemical Composition

Fat, protein, and lactose concentration (g/100 g of milk) of milks and FSM were measured by a mid-infrared milk analyzer before thermal processing to verify formulation and process control compared with raw skim milk. Pre-calibration (Lynch et al., 2006) and calibration (Kaylegian et al., 2006) of the mid-infrared milk analyzer with modified milks were performed as described (Wojciechowski et al., 2016). Milk pasteurization was confirmed by alkaline phosphatase test (AOAC method 946.03, Phos-kit, Weber Scientific, Hamilton, NJ). All pasteurized milks were tested for aerobic plate count (AOAC International, 2012; method 990.12) and coliform count (AOAC International, 2012; method 991.14; Petrifilm, 3M, St. Paul, MN) 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 milk and FSM were evaluated by 7 trained panelists at d 1 postprocessing. Each panelist (4 females, 3 males, ages 21 to 55 y) had a minimum of 80 h of 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; McCarthy et al., 2017;

Jo et al., 2018). One addition to the current study was that sulfur/eggy flavor was split into 2 distinct aromatics: sulfur/burnt flavor (burnt/cooked flavor not eggy in nature) and eggy flavor (sulfur aromatic like hard-boiled egg), to further clarify the nature of the specific cooked flavors that were only encountered in UP milk. Milks (30 mL) were dispensed into 59-mL soufflé cups with lids (Dart Container Corp., Mason, MI) with random 3-digit blinding codes. Samples were prepared with overhead lights off to prevent light oxidation, and tempered to 10°C. Each panelist evaluated each milk in duplicate using Compusense Cloud (Compusense, Guelph, Canada).

Volatile Sulfur Compound Analysis: Headspace-Solid-Phase Microextraction GC-MS/MS

Selected sulfur compounds including H₂S, CS₂, DMS, DMDS, dimethyl sulfoxide (DMSO), methional, and DMTS were measured using an Agilent 7890B gas chromatograph applied to an Agilent 7000C triple quad mass spectroscopy (GC-MS/MS) and sulfur selective flame photometric detector (Agilent Technologies Inc., Santa Clara, CA) equipped with a ZB-5ms column (30 m length × 0.25 mm i.d. × 0.25 µm film thickness; Phenomenex, Torrance, CA). Sample introduction was accomplished using a CTC Analytics CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland). Five milliliters of skim milk or FSM along with 20 µL of internal standard (ethyl methyl sulfide in ethyl ether at 1.65 mg/kg; Sigma-Aldrich, St. Louis, MO) was added to 20-mL solid-phase microextraction autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical, Suwanee, GA). The analytical conditions and the multiple reaction monitoring transition for selected sulfur compounds (Table 2) 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. MassHunter Qualitative and Quantitative Analysis software (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.

Orthonasal Threshold Analysis

The compounds H₂S and CS₂ were selected for orthonasal threshold testing to align with the volatile compound analysis and sensory intensities of sulfur/burnt and eggy flavors in UP milk. A modification of the American Society for Testing and Materials procedure E679-9 (ASTM, 2004), an ascending forced-choice

method of limits, was used to determine best estimate orthonasal threshold values for each compound as a 7-series 3-ascending forced choice. Stock solutions of each compound were prepared in 95% ethyl alcohol (Sigma-Aldrich). Aliquots of the stock solutions were placed into the selected medium (skim milk, HTST pasteurized at 77°C for 25 s). The HTST skim milk for threshold testing was obtained from North Carolina State University Dairy Enterprise System within 1 d of postprocessing. The concentrations of H₂S and CS₂ in HTST skim milk are below the limit of quantification and this milk has no discernable sulfur/eggy flavor (Jo et al., 2018). Blank solutions were skim milk with no added compound. The orthonasal detection thresholds of each of each compound were determined individually. Stock solutions were serially diluted with the respective diluent (step factor of 1.5), and 30 mL of each was poured into clean, 3-digit-coded, 60-mL lidded soufflé cups (Dart Container Corp.). The samples for each threshold test were prepared 3 h before the test to achieve equilibrium for each compound in the sample cup (Leksrisompong et al., 2010). Subjects (n = 38) were instructed on the appropriate sniffing procedure before testing as described by Leksrisompong et al. (2010). Seven ascending series were presented with sample presentation randomized within each series. A 1-min rest was enforced between each set of 3 samples. Responses were collected using paper ballots.

Descriptive Analysis of Model Systems with Sulfur Compounds

Skim milk model systems containing spiked additions of targeted sulfur compounds were prepared to further confirm specific contributions to eggy and sulfur/burnt flavors of UP milk. The H₂S and CS₂ were selected as primary contributors to the eggy and sulfur/burnt fla-

vors, respectively. Stock solutions of each compound were prepared individually in 95% ethyl alcohol (Sigma-Aldrich) and spiked into HTST pasteurized skim milk (77°C for 25 s) at the range of orthonasal threshold and average concentration documented in UP milk (Jo et al., 2018). A control with no added compounds was also prepared. Eggy and sulfur/burnt flavor intensities were documented in model systems in duplicate by a trained panel as described previously. The experiment was replicated twice.

Statistical Analysis

The data from the experiment with FSM and different heat treatment were analyzed by 2-way ANOVA (heat treatment × protein ratio) to determine the effect of casein as a percentage of true protein in FSM (skim milk, FSM95, FSM80, and FSM60) and heat treatment (HTST, DSI-UP, IND-UP) with means separation using Fisher's least significant difference test. For threshold results, the individual best estimate threshold (**BET**) was taken as the geometric mean of the last concentration with an incorrect response and the first concentration with a correct response, except for the following sequence: if the subject indicated a "not sure" response for the correct choice, that concentration was increased by a factor of 1.41 to adjust for the possibility of a chance correct response (Lawless et al., 2000). The group BET was taken as the geometric mean of the individual BET values. Sensory results from model systems were evaluated by ANOVA with Fisher's least significant difference test. Partial least squares regression was performed to correlate instrument and sensory data and determine the ability of volatile sulfur compounds to predict sensory attributes. All statistical analyses were performed using XLSTAT (version 2018, Addinsoft, New York, NY) at *P* < 0.05 significance.

Table 2. Multiple reaction monitoring transition parameters for selected compounds for GC-triple quadrupole-MS analytical conditions

No.	Compound	Precursor ion (<i>m/z</i>)	Production			
			Quantifier (<i>m/z</i>)	CE ¹ (V)	Qualifier (<i>m/z</i>)	CE (V)
1	Hydrogen sulfide	34	32	3		
2	Dimethyl sulfide	62	47	4	46	4
3	Carbon disulfide	76	44	3	46	3
ISTD ²	Ethyl methyl sulfide	76	61	2	48	2
4	Dimethyl disulfide	94	79	10	64	15
5	Dimethyl sulfoxide	78	63	3	61	3
6	Methional	104	76	1	48	4
7	Dimethyl trisulfide	126	80	15	61	5

¹CE = collision energy.

²ISTD = internal standard.

RESULTS AND DISCUSSION

Chemical Composition

The composition of the skim milk, FSM95, FSM80, and FSM60 are shown in Table 1. All FSM had similar fat and protein content to the skim milk control. All milks were negative for alkaline phosphatase, indicating complete pasteurization. No coliforms were detected in pasteurized skim milks and FSM. All milks had aerobic plate count less than 10^2 cfu/mL.

Descriptive Sensory Analysis

Trained panel profiling of milks and FSM demonstrated that the milks were different in overall aroma intensity, sweet aromatic, cooked/milky, sulfur/burnt and eggy flavors, and astringency by both protein ratio and heat treatment ($P < 0.05$; Table 3). As expected, UP treatments by DSI or IND were distinguished by higher overall aroma, cooked/milky flavor, and astringency compared with HTST in all skim milk and FSM ($P < 0.05$). Regardless of SPI content in FSM, HTST products had no changes in cooked/milky flavor and no detectable sulfur/burnt or eggy flavors. This result confirmed that the development of sulfur/burnt and eggy flavors were due to UP heat treatment, in agreement with previous studies of UP milk flavors (Lee et

al., 2017; Jo et al., 2018). Astringency was increased in all UP milk and FSM compared with HTST products but was also distinct within the UP treatments and was higher in FSM with increased serum protein ratios. Higher astringency in UP treatments compared with HTST was consistent with previous studies (Liem et al., 2016; Lee et al., 2017). Josephson et al. (1967) reported a higher astringency in whey than skim milk in a given heat treatment. The development of astringency in milk could be due to serum protein denaturation and aggregation with UP heat treatment (Josephson et al., 1967; Beecher et al., 2008). Hutton and Josephson (1951) suggested that β -LG was presumed to be a significant contributor to the astringency in heat-treated milk. In addition, the order of homogenization and thermal process may have an influence on astringency of milk and milk protein-based beverages (Lee et al., 2017). Different ratios of milk protein fractions (MCC and SPI) affected other flavors of the FSM processed with UP ($P < 0.05$, Table 3). Cooked/milky flavor increased with heat treatment and SPI ratio. Higher serum protein content FSM had higher intensities of cooked/milky flavor in both UP treatments, DSI and IND, compared with HTST. The development of cooked flavor in UP milk has been suggested to be derived from milk protein denaturation, particularly serum protein, possibly in relation to the formation of significant amounts of volatile sulfur compounds (Hutton and Patton, 1952;

Table 3. Mean ($n = 2$) descriptive sensory profiles of skim milks and formulated skim milks with different thermal treatments at d 1 postprocessing at 4°C

Treatment ¹	Type ²	Sensory attribute ³						
		Overall aroma	Sweet aromatic	Cooked/milky	Sulfur/burnt	Eggy	Sweet taste	Astringent
HTST	Skim milk	2.3 ^c	1.5 ^{cde}	3.3 ^d	ND ⁴	ND	2.1 ^a	2.0 ^d
	FSM95	1.6 ^d	1.7 ^{bcd}	3.0 ^d	ND	ND	2.0 ^a	2.0 ^d
	FSM80	1.8 ^{cd}	1.9 ^{ab}	3.1 ^d	ND	ND	2.1 ^a	2.0 ^d
	FSM60	1.6 ^d	1.6 ^{bcd}	3.0 ^d	ND	ND	2.0 ^a	2.2 ^{cd}
DSI-UP	Skim milk	3.5 ^b	1.1 ^{ef}	4.2 ^{bc}	1.5 ^b	1.5 ^c	2.0 ^a	3.4 ^a
	FSM95	2.0 ^{cd}	1.5 ^{cde}	3.9 ^c	0.8 ^c	ND	2.0 ^a	2.5 ^c
	FSM80	4.0 ^b	1.3 ^{def}	4.5 ^{ab}	2.0 ^a	2.5 ^b	2.0 ^a	3.0 ^{ab}
	FSM60	4.0 ^b	1.0 ^f	4.6 ^{ab}	2.3 ^a	3.1 ^a	2.0 ^a	3.2 ^a
IND-UP	Skim milk	3.5 ^b	1.8 ^{bc}	4.2 ^{bc}	1.8 ^b	1.8 ^c	2.0 ^a	3.0 ^{ab}
	FSM95	2.3 ^c	2.2 ^a	3.9 ^c	0.5 ^c	ND	2.0 ^a	2.7 ^{bc}
	FSM80	4.0 ^b	1.8 ^{abc}	4.5 ^{ab}	0.7 ^c	2.8 ^b	2.0 ^a	3.1 ^{ab}
	FSM60	4.8 ^a	1.7 ^{bcd}	4.7 ^a	0.9 ^c	3.5 ^a	2.0 ^a	3.4 ^a
<i>P</i> -value	Heat	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.003	<0.0001
	Protein	0.003	<0.0001	<0.0001	<0.0001	<0.0001	0.008	<0.0001
	Heat × protein	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

^{a-f}Means in the same column followed by a different superscript differ ($P < 0.05$).

¹DSI-UP = ultrapasteurization by direct steam injection; IND-UP = ultrapasteurization by indirect heating.

²FSM = formulated skim milks that were made as blends of micellar casein concentrate and serum protein isolate. FSM95, FSM80, and FSM60 = FSM with casein as a percent of true protein of 95, 80, and 60%, respectively.

³Attribute intensities were scored on a 0- to 15-point universal intensity scale (Meilgaard et al., 2007). Fluid milk flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Drake, 2007; McCarthy et al., 2017).

⁴ND = not detected.

Patrick and Swaisgood, 1976; Mehta, 1980; Calvo and de la Hoz, 1992).

Sulfur/eggy flavor was previously documented in studies with UP milk. Lee et al. (2017) and Jo et al. (2018) documented distinct differences in the sensory profiles, especially sulfur/eggy flavor intensity of DSI-UP and IND-UP skim and 2% milk fat milks. In the current study, this flavor was split into 2 distinct aromatics: sulfur/burnt flavor (burnt/cooked flavor not eggy in nature) and eggy flavor (sulfur aromatic like hard-boiled eggs), in an effort to clarify the nature of these cooked flavors that were only encountered in UP milk. This process could also help clarify flavor differences generated from each heat treatment (DSI vs. IND). The distinct differences in the sensory profiles of DSI and IND in previous studies were the intensities of sulfur/eggy and sweet aromatic flavors. In the current study, DSI-UP skim had a lower sweet aromatic flavor than IND-UP skim, consistent with previous studies (Table 3). Cooked/milky, sulfur/burnt, and eggy flavors were not distinct between DSI-UP skim and IND-UP skim, but DSI and IND FSM with 20 or 40% SPI (i.e., FSM80 and FSM60) as a proportion of the protein had distinct eggy flavor intensities that were higher than those in the FSM with 5% SPI as a proportion of the protein ($P < 0.05$). The DSI FSM also had higher intensities of sulfur/burnt flavor than IND FSM. The differences in the intensities of sulfur/burnt and eggy flavors could be associated with specific milk protein denaturation in relation to the differences in heat treatment. The DSI process can denature serum proteins in a short time period due to the rapid temperature increase, whereas serum protein denaturation during the IND process can take a longer period of time (Oldfield et al., 1998; Lee et al., 2017). This difference may affect the formation of sulfur/burnt and eggy flavors in UP milks.

The differences in these 2 flavors (sulfur/burnt and eggy) between DSI and IND were not significant when skim milks were compared. Although FSM80 had equivalent casein as a percentage of true protein to skim milk, the sulfur/burnt and eggy flavors were more distinct in FSM80 than skim milk. It is possible that the recombination of milk protein fractions have increased available sulfhydryl groups at the time of heat treatments, because the properties of the β -LG and casein interactions in milk are not the same as those interactions formed in isolated systems of serum protein and casein (Sawyer, 1969). The interaction between serum protein and casein when milk or individual milk proteins are heated can influence the flavor character of milk, which can also be important to the applications of milk proteins. In addition, the relative rates of these interactions would be dependent on temperature and

heating profile of the heat treatment (Oldfield et al., 1998), which can affect the relative rates of unfolding and the formation of sulfur compounds. Future studies should investigate the interaction between thermally processed milk proteins and flavor compounds in relation to the flavor development or retention of flavors of milk-based beverages.

It has been recognized that cooked and sulfur flavors (sulfur/burnt and eggy) in UP milks are formed by milk proteins during heat treatments, but the direct evidence to the source of off-flavors has not been addressed. Previous studies investigated structural, functional, and physical changes of individual milk protein fractions (casein and serum) following heat treatment (Hutton and Patton, 1952; Jenness, 1954; Oldfield et al., 1998; Anema and Li, 2003); however, it is difficult to determine the off-flavor sources in milk without understanding the sensory and flavor chemistry perspective. The formulated skim milks used in the current study provide clarification of sensory attributes of milk and of how processing conditions interact with different proportions of casein and serum proteins at constant true protein concentration to affect flavor formation in UP milk.

Volatile Compound Analysis

Concentrations of volatile sulfur compounds, except DMSO, were distinct in skim milk and FSM across different heat treatments ($P < 0.05$, Table 4). The concentration of H_2S and CS_2 increased in FSM with increased SPI ratio ($P < 0.05$, Table 4). Volatile sulfur compound profiles also differed by UP treatment: DSI or IND. The DSI-UP had higher concentration of CS_2 , DMTS, methional, and DMSO, whereas IND-UP was higher in DMS. This observation can be directly linked to the fact that differences in heating mechanisms affect the formation of sulfur compounds. Higher concentrations of DMS in IND-UP would be expected as IND-UP milk contains a higher level of dissolved oxygen and requires longer cooling times, which can lead to further oxidation of other sulfur compounds including methanethiol, methional, and DMDS (Datta et al., 2002; Jo et al., 2018). Methanethiol, another sulfur compound previously found in UHT milk (Al-Attabi et al., 2014), was not included in this study. This compound is derived from Strecker degradation of methionine; however, there are severe limitations to measure methanethiol because it forms artifacts and is quickly oxidized to other compounds such as DMS, DMDS, or DMTS (Vazquez-Landaverde et al., 2006). Vazquez-Landaverde et al. (2006) used acidified milk matrix for methanethiol measurement to minimize artifact formation. As such, it would be more reasonable to observe DMS, DMDS, or DMTS in UP

or UHT milk as oxidized forms of methanethiol. The current results are consistent with a previous study, which reported higher amounts of DMS in IND-UP milk compared with DSI-UP milk (Jo et al., 2018). Methional is a high impact aroma compound in UP and UHT milk (Colahan-Sederstrom and Peterson, 2005; Jo et al., 2018), derived from Strecker degradation of methionine (Belitz et al., 2009). Higher concentrations of H₂S and CS₂ were attributed to both UP treatments compared with HTST treatments. These compounds were reported as the main sulfur compounds in UP milks contributing to sulfur/eggy and cooked flavors (Jo et al., 2018). The IND-UP milks had lower concentrations of CS₂ compared with DSI-UP. This could be explained by dissolved oxygen levels during the IND process. Extended heat treatment activates sulfhydryl groups in serum protein, which generates CS₂, and makes them more oxidizable by atmospheric oxygen (Jenness, 1954). The reactive sulfhydryl group is more readily oxidized in a liquid system including fluid milk (Jenness, 1954). The origin and formation of CS₂ in milk has not been clearly elucidated, and it is presumably generated from a breakdown product of other sulfur compounds (Urbach, 1993).

Volatile sulfur compounds were affected by serum protein ratio (Table 4). Changes in H₂S and CS₂ were specifically attributed to higher serum protein content ($P < 0.05$). However, DMS, DMDS, DMTS, and methional were not affected by serum protein ratio in

FSM ($P > 0.05$). This may be related to the content of sulfur-containing AA in milk protein fractions, casein, and serum protein. Sulfur compounds including DMS, DMDS, and DMTS originate from the degradation of methionine (Pripis-Nicolau et al., 2000), one of the main sulfur-containing AA in milk proteins. Serum protein and casein contain relatively equivalent amounts of methionine (Belitz et al., 2009). As such, changes in protein ratio did not affect amounts of methionine or compounds purportedly sourced to methionine.

The increase of H₂S and CS₂ in FSM with higher SPI as a proportion of total protein can be linked to the higher amount of cysteine in serum protein (Belitz et al., 2009). The elevated levels of these sulfur compounds, especially in FSM with higher SPI, directly support the assumption that serum protein is the source of sulfur/burnt and eggy off-flavors in milk treated with severe heat treatment such as UP, as those flavors were not detected in HTST milk and those compounds were not associated with HTST milk (Table 3, Figure 1). Studies reported that the degradation of cysteine alone produced the greatest amount of volatile compounds compared with other sulfur-containing precursors (Zhang and Ho, 1991; Pripis-Nicolau et al., 2000). Pripis-Nicolau et al. (2000) noted that greater olfactory contributions from cysteine were due to cysteine undergoing competitive reactions between Strecker degradation and decarboxylation, and release of sulfur compounds. As such, it is assumed that cysteine in se-

Table 4. Mean ($n = 2$) volatile sulfur compounds in skim milks and formulated skim milk with different thermal treatments at d 1 postprocessing at 4°C

Treatment ¹	Type ²	Volatile sulfur compound ³ (μg/kg)						
		Hydrogen sulfide	Dimethyl sulfide	Carbon disulfide	Dimethyl disulfide	Dimethyl trisulfide	Methional	Dimethyl sulfoxide
HTST	Skim milk	6.12 ^f	5.45 ^c	15.9 ^g	0.04 ^a	0.03 ^{cd}	0.30 ^c	0.002 ^a
	FSM95	4.89 ^f	5.37 ^c	13.1 ^g	0.01 ^{bc}	0.07 ^{ab}	0.63 ^{bc}	0.002 ^a
	FSM80	6.20 ^f	5.20 ^c	15.7 ^g	0.01 ^{bc}	0.05 ^{bc}	0.72 ^b	0.003 ^a
	FSM60	7.59 ^f	5.05 ^c	19.6 ^g	0.02 ^{bc}	0.05 ^{bc}	0.81 ^b	0.002 ^a
DSI-UP	Skim milk	56.5 ^d	0.35 ^e	103 ^d	0.01 ^{bc}	0.02 ^d	1.05 ^a	0.01 ^a
	FSM95	16.4 ^e	0.51 ^d	34.3 ^{cd}	0.005 ^c	0.10 ^a	1.20 ^a	0.01 ^a
	FSM80	70.3 ^c	0.37 ^e	183 ^c	0.01 ^{bc}	0.07 ^{ab}	1.11 ^a	0.01 ^a
	FSM60	143 ^a	0.37 ^e	383 ^a	0.01 ^{bc}	0.05 ^{bc}	1.06 ^a	0.01 ^a
IND-UP	Skim milk	47.0 ^d	19.7 ^a	81.7 ^e	0.01 ^{bc}	0.02 ^d	1.03 ^a	0.002 ^a
	FSM95	16.1 ^e	17.2 ^{ab}	38.4 ^f	0.01 ^{bc}	0.04 ^{bc}	0.92 ^{ab}	0.002 ^a
	FSM80	67.1 ^c	19.8 ^a	116 ^b	0.02 ^{ab}	0.04 ^{bc}	0.90 ^{ab}	0.003 ^a
	FSM60	123 ^b	14.0 ^b	310 ^b	0.02 ^{bc}	0.04 ^{bc}	0.85 ^{ab}	0.002 ^a
<i>P</i> -value	Heat	<0.0001	<0.0001	<0.0001	0.049	0.013	0.001	0.157
	Protein	<0.0001	0.771	<0.0001	0.124	<0.0001	0.277	0.110
	Heat × protein	<0.0001	<0.0001	<0.0001	0.008	<0.0001	0.005	0.335

^{a-g}Means in the same column followed by a different superscript differ ($P < 0.05$).

¹DSI-UP = ultrapasteurization by direct steam injection; IND-UP = ultrapasteurization by indirect heating.

²FSM = formulated skim milks that were made as blends of micellar casein concentrate and serum protein isolate. FSM95, FSM80, and FSM60 = FSM with casein as a percent of true protein of 95, 80, and 60%, respectively.

³Relative mean concentration triplicate (μg/kg).

rum protein could contribute to sulfur/burnt and eggy off-flavors more than methionine. This result further suggests that sulfur-containing AA in serum protein play an important role in determination of the source of sulfur/burnt and eggy off-flavors in milks.

Orthonasal Threshold of Selected Sulfur Compounds

Orthonasal detection BET values for H_2S and CS_2 were calculated in skim milk (Table 5). The values were higher than published values in water. Threshold values for these compounds in milk have not been previously reported. Although water and skim milk have similar pH, the protein or fat content (or both) in milk affects thresholds (Hofmann et al., 2001). As such, it is critical to evaluate the threshold value of these compounds in

skim milk because thresholds can vary considerably in different of matrices or by different methodology. The current study expands the sensory and flavor perspective of sulfur/burnt and eggy flavors in milk because sensory threshold determination can be more significant than the concentration of the chemicals (Teranishi et al., 1991).

Confirmation of Sulfur Compounds in Skim Milk

Following threshold determination of H_2S and CS_2 , HTST skim milk models spiked with those compounds were evaluated to verify specific eggy and sulfur/burnt flavors of UP milk (Table 6). The descriptive panel documented the eggy and sulfur/burnt flavors in the skim milk models containing H_2S and CS_2 , and the intensities of those specific attributes were similar to DSI-UP or

Figure 1. Partial least squares correlation biplot [principal components (PC) 1 and 2] of volatile sulfur compounds and sensory attributes in skim milks and formulated skim milks (FSM) at d 1 postprocessing at 4°C. DSI = ultrapasteurization by direct steam injection; IND = ultra-pasteurization by indirect heating. FSM95, FSM80, and FSM60 = FSM with casein as a percent of true protein of 95, 80, and 60%, respectively.

Table 5. Best estimate threshold (BET) values for each odorant in skim milk¹

Compound	BET ² (μg/L)	Previously reported threshold value	
	Skim milk	Water (μg/kg)	Other (μg/L)
Hydrogen sulfide	22.5	10 ³	0.41 in odor bag ⁴
Carbon disulfide	35.2	5 ⁵	16.1 in air ⁶

¹Skim milk was HTST pasteurized at 77°C for 25 s.²Geometric means of group BET values from 38 consumers.³Pippen and Mecchi, 1969.⁴Nagata, 2003.⁵Vazquez-Landaverde et al., 2006.⁶EPA, 2016. The unit was converted from mg/m³.

IND-UP milks (Tables 3 and 6). The skim milk model system confirmed the contribution of H₂S and CS₂ to characteristic eggy and sulfur/burnt flavors in milk.

Relationship Between Volatile Sulfur Compounds and Sulfur Off-Flavors

Partial least squares regression further demonstrates the changes in volatile sulfur compound profiles in milks and FSM and how they were affected by both heat treatment and the increase of serum protein as a percentage of total protein, which pinpoints the distinct eggy and sulfur/burnt flavors and the higher concentration of H₂S and CS₂ (Figure 1). These flavors and compounds have been discussed in previous studies on fluid milk flavor as a general aspect and the possibilities of sulfur compounds contributing to the cooked and sulfur off-flavors in UP or UHT milks (Vazquez-Landaverde et al., 2006; Al-Attabi et al., 2009, 2014). However, these studies had insufficient information to conclude which specific compounds were responsible for specific sulfur off-flavors in UP or UHT milks. Since distinct eggy and sulfur/burnt flavors have been demonstrated as consumer defects for UP milk (Lee et al., 2017), a clear understanding of the nature and source of these

flavors is crucial. The differentiation of DMS, DMDS, DMTS, DMSO, and methional is likely derived from the differences in processing mechanisms between DSI and IND, such as heat transfer and dissolved oxygen during the heat process. These compounds were previously described as sulfur notes related to characteristic cabbage and vegetable odors in UP milk (Jo et al., 2018), which may not be directly linked to eggy and sulfur/burnt off-flavors encountered in the current study. These compounds can be possibly imparting overall aroma or cooked/milky flavor; however, no clear relationships were found by partial least squares or correlation analysis. A primary precursor of these compounds is known to be methionine, which is present in similar amounts in both casein and serum protein (Belitz et al., 2009). Increases in H₂S and CS₂ contributed to eggy and sulfur/burnt off-flavors in UP milk and originate from serum protein. Correlation analysis confirmed that eggy and sulfur/burnt flavors were correlated with H₂S ($R^2 = 0.94$, $P < 0.05$; $R^2 = 0.72$, $P < 0.05$) and CS₂ ($R^2 = 0.88$, $P < 0.05$; $R^2 = 0.70$, $P < 0.05$, respectively). The correlation coefficients for DMS, DMDS, DMTS, DMSO, and methional for eggy and sulfur/burnt flavors were below 0.50 ($P < 0.05$), which along with their aroma properties, suggests that

Table 6. Mean (n = 2) descriptive sensory profiles of skim milk model system

Sample	Sensory attribute ¹						
	Overall aroma	Sweet aromatic	Cooked/milky	Sulfur/burnt	Eggy	Sweet taste	Astringent
Skim milk ²	2.0 ^d	1.2 ^a	3.2 ^b	ND ³	ND	2.3 ^a	2.0 ^a
Skim + CS ₂ added ⁴	3.0 ^c	0.9 ^b	3.7 ^a	1.1 ^a	ND	2.3 ^a	2.1 ^a
Skim + H ₂ S added ⁴	3.5 ^b	1.0 ^{ab}	3.6 ^a	ND	2.0 ^a	2.3 ^a	2.0 ^a
Skim + CS ₂ and H ₂ S added ⁴	4.3 ^a	0.5 ^c	3.7 ^a	0.8 ^a	1.9 ^a	2.3 ^a	2.0 ^a

^{a-d}Means in the same column followed by a different superscript differ ($P < 0.05$).¹Attribute intensities were scored on a 0- to 15-point universal intensity scale (Meilgaard et al., 2007). Fluid milk flavors fall between 0 and 4 on this scale (Croissant et al., 2007; Drake, 2007; McCarthy et al., 2017).²Skim milk was HTST pasteurized at 77°C for 25 s.³ND = not detected.⁴Carbon disulfide (CS₂), hydrogen sulfide (H₂S), or both, were added in skim milk at 50 and 30 μg/L, respectively.

these compounds are not primary contributors to the eggy and sulfur/burnt off-flavors in UP milks. As such, the general trend of increased H₂S was associated with eggy flavor and higher intensity of sulfur/burnt flavor could be attributed to increases in both H₂S and CS₂. Higher concentrations of both H₂S and CS₂ intensify sulfur flavors of UP milk, documented as sulfur/eggy flavor in previous studies.

CONCLUSIONS

The approach of manufacturing FSM by mixing MCC and SPI in different ratios allowed further clarification of the source of sulfur off-flavors in skim milk processed by UP. Heat treatment and serum protein ratio had significant effects on eggy and sulfur/burnt flavors and volatile sulfur compound profiles. Sensory and instrumental analysis results suggested that sulfur compounds including DMS, DMDS, DMTS, DMSO, and methional originated from the differences in processing mechanisms between DSI and IND. Increased H₂S and CS₂ were correlated with distinct eggy and sulfur/burnt off-flavors derived from serum protein and attributed to UP treatment. A potential interaction between milk proteins and sulfur compounds affected by serum protein associated with casein during UP treatment was also documented. Orthonasal threshold calculations and descriptive analysis of model systems confirmed that H₂S and CS₂ were responsible for eggy and sulfur/burnt off-flavors, respectively. These results suggest that those compounds are critical indicators to determine sulfur off-flavors in UP milk. These findings also provide a practical insight into the source of heat-generated sulfur flavors in UP milk as well as milk protein-based beverages with altered casein as a percent of true protein that may require UP.

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