ABSTRACT

LISTIYANI, MARIA ANGELICA DIANA. Influence of Bleaching on Flavor of Fluid Whey and Whey Protein Concentrate and Benzoic Acid Concentration in Dried Whey Proteins. (Under the direction of Dr. MaryAnne Drake.)

Increasing use of whey protein ingredients in foods and beverages increases the demand for colorless and mild tasting whey protein. Colored fluid whey is frequently bleached to remove residual annatto colorant at different temperatures and bleach concentrations. Bleaching effects on WPC34 have not been established in previous studies. Benzoyl peroxide (BP), a bleaching agent for whey, degrades to benzoic acid (BA) and water and may elevate BA concentrations in dried whey products, which give rise to international concerns. Two studies were designed to look at different bleaching parameters and BA residue in dried whey products.

The objectives of the first study were to compare the effects of hot and cold bleaching, point of bleaching (before and after fat separation), and bleaching agent on bleaching efficacy and volatile components of liquid Cheddar whey. Liquid colored Cheddar whey was manufactured in triplicate and pasteurized. Part of the whey was collected (no separation, NSE) and the rest was subjected to fat separation (FSE). NSE and FSE whey were then subdivided and bleaching treatments (BP 50 or 100 mg/kg, hydrogen peroxide (HP) 250 or 500 mg/kg, and no bleach) at 68°C for 30 min or 4°C for 16 h were applied. Norbixin was extracted to compare bleaching efficacy and volatile compounds were evaluated by gas chromatography-mass spectrometry (GC-MS). BP bleached wheys had lower norbixin content compared to HP bleached wheys (p<0.05).
HP bleaching efficacy was decreased at 4°C compared to 68°C (p<0.05), but not for BP (p>0.05). Liquid whey subjected to hot bleaching at both concentrations of HP or 100 mg/kg BP had higher lipid oxidation products (aldehydes) compared to unbleached, 50 mg/kg BP hot bleached, or cold bleached wheys. Fat separation had no impact on the relative abundance of volatile lipid oxidation products (p>0.05).

The objectives of the second study were to determine the impact of HP or BP bleaching on bleaching efficacy and flavor of WPC34 and to evaluate residual BA in commercial and experimental WPC manufactured with and without BP bleaching. Cheddar cheese whey was manufactured in duplicate. Pasteurized fat-separated whey was subjected to hot bleaching with either HP at 500 mg/kg, BP at 50 or 100 mg/kg, or no bleach. Bleached and unbleached whey was ultrafiltered and spray dried into WPC34. Color (L*a*b*) measurements and norbixin extractions were conducted to compare bleaching efficacy. Descriptive sensory and instrumental analyses were used to evaluate bleaching effects on flavor. Benzoic acid was extracted from experimental and commercial WPC34 and WPC80 and quantified by high performance liquid chromatography (HPLC). The b* value and norbixin concentration of BP bleached WPC34 were lower than unbleached and HP bleached WPC34. Descriptive sensory analysis demonstrated that HP bleached WPC34 had higher cardboard flavor and relative abundances of volatile lipid oxidation products than BP bleached or unbleached WPC34. BP bleached WPC34 and WPC80 had higher BA concentrations than unbleached and HP bleached samples, in which WPC80 had smaller differences than those observed in
WPC34. Benzoic acid extraction from permeate demonstrated that WPC80 permeate contained more BA than WPC34 permeate (p<0.05).

These studies suggest that fat separation had no measurable impact on bleaching efficacy or initial lipid oxidation and hot bleaching may result in increased lipid oxidation in fluid whey. In addition, BP is a more effective bleaching agent compared to HP both in color removal and flavor side effects and BA concentration is decreased by ultrafiltration and diafiltration.
Influence of Bleaching on Flavor of Fluid Whey and Whey Protein Concentrate and Benzoic Acid Concentration in Dried Whey Proteins

by

Maria Angelica Diana Listiyani

A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science

Food Science

Raleigh, North Carolina

2011

APPROVED BY:

Dr. MaryAnne Drake
Chair of Advisory Committee

Dr. Lisa Dean
Committee Member

Dr. Brian Farkas
Committee Member

Dr. Timothy Sanders
Committee Member
DEDICATION

To mom, dad, and my brother, who have always been very supportive.
BIOGRAPHY

Maria Angelica Diana Listiyani was born in Jakarta, Indonesia on April 4, 1986. Her parents, Sutoyo Gunoharjo, who is a dentist; and Tinawati, who is a housewife, still reside in Jakarta, Indonesia. She has a younger brother, Felix Adisaputra, who was awarded a bachelors degree in electrical engineering with high honor from Michigan State University, and now is working as an engineer in a packaging company in Wyoming, MI.

Maria graduated from Santa Ursula High, Jakarta, Indonesia in June 2004 with honors in natural science. She then went to the U.S. to pursue her bachelor degree in food science with concentrations in food chemistry and food technology from Michigan State University. While in college, she got her first internship experience in quality assurance at Gray & Company at Hart, MI. Then she worked as a summer scholar at Cornell University in dairy microbiology research area. During her last semester in undergrad, she was a TA for dairy processing class. In the same semester, she joined MSU dairy judging team, where she was able to participate in the regional competition, tasted many dairy products, and developed her passion in sensory and flavor chemistry. She graduated from Michigan State University with honor in May 2008. She was fortunate to obtain an internship as a product developer at Kellogg Company right after she graduated from college. There, she developed even more desire to obtain her master degree in food science.
Maria worked as a laboratory specialist in sensory and flavor chemistry lab at North Carolina State University before she started her graduate study there. She started her master study under the direction of Dr. MaryAnne Drake in August 2009. During her graduate study, she joined NCSU product development team and was able to help the team win the national competition at IFT. She also had the opportunity to participate in the ADSA conference and won third place in dairy foods poster competition, presenting her research on “Impact of Fat Separation, Temperature, and Bleaching Agent on Bleaching of Liquid Cheddar Whey”.
ACKNOWLEDGMENTS

I would like to thank everyone who has helped me finishing up my master degree. First and foremost, I would like to thank God who has guided me through this whole journey. To my dad, Sutoyo Gunoharjo and my mom, Tinawati who have always been supportive from the beginning. Thank you for even giving me the opportunity to study in the U.S., for supporting my idea about going into grad school, for listening to my complaints, and for believing in me even when I felt like I could not finish this. To my brother, Felix Adisaputra, who is always there when I need him and who has been so supportive with my job searching and always encourages me to say a prayer. I am so proud of you. To Dr. Drake for taking me as her graduate student, for supporting me, and for believing in me. I hope I do not disappoint you. To Dr. Dean who was always willing to help me and answering all of my HPLC questions patiently. To Evan Miracle, thank you for spending so much time, helping me with GC analysis and benzoic acid calculations. To every single member of MAD Lab, thank you for helping me with pilot plant runs, especially for always showing up early in the morning, staying up so late, lifting heavy stuff, and making sure I kept my head straight while running in the pilot plant for 18 hours. To NCSU Dairy people who were always willing to help me fix things up in the pilot plant. To Dr. Barbano and his lab members at Cornell University, thank you for helping me with SAS problems and proximate analysis. Last but not least, to Andre Legowo, thank you for always being there for me, for listening to all of my complaints, for telling me that I needed to relax sometimes, for making me laugh even
when I got stressed out, for visiting me here, and of course for always being a good cook and taking care of me.
# TABLE OF CONTENTS

LIST OF TABLES ................................................................................................................................. ix

LIST OF FIGURES ................................................................................................................................. xi

Chapter 1. Literature Review .............................................................................................................. 1

- Whey ................................................................................................................................. 2
- Types of Fluid Whey ........................................................................................................... 2
- Dried Whey Color ................................................................................................................ 3
  - Hydrogen peroxide ........................................................................................................... 6
  - Benzoyl peroxide ............................................................................................................. 7
- Whey Flavor ......................................................................................................................... 11
  - Descriptive sensory analysis ........................................................................................... 12
  - Instrumental volatile analysis .......................................................................................... 13
  - Lipid oxidation ................................................................................................................ 18
  - Phospholipids ................................................................................................................ 19
  - Types of phospholipids in milk fat globule membrane (MFGM) ...................................... 21
  - Functions of phospholipids and their roles in foods ......................................................... 23
  - Phospholipids analysis in foods ....................................................................................... 24
  - Roles of phospholipids in flavor ...................................................................................... 25
- Whey Processing ....................................................................................................................... 27
- Whey Protein Functionality .................................................................................................... 29
- Application of Whey Protein ................................................................................................. 31
- References ............................................................................................................................. 33

Chapter 2. Impact of fat separation, temperature, and bleaching agent on bleaching of liquid Cheddar whey .......................................................................................................................... 48

- Abstract ............................................................................................................................... 50
- Introduction .......................................................................................................................... 52
- Materials and Methods ........................................................................................................ 53
- Results and Discussion ........................................................................................................ 64
- Conclusions ........................................................................................................................ 69
- Acknowledgments ................................................................................................................. 71
- References ........................................................................................................................... 72
Chapter 3. Influence of bleaching on flavor of 34% whey protein concentrate and residual benzoic acid concentrations in dried whey proteins ................................................. 89

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>91</td>
</tr>
<tr>
<td>Introduction</td>
<td>93</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>97</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>107</td>
</tr>
<tr>
<td>Conclusions</td>
<td>113</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>114</td>
</tr>
<tr>
<td>References</td>
<td>115</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Chapter 1.

Table 1.1 Composition of fluid and dried sweet and acid wheys (in percentages) ................................................................. 3

Chapter 2.

Table 2.1 Sums of the squares (Type III SS) for the analysis of variance (ANOVA) for the impact of temperature and bleach type on the norbixin concentration and yellowness in colored and uncolored liquid Cheddar whey (experiment I) that were bleached with benzoyl peroxide or hydrogen peroxide at 4 and 68°C .............. 76

Table 2.2 Least square means values of bleaching temperature by bleach type interaction for norbixin concentrations (mg/kg) and yellowness in colored liquid Cheddar whey (experiment I) ...... 77

Table 2.3 Sums of the squares (Type III SS) for the analysis of variance (ANOVA) for the impact of fat separation, temperature, and bleach type on the norbixin concentration in colored liquid Cheddar whey (experiments I and II) ................................................. 78

Table 2.4 Least squares mean values of bleaching temperature by bleach type interaction for norbixin concentrations (mg/kg) in colored liquid Cheddar whey (experiment II) ................................................. 79

Table 2.5 Relative abundance (μg/kg) of selected aroma active compounds in bleached and unbleached Cheddar liquid whey (experiment II) ................................................................. 80

Table 2.6 Sums of the squares (Type III SS) for the ANOVA analysis of the impact of fat separation, bleaching temperature, and bleach type on the concentration of volatile flavor compounds identified in liquid Cheddar whey (experiment II) ............... 81

Table 2.7 Least squares mean values of bleaching temperature by bleach type interaction of volatile flavor compounds (μg/kg) identified in liquid Cheddar whey (experiment II) ................................. 82
Chapter 3.

Table 3.1 Mineral analysis of experimental WPC34 ......................................... 120

Table 3.2 Hunter L*, a*, b* color values of experimental bleached and unbleached Cheddar WPC34 ................................................................. 121

Table 3.3 Descriptive sensory analysis of bleached and unbleached Cheddar WPC34 .......................................................................... 122

Table 3.4 Relative abundance (μg/kg) of selected volatile compounds found in bleached and unbleached Cheddar WPC34 ..........................123

Table 3.5 Benzoic acid concentration (mg/kg) in experimental and commercial WPC34 ................................................................. 124

Table 3.6 Benzoic acid concentration (mg/kg) in experimental and commercial WPC80 ................................................................. 125

Table 3.7 Benzoic acid concentration (mg/kg) in experimental WPC34 and WPC80 permeates ............................................................. 126
# LIST OF FIGURES

## Chapter 1.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Chemical structures of bixin and norbixin</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>Break down of hydrogen peroxide to oxygen and water</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Breakdown of benzoyl peroxide to two molecules of benzoic acid</td>
<td>9</td>
</tr>
<tr>
<td>1.4</td>
<td>Lipid oxidation mechanism</td>
<td>19</td>
</tr>
<tr>
<td>1.5</td>
<td>General phospholipid structure</td>
<td>20</td>
</tr>
<tr>
<td>1.6</td>
<td>Common phospholipids found in milk fat globule membrane (MFGM)</td>
<td>21</td>
</tr>
<tr>
<td>1.7</td>
<td>Membrane separation technologies</td>
<td>28</td>
</tr>
</tbody>
</table>

## Chapter 2.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The impact of concentration of HP and bleaching temperature on yellowness (A) and norbixin concentration (B) of whey made from cheese colored with annatto and whey from cheese not colored with annatto bleached at 4 and 68°C</td>
<td>83</td>
</tr>
<tr>
<td>2.2</td>
<td>The impact of concentration of BP and bleaching temperature on yellowness (A) and norbixin concentration (B) of whey made from cheese colored with annatto and whey from cheese not colored with annatto bleached at 4 and 68°C</td>
<td>84</td>
</tr>
<tr>
<td>2.3</td>
<td>Norbixin concentration in fat separated (FSE) and non fat separated (NSE) hydrogen peroxide (HP) bleached liquid Cheddar whey at two different bleaching temperatures (4°C and 68°C) (experiment II)</td>
<td>85</td>
</tr>
<tr>
<td>2.4</td>
<td>Norbixin concentration in fat separated (FSE) and non fat separated (NSE) benzoyl peroxide (BP) bleached liquid Cheddar whey at two different bleaching temperatures (4°C and 68°C) (experiment II)</td>
<td>86</td>
</tr>
</tbody>
</table>
Figure 2.5 Norbixin concentration (mg/kg) in fat separated (FSE) hydrogen peroxide (HP) and benzoyl peroxide (BP) bleached liquid Cheddar whey at two different bleaching temperatures (4°C and 68°C) (experiment II) ................................................... 87

Figure 2.6 PCA biplot of volatile flavor compounds from all bleaching treatments in liquid Cheddar whey (experiment II) .................... 88

Chapter 3.

Figure 3.1 Experimental WPC34 at all bleaching treatments ................... 127

Figure 3.2 Experimental reconstituted WPC34 at 10% solids at all bleaching treatments ................................................................. 128

Figure 3.3 Norbixin recovery (mg/kg) from liquid whey bleached or unbleached WPC 34 ................................................................. 129

Figure 3.4 PCA biplot of volatile compounds in bleached and unbleached Cheddar WPC 34 ................................................................. 130
CHAPTER 1: LITERATURE REVIEW
**Whey**

Whey is the liquid by-product that is separated during cheese making (USDA, 2000; Gallardo-Escamilla et al., 2005; Liaw et al., 2010) and is composed of water, lactose, proteins, and residual lipids (Morr and Ha, 1993). Compositions of the cheese milk and processing conditions, including pasteurization, type and amount of bacterial starter cultures and casein coagulant, removal of residual whey lipids, and conditions of ultrafiltration (UF), diafiltration (DF), and spray drying may influence the composition and functionality of the whey protein concentrate (WPC) and whey protein isolate (WPI) (Schmidt et al., 1984; Johansen et al., 2002). Whey was historically a waste product, but it has become a valuable and versatile dried ingredient in recent years (Foegeding et al., 2002; Pesta et al., 2007; Smithers, 2008). In 2010, United States dry whey production was at 78.3 million pounds and WPC production was at 35.6 million pounds (ADPI, 2010b). U.S. dairy exports, including milk powders, whey proteins, lactose, cheese, and butterfat, have reached 289.7 million pounds, 14.4% of which is dried whey and 8.4% is whey protein concentrate (WPC) (ADPI, 2010b).

**Types of Fluid Whey**

There are two types of fluid whey: sweet whey (pH 6 to 6.3) and acid whey (pH 4.3 to 4.6) (Morr and Ha, 1993). Sweet whey is derived from rennet coagulation such as Cheddar, Mozzarella, and Swiss, while acid whey is derived from cheeses manufactured by direct acidification such as cottage, cream cheese, and ricotta (Gallardo-Escamilla et al., 2005; USDEC, 2004). Fluid whey can be further processed to make condensed,
dried, fermented, delactosed, demineralized, or deproteinated whey through ultrafiltration, reverse osmosis, ion exchange, electrodialysis, and nanofiltration (Kosikowski, 1997). Whey processing is further discussed in a later section. The composition of fluid and dried sweet and acid wheys is shown in Table 1.

Table 1. Composition of fluid and dried sweet and acid wheys (in percentages)

<table>
<thead>
<tr>
<th>Component</th>
<th>Fluid Sweet Whey</th>
<th>Fluid Acid Whey</th>
<th>Dried Sweet Whey</th>
<th>Dried Acid Whey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Solids</td>
<td>6.35</td>
<td>6.5</td>
<td>96.5</td>
<td>96.0</td>
</tr>
<tr>
<td>Moisture</td>
<td>93.7</td>
<td>93.5</td>
<td>3.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Fat</td>
<td>0.5</td>
<td>0.04</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Total Protein</td>
<td>0.8</td>
<td>0.75</td>
<td>13.1</td>
<td>12.5</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.85</td>
<td>4.90</td>
<td>75.0</td>
<td>67.4</td>
</tr>
<tr>
<td>Ash</td>
<td>0.50</td>
<td>0.80</td>
<td>7.30</td>
<td>11.8</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>0.05</td>
<td>0.40</td>
<td>0.20</td>
<td>4.2</td>
</tr>
</tbody>
</table>

(Kosikowski, 1997)

**Dried Whey Color**

A colorless and bland dried ingredient is desired to minimize flavor carry through to finished products (Childs et al., 2007; Drake, 2006; Drake et al., 2009). Most of the whey produced in the United States comes from yellow cheese, such as Cheddar. This yellow color primarily comes from annatto, which is a natural colorant that comes from the seeds of the tropical tree fruit *Bixa orellana* (Guiliano et al., 2003; Scotter, 2009). Annatto consists of the carotenoids, bixin and norbixin and these have an orange color. The primary component of water-soluble annatto used in cheesemaking is the carotenoid norbixin (Scotter et al., 2002; Kang et al., 2010). The chemical structures of bixin and norbixin are shown in Figure 1. Naturally, bixin and norbixin occur in the cis form
(Smith, 2008). However, they can be converted to trans form by light and heat (Smith, 2008). The cis forms of both bixin and norbixin have a more red color than the trans forms (Smith, 2008). Due to annatto addition, some of this colorant is carried through into the whey in addition to the cheese.
Cis-bixin

Trans-bixin

Cis-norbixin

Trans-norbixin

Figure 1. Chemical structures of bixin and norbixin (Smith, 2008).
Since a colorless dried whey ingredient is desirable, bleaching of fluid whey is a common whey processing step (Kang et al., 2010). Two bleaching agents that are allowed for bleaching whey in the U.S. are hydrogen peroxide (maximum usage rate at 500 mg/kg) (U.S. FDA, 2010c) and benzoyl peroxide (U.S. FDA, 2010a). While hydrogen peroxide has been used as a bleaching agent worldwide, benzoyl peroxide does not have acceptance as a bleaching agent in many countries outside the U.S.

**Hydrogen peroxide**

Hydrogen peroxide (H$_2$O$_2$) is a clear, colorless liquid, with a molecular weight of 34.0 (Smith, 2008). The regulation covering use of hydrogen peroxide as a bleaching agent is 21CFR 184.1366 (U.S. FDA, 2010d). Hydrogen peroxide breaks down to oxygen and water (Figure 2). The residual hydrogen peroxide in whey must be removed using catalase. The use of catalase is regulated by 21CFR 133.113 (U.S. FDA, 2010c) which it is stated that 20 mg/kg of the sample treated is allowed. The advantages of hydrogen peroxide as a bleaching agent are its purported effectiveness over a wide range of temperatures and total solids (Smith, 2008), and little or no effect on the nutritional value of the products (Teply et al., 1958). However, hydrogen peroxide has a corrosive nature, its bleaching requires catalase addition to remove the residual hydrogen peroxide, and it requires long hold times to remove color (Gilliland, 1969; Smith, 2008). More importantly, hydrogen peroxide bleaching results in oxidized flavors in fluid whey and dried whey proteins (Croissant et al., 2009).
Benzoyl peroxide

Benzoyl peroxide (C\textsubscript{14}H\textsubscript{10}O\textsubscript{4}) is colorless, crystalline solid that is generally recognized as safe (GRAS) when used as bleaching agent following current GMP rules (U.S. FDA, 2010a). It is used as a bleaching agent in certain foods, such as flour, whey, and milk for cheese making, but is not approved for use in infant formula (JECFA, 2004a; Kang et al., 2010). It is one of the two currently approved bleaching agents that can be used to bleach whey in the U.S. However, unlike hydrogen peroxide, there is no legal limit for benzoyl peroxide use in whey bleaching established in the U.S. At their 63\textsuperscript{rd} meeting in 2004, Joint FAO/WHO Expert Committee on Food Additives (JECFA) stated that there was no safety concern posed when as much as 100 mg/kg of benzoyl peroxide was used to bleach whey. In Codex Alimentarius, it was stated that 100 mg/kg was the maximum limit of benzoyl peroxide that can be used for liquid whey, dried whey, and whey products bleaching, except for infant applications, where its use is prohibited (Codex, 2008). Australia and New Zealand have set the maximum level on benzoyl peroxide (BP) used at 40 mg/kg in all food, whereas Canada has set the maximum level of BP at 100 mg/kg to bleach liquid whey intended for the manufacture of dried whey products, other than for infant formula (JECFA, 2004a).
hydrogen peroxide, bleaching with benzoyl peroxide requires less peroxide for satisfactory bleaching, it does not need catalase addition to deactivate it, and it is less corrosive to stainless steel equipment (Chang et al., 1977; Smith, 2004).

Benzoyl peroxide breaks down to benzoic acid when it reacts with oxidizable substances present, such as annatto or carotenoid pigments during processing (JECFA, 2004a; Smith, 2004; Kang et al., 2010). The rate of this decomposition increases as the temperature increases (JECFA, 2004a). The break down reaction of benzoyl peroxide to two molecules of benzoic acids is shown in Figure 3. Benzoic acid is considered GRAS (U.S. FDA, 2010b) and it is a common preservative used widely in foods, drugs, and cosmetics (Sieber et al., 1995; Qi et al., 2009). As food preservatives, benzoic acid is used mostly in food products that are naturally in the acidic pH range, such as fruit juices and soft drinks (WHO, 2000). The maximum allowable concentrations of benzoic acid in China are 1,000 mg/kg in fruit juice, sauce, and jam, and 200 mg/kg in carbonated beverage (State Bureau for Quality Supervision, Inspection and Quarantine, 1996). Other than foods, drugs, and cosmetics, benzoic acid is also widely used as preservative in toothpastes, mouthwashes, and dentifrices (WHO, 2000).

Besides being a preservative, benzoic acid also occurs naturally in many plants and animals (WHO, 2000). Benzoic acid is produced as fermentation product of hippuric acid which is naturally present in the milk (Sieber et al., 1995). Therefore, cultured dairy products, such as yogurt, sour cream, buttermilk, kefir, and smear-ripened cheese contain some benzoic acids (Sieber et al., 1995). In yogurt, benzoic acid was detected at various levels between 12 – 47 mg/kg (plain yogurt) and between 5 – 39 mg/kg (fruit yogurt)
Benzoic acid content varied between 10 – 18 mg/kg in sour cream, 10 – 19 mg/kg in buttermilk, 8 – 23 mg/kg in kefir, and 2 – 18 mg/kg in cottage cheese (Sieber et al., 1995). Benzoic acid concentrations were 12 – 13 mg/kg in whey and 23 – 75 mg/kg in whey powder (Sieber et al., 1995), but bleaching conditions were unknown. In addition, benzoic acid occurs naturally in many other foods such as nuts, fruits, and vegetables (Sieber et al., 1995).

![Breakdown of benzoyl peroxide to two molecules of benzoic acid](image)

Figure 3. Breakdown of benzoyl peroxide to two molecules of benzoic acid (Smith, 2008).

Benzoic acid has been reported to result in adverse health effects, such as skin and eye irritation, asthma, urticaria, metabolic acidosis, and convulsions (WHO, 2000; Tfouni and Toledo, 2002; Qi et al., 2009; Lino and Pena, 2010). Many Asian and European countries consider benzoic acid and other breakdown products of benzoyl peroxide harmful (USDEC, 2009; Smith, 2008; Kang et al., 2010). Benzoic acid levels in dried whey ingredients are therefore a concern in China (Dairy Management Inc., 2009) and there have been some problems with exporting products with benzoic acid residues in them. Many European countries and China have regulated the maximum level of benzoic
acid in many products (EC, 1995; State Bureau for Quality Supervision, Inspection and Quarantine, 1996). The European Commission (EC) has allowed the use of benzoyl peroxide as a bleaching agent; however, the regulation per country provisions may vary (Kang et al., 2010). Taiwan also has permitted the use of benzoyl peroxide as bleaching agent in whey powder after a petition submitted by the USDEC was accepted by the Taiwan Department of Health, Food Sanitation and Safety in 1999 (Johnson, 2006). Chinese authorities, on the other hand, have banned the use of benzoyl peroxide as a bleaching agent and do not allow the presence of benzoic acid in whey products (USDEC, 2009; McKnight, 2010). Currently, USDEC has requested China’s Ministry of Health to permit benzoyl peroxide usage as a bleaching agent (McKnight, 2010). China’s Ministry of Health is currently in the process of developing new standards for whey protein concentrate (WPC) and whey protein isolate (WPI), and the permitted level of benzoic acid has not been determined (NMPF and USDEC, 2009; McKnight, 2010).

Methods for benzoic acid testing in whey powder involve reconstitution of the powdered sample, precipitation of fats and proteins using zinc acetate and potassium hexacyanoferrate(II) trihydrate, filtration of the sample using a 0.45 µm membrane, and analysis using a C-18 HPLC column (ISO, 2008; Qi et al., 2009). High performance liquid chromatography (HPLC) is the most common separation technique used to quantify benzoic acid since it has a high sensitivity, and it only requires minimum sample preparation and no sample derivatization is needed (WHO, 2000). Benzoic acid is detected using UV detector set at 230 nm, with the limit of detection (LOD) of 0.2 mg/kg.
for pasteurized and UHT milk and 2 mg/kg for milk powder and infant formula (Qi et al.,
2009) and 1.2 ng/kg (Wen et al., 2007).

In experimental animals and humans, ingested benzoyl peroxide is degraded to
benzoic acid in the intestine (WHO, 2000; JECFA, 2004b). The benzoyl peroxide
absorbed, will be metabolized to benzoic acid in the liver (WHO, 2000; JECFA, 2004b).
Benzoic acid is then excreted through urine in the form of benzoate or hippuric acid
(WHO, 2000; JECFA, 2004b). Benzoyl peroxide may cause skin irritation and
sensitization, however, it is not carcinogenic and it has a low acute toxicity (WHO, 2000;
JECFA, 2004b). The total daily exposure to benzoic acid in the U.S. is 0.01 mg/kg body
weight (JECFA, 2004a) and 0.18 – 2.3 mg/kg body weight of benzoate in several
countries (WHO, 2000). This number is about 2 – 28 times lower than the acceptable
dietary intake (ADI) (5 mg/kg body weight) (WHO, 2000). According to JECFA
(2004b) the adverse reactions to benzoic acid-related compounds are rare, and life-
threatening reactions are extremely rare. The ADI for benzoic acid and its calcium,
potassium and sodium salts, benzyl acetate, benzyl alcohol, benzaldehyde and benzyl
benzoate was established at 0 – 5 mg/kg of body weight (EC, 1995; JECFA, 2004b).

Whey Flavor

Besides a lack of color, a bland flavor in whey ingredients is also desirable. Dried
whey proteins contain lipid oxidation compounds, including aldehydes, ketones, and free
fatty acids, that are often found as the common sources of off-flavors (Carunchia
Whetstine et al., 2005; Croissant et al., 2009; Wright et al., 2009). Studies have linked
several factors and processing steps such as whey source, starter culture used, storage, and bleaching to the generation of off-flavors in whey products (Mahajan et al., 2004; Carunchia Whetstine et al., 2005; Gallardo-Escamilla et al., 2005; Wright et al., 2006; 2008; Croissant et al., 2009; Liaw et al., 2010; Whitson et al., in press). In addition, phospholipids that are present in milk fat globule membrane (MFGF) may also contribute to the off-flavors due to oxidation (Szuhaj and Sipos, 1989). Studies show that off-flavors in the ingredients may carry through to the finished products and limit food applications (Drake, 2006; Drake et al., 2009; Wright et al., 2009). Descriptive sensory analysis and instrumental volatile analysis are often used as techniques to pinpoint specific compounds that are responsible for the flavors in the whey products.

Descriptive sensory analysis

Descriptive sensory analysis is a sophisticated analytical sensory tool that utilizes a group of trained panelists as an instrument to identify sensory attributes of a product (Murray et al., 2001; Drake and Civille, 2003; Meilgaard et al., 2007; Lawless and Heymann, 2010). Sensory attributes that are profiled using descriptive analysis are appearance, aroma, flavor, and texture (Meilgaard et al., 2007). In order to perform descriptive sensory analysis, a sensory language (lexicon) and intensity scale are needed. Flavor lexicons are sets of words used to document and describe the flavor of a product (Drake and Civille, 2003). A clearly defined and anchored lexicon is used to train and calibrate the panelists and serves to interface descriptive analysis results with consumer acceptance and instrumental analysis (Drake and Civille, 2003). Drake et al. (2003)
developed a flavor lexicon for milk powders and dried dairy ingredients. In addition, Russell et al. (2006) developed flavor lexicons for whey and soy proteins including WPC80, WPI, SPC70, and SPI. In whey proteins, sweet aromatic, cooked/milky, doughy/fatty/oxidized, cucumber, brothy, cardboard/wet paper, animal/wet dog, pasta water, soapy, cabbage, and metallic flavors, bitter taste and astringency have been documented in previous studies (Carunchia Whetstine et al., 2003, 2005; Wright et al., 2006; Croissant et al., 2009; Liaw et al., 2010; Campbell et al., 2011). Cardboard is a common off-flavor present in whey proteins (Whitson et al., 2010). This flavor is associated with lipid oxidation products and increases during storage (Tomaino et al., 2004). Intensity scales used in descriptive analysis include category, line, and magnitude estimation scales (Meilgaard et al., 2007). Category scales are the most common scale used in sensory analysis, which include 0 to 10 and 0 to 15 point scales used for descriptive analysis and the 9-point hedonic scale used for consumer acceptance testing. Line scales use 6 in or 15 cm lines instead of numbers. This scale is purportedly used to reduce the central tendency habit that often happens with category scales. Magnitude estimation scales allow the panelists to choose the first number freely and the subsequent numbers are assigned in proportion (Meilgaard et al., 2007).

**Instrumental volatile analysis**

In order to identify volatile flavor compounds instrumentally, gas chromatography (GC) is often used. Since some volatile compounds found in foods are usually present at very low concentrations, sample preparation in GC, including isolation of volatiles from
foods using either headspace analysis, distillation, preparative chromatography (solid-phase extraction), or extraction (liquid-liquid) is usually required (Qian et al., 2010). The gas chromatograph consists of a gas supply and regulators, injection port, column and column oven, detector, electronics, and a data recording and processing system (Qian et al., 2010). The mobile phase in GC is gas and the stationary phase is either immobilized liquid or solid (Ismail and Nielsen, 2010). In GC, the sample is volatilized, loaded onto the head of the column, and is moved through the column by the mobile phase under a controlled temperature gradient (Ismail and Nielsen, 2010). In the column, the volatile compound mixtures are separated based on their boiling points, molecular sizes, and polarity (Ismail and Nielsen, 2010). Compounds eluting from a GC column have a specific retention time (RT) for a given column. However, since retention indices (RIs) are more stable and comparable across time, instrument, and location than RT, RI is used to normalize RT by comparing it to an alkane series (Van den Dool and Kratz, 1963).

In identifying flavor compounds, GC is coupled with a detector, such as the human nose in gas chromatography-olfactometry (GC-O) or a mass spectrometer (MS). Gas chromatography-olfactometry (GC-O) is a technique which combines a human nose as a detector (olfactometry) with the gas chromatographic (GC) separation of volatiles to access odor activity of specific separated compounds (Friedrich and Acree, 1998). Using GC-O, one can determine the odor activity of volatile compounds in a sample extract (Delahunty et al., 2006). In GC-O, once the sample is injected to the column, chromatographic separation occurs, and the column effluent is split between a physical detector such as a flame ionization detector (FID) and the human nose detector at the
sniffer port (Friedrich and Acree, 1998; Reineccius, 2006). A chromatogram is established by the physical detector and the human detector sniffs the compounds eluted in order to detect, measure the duration, and describe the qualities and quantities of the odor-active compounds via the sniffer port (Delahunty et al., 2006; Croissant et al., 2011). There are different methods that are used to determine the odor active compounds in the sample extract including detection frequency, dilution to threshold, and direct intensity (van Ruth, 2001; Delahunty et al., 2006). In detection frequency, a group of panelists sniff the same sample extract and the number of panelists that detect the odor active compounds (frequency) and their duration are recorded (van Ruth, 2001; Delahunty, 2006). From this, an “aromagram” is constructed with % frequency as the height and % frequency x duration as the area (Pollien et al., 1997). The peak height is called nasal impact frequency (NIF) and the area is called surface of nasal impact frequency (SNIF) (Pollien et al., 1997). The advantage of using this method is that not much training is required for the panelists because of its simplicity (Delahunty et al., 2006). The limitation of this method is that it assumes that the actual odor intensity is related to the detection frequency (van Ruth and O’Connor, 2001). In dilution to threshold, a sample extract is serially diluted until no odor is detected (van Ruth, 2001). Included in this method are CharmAnalysis™ (Acree et al., 1984) and aroma extraction dilution analysis (AEDA) (Ulrich and Grosch, 1987). In CharmAnalysis™, besides the dilution factor, the duration of the odors is also recorded to generate peaks. The peak areas are then utilized to generate Charm values (Acree, 1993). In AEDA, flavor dilution factors are calculated based on the maximum dilution of the sample extract that an odor
can be perceived (Ulrich and Grosch, 1987; Grosch, 1993). The drawbacks of dilution methods are that they are very time consuming and they assume that all compounds have the same response slopes as the concentrations increase (van Ruth, 2001). Direct intensity requires the panelists to record the intensity of the compounds as they elute (Delahunty et al., 2006). Post peak intensity and Osme are included in this method. In post peak intensity, panelists record the maximum odor intensity as the compound elutes (van Ruth, 2001). In Osme, using a 16-point scale from 0 (none) to 15 (extreme), the panelists rate the intensity and the duration of odor perceived using a time-intensity device (Miranda-Lopez et al., 1992). An osmogram is built based on the odor intensity (peak height) and its duration (peak width) to determine the total response (peak area) (da Silva et al., 1994). The advantage of utilizing GC-O is the use of the human nose may detect the aroma of compounds at a concentration below instrumental detection, which aid in compound identity and determination of aroma quality and its significance to the flavor of food (Mistry et al. 1997; Croissant et al., 2011). However, human responses are subject to inconsistencies. Therefore, panel training, use of at least two sniffers, and sniffing on at least two different columns are necessary to reduce these inconsistencies (Mistry et al., 1997; Croissant et al., 2011).

Gas chromatography-mass spectrometry (GC-MS) is capable of separating compounds mixtures and identifying them and also provides quantitative and qualitative information on the amount and chemical structure of each compound (McMaster, 2008). Mass spectrometry uses an electrostatic field to ionize and resolve the volatile compounds based on mass-to-charge ratios (Smith and Thakur, 2010).
separated compounds flow through the detector cell in the order of their separation (McMaster, 2008). Since each compound breaks down into a unique and predictable ion pattern, the presence of particular spectra in a sample chromatogram is evidence of the presence of that compound in a sample. In addition to the RIs, mass spectra are compared to a computer database and also to spectra of authentic standards for precise identification of compounds (Smith and Thakur, 2010). There are two modes that can be used to detect the ions present: scan mode and selective ion monitoring (SIM) mode. In scan mode, the electrodes focus all of the ions to the detector, and the detector detects all ions present. In SIM mode, the detector is only looking for certain mass to charge ratios \((m/z)\), and therefore sensitivity is increased (McMaster, 2008).

Instrumental analysis of liquid whey, WPC, and WPI showed that 2,3-butanedione (butter), acetic acid (sour), methional (boiled potato), 2-acetyl-1-pyrroline (popcorn), 2-pentylfuran (green bean, butter), 2- and 3-methylbutanal (cocoa, almond), 1-octen-3-one (mushroom, metal), dimethylsulfide (cabbage, sulfur), pentanal (malt), hexanal (grassy), Z-4-heptenal (fatty), heptanal (fatty), octanal (fatty, soapy), nonanal (fatty), decanal (soapy, tallow), (E,E)-2,4-nonadienal (fatty) and (E,E)-2,4-decadienal (fryer oil) are the major players in whey protein flavor (Carunchia Whetstine et al., 2003, 2005; Mahajan et al., 2004; Tomaino et al., 2004; Gallardo-Escamilla et al., 2005; Mortenson et al., 2008; Croissant et al., 2009; Evans et al., 2009, 2010; Liaw et al., 2010; Campbell et al., 2011; Campbell et al., in press). Hexanal, heptanal, and octanal are common aldehydes lipid oxidation products found in whey (Tomaino et al., 2004).
Lipid oxidation

Lipid oxidation is one of the major concerns in food industries. Lipid oxidation causes deterioration in foods, including the development of rancid flavor and decrease of nutritional quality (Frankel, 1998). In addition, the oxidation of lipids leads to the formation of peroxides, which are susceptible to further decomposition to secondary oxidation byproducts, such as short-chain aldehydes and ketones (Wong and Kitts, 2003). These compounds will adversely affect flavor, taste, nutritional value, and overall quality of the foods (Vercellotti et al., 1992). Oxidative rancidity involves the presence of oxygen and lipid materials. The reaction is called autoxidation (Belitz et al., 2009).

Autoxidation (peroxidation) of lipids proceeds by a free radical chain reaction, which involves 3 steps: initiation, propagation, and termination (Frankel, 1998). In the initiation step, the unsaturated lipids (LH) form lipid free radicals (L·) by losing a hydrogen radical (H·) in the presence of initiators. The initiators act as catalysts which can produce radicals by different mechanisms, such as thermal dissociation of hydroperoxide, metal catalysis, or exposure to light (Frankel, 1998). In propagation, peroxyl radicals (LOO·) are formed from lipid radicals (L·) and molecular oxygen. Hydrogen abstraction reaction is selective to the most weakly bound hydrogen (Frankel, 1998). Therefore, this reaction occurs around the fatty acid double bonds in unsaturated fatty acids, creating more free radical species (Nawar, 1996). In termination, the peroxyl radicals (LOO·) react with each other to form non-radical products (Frankel, 1998). This lipid oxidation mechanism is shown in Figure 4. Hydroperoxides formed from the oxidation of long unsaturated fatty acids are unstable, but they are odorless and tasteless (Belitz et al., 2009). However,
once they further decompose to secondary products, including aldehydes and ketones, they can give negative impacts on flavor and quality of the foods (Wong and Kitts, 2003).

\[ \text{Initiation: } \text{LH} \xrightarrow{R_i} \text{IH} + \text{L}^- \]

\[ \text{Propagation: } \text{L}^- + \text{O}_2 \xrightarrow{k_p} \text{LOO}^- \]

\[ \text{LOO}^- + \text{LH} \xrightarrow{k_p} \text{LOOH} + \text{L}^- \]

\[ \text{Termination: } \text{LOO}^- + \text{LOO}^- \xrightarrow{k_t} \text{non-radical products} \]

Figure 4. Lipid oxidation mechanism (Frankel, 1998).

**Phospholipids**

Phospholipids are polar lipids that have amphiphilic characteristic with one or two hydrophobic tails and hydrophilic head groups (Rombaut et al., 2006; Rombaut and Dewettinck, 2006). Phospholipids consist of two fatty acids esterified at the first and second positions (sn-1 and sn-2 positions respectively) on a glycerol backbone which make hydrophobic tails and a phosphoric acid at the third position through a phosphate ester bond (Ophardt, 2003; Rombaut and Dewettinck, 2006). In addition, an organic group, such as choline, ethanolamine or serine, is usually attached to the phosphate through another phosphate ester bond (Pokorný, 2002). The fatty acid attached on the sn-1 position is usually more saturated than that on the sn-2 position (Rombaut and Dewettinck, 2006). A polyunsaturated fatty acid (PUFA) is usually positioned on the sn-2 position of the glycerol backbone (Christie, 2010). A general phospholipid structure is shown in Figure 5. The negatively charged phosphate group (phospholipid head) gives a
hydrophilic end to the phospholipid, while the long hydrocarbon fatty acids (phospholipid tail) give a hydrophobic characteristic to the other end. This amphiphilic property makes phospholipids play important roles in lipid bilayers of cell membranes in human, animal and plant tissues (Weihrauch and Son, 1983; Campbell et al., 2006).

Figure 5. General phospholipid structure. Both R groups represent fatty acids and the X group represents the organic group such as choline, ethanolamine, or serine group (Pokorný, 2002).

The synthesis of phospholipids in plants and animals starts at the smooth endoplasmic reticulum of a cell (Champe and Harvey, 2007). The phospholipids are transferred to the Golgi apparatus and finally to membranes of organelles, plasma membrane, or they undergo exocytosis from the cell (Champe and Harvey, 2007). Polar lipids, including phospholipids and sphingolipids in milk are located in and comprise about one-third portion of the milk fat globule membrane (MFGM) (Fox and McSweeney, 1998; Rombaut et al., 2005; Rombaut and Dewettinck, 2006). The milk fat globule membrane (MFGM) stabilizes the milk fat globules in the serum phase of milk
and prevents enzymatic degradation of milk fat globules by the endogenous lipases (Danthine et al., 2000; Rombaut et al., 2005). The four major phospholipids found in dairy products are phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC) (Rombaut et al., 2005). The structures on these phospholipids are shown in Figure 6. PE, PI, and PS are concentrated on the inner surface of MFGM, while PC is concentrated in the outside surface of the MFGM (Deeth, 1997). The amount of phospholipids in the MFGM makes dairy products a good source of these phospholipids (Vesper et al., 1999).

![Figure 6. Common phospholipids found in milk fat globule membrane (MFGM)](image)

Types of phospholipids in milk fat globule membrane (MFGM)

Phosphatidylethanolamine is one of the most abundant phospholipids found in the eukaryotic cells (Champe and Harvey, 2007). It is found in liver and brain cells and mostly in the mitochondria compared to other organelles (Christie, 2010). Therefore, it is
the key to generate the building block of membrane bilayers (Christie, 2010). Phosphatidylinositol is an important membrane component that participates in the metabolism of plants and animals (Christie, 2010). Phosphatidylinositol is present in all cell types and tissues, but mostly in the brain (Christie, 2010). Phosphatidylserine is distributed among all cell types and is less than 10% of the total phospholipids. The most abundant PS is located in the myelin of the brain tissue (Christie, 2010). Phosphatidylcholine is commonly the most abundant phospholipid in animals and plants, which like PE, it also is the key building block to the generation of cell membranes (Christie, 2010).

The phospholipid content of the total bovine milk lipids is about 1% (Bitman and Wood, 1990). About 60% of these phospholipids are found on the MFGM (Gallier et al., 2010). Since most phospholipids are located on the MFGM, fat rich products are rich in polar lipids (Rombaut et al., 2005). Through processing (heating, homogenization, aeration, and agitation), however, some MFGM are ruptured and phospholipids are released to the aqueous phase (Michalski et al., 2006; Rombaut et al., 2006; Rombaut and Dewettinck, 2006; Vandergem et al., 2010). Therefore, low fat products such as skim milk, buttermilk, butterserum, and whey also become rich in polar lipids (Christie et al., 1987; Rombaut et al., 2006; Rombaut and Dewettinck, 2006; Singh, 2006; Rombaut et al., 2007; Belitz et al., 2009). Rombaut and Dewettinck (2006) have documented that fat rich products such as cream, butter, and cheese have less than 1% w/w of polar lipids in total lipids, whereas in products like skim milk, buttermilk, butterserum, and whey, the polar lipids content is much higher. Phospholipids contents (g/100g of total lipids)
reported by Rombaut and Dewettinck (2006) were 0.35 – 0.86 in cream, 0.20 – 0.27 in butter, 0.47 in Cheddar cheese, 19.06 in skim milk, 21.66 – 33.05 in buttermilk, 14.8 – 48.39 in butterserum, and 5.32 in Cheddar whey.

**Functions of phospholipids and their roles in foods**

Phospholipids play a major role in cell membranes. They are able to interact with metabolites, ions, hormones, antibodies and other cells (Weihrauch and Son, 1983). Phospholipids also act as emulsifiers in living tissues, where they are bound to lipoproteins, and help to transfer nonpolar lipids in blood and other intercellular fluids (Pokorný, 2002). Phospholipids are also found in blood platelets and help the blood clotting process (Ophardt, 2003). In addition, phospholipids are able to reduce blood cholesterol levels and enhance brain function (Pepeu et al., 1996; Eckhardt et al., 2002; Spitsberg, 2005).

Some food products that contain phospholipids include egg yolk, organ meats, lean meats, fish, shellfish, cereal grains, oilseeds, soybean, and some dairy products such as milk, cream, butter, cheese and whey (Weihrauch and Son, 1983; Rombaut and Dewettinck, 2006; Sánchez-Juanes et al., 2009). Phospholipid content in foods varies. Egg yolks contain 23% phospholipids and other polar lipids out of the total lipids (Kuksis, 1985). However, oilseeds contain only between 1 to 3% phospholipids of the total lipids (Pokorný, 2002). Bovine milk phospholipids play a dominant role in lipid metabolism, cell proliferation, and maturation of intestinal cells in infants (Miura et al., 2004). Therefore, milk phospholipids have been used in food and nutrition supplements...
for infants and adults (Sánchez-Juanes et al., 2009). In food systems, phospholipids are used for their antioxidant, bacteriostatic, and their functional and emulsifying properties due to surfactant properties (Saito and Ishihara, 1997; Gunstone, 2001; Schneider, 2001; Sprong et al., 2002). As emulsification and wetting agents, phospholipids are used widely in a variety of foods including margarines, mayonnaises, chocolates, instant products, and pharmaceuticals (Szuhaj, 1983; Vanhoutte et al., 2004).

**Phospholipids analysis in foods**

Determination of phospholipids in foods includes extraction, separation, and instrumental analysis (Rombaut et al., 2005). In order to extract lipids in the organic phase, lipid-protein complexes are broken down using a polar solvent (Van der Meeren and Vanderdeelen, 2000). Cold-extraction methods using chloroform-methanol are preferred to acid or base extractions since acids and bases may cause oxidation and hydrolysis of phospholipids (Boyd et al., 1999; Rombaut et al., 2005; Braun et al., 2010; Lee et al., 2010). Phospholipids are then subjected to fractionation and pre-concentration using solid phase extraction (SPE) as a rapid and efficient technique (Van der Meeren and Vanderdeelen, 2000; Rombaut et al., 2005). Some separation methods that can be used to separate phospholipids are P-NMR, HPLC, TLC, and FTIR (Rombaut et al., 2005). HPLC is the most common method used to separate phospholipids since it is able to determine phospholipids quantitatively and qualitatively and it is less costly than P-NMR (Vaghela and Killara, 1995; Van der Meeren and Vanderdeelen, 2000; Vanhoutte et al., 2004; Rombaut et al., 2005; Braun et al., 2010). Evaporative light scattering
detection (ELSD) is commonly used as a method for phospholipids detection coupled with HPLC (Vaghela and Kilara, 1995; Van der Meeren and Vanderdeelen, 2000; Rombaut et al., 2005). The amount of scattered light detected by a photodiode is directly related to the quantity of the analyte (Rombaut et al., 2005). Some dairy products, including raw and pasteurized milk, cream, condensed milk, butter, buttermilk, butterserum yogurt, kefir, various cheeses (Ricotta, Quark, Cottage, Mozzarella, Gouda, Cheddar, Camembert, Emmenthal, Parmigiano), and Mozzarella and Cheddar wheys have been analyzed for their phospholipids content (Rombaut et al., 2005; Rombaut and Dewettinck, 2006; Rombaut et al., 2007). From studies by Rombaut and Dewettinck (2006), butterserum (the aqueous phase of butter) was known to have the highest polar lipids content, which was 28.4% of the original raw milk polar lipids. Besides butterserum, buttermilk also has high phospholipids content in comparison to milk due to its high content in MFGM (Mulder and Walstra, 1974). Elling et al. (1996) reported that buttermilk has 7 times more phospholipids compared to whole milk (0.89 mg/g vs. 0.12 mg/g, respectively). In addition, Christie et al. (1987) reported a 4-fold increase of phospholipids in buttermilk compared to whole milk (0.72 mg/mL vs. 0.15 mg/mL, respectively).

Roles of phospholipids in flavor

Phospholipids have been documented to impact flavor of foods as pro-oxidants as well as antioxidants (Szuhaj and Sipos, 1989). Phospholipids have the potential to contribute to the generation of off-flavors and off-colors in foods through oxidation.
(Sessa, 1985). Some factors contributing to the oxidation of phospholipids are the amount of polyunsaturated fatty acids (PUFA) and the degree of unsaturation (Corliss and Dugan, 1970). Phospholipids that contain unsaturated fatty acids are subjected to oxidation and therefore to oxidized flavor (Bodyfelt et al., 1988). Xanthine oxidase, as a major component of MFGM acts as a catalyst in lipid oxidation (Fox et al., 2000; Fox and Kelly, 2006). Hydrolysis of fatty acid ester bonds of phospholipids by phospholipases produces free fatty acids which generate rancid flavors (Szuhaj and Sipos, 1989). In addition to the phospholipases, heating and storage contribute to the oxidation of phospholipids (Mielche and Bertelsen, 1995; Frankel, 1998; Rahman and Velez-Ruiz, 2007; Erickson, 2008). The end product of this lipid oxidation is carbonyls, which cause flavor defects (Fox and Kelly, 2006).

Studies conducted by Spill et al. (2002) suggested that high heat storage and storage time contributed to oxidized, cardboard flavors, and astringent mouthfeel in buttermilk. Other than oxidized flavor, metallic flavor is also developed during buttermilk storage. The key flavor compound that is responsible for this metallic flavor is (E,Z)-2,6-nonadienol (Heiler and Schieberle, 1997b). The mechanism of metallic flavor development in buttermilk was described in Heiler and Schieberle (1997a).

During cream fermentation, prior to buttermaking process, oxygenases from the starter cultures peroxidize \( \alpha \)-linolenic acid, in the neutral triacylglycerides, resulting in glycerol-bound 9-hydroperoxy-10,12,15-octadecatrienoate. In the process of buttermaking, this polar hydroperoxide is transferred into the buttermilk and then it is degraded into (E,Z)-2,6-nonadienal by acid catalysis. During storage, the dienal is reduced by the reductase
from the starter cultures, which are still active during storage, to the off-odorant (E,Z)-
2,6-nonadienol (Heiler and Schieberle, 1997a).

Others, however, have reported that phospholipids have synergistic antioxidant
capability in the presence of other antioxidants such as tocopherols and flavonoids
(Hudson and Mahgoub, 1981). The main source of the antioxidant property of
phospholipids is the basic amine groups which decompose the hydroperoxides to alcohols
by means of electron donation (Saito and Ishihara, 1997).

**Whey Processing**

The first step in whey processing is clarification. In clarification, fluid whey is
separated from cheese fines by centrifugal motion (USDEC, 2004). The next step is to
run the fluid whey through a separator to separate the fat. This fat is called whey cream.
This fat could be oxidized and cause off flavor in the finished product. After undergoing
clarification and fat separation, the fluid whey is then pasteurized. The common
time/temperature combination for whey pasteurization that is used in the industry is
72°C/15 sec. This step is necessary to eliminate any pathogens and/or bacteria that might
be present in the whey and also to inactivate the starter culture bacteria used in cheese
making (Spreer, 1998). After the removal of cheese fines and the fat, and after
pasteurization, the fluid whey is ready to be converted to the desired whey products
through ultrafiltration (UF) and spray drying processes.

Some filtration techniques using membrane technology that have been used in the
dairy industry are microfiltration, ultrafiltration, nanofiltration, and reverse osmosis (RO)
(Marcello and Rizvi, 2008). These filtration techniques are used to separate components based on their sizes (Henning, 2006). Microfiltration is used to separate suspended particles in the micron range, while ultrafiltration retains both macromolecules like proteins and suspended particles. Nanofiltration retains those retained by both microfiltration and ultrafiltration in addition to sugars, divalent salts, and dissociated acids. Reverse osmosis on the other hand, retains everything except the solvent such as water (Cheryan, 1998). The diagram for membrane separations is shown in Figure 7.

Figure 7. Membrane separation technologies (Henning et al., 2006).

In whey processing, ultrafiltration (UF) is commonly practiced. The ultrafiltration process is used to separate components by their sizes, shapes, charges, and affinity to the membrane (Bastian et al., 1991; Marcello and Rizvi, 2008). This process
has been used in the dairy industry since the 1970s as a technique to separate whey protein to high protein retentate and lactose containing permeate (Atra et al., 2005; Marcello and Rizvi, 2008). In order to manufacture whey protein concentrate 80% protein (WPC80), following UF, diafiltration (DF) is conducted. In DF, deionized water is added to the retentate to remove the residual lactose and minerals (Morr and Ha, 1993). This DF step is added to purify the UF retentate to the level of protein desired (Marcello and Rizvi, 2008). After the whey is run through the UF/DF step, it is then spray dried to make a dried whey product. Spray drying has commonly been used as a drying method since the early 1900s (Masters, 1997). In spray drying, the concentrated whey is pumped to the drying chamber where it is atomized and mixed with hot air to create a dried whey product (Henning et al., 2006).

**Whey Protein Functionality**

Protein functionality is the ability of protein to provide various functions in food quality and stability, including solubility, gelation, and surface properties (emulsions and foams) (Morr and Ha, 1993; Foegeding et al., 2002; Davis et al., 2007). Physicochemical properties of protein, such as molecular weight, protein structure, electrostatic charge, hydrophobicity, hydrophilicity, pH, temperature, and ion composition affect protein solubility (Morr and Ha, 1993). In terms of pH, native whey is soluble at its pI (pH 4.6), however, once the protein is heat-denatured, it becomes insoluble at its pI (Morr and Ha, 1993). This property is often used to indicate protein denaturation.
Another functionality of whey protein is its ability to form gels. The first step of gel formation is when the hydrogen and disulfide bonds are broken, disrupting the native protein structure, and causing protein denaturation by heat. Polymerization between the denatured proteins causes them to aggregate and form an elastic network. Cooling will then allow the protein to continue polymerization and form more hydrogen bonds with water, which then create a gel structure (Morr and Ha, 1993; Foegeding et al., 2002).

Some factors that affect protein gelling are protein composition, environmental conditions (pH and ionic strength), and processing conditions (rate of heating and cooling) (Hines and Foegeding, 1993; Morr and Ha, 1993; Aguilara, 1995; Turgeon and Beaulieu, 2001).

Surface properties of whey protein include emulsions and foams. Emulsions are dispersion of at least one immiscible liquid in another in a colloidal system (Yoshida and Antunes, 2004). In an emulsion system, emulsifying agents or surfactants are needed to lower the surface tension between the interface of a protein and lipid (Baldwin et al., 1997). Whey protein, which is known to have surface properties, can act as an emulsifier in many food products. Factors that affected emulsion stability were film characteristics, viscosity, electrical charge and size of the fat globules (Morr and Ha, 1993; Pérez-Gago and Krochta, 1999).

Foam is generated by dispersing air through whipping or blending into a solution of protein, emulsifier, and polysaccharide food stabilizer gum (Morr and Ha, 1993). Foaming in whey protein concentrate (WPC) is usually measured as overrun and foam stability (Campbell and Mougeot, 1999; Pernell et al., 2000). In foam film formation,
protein functions to lower the interfacial tension by partially unfolding and interacting with the foam cell interface via intermolecular bonding to form a cohesive film (Morr and Ha, 1993). One way to modify foam yield stress is to increase the ratio of β-lactoglobulin to α-lactalbumin or by hydrolysis (Luck et al., 2002).

**Application of Whey Protein**

Whey protein has been used in food products, not only for its high nutrition content, but also as a functional food ingredient due to its ability to reduce the risk of cardiovascular disease, increase muscle strength, and act as antimicrobial (Marshall, 2004). Therefore, whey protein has been used in many pharmaceutical and food products, ranging from infant formula to frozen foods (Morr and Ha, 1993; de Wit, 1998). The growing applications of whey proteins include sports and nutrition bars and beverages (Beucler et al., 2005; Childs and Drake, 2010; Koski, 2010). Besides containing protein, lactose, and minerals, whey is low in calories and contains a very low amount of fat which makes whey proteins a suitable ingredient in low-calorie, dietary-restricted meals, or athletic protein weight gain products (Kosikowski, 1979; Sienkiewicz, 1990; Dahm, 2005; ADPI, 2010a). The gelling property of whey proteins has been used in meat and bakery products (Morr and Ha, 1993). In addition, whey proteins have been used as thickeners and gelling agents to give the desired texture to yogurt (Mleko, 1999). Other whey proteins applications as a functional food ingredient are as a fat substitute, an egg replacer, a stabilizer, and a texturizer (Morr and Ha, 1993).
Surface active properties (emulsion and foaming) of whey proteins give them the ability to act as surfactants and to be used in many food products to give unique textures and sensations (Campbell and Mougeot, 1999). Vegetable oil-based emulsions use whey proteins as emulsifiers (Huang et al., 1996; Agboola et al., 1998; Singh and Dalgleish, 1998). Whey protein also can create oxygen barrier films with good mechanical properties (Chen, 1995; Yoshida and Antunes, 2004). Examples of food products that use whey protein foaming properties range from soufflés, dressings, and frothed drinks such as beer (light and airy texture) to bread and ice cream (dense and solid texture) (Bryant and Mc Clements, 1998; Campbell and Mougeot, 1999).

The use of whey protein as an ingredient is increasing. Therefore the demand for bland tasting and colorless dried whey ingredients is also in the rise. However, the addition of bleaching agents negatively impact the flavor profile of whey products, and therefore additional studies on bleaching parameters and their influence on whey flavor are needed. In addition, studies on benzoic acid residues in whey products are also needed given the current international concern. The objectives of this study were to compare the effects of bleaching parameters, including point of bleaching (before vs. after fat separation), temperature (68°C vs. 4°C), and bleaching agent (HP vs. BP), on bleaching efficacy and volatile compounds in liquid Cheddar whey and also to determine the impact of hydrogen peroxide or benzoyl peroxide bleaching on flavor of whey protein concentrate 34% protein and to evaluate residual benzoic acid in commercial and experimental whey protein concentrates manufactured with and without BP bleaching.
REFERENCES


Smith, K. 2008. Bleaching. Wisconsin Center for Dairy Research, Madison, WI.


U.S. FDA. 2010c. 21 CFR 133.113: Cheddar cheese.  


CHAPTER 2: IMPACT OF FAT SEPARATION, TEMPERATURE, AND BLEACHING AGENT ON BLEACHING OF LIQUID CHEDDAR WHEY
Impact of fat separation, temperature, and bleaching agent on bleaching of liquid Cheddar whey

M. A. D. Listiyani,* R. E. Campbell,* R. E. Miracle,* D. M. Barbano,† P. D. Gerard,‡ and M. A. Drake*

*Southeast Dairy Foods Research Center, Department of Food, Bioprocessing & Nutrition Sciences, North Carolina State University, Raleigh, NC 27695
†Northeast Dairy Foods Research Center, Department of Food Science, Cornell University, Ithaca, NY 14853
‡Department of Applied Economics and Statistics, Clemson University, Clemson, SC 29634

To be submitted for publication into the Journal of Dairy Science

*Corresponding author:
MaryAnne Drake
E-mail: mdrake@unity.ncsu.edu
Phone: 919-513-4598
Fax: 919-515-7124
ABSTRACT

The use of whey protein as an ingredient in foods and beverages is increasing, and thus demands for colorless and mild tasting whey protein are rising. Bleaching is commonly applied to fluid colored cheese whey to decrease color, and different temperatures and bleach concentrations are used. The objectives of this study were to compare the effects of hot and cold bleaching, the point of bleaching (before and after fat separation), and bleaching agent on bleaching efficacy and volatile components of liquid colored and uncolored Cheddar whey. Liquid colored Cheddar whey was manufactured in triplicate and pasteurized. Part of the whey was collected (no separation, NSE) and the rest was subjected to fat separation (FSE). NSE and FSE whey were then subdivided and bleaching treatments (benzoyl peroxide (BP) 50 or 100 mg/kg and hydrogen peroxide (HP) 250 or 500 mg/kg) at 68°C for 30 min or 4°C for 16 h were applied. Control NSE and FSE with no added bleach were also subjected to each time temperature combination. Volatile compounds from wheys were evaluated by gas chromatography mass spectrometry (GC-MS) and norbixin (annatto) was extracted and quantified to compare bleaching efficacy. Proximate analysis, including total solids, protein, and fat content were also conducted. Liquid whey subjected to hot bleaching at both concentrations of HP or 100 mg/kg BP had higher lipid oxidation products (aldehydes) compared to unbleached wheys, 50 mg/kg BP hot-bleached whey, or cold-bleached wheys. No impact was detected between NSE and FSE liquid Cheddar whey on the relative abundance of volatile lipid oxidation products. Wheys bleached with BP had lower norbixin content.
compared to wheys bleached with HP. HP bleaching efficacy was decreased at 4°C compared to 68°C, BP bleaching efficacy was not impacted by temperature. These results suggest that no impact was detected between NSE and FSE liquid Cheddar whey on bleaching efficacy or lipid oxidation and that hot bleaching may result in increased lipid oxidation in fluid whey.

**Key words:** whey, bleach, norbixin, flavor
INTRODUCTION

Whey is a by-product from cheese making, but has become a valuable and versatile dried ingredient in recent years (Foegeding et al., 2002; Smithers, 2008). A bland and colorless dried ingredient is desired to minimize flavor carry through to finished products (Childs et al., 2007; Drake, 2006; Drake et al., 2009). Lipid oxidation products, such as aldehydes, ketones, and free fatty acids, are the common sources of off-flavors found in dried whey proteins (Carunchia Whetstine et al., 2005; Croissant et al., 2009; Wright et al., 2009). Several factors and processing steps contribute to off-flavors including starter culture selection, starter culture type, fluid storage, dried product storage, and bleaching (Croissant et al., 2009; Liaw et al., 2010; Whitson et al., in press).

In addition to bland flavor, colorless whey ingredients are also desirable. Therefore, bleaching is a common whey processing step (Kang et al., 2010). The yellow color in Cheddar whey primarily comes from added annatto. Annatto comes from seeds of the tropical tree fruit *Bixa orellana*, which are covered in a bright red pulp (Giuliano et al., 2003). The primary component of water-soluble annatto used in cheesemaking is the carotenoid norbixin (Kang et al., 2010). Two bleaching agents that are allowed for whey bleaching in the U.S. are hydrogen peroxide (maximum usage rate at 500 mg/kg) (US FDA, 2009b) and benzoyl peroxide (US FDA, 2009a). There is no current regulation for the maximum usage of benzoyl peroxide in whey bleaching. FAO/WHO has stated that whey treated with benzoyl peroxide at 100 mg/kg does not pose a safety concern.
In addition, CODEX has adopted the use of benzoyl peroxide in dried whey products and liquid whey in 2006 and 2007 respectively (CODEX, 2008).

Processors can employ hot or cold bleaching processes and may apply bleaching before or after fat separation of whey. Croissant et al. (2009) demonstrated that bleaching, bleaching agent, and bleach concentration influenced lipid oxidation and flavor of fluid whey and dried whey products under hot bleaching conditions. They also demonstrated that sensory and volatile compound profiles in fluid whey were indicative of sensory and volatile compound profiles in dried whey protein. These results demonstrated that bleaching trials could be conducted in fluid whey rather than dried whey proteins. Recent studies have not compared bleaching temperatures or fat separation of whey on bleaching efficacy or flavor of fluid whey. The objectives of this study were to compare the effects of bleaching agent, bleaching temperature, and the impact of point of bleaching (before or after fat separation) on bleaching efficacy and volatile compounds in liquid Cheddar whey.

MATERIALS AND METHODS

Experimental Overview

Two separate experiments were conducted to achieve the experimental objectives. In the first study, Cheddar whey was manufactured, pasteurized, fat-separated, and subjected to one of a range of hot or cold bleaching applications followed by measurement of residual norbixin and color by reflectance. In the second experiment,
bleaching treatments from the first experiment were selected and then incorporated into pasteurized Cheddar whey before or after fat separation followed by volatile compound analysis and measurement of residual norbixin. All experiments were conducted in triplicate.

**Experiment I – Bleaching of fluid Cheddar whey.** Raw whole milk was obtained from the Cornell University dairy plant. The milk was then pasteurized using a plate heat exchanger (model 080-S, AGC Engineering, Manassas, VA) at 72°C for 16 s. The pasteurized milk was cooled to 31°C and transferred to the cheese vat (model DLHD8SSS, Kusel Equipment Company, Watertown, WI). Starter culture *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* (911 DVS, Chr. Hansen Inc., Milwaukee, WI) was added at a rate of 0.1 g/kg. The milk was agitated for 5 min and ripened for 30 min. No annatto or double strength annatto (Chr. Hansen, Inc., Milwaukee, WI) at 0.066 mL/kg of milk was added. Double strength chymosin (Chymax Extra, Chr. Hansen, Inc., Milwaukee, WI) was added to coagulate the ripened milk at a rate of 0.1 mL/kg of milk for 30 min. The coagulum was then cut using 1.6 cm wire knives and healing process took place for 5 min. Cheese curds and whey was gently stirred for 10 min without heating, then the temperature was increased from 31°C to 33°C over 15 min and from 33°C to 38°C over an additional 15 min with continuous stirring. The whey was drained at pH 6.3 and pasteurized using a 3-section (regeneration, heating, and cooling) plate heat exchanger at 72°C for 16 s. The pasteurized whey was then cooled to 50°C and run through a hot bowl separator (model 619, DeLaval, Inc., Kansas,
MO) to separate the fat. After separation, some of the whey was subjected to bleaching at 68°C (hot bleaching) and some was cooled to 4°C before the bleaching process was applied (cold bleaching).

Bleaching agents used in this experiment were hydrogen peroxide (HP) (35%, w/v, Nelson Jameson, Inc., Marshfield, WI) at 50, 100, 250, and 500 mg/kg; benzoyl peroxide (BP) (32%, w/w, Oxylite Type XX, Nelson Jameson, Inc., Marshfield, WI) at 25, 50, 75, and 100 mg/kg. Each bleach treatment was applied to whey under hot and cold bleaching, and also to whey with and without annatto (Chr. Hansen, Inc., Milwaukee, WI at 0.066 mL/kg of milk). Unbleached liquid Cheddar whey with and without annatto was used as a control. Catalase (20 mg/kg, FoodPro CAT, Danisco, New Century, NJ) was added to the HP treated whey. All whey samples were cooled to 4°C, frozen and shipped by overnight carrier on ice packs to North Carolina State University (Raleigh, NC). Upon receipt, samples were thawed and subjected to norbixin extraction and measurement (described below). This experiment was replicated three times. Following data analysis, selected treatments from this experiment were then utilized in experiment II.

**Experiment II – Bleaching of fluid Cheddar whey before or after fat separation.**

Raw whole milk was obtained from the North Carolina State University Dairy Plant. Eighty seven kg of milk were vat-pasteurized (Model MPD1050, Micro Process Design, D & F Equipment Co, McLeansville, NC) at 63°C for 30 min, cooled, and held at 4°C overnight. Liquid whey was manufactured the following day. Pasteurized milk was
heated to 31°C in a cheese vat (model MX4, Kusel Equipment Company, Watertown, WI). Calcium chloride (50% w/v, Dairy Connection Inc., Madison, WI) at the rate of 0.39 mL/kg of milk and mesophilic lactic starter (Choozit MA 11, Danisco, New Century, NJ) at 50 DCU/454 kg of milk were added and the cheese was ripened for 60 min. After the first 30 min of ripening, 1:20 diluted double strength annatto (Chr. Hansen, Inc., Milwaukee, WI) was added at 0.066 mL/kg of milk. The milk was then coagulated for 30 min using 1:80 diluted rennet (Dairy Connection Inc., Madison, WI) at the rate of 0.09 mL/kg of milk at the end of the ripening period. The cheese curd was then cut using 0.95 cm knives and healed for 5 min. The curd was then stirred gently for 10 min and cooked by increasing the temperature to 39°C across 30 min. The pH and titratable acidity were monitored closely, and the liquid whey was drained at pH 6.4. The liquid Cheddar whey obtained was then pasteurized at 63°C for 30 min.

After pasteurization, half of the hot whey (ca. 22.7 L) was separated using a hot bowl separator (Westfalia Separator, C.A. De Fehr & Sons Ltd., Winnipeg, Manitoba, Canada) to obtain 19 L of fat separated whey (FSE). From the 19 L of fat separated whey, 9.5 L was cooled in a glycol-cooled ice water bath with occasional stirring to 12°C for cold bleaching. This process was repeated with pasteurized whey not subjected to fat separation yielding fat separated (FSE) and not separated whey (NSE) for hot or cold bleaching. For cooled FSE and NSE wheys, 300 mL was aliquoted into 500 mL media glass bottles (VWR International, West Chester, PA) and assigned to control (no bleach) or one of 4 different bleaching treatments. Before the bleaching agent was added, the whey was cooled to 4°C by immersion in an ice water bath. After the whey reached 4°C,
each bleaching agent was added to each bottle to yield control (no bleaching agent), BP (32%, w/w, Oxylite Type XX, Nelson Jameson, Inc., Marshfield, WI) 50 mg/kg, BP 100 mg/kg, HP (35%, w/v, Nelson Jameson, Inc., Marshfield, WI) 250 mg/kg, and HP 500 mg/kg, followed by a 16 h holding time. Catalase (FoodPro CAT, Danisco, New Century, NJ) at 20 mg/kg was added to all of the HP bleached whey following 16 h. For hot bleach treatments, bleaching agents were added right after fat separation (FSE) for separated treatments or following pasteurization for no fat separation (NSE) treatments. The same bleaching agents and concentrations were used for both hot and cold bleaching treatments. The hot treatments were heated to 68°C for 30 min in a shaker water bath (VWR International, West Chester, PA) and then cooled to 40°C for catalase addition to HP bleached whey. Wheys were then cooled to 4°C. EM Quant peroxide testing strips (EMD Chemicals Inc., Gibbstown, NJ) was used to confirm inactivation of the hydrogen peroxide after catalase addition. This entire experiment was repeated three times. All samples were evaluated for proximate analysis (total solids, fat, and protein), norbixin content, and volatile compound analysis (SPME GC-MS).

**Proximate Analysis**

*Experiment I.* Total solids, fat, crude protein, and non protein nitrogen (NPN) analyses were conducted in duplicate by Cornell University. The total solids was analyzed using forced-air oven drying (AOAC, 2000; method 990.20; 33.2.44), the fat content was analyzed using modified Mojonnier ether extraction (AOAC, 2000; method 989.05; 33.2.26), total nitrogen (TN) was analyzed using Kjeldahl (AOAC 2000; method
991.20; 33.2.11), and non protein nitrogen (NPN) was analyzed using Kjeldahl (AOAC 2000; method 991.21; 33.2.12). Crude protein was calculated by multiplying the TN by 6.38.

**Experiment II.** Total solids were conducted on the SMART Trac System5 (CEM, Matthews, NC). The fat content of fluid whey was analyzed using the Pennsylvania modified Babcock method (Bradley et al., 1992). Protein analysis was conducted using a SPRINT™ Rapid Protein Analyzer (CEM, Matthews, NC) and reported as percent crude protein. All proximate analyses were conducted in duplicate.

**Norbixin Extraction and Measurement**

Norbixin extraction and measurement methods were adapted from Croissant et al. (2009). Briefly, ten grams of liquid whey was weighed into a 20 mL centrifuge tube (Nalgene, Rochester, NY). Six milliliters of ethanol (99.5% v/v, EMD Chemicals, Gibbstown, NJ) was then added to the sample and it was vortexed for 30 sec. The sample was held for 30 min at room temperature and then 3 mL of chloroform (EMD Chemicals) was added. The sample was vortexed (Labnet International, Inc., Woodbridge, NJ) and centrifuged (Sorvall RC-5B centrifuge, Thermo Scientific, DuPont Instruments, Wilmington, DE) at 747.8 x g for 10 min at 4°C. The supernatant was removed to another centrifuge tube and the remaining volume was centrifuged again at 11,970 x g for another 10 min at 4°C. Supernatants were pooled. Two milliliters of 1% acetic acid (99.5% v/v, J.T.Baker, Phillipsburg, NJ) was added to the collected supernatant and it
was vortexed for 30 sec. The sample was centrifuged at 11,970 x g for 10 min at 4°C. The bottom chloroform layer, which contained the norbixin, was collected and subjected to solid phase extraction (SPE).

A solid phase extraction (SPE) column (Strata NH₂ (55μm, 70Å) 500 mg/3 mL, Phenomenex, Torrance, CA) was conditioned with 7 mL of n-hexane (Fisher Scientific, Pittsburgh, PA) before 1 mL of sample was added to the column. The column was then rinsed with 5 mL of 1:1 (v/v) n-hexane:diethyl ether (EMD Chemicals) and 1 mL of acetone (EMD Chemicals) to wash out the fat and β-carotene. The annatto was then eluted with 3 mL of 7:3 (v/v) methanol (EMD Chemicals) : glacial acetic acid (99.5%, J.T.Baker) and collected.

The extracted sample was transferred to a rectangular quartz spectrophotometer 10 mm cuvette (Starna cells, Inc., Atascadero, CA). The absorbance of the sample was measured in duplicate using a UV-Visible Spectrophotometer (UV-1700 PharmaSpec, Shimadzu Scientific Instruments, Columbia, MD) at 458 nm. A standard curve was generated from 0 to 10 mg/kg norbixin (45% w/w, Chr. Hansen, Milwaukee, WI), first dissolved in 2.5% potassium hydroxide (J.T.Baker) and then in 7:3 methanol:glacial acetic acid. The concentration of norbixin recovery was calculated based on the absorbance and the standard curve. The annatto extractions were conducted in duplicate for each sample in experiments I and II, and absorbance measurements were conducted in duplicate for each duplicate sample.

In addition to the annatto extractions, samples from experiment I were evaluated for yellowness. Samples were measured in duplicate using a MacBeth Color-Eye
spectrophotometer (model 2020, Kollmorgen Instruments Corp., Newburgh, NY) equipped with Optiview software. The reflectance method was based on illuminant A and the wavelength was set up at 380 to 760 nm range at 20 nm intervals. A white reference tile was used as the background when measuring. This provides a double reflectance measurement and increases sensitivity of the method to detect differences (in both reflectance and light absorbance expressed as a-value and b-value) among treatments because the path length of the cuvette has effective increased by a factor of 2 by reflecting the transmitted light off a white color tile behind the sample back to the detector. Care must be taken when interpreting these results because they are not the classical b-values. Color was measured at a sample temperature of 20 ± 1 °C.

From experiment I results, BP and HP at their highest concentrations (100 mg/kg and 500 mg/kg respectively) and at their mid concentrations (50 mg/kg and 250 mg/kg respectively) were chosen to be the bleach concentrations used for experiment II. The bleaching temperatures were the same as those used in experiment I.

**Instrumental Volatile Compound Analysis**

Volatile compounds were extracted by solid phase micro extraction (SPME) followed by separation and identification by gas chromatography mass spectrometry (GC-MS) (Croissant et al., 2009). Ten percent (w/w) sodium chloride (VWR International, West Chester, PA) was added to 20 mL SPME autosampler vials (MicroLiter Analytical Supplies, Inc., Sawanee, FL). Five grams of sample was then added to the vial, followed by 10μL of 8.1 mg/kg internal standard (2-methyl-3-
heptanone (Sigma-Aldrich, St. Louis, MO) dissolved in methanol (EMD Chemicals). The vial was then capped with a screw top cap equipped with Teflon septa (MicroLiter Analytical Supplies, Inc., Sawanee, FL). The extracted volatiles were separated by GC-MS (model 6890N, Agilent Technologies Inc., Cary, NC), equipped with 5973 mass selective detector, and a DB-5 column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness, Restek U.S., Bellefonte, PA). Samples were maintained at 5°C prior to DVB/Carboxen/PDMS StableFlex fiber (Supelco, Bellefonte, PA) exposure. Samples were equilibrated at 40°C for 25 min before 1 cm of the fiber was exposed for 30 min at 31 mm. While the fiber was exposed to the sample, the vial was agitated at 250 rpm with 4 s agitation pulse. Then the fiber was inserted into the injector at 50 mm for 5 min at 250°C split/splitless injector. Helium gas was used as the carrier gas at a rate of 1 mL/min. The initial oven condition in the GC was 40°C for 3 min. The temperature was then ramped to 90°C at a rate of 10°C/min and then to 200°C at 5°C/min. This temperature was held for 10 min. The temperature was then increased again to 250°C at 20°C/min and was held for 5 min. The total run time was 52 min per sample. Retention indices (RI) were calculated (Van den Dool and Kratz, 1963) based on an alkane series (Sigma-Aldrich, St. Louis, MO). Scan mode and National Institute of Standards and Technology (NIST) 2005 mass spectral library were used to initially identify the volatile flavor compounds of interest. Selective ion monitoring (SIM) mode was then used for higher sensitivity to pick out specific compounds of interest (Croissant et al., 2009; Liaw et al., 2010). In addition, authentic standards were injected under identical conditions to verify the identity of volatile compounds. Relative abundance of compounds was
determined by comparing their area to the area of the known concentration of the
recovered internal standard.

**Statistical Analyses**

Proximate analyses results were evaluated by analysis of variance (ANOVA) using the PROC GLM procedure of the statistical analysis software (SAS, version 9.2, SAS Institute, Cary, NC) to determine if there were differences in total solids, fat, and crude protein between FSE and NSE liquid Cheddar whey (experiment II) and also in non protein nitrogen (NPN) between liquid Cheddar whey with and without annatto (experiment I). Norbixin in liquid Cheddar whey with annatto (experiment I), both FSE and NSE liquid Cheddar whey, and also the instrumental volatile compounds (experiment II) were analyzed using a split-plot model in PROC GLM (SAS). In experiment I, color (n = 2), temperature (n = 2), bleach type (n = 2), and replicate (n = 3) were used as the whole-plot category variables with the interaction term of the 4 whole plot variables used as the error term to test for significance of the whole plot variables. There were 5 different concentrations used for each different bleach type. Bleach concentration was treated as a continuous variable in the split-plot ANOVA models. Distortion of the ANOVA by multicollinearity in the model was minimized by centering the bleach concentration using a mathematical transformation (Glantz and Slinker, 2001). Bleach concentration was transformed as follows: bleach concentration = bleach concentration – [(highest bleach concentration – lowest bleach concentration) / 2]. This transformation was done separately for each bleach type made the data set zero centered and orthogonal.
with respect to beach concentration. In experiment II, fat separation (n = 2), bleaching temperature (n = 2), bleach type (n = 2), and replicate (n = 3) were used as whole-plot category variables. In addition to the interactions of each of these variables, the interactions of bleach concentrations with each of these variables were treated as subplot variables with bleach concentration as a centered continuous variable. The impact of bleach concentration was also evaluated for both linear and quadratic effects (experiment I – 5 bleach concentrations) or only for linear effects (experiment II) because only 3 bleaching concentrations were applied. The effect of bleach concentration was determined using the full model error term. A full model with the main and interaction effects was run for each parameter including norbixin concentration and volatile compounds. From the full model, terms that were not significant were removed from the model step by step, starting from the highest interaction terms in the sub-plot with the lowest type III sum of squares. Least square means were reported for bleaching temperature by bleach type interaction. This analysis allowed the determination of the effect of bleaching temperature (68°C vs. 4°C) and bleach type (HP vs. BP) on the bleaching efficacy and volatile compounds. In addition, principal component analysis (PCA) was also conducted to visualize how the liquid Cheddar wheys with different bleaching treatments were differentiated across the volatile flavor compounds.
RESULTS AND DISCUSSION

Proximate Analysis

No significant difference ($P > 0.05$) in liquid whey compositions between non-colored and annatto colored wheys from experiment I was detected. Total solids were $6.76 \pm 0.062\%$ and $6.79 \pm 0.083\%$; fat contents were $0.063 \pm 0.013\%$ and $0.058 \pm 0.005\%$; crude protein were $0.945 \pm 0.041\%$ and $0.924 \pm 0.029\%$; and non protein nitrogen (NPN) were $0.226 \pm 0.006\%$ and $0.229 \pm 0.003\%$ for non-colored and annatto colored wheys, respectively. Similarly, no significant difference in protein content ($P > 0.05$, $0.898 \pm 0.034\%$ and $0.922 \pm 0.030\%$ for FSE and NSE liquid wheys, respectively) of wheys from experiment II was detected. Total solids and fat contents of FSE and NSE liquid wheys from experiment II were distinct ($P < 0.05$) as expected (total solids: $6.80 \pm 0.004\%$ vs. $7.02 \pm 0.005\%$; fat: $0.007 \pm 0.008\%$ vs. $0.240 \pm 0.064\%$, respectively).

Norbixin Concentration and Yellowness

From experiment I, there were significant effects for color, temperature of bleaching, bleach type, and bleach level on both norbixin concentration and yellowness (Table 1). Colored whey at 0 ppm of added beach was more yellow (Figures 1A and 2A) and had a higher norbixin content (Figure 1B and 2B) than uncolored whey. Hydrogen peroxide decreased the yellowness and norbixin concentration in colored whey and the decrease was larger at higher temperature. Both HP and BP decreased the yellowness of colored whey but the BP was more effective at reducing yellowness than HP. The HP did
not reduce the yellowness to value of uncolored whey at 0 ppm bleach concentration (Figure 1A) while BP did (Figure 2A). The influence of bleaching temperature on the ability of HP to reduce the yellowness and norbixin content of colored whey (Figures 1A and 1B) was large while the influence of bleaching temperature on the effectiveness of BP to reduce yellowness and norbixin content was much less (Figures 2A and 2B) and is supported by the significant ($P < 0.01$) color by bleach type in Table 1. In addition, there were also significant temperature by bleach type interactions, linear and quadratic effects ($P < 0.01$) of bleach concentration, and linear and quadratic effects of bleach concentration by bleach type for norbixin concentration and yellowness (Table 1, Figures 1 and 2). From experiment I, liquid Cheddar whey bleached with HP at 4°C had significantly higher norbixin concentrations and yellowness compared to whey bleached by other temperature – bleach type treatments ($P < 0.01$) (Table 2). Fluid wheys bleached with BP at 4°C or 68°C had lower norbixin and yellowness compared to wheys bleached with HP (Table 2) and the most of the color reduction due to BP was gained at 25 mg/kg or less (Figure 2A). Croissant et al. (2009) also stated that BP bleached WPC had a lower b* value, which means that BP reduced the yellowness of the whey more than HP. Cold (4°C) bleaching of colored whey with HP was relatively ineffective at reducing norbixin concentration and the yellowness of whey (Figures 1, 2). Hot (68°C) bleaching with HP had linear and quadratic effects on decreasing the norbixin concentration and the yellowness of whey (Table 1; Figures 1, 2). When no annatto was added to the whey, no effect of bleaching (statistical analysis of uncolored whey data alone not shown) with either HP or BP on yellowness of uncolored whey was detected.
but holding the whey at 68°C with or without added bleach caused yellowness of whey to
decrease ($P < 0.01$) from 10.29 to 9.28. The quadratic relationship of improved
bleaching was much more pronounced in BP bleaching compared to HP bleaching
(Figures 1, 2). Overall, BP bleaching reduced the norbixin concentration and yellowness
of colored whey more than HP bleaching (Figures 1, 2).

Experiment II results were consistent with trends observed in experiment I. The
type III sum of squares of the norbixin concentration had the highest significant
interaction in bleaching temperature by bleach type (Table 3) and liquid Cheddar whey
bleached with HP at 4°C had a higher norbixin concentration compared to other
bleaching temperature – bleach type treatment ($P < 0.01$). Once again, BP bleached
wheys had lower norbixin concentration ($P < 0.01$) at both bleaching temperatures
compared to HP bleached wheys (Table 4). There was no difference in the norbixin
concentration between fat separated (FSE) and non fat separated (NSE) wheys of the
same bleaching temperatures and bleaching agents (Figures 3, 4). Bleaching efficacy was
not affected by the fat content of whey. Both concentrations of HP used (250 and 500
mg/kg) to bleach the liquid whey were not different from each other in bleaching efficacy
(Figure 3). Hot bleaching with HP at either concentration (250 or 500 mg/kg) was more
effective in reducing the norbixin content in whey compared to cold bleaching with HP
(Figure 3). Bleaching of whey at either BP concentration (50 or 100 mg/kg) was not
different in bleaching efficacy (Figure 4). BP reduced the norbixin concentration in whey
when bleached at either hot or cold temperature to a similar degree. Bleaching
temperature affected the bleaching efficacy of HP, wheys were bleached more at 68°C
compared to 4°C (Figure 5). However, bleaching temperature did not affect BP bleaching efficacy (Figure 5). These results were consistent with the study conducted by McDonough et al. (1968), in which the rate and extent of whey decoloration increased as the temperature increased. In addition, they also stated that BP was a more effective bleaching agent at all temperatures tested compared to HP (McDonough et al., 1968). Overall, BP bleaching resulted in lower norbixin content in whey compared to HP at all concentrations tested (Figure 5).

**Instrumental Volatile Analysis**

Ten volatile compounds were selected based on previous studies on whey bleaching and whey flavor and stability (Carunchia Whetstine et al., 2007; Croissant et al., 2009; Evans et al., 2009; Evans et al., 2010; Liaw et al., 2010; Campbell et al., in press). These compounds included a range of fermentation flavor compounds (i.e., diacetyl and acetic acid) as well as Strecker degradation products (i.e., 3-methylbutanal, 2-methylbutanal) and lipid oxidation products (i.e, hexanal, Z-4-heptenal, heptanal, 2-pentylfuran, octanal, and nonanal). Fermentation flavor compounds were produced by the starter culture (lactic acid bacteria) during cheese making (Singh et al., 2003). The fermentation products include 2,3-butanedione (diacetyl) and acetic acid (Singh et al., 2003; Gallardo-Escamilla et al., 2005). Strecker degradation is the reaction of α-amino acids with lipid oxidation products that contain two oxygenated functions, resulted in Strecker aldehydes and α-keto acids (Zamora et al., 2007). This reaction is always accompanied by other reactions that developed browning, fluorescence, and the
pyrrolization of the amino group of the amino acid (Zamora et al., 2007). Strecker degradation reaction occurs with Maillard browning and generates specific Strecker aldehydes, such as 2- and 3-methylbutanal (Leksrisompong et al., 2010). 2-methyl butanal was derived from the degradation of the amino acid isoleucine, while 3-methyl butanal was derived from leucine (Yvon and Rijnen, 2001). Lipid oxidation is initiated in the cheese making process and whey pasteurization (Tomaino et al., 2004). The unsaturated fatty acids created unstable hydroperoxides, which decomposed to carbonyl products, such as short chain fatty acids and aldehydes (Badings, 1991; O’Connor and O’Brien, 2006). Aldehydes, including hexanal, heptanal, octanal, and nonanal have been detected in liquid Cheddar whey and documented in previous literature (Carunchia-Whetstine et al., 2003; Karagul-Yuceer et al., 2003; Tomaino et al., 2004; Gallardo-Escamilla et al., 2005; Croissant et al., 2009; Liaw et al., 2010). These compounds can contribute to off-flavors in dairy products (Carunchia-Whetstine et al., 2005; Drake et al., 2009; Wright et al., 2009).

Liquid Cheddar whey with no bleach added (control) was characterized by a lack of lipid oxidation products (i.e., aldehydes), whereas bleached liquid Cheddar wheys, especially those treated with BP 100 mg/kg or HP 500 mg/kg were more characterized by higher concentrations of lipid oxidation compounds (Table 5, Figure 6). Most volatile compounds in liquid Cheddar whey had the highest interaction between bleaching temperature and bleach type (Table 6). The interaction of bleaching temperature with bleach type confirms that bleaching at hot temperature causes higher concentrations of lipid oxidation products, with hexanal highest in hot HP bleached whey, and Z-4-
heptenal, heptanal, 2-pentylfuran, and nonanal highest in hot BP bleached whey (Table 7). Croissant et al. (2009) suggested that HP hot bleaching in liquid Cheddar whey resulted in higher lipid oxidation compared to the control whey with no bleach added and BP hot bleached whey. However, the concentrations of BP used were 10 mg/kg and 20 mg/kg, whereas in this study, BP at 50 mg/kg and 100 mg/kg were used in liquid whey bleaching. In addition, Croissant et al. (2009) also suggested that higher concentration of bleach used in liquid Cheddar whey bleaching resulted in higher lipid oxidation compounds, which correlated with what was found in this paper. Overall, these results were consistent with McDonough et al. (1968) and Croissant et al. (2009), in which the addition of bleaching agent (HP or BP) in liquid Cheddar whey resulted in higher lipid oxidation compounds.

CONCLUSIONS

The impact of temperature on benzoyl peroxide (BP) bleaching was minor compared to the effect of temperature on hydrogen peroxide (HP) bleaching. Cold (4°C) HP bleaching of colored whey was relatively ineffective at reducing the color of whey; however, HP hot (68°C) bleaching was linear with increasing concentrations in decreasing the yellowness of whey. BP reduced the color of whey to nearly uncolored whey levels and most of the color reduction was gained at 25 mg/kg or less. These results demonstrated that BP was a more effective bleaching agent compared to HP in bleaching colored whey. Bleach type, temperature of bleaching, and bleach levels
impacted both yellowness and norbixin concentration. Overall, our results suggest that bleaching treatments with benzoyl peroxide are more effective than those with the hydrogen peroxide in fluid whey at all concentrations tested. Hydrogen peroxide bleaching was temperature dependent. This study also suggests that fat separation has no impact on bleaching efficacy and that hot bleaching at BP concentration $\geq 100$ mg/kg and HP concentration $\geq 500$ mg/kg result in increased lipid oxidation in fluid whey. Since it has been previously documented that flavor in fluid whey is carried through to the finished dried whey product, these results confirm that careful selection and optimization of bleach type, concentration, and temperature are warranted to maximize quality of dried ingredients.
ACKNOWLEDGEMENTS

Funding is provided by Dairy Management Inc. (Rosemont, IL). Paper FSR XX 11 of the Department of Food, Bioprocessing & Nutrition Sciences, North Carolina State University. The use of trade names does not imply endorsement nor lack of endorsement by those not mentioned.
REFERENCES


### Table 1. Sums of the squares (Type III SS) for the analysis of variance (ANOVA) for the impact of temperature and bleach type on the norbixin concentration and yellowness in colored and uncolored liquid Cheddar whey (experiment I) that were bleached with benzoyl peroxide or hydrogen peroxide at 4 and 68°C

<table>
<thead>
<tr>
<th>Factors</th>
<th>df</th>
<th>Norbixin concentration (Type III SS)</th>
<th>Yellowness (Type III SS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>1</td>
<td>35.9</td>
<td>77.1</td>
</tr>
<tr>
<td>Temp</td>
<td>1</td>
<td>5.0</td>
<td>53.1</td>
</tr>
<tr>
<td>Bleach type</td>
<td>1</td>
<td>62.9</td>
<td>34.1</td>
</tr>
<tr>
<td>Rep</td>
<td>2</td>
<td>NS²</td>
<td>12.7</td>
</tr>
<tr>
<td>Color*temp</td>
<td>1</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Color*bleach type</td>
<td>1</td>
<td>82.1</td>
<td>29.9</td>
</tr>
<tr>
<td>Color*rep</td>
<td>2</td>
<td>2.9</td>
<td>7.1</td>
</tr>
<tr>
<td>Temp*bleach type</td>
<td>1</td>
<td>6.9</td>
<td>7.0</td>
</tr>
<tr>
<td>Temp*rep</td>
<td>2</td>
<td>NS</td>
<td>3.0</td>
</tr>
<tr>
<td>Color<em>temp</em>bleach type</td>
<td>1</td>
<td>6.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Color<em>temp</em>bleach type*rep</td>
<td>10</td>
<td>NS²</td>
<td>NS²</td>
</tr>
<tr>
<td>BConT</td>
<td>1</td>
<td>44.6</td>
<td>64.9</td>
</tr>
<tr>
<td>BConT*color</td>
<td>1</td>
<td>60.8</td>
<td>21.4</td>
</tr>
<tr>
<td>BConT*temp</td>
<td>1</td>
<td>NS</td>
<td>1.5</td>
</tr>
<tr>
<td>BConT*bleach type</td>
<td>1</td>
<td>7.1</td>
<td>14.4</td>
</tr>
<tr>
<td>BConT<em>color</em>bleach type</td>
<td>1</td>
<td>7.7</td>
<td>4.8</td>
</tr>
<tr>
<td>BConT*BConT</td>
<td>1</td>
<td>17.6</td>
<td>12.0</td>
</tr>
<tr>
<td>BConT<em>BConT</em>color</td>
<td>1</td>
<td>17.7</td>
<td>9.3</td>
</tr>
<tr>
<td>BConT<em>BConT</em>bleach type</td>
<td>1</td>
<td>11.2</td>
<td>3.2</td>
</tr>
<tr>
<td>R-square</td>
<td></td>
<td>0.85</td>
<td>0.96</td>
</tr>
</tbody>
</table>

¹Temp = temperature; Rep = replicate; BConT = bleach concentration continuous variable
²Not significant (all other means significant at $P < 0.05$)
Table 2. Least square means values of bleaching temperature by bleach type interaction for norbixin concentrations (mg/kg) and yellowness in colored liquid Cheddar whey (experiment I)

<table>
<thead>
<tr>
<th>Temp*bleach type</th>
<th>Norbixin concentration (mg/kg)</th>
<th>Yellowness</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C*HP</td>
<td>3.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4°C*BP</td>
<td>1.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>68°C*HP</td>
<td>2.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>68°C*BP</td>
<td>1.62&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temp</th>
<th>Norbixin concentration (mg/kg)</th>
<th>Yellowness</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>2.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>68°C</td>
<td>2.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bleach type</th>
<th>Norbixin concentration (mg/kg)</th>
<th>Yellowness</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>3.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP</td>
<td>1.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-d</sup>Means in the same column that do not share the common superscript are different (P < 0.01)

<sup>1</sup>Temp = temperature; HP = hydrogen peroxide; BP = benzoyl peroxide
<table>
<thead>
<tr>
<th>Factors</th>
<th>df</th>
<th>Norbixin (Type III SS) concentration (Experiment I)</th>
<th>Norbixin (Type III SS) concentration (Experiment II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep</td>
<td>1</td>
<td>NA&lt;sup&gt;2&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Temp</td>
<td>1</td>
<td>5.87</td>
<td>4.86</td>
</tr>
<tr>
<td>Bleach type</td>
<td>1</td>
<td>148.6</td>
<td>44.0</td>
</tr>
<tr>
<td>Rep</td>
<td>2</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Temp*Bleach type</td>
<td>1</td>
<td>13.22</td>
<td>2.51</td>
</tr>
<tr>
<td>Sep<em>temp</em>Bleach type*rep</td>
<td>2</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BConT</td>
<td>1</td>
<td>102.5</td>
<td>56.3</td>
</tr>
<tr>
<td>BConT*bleach</td>
<td>1</td>
<td>15.7</td>
<td>16.4</td>
</tr>
<tr>
<td>R-square</td>
<td></td>
<td>0.88</td>
<td>0.84</td>
</tr>
</tbody>
</table>

<sup>1</sup> Sep = fat separation; Temp = temperature; Rep = replicate; BConT = bleach concentration continuous variable  
<sup>2</sup> Not applicable  
<sup>3</sup> Not significant (all other means significant at $P < 0.05$)
Table 4. Least squares mean values of bleaching temperature by bleach type interaction for norbixin concentrations (mg/kg) in colored liquid Cheddar whey (experiment II)

<table>
<thead>
<tr>
<th>Temp*Bleach type</th>
<th>Norbixin Concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C*HP</td>
<td>3.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4°C*BP</td>
<td>1.69&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>68°C*HP</td>
<td>2.74&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>68°C*BP</td>
<td>1.55&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temp</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>2.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>68°C</td>
<td>2.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bleach type</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>3.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP</td>
<td>1.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup>Means in the same column that do not share the common superscript are different (<i>P</i> < 0.01)

<sup>1</sup>Temp = temperature; HP = hydrogen peroxide; BP = benzoyl peroxide
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment</th>
<th>Acetic Acid</th>
<th>3- Methylobutanal</th>
<th>2-Methylbutanal</th>
<th>Hexanal</th>
<th>Z-4-Heptenal</th>
<th>Heptanal</th>
<th>2-Pentylfuran</th>
<th>Octanal</th>
<th>Nonanal</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI on DB-5 column</td>
<td>600</td>
<td>646</td>
<td>654</td>
<td>665</td>
<td>807</td>
<td>888</td>
<td>913</td>
<td>991</td>
<td>1005</td>
<td>1108</td>
</tr>
<tr>
<td>FSE Hot 0</td>
<td>0.231&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.070&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.071&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.067&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>0.245&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.004&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.037&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.065&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>0.081&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.507&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FSE Hot BP 50</td>
<td>0.181&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.088&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.086&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.069&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>2.62&lt;sup&gt;fghi&lt;/sup&gt;</td>
<td>0.026&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>0.136&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.294&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.112&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.448&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FSE Hot BP 100</td>
<td>0.254&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.100&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.110&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.084&lt;sup&gt;def&lt;/sup&gt;</td>
<td>6.02&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.123&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.372&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.585&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.125&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.80&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>FSE Hot HP 250</td>
<td>0.151&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.092&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.612&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.126&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>6.90&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.055&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.224&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.312&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.134&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.449&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FSE Hot HP 500</td>
<td>0.076&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.117&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.09&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.159&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.87&lt;sup&gt;bde&lt;/sup&gt;</td>
<td>0.045&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.216&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.264&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.117&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.341&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FSE Cold 0</td>
<td>0.282&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.092&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.112&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.089&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.825&lt;sup&gt;ahi&lt;/sup&gt;</td>
<td>0.002&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.033&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.075&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.202&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.221&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FSE Cold BP 50</td>
<td>0.228&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.105&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.077&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.068&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3.52&lt;sup&gt;fghi&lt;/sup&gt;</td>
<td>0.025&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.077&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.181&lt;sup&gt;bde&lt;/sup&gt;</td>
<td>0.139&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.234&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Cold 0</td>
<td>0.294&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.143&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.157&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.141&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>1.58&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>0.020&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.075&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.077&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.162&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.430&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Cold BP 50</td>
<td>0.416&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.211&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.168&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.107&lt;sup&gt;def&lt;/sup&gt;</td>
<td>5.63&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.153&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.739&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.216&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>0.218&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.524&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Hot 0</td>
<td>0.460&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.250&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.194&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.133&lt;sup&gt;ed&lt;/sup&gt;</td>
<td>11.8&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.448&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.479&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.168&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.811&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Hot BP 50</td>
<td>0.201&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.112&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.944&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.201&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.22&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.070&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.315&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.180&lt;sup&gt;bde&lt;/sup&gt;</td>
<td>0.169&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.205&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Hot HP 500</td>
<td>0.136&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.148&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.22&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.220&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.73&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.105&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>0.273&lt;sup&gt;ed&lt;/sup&gt;</td>
<td>0.171&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.187&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.453&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Cold 0</td>
<td>0.435&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.071&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.091&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.094&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.397&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.029&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.034&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.132&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.255&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Cold BP 50</td>
<td>0.343&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>0.100&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.098&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.110&lt;sup&gt;def&lt;/sup&gt;</td>
<td>1.82&lt;sup&gt;fghi&lt;/sup&gt;</td>
<td>0.010&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.045&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.064&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>0.260&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.472&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Cold BP 100</td>
<td>0.382&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>0.143&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.108&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.103&lt;sup&gt;def&lt;/sup&gt;</td>
<td>3.19&lt;sup&gt;fghi&lt;/sup&gt;</td>
<td>0.025&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.112&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.093&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.211&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.349&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Cold HP 250</td>
<td>0.299&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.120&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.799&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.087&lt;sup&gt;def&lt;/sup&gt;</td>
<td>1.89&lt;sup&gt;fghi&lt;/sup&gt;</td>
<td>0.009&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.050&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.061&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>0.230&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.117&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NSE Cold HP 500</td>
<td>0.240&lt;sup&gt;bde&lt;/sup&gt;</td>
<td>0.241&lt;sup&gt;i&lt;/sup&gt;</td>
<td>1.51&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.081&lt;sup&gt;df&lt;/sup&gt;</td>
<td>2.78&lt;sup&gt;fghi&lt;/sup&gt;</td>
<td>0.026&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>0.076&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.107&lt;sup&gt;def&lt;/sup&gt;</td>
<td>0.108&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.120&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-b</sup>Means in the same column that do not share the common superscript are different (P < 0.05)

<sup>1</sup>FSE = fat separation, NSE = no fat separation; Hot = 68°C, Cold = 4°C; BP = benzoyl peroxide, HP = hydrogen peroxide; Numerals indicate bleach concentration in mg/kg
Table 6. Sums of the squares (Type III SS) for the ANOVA analysis of the impact of fat separation, bleaching temperature, and bleach type on the concentration of volatile flavor compounds identified in liquid Cheddar whey (experiment II)

<table>
<thead>
<tr>
<th>Factors</th>
<th>df</th>
<th>Diacetyl</th>
<th>Acetic Acid</th>
<th>3-methylbutanal</th>
<th>2-methylbutanal</th>
<th>Hexanal</th>
<th>Z-4-heptenal</th>
<th>Heptanal</th>
<th>2-pentylfuran</th>
<th>Octanal</th>
<th>Nonanal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sep</td>
<td>1</td>
<td>0.729</td>
<td>0.113</td>
<td>0.641</td>
<td>0.010</td>
<td>56.0</td>
<td>0.101</td>
<td>2.48</td>
<td>0.208</td>
<td>0.136</td>
<td>NS</td>
</tr>
<tr>
<td>Temp</td>
<td>1</td>
<td>0.130</td>
<td>NS</td>
<td>NS</td>
<td>0.101</td>
<td>456</td>
<td>0.284</td>
<td>5.76</td>
<td>0.946</td>
<td>NS</td>
<td>0.060</td>
</tr>
<tr>
<td>Bleach</td>
<td>1</td>
<td>0.336</td>
<td>NS</td>
<td>18.5</td>
<td>0.023</td>
<td>16.9</td>
<td>0.096</td>
<td>2.10</td>
<td>0.198</td>
<td>NS</td>
<td>0.035</td>
</tr>
<tr>
<td>Rep</td>
<td>2</td>
<td>1.40</td>
<td>0.140</td>
<td>0.198</td>
<td>0.025</td>
<td>188</td>
<td>NS</td>
<td>NS</td>
<td>0.052</td>
<td>0.063</td>
<td>0.023</td>
</tr>
<tr>
<td>Sep*Temp</td>
<td>1</td>
<td>NS</td>
<td>0.058</td>
<td>NS</td>
<td>0.020</td>
<td>184</td>
<td>0.130</td>
<td>2.58</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sep*Bleach</td>
<td>1</td>
<td>0.071</td>
<td>0.010</td>
<td>0.202</td>
<td>NS</td>
<td>NS</td>
<td>0.043</td>
<td>1.95</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sep*Rep</td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.007</td>
<td>42.9</td>
<td>0.048</td>
<td>0.401</td>
<td>0.116</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Temp*Bleach</td>
<td>1</td>
<td>0.096</td>
<td>0.045</td>
<td>0.079</td>
<td>0.049</td>
<td>14.7</td>
<td>0.070</td>
<td>2.07</td>
<td>0.107</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Temp*Rep</td>
<td>2</td>
<td>0.310</td>
<td>NS</td>
<td>0.429</td>
<td>0.014</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.058</td>
<td>0.106</td>
<td>NS</td>
</tr>
<tr>
<td>Bleach*Rep</td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>25.8</td>
<td>NS</td>
<td>0.352</td>
<td>0.029</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sep<em>Temp</em>Bleach</td>
<td>1</td>
<td>NS</td>
<td>0.030</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.063</td>
<td>1.78</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sep<em>Temp</em>Rep</td>
<td>2</td>
<td>NS</td>
<td>0.037</td>
<td>NS</td>
<td>0.017</td>
<td>79.0</td>
<td>0.033</td>
<td>0.273</td>
<td>0.140</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sep<em>Bleach</em>Rep</td>
<td>2</td>
<td>NS</td>
<td>0.015</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Temp<em>Bleach</em>Rep</td>
<td>2</td>
<td>NS</td>
<td>0.031</td>
<td>0.396</td>
<td>NS</td>
<td>37.2</td>
<td>NS</td>
<td>0.392</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sep<em>Temp</em>Bleach*Rep</td>
<td>2</td>
<td>NS</td>
<td>0.022</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.340</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BConT</td>
<td>1</td>
<td>0.181</td>
<td>0.156</td>
<td>11.7</td>
<td>NS</td>
<td>932</td>
<td>0.335</td>
<td>5.12</td>
<td>1.32</td>
<td>NS</td>
<td>0.014</td>
</tr>
<tr>
<td>BConT*Sep</td>
<td>1</td>
<td>NS</td>
<td>0.018</td>
<td>0.133</td>
<td>0.007</td>
<td>12.0</td>
<td>0.059</td>
<td>1.84</td>
<td>0.045</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BConT*Temp</td>
<td>1</td>
<td>0.059</td>
<td>0.013</td>
<td>NS</td>
<td>0.034</td>
<td>173</td>
<td>0.169</td>
<td>3.73</td>
<td>0.440</td>
<td>NS</td>
<td>0.012</td>
</tr>
<tr>
<td>BConT*Bleach</td>
<td>1</td>
<td>0.247</td>
<td>NS</td>
<td>11.2</td>
<td>0.009</td>
<td>NS</td>
<td>0.109</td>
<td>2.36</td>
<td>0.233</td>
<td>NS</td>
<td>0.029</td>
</tr>
<tr>
<td>BConT*Rep</td>
<td>2</td>
<td>0.134</td>
<td>0.259</td>
<td>NS</td>
<td>NS</td>
<td>78.3</td>
<td>NS</td>
<td>NS</td>
<td>0.037</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BConT<em>Sep</em>Temp</td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>0.089</td>
<td>NS</td>
<td>17.5</td>
<td>0.076</td>
<td>1.68</td>
<td>NS</td>
<td>0.109</td>
<td>NS</td>
</tr>
<tr>
<td>BConT<em>Sep</em>Bleach</td>
<td>1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.031</td>
<td>1.82</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BConT<em>Sep</em>Rep</td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>21.4</td>
<td>0.033</td>
<td>0.188</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BConT<em>Temp</em>Bleach</td>
<td>1</td>
<td>0.064</td>
<td>0.037</td>
<td>0.131</td>
<td>0.021</td>
<td>NS</td>
<td>0.085</td>
<td>2.35</td>
<td>0.208</td>
<td>NS</td>
<td>0.010</td>
</tr>
<tr>
<td>BConT<em>Temp</em>Rep</td>
<td>2</td>
<td>0.152</td>
<td>0.016</td>
<td>0.371</td>
<td>0.011</td>
<td>50.9</td>
<td>NS</td>
<td>NS</td>
<td>0.060</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BConT<em>Bleach</em>Rep</td>
<td>2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>R²</td>
<td></td>
<td>0.65</td>
<td>0.69</td>
<td>0.93</td>
<td>0.71</td>
<td>0.86</td>
<td>0.81</td>
<td>0.89</td>
<td>0.84</td>
<td>0.28</td>
<td>0.38</td>
</tr>
</tbody>
</table>

1-Sep = fat separation; Temp = temperature; Rep = replicate; BConT = bleach concentration
2-Not significant (all other means significant at $P < 0.05$)
Table 7. Least squares mean values of bleaching temperature by bleach type interaction of volatile flavor compounds (µg/kg) identified in liquid Cheddar whey (experiment II)

<table>
<thead>
<tr>
<th>Temp*Bleach¹</th>
<th>Diacetyl</th>
<th>Acetic Acid</th>
<th>3-methylbutanal</th>
<th>2-methylbutanal</th>
<th>Hexanal</th>
<th>Z-4-heptenal</th>
<th>Heptanal</th>
<th>2-pentylfuran</th>
<th>Octanal</th>
<th>Nonanal</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C*HP</td>
<td>0.274&lt;sup&gt;a&lt;/sup&gt; 0.132&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.716&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.078&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.013&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.058&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.089&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.151&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.017&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4°C*BP</td>
<td>0.311&lt;sup&gt;a&lt;/sup&gt; 0.103&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.091&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.088&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.24&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.019&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.060&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.106&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.173&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.032&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>68°C*HP</td>
<td>0.182&lt;sup&gt;b&lt;/sup&gt; 0.114&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.679&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.152&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.050&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.190&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.178&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.142&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.040&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>68°C*BP</td>
<td>0.303&lt;sup&gt;a&lt;/sup&gt; 0.142&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.131&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.101&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.130&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.583&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.283&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.144&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.076&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a-b</sup>Means in the same column that do not share the common superscript are different (P < 0.05)

¹Temp = temperature; HP = hydrogen peroxide; BP = benzoyl peroxide
Figure 1. The impact of concentration of HP and bleaching temperature on yellowness (A) and norbixin concentration (B) of whey made from cheese colored with annatto and whey from cheese not colored with annatto bleached at 4 and 68°C.
Figure 2. The impact of concentration of BP and bleaching temperature on yellowness (A) and norbixin concentration (B) of whey made from cheese colored with annatto and whey from cheese not colored with annatto bleached at 4 and 68°C.
Figure 3. Norbixin concentration in fat separated (FSE) and non fat separated (NSE) hydrogen peroxide (HP) bleached liquid Cheddar whey at two different bleaching temperatures (4°C and 68°C) (experiment II).
Figure 4. Norbixin concentration in fat separated (FSE) and non fat separated (NSE) benzoyl peroxide (BP) bleached liquid Cheddar whey at two different bleaching temperatures (4°C and 68°C) (experiment II).
Figure 5. Norbixin concentration (mg/kg) in fat separated (FSE) hydrogen peroxide (HP) and benzoyl peroxide (BP) bleached liquid Cheddar whey at two different bleaching temperatures (4°C and 68°C) (experiment II).

10 = 0 mg/kg; 1 = 250 mg/kg HP or 50 mg/kg BP; 2 = 500 mg/kg HP or 100 mg/kg BP.
Figure 6. PCA biplot of volatile flavor compounds from all bleaching treatments in liquid Cheddar whey (experiment II).
CHAPTER 3: INFLUENCE OF BLEACHING ON FLAVOR OF 34% WHEY PROTEIN CONCENTRATE AND RESIDUAL BENZOIC ACID CONCENTRATIONS IN DRIED WHEY PROTEINS
Influence of bleaching on flavor of 34% whey protein concentrate and residual benzoic acid concentrations in dried whey proteins

M. A. D. Listiyani,* R. E. Campbell,* R. E. Miracle,* L. O. Dean,† and M. A. Drake *

*Southeast Dairy Foods Research Center, Department of Food, Bioprocessing & Nutrition Sciences, North Carolina State University, Raleigh, NC 27695
†Market Quality and Handling Research Unit, Agricultural Research Service U. S. Department of Agriculture, North Carolina State University, Raleigh, NC 27695

Submitted for publication into the Journal of Dairy Science

*Corresponding author:
MaryAnne Drake
E-mail: mdrake@unity.ncsu.edu
Phone: 919-513-4598
Fax: 919-515-7124
ABSTRACT

Previous studies have shown that bleaching negatively impacts the flavor of 70% whey protein concentrate (WPC70), but bleaching effects on lower protein products have not been established. Benzoyl peroxide (BP), a whey bleaching agent, degrades to benzoic acid (BA) and may elevate BA concentrations in dried whey products. There is no legal limit in the U.S. for BP use in whey, but international concerns exist. The objectives of this study were to determine the impact of hydrogen peroxide (HP) or BP bleaching on the flavor of WPC34 and to evaluate residual BA in commercial and experimental WPC bleached with and without BP. Cheddar whey was manufactured in duplicate. Pasteurized fat-separated whey was subjected to hot bleaching with either HP at 500 mg/kg, BP at 50 or 100 mg/kg, or no bleach. Whey was ultrafiltered and spray dried into WPC34. Color (L*a*b*) measurements and norbixin extractions were conducted to compare bleaching efficacy. Descriptive sensory and instrumental volatile analyses were used to evaluate bleaching effects on flavor. Benzoic acid was extracted from experimental and commercial WPC34 and WPC80 and quantified by high performance liquid chromatography (HPLC). The b* value and norbixin concentration of BP bleached WPC34 were lower than HP bleached and control WPC34. HP bleached WPC34 displayed higher cardboard flavor and had higher volatile lipid oxidation products than BP bleached or control WPC34. BP bleached WPC34 had higher BA concentrations than unbleached and HP bleached WPC34 and BA concentrations were also higher in BP bleached WPC80 compared to unbleached and HP bleached WPC80,
with smaller differences than those observed in WPC34. Benzoic acid extraction from permeate showed that WPC80 permeate contained more BA than WPC34 permeate. These results suggest that BP is more effective in color removal of whey and results in fewer flavor side effects compared to HP and that BA is decreased by ultrafiltration and diafiltration.

**Key words:** whey, benzoic acid, bleach, norbixin, flavor
INTRODUCTION

Benzoyl peroxide (C₁₄H₁₀O₄) (BP) is a colorless, crystalline solid used as a bleaching agent in certain foods, such as flour, whey, and milk used for certain types of cheese (JECFA, 2004a). It is also one of two currently approved bleaching agents that can be used to bleach whey (Kang et al., 2010). BP is generally recognized as safe (GRAS) when used as a bleaching agent following current Good Manufacturing Practices (GMP) (U.S. FDA, 2010a). Currently there are no legal limits for BP use established in the U.S. At the 63rd meeting, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) stated that there was no safety concern for BP in whey bleaching when used at up to 100 mg/kg (JEFCa, 2004b). In Codex Alimentarius, 100 mg/kg was the maximum limit for BP bleaching of liquid whey, dried whey, and whey products, except for infant applications where it is prohibited (Codex, 2008). Currently, there are two bleaching agents that are allowed for whey bleaching in the U.S.: hydrogen peroxide (maximum usage rate at 500 mg/kg) (U.S. FDA, 2010c) and benzoyl peroxide (U.S. FDA, 2010b). In comparison to hydrogen peroxide (HP), bleaching with BP requires less peroxide for satisfactory bleaching, does not need catalase addition to deactivate, and it is less corrosive to stainless steel equipment (Chang et al., 1977; Kang et al., 2010). Croissant et al. (2009) suggested that when applied to liquid whey, BP bleaching resulted in less lipid oxidation products and off-flavors compared to HP.

BP breaks down to benzoic acid when it reacts with oxidizable substances present, such as annatto or carotenoid pigments during processing (JECFA, 2004a; Kang
et al., 2010). Benzoic acid occurs naturally in many foods such as dairy products, nuts, fruits, and vegetables (Sieber et al., 1995). In fermented dairy products, it is produced from hippuric acid in milk during fermentation and therefore cultured dairy products, such as yogurt and smear-ripened cheeses contain some benzoic acid (Sieber et al., 1995). In yogurt, benzoic acid was detected at various levels between 12 – 47 mg/kg (plain yogurt) and between 5 – 39 mg/kg (fruit yogurt) (Sieber et al., 1995). Benzoic acid content varied between 10 – 18 mg/kg in sour cream, 10 – 19 mg/kg in buttermilk, 8 – 23 mg/kg in kefir, and 2 – 18 mg/kg in cottage cheese (Sieber et al., 1995). Sieber et al. (1995) also reported benzoic acid concentrations between 12 – 13 mg/kg in fluid whey and between 23 – 75 mg/kg in whey powder but bleaching conditions for these products were not known. Benzoic acid is considered GRAS (U.S. FDA, 2010b) and it is a common preservative used widely in foods, drugs, and cosmetics (Sieber et al., 1995; Chipley, 2005; Qi et al., 2009). As a food preservative, benzoic acid is used mostly in food products that are naturally in an acidic pH range, such as fruit juices and soft drinks (WHO, 2000; Chipley, 2005). Benzoic acid is also widely used as a preservative in toothpastes, mouthwashes, and dentifrices (WHO, 2000). Benzoic acid concentration found in foods, where it was used as a preservative did not exceed 2,000 mg/kg of food; and in foods in which it occurred naturally, its concentration did not exceed 40 mg/kg (WHO, 2000).

Although benzoic acid is GRAS, it has been reported to give adverse health effects, such as skin and eye irritation, asthma, urticaria, metabolic acidosis, and convulsions (WHO, 2000; Tfouni and Toledo, 2002; Qi et al., 2009; Lino and Pena,
According to JECFA (2004b) the adverse reactions to benzoic acid-related compounds are rare, and life-threatening reactions are extremely rare. Many Asian and European countries consider benzoic acid harmful (USDEC, 2009; Kang et al., 2010). Benzoic acid levels in dried whey ingredients are concerns in China (Dairy Management Inc., 2009) and therefore, there have been problems with exporting whey products bleached with BP. The maximum level of benzoic acid in many products has also been regulated in Europe and China (EC, 1995; State Bureau for Quality Supervision, Inspection and Quarantine, 1996). In China, the maximum concentrations of benzoic acid permitted are 200 mg/kg in carbonated drinks, and 1,000 mg/kg in juice drinks, sauces, and jams (State Bureau for Quality Supervision, Inspection and Quarantine, 1996). The European Commission (EC) has allowed the use of BP as a bleaching agent, however, the regulations per individual country may vary (Kang et al., 2010). Taiwan has permitted the use on BP as a bleaching agent in whey powder since 1999 (Johnson, 2006). Chinese authorities, on the other hand, have banned the use of BP as a bleaching agent and do not allow the presence of benzoic acid in whey products (USDEC, 2009; McKnight, 2010). Currently, the U.S. Dairy Export Council (USDEC) has requested China’s Ministry of Health for permission to use BP as a bleaching agent (McKnight, 2010). China’s Ministry of Health is currently in the process of developing new standards for whey protein concentrate (WPC) and whey protein isolate (WPI), and the specific permitted level of benzoic acid has not been determined (NMPF and USDEC, 2009; McKnight, 2010).
A method for the determination of benzoic acid in foods using liquid chromatography (LC) was developed in the 1980s (Bui and Cooper, 1987). More current methods for benzoic acid testing in whey powder involve reconstitution of the powdered sample, precipitation of fats and proteins using zinc acetate and potassium hexacyanoferrate(II) trihydrate, filtration of the sample, and separation using a C-18 HPLC column with UV-VIS detection (ISO, 2008; Qi et al., 2009). High performance liquid chromatography (HPLC) is the most common separation technique used since it has a high sensitivity, requires minimum sample preparation, and no sample derivatization is needed (WHO, 2000).

Previous studies have suggested that BP bleaching of fluid whey results in fewer off-flavors in WPC70 compared to HP bleaching of fluid whey. However, specific effects on WPC34 have not been evaluated. Further, comparisons of benzoic acid residues in WPC34 and WP80 where the specific bleaching agent is known have not been published. The objectives of this study were to evaluate the impact of bleaching agent on bleaching efficacy and flavor of WPC34; and to determine the influence of bleaching agent on benzoic acid concentration in dried whey proteins.
MATERIALS AND METHODS

Experimental Design

Influence of bleaching on color and flavor of WPC34

WPC34 manufactured from colored Cheddar whey with no bleach, hydrogen peroxide (HP) bleach, and benzoyl peroxide (BP) bleach were manufactured in duplicate at the NC State University Dairy Plant. Color, sensory, and volatile analyses were conducted to determine the influence of bleaching and bleaching agent on WPC34 color and flavor.

Benzoic acid residues

In a subsequent experiment, benzoic acid residues in dried whey proteins were compared. Commercial WPC34 and WPC80 (less than 60 d old, duplicate lots) were obtained from at least 3 different manufacturing facilities. Pilot plant manufactured WPC80 were obtained in triplicate from Dr. D. M. Barbano (Cornell University, Ithaca, NY). The WPC34 manufactured for the first part of this study were also used as samples. The bleaching agents used and bleaching conditions of pilot plant manufactured WPC34 and WPC80 were known. In the case of commercial whey proteins, the bleaching agent (HP or BP or no bleach) was known, but the bleaching conditions and concentrations were proprietary.
Experimental WPC34 Manufacture

Raw whole milk was obtained from the North Carolina State University Dairy Plant (Raleigh, NC). Milk (150 kg) was vat-pasteurized (Model MPD1050, Micro Process Design, D & F Equipment Co, McLeansville, NC) at 63°C for 30 min. The milk was cooled to 35°C and Cheddar cheese manufacture was initiated. The pasteurized milk was adjusted to 31°C in a cheese vat (model MX4, Kusel Equipment Company, Watertown, WI). Calcium chloride (50% w/v, Dairy Connection Inc., Madison, WI) at the rate of 0.39 mL/kg of milk and mesophilic lactic starter (Choozit MA 11, Danisco, New Century, NJ) at 50 DCU/454 kg of milk were added and the cheese was ripened for 60 min. After the first 30 min of ripening, 1:20 diluted double strength annatto (Chr. Hansen, Inc., Milwaukee, WI) was added at 0.066 mL/kg of milk. The milk was then coagulated for 30 min using 1:80 diluted recombinant rennet (Dairy Connection Inc., Madison, WI) at the rate of 0.09 mL/kg of milk at the end of the ripening period. Following coagulation, the cheese curd was cut using 0.95 cm knives and healed for 5 min. The curds were stirred gently for 10 min and cooked by increasing the temperature to 39°C across 30 min. The pH and titratable acidity were monitored closely, and the liquid whey was drained at pH 6.4. The liquid Cheddar whey obtained was then pasteurized at 63°C for 30 min followed by fat removal using a hot bowl separator (Westfalia Separator, C.A. De Fehr & Sons Ltd., Winnipeg, Manitoba, Canada) to obtain 90 kg of fat separated whey.

The separated whey was portioned into 2 batches of 45 kg each. Each portion was transferred into a stainless vat (Fermenator™, Blichmann Engineering™, Lafayette,
IN) equipped with a coil heater. The whey was heated to 60°C and one of four treatments was conducted, no bleach for the control whey or the corresponding bleaching agent (hydrogen peroxide (HP; 35% w/v, Nelson Jameson, Inc., Marshfield, WI) 500 mg/kg or benzoyl peroxide (BP; 32% w/w, Oxylite Type XX, Nelson Jameson, Inc., Marshfield, WI) 50 mg/kg or BP 100 mg/kg. The bleaching time was 1 h. The liquid whey was bleached during ultrafiltration. The ultrafiltration (UF) system (Model Pellicon 2, Millipore Inc., Billerica, MA) was equipped with 4 polyethersulfone cartridge membrane filters (Model P2B010V05, 10 kDa nominal separation cutoffs, 0.5 m² surface area, Millipore Inc., Billerica, MA). Each sample was run through a peristaltic pump (Model 77410-10) into separate pump heads (Model 77601-00) and the UF assembly using silicone tubing (Model 96440-73) that was connected to the Fermentor™. Pumps, pumpheads, and tubing were obtained from Cole-Palmer (Vernon Hills, IL). This process continued until the retentate reached 38 – 39% protein content confirmed by a Sprint™ rapid protein analyzer (CEM, Matthews, NC). Catalase was added to the HP bleached whey (20 mg/kg, FoodPro CAT, Danisco, New Century, NJ). The absence of HP was confirmed by testing with a peroxide strip (EMD Chemicals, VWR International, West Chester, PA). Three liters of retentate were then collected and spray dried (Model Lab 1, Anhydro Inc., Soeberg, Denmark).

The retentate was sent to the spray drier through a hose (Model 06402-73) that went through a peristaltic pump (Model 85MHP40, Stenner Pump Company, Jacksonville, FL) and connected to a valve (Pneu-Trol, Bellwood, IL) to regulate a continuous flow for spray drying at a rate of approximately 1 kg/h. The inlet temperature
was at 150°C and the outlet temperature was at 80°C. Total spray drying time was approximately 1.5 h. The spray dried whey was evaluated on a Sprint™ rapid protein analyzer (CEM, Matthews, NC) to ensure that the protein content was 34% w/w. The powder was sealed in mylar bags and stored at -80°C. Two treatments were conducted from one lot of milk and cheese, so it took 2 days to complete the entire experimental replication. The order of treatment manufacture was randomized and the entire experiment was replicated twice. The experimental WPC34 were evaluated in duplicate for composition analysis (moisture, fat, protein, and mineral), norbixin content, L*, a*, b* color values, descriptive sensory analysis, and volatile compound analysis (SPME GC-MS), in addition to benzoic acid analysis. Benzoic acid analysis was conducted on experimental WPC34, WPC80 from Cornell University, and commercial whey proteins.

**Composition Analysis**

Total solids, fat, and protein analyses were conducted in duplicate on experimental WPC34. The samples were reconstituted to 10% solids. The total solids were analyzed using forced-air oven drying (AOAC, 2000; method 990.20; 33.2.44), fat content was analyzed by modified Mojonnier ether extraction (AOAC, 2000; method 989.05; 33.2.26), and the protein (total nitrogen or TN) was analyzed using Kjeldahl (AOAC 2000; method 991.20; 33.2.11). Mineral analysis (phosphorus, calcium, magnesium, potassium, sulfur, iron, sodium, and ash) was determined by the NCSU Analytical Services Laboratory (Raleigh, NC) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy (Lloyd et al., 2009). The
moisture content of each sample was calculated from the total solids and the protein and fat were calculated on both dry and wet weight bases. Percent lactose and mineral contents were calculated by subtracting moisture, protein, and fat from 100.

*Color Analysis and Norbixin Extraction and Measurement*

L* (lightness), a* (red-green), b* (yellow-blue) color values were measured in duplicate using a MacBeth Color-Eye spectrophotometer (model 2020, Kollmorgen Instruments Corp., Newburgh, NY) equipped with Optiview software. The reflectance method utilized illuminant A and the wavelength was set at 380 to 760 nm range at 20 nm intervals. A white reference tile was used as the background when measuring the reflectance. Duplicate measurements were conducted on both the powder and the 10% (w/v) reconstituted powders.

Norbixin extractions and measurements were conducted in duplicate on experimental WPC34 as described by Campbell et al. (2011). Briefly, two milliliters of HPLC grade water (Honeywell, Burdick & Jackson, Muskegon, MI) was added to one gram of WPC34 sample and vortexed (Labnet International, Inc., Woodbridge, NJ). Six milliliters of ethanol (99.5%, EMD Chemicals, Gibbstown, NJ) was then added to the sample and vortexed for 30 sec. The sample was held for 30 min at room temperature and then 3 mL of chloroform (EMD Chemicals, Gibbstown, NJ) was added. The sample was vortexed and centrifuged (Sorvall RC-5B refrigerated superspeed centrifuge, Thermo Scientific, DuPont Instruments, Wilmington, DE) at 11,970 x g for 10 min at 4°C. The supernatant was collected into another centrifuge tube and another 3 mL of chloroform
was added to the remaining volume which was then centrifuged again. More supernatant was collected and combined with previously collected supernatant. Two milliliters of 1% acetic acid (99.5%, J.T.Baker, Phillipsburg, NJ) was added to the collected supernatant and it was vortexed and centrifuged. The volume of the bottom layer, which was the chloroform layer containing norbixin, was measured, collected, and subjected to solid phase extraction (SPE). The solid phase extraction (SPE) column (Strata NH₂ (55μm, 70A) 500 mg/3 mL, Phenomenex, Torrance, CA) was conditioned with 7 mL of n-hexane (Fisher Scientific, Pittsburg, PA) before 1 mL of sample was added to the column. Then the column was rinsed with 5 mL of 1:1 (v/v) n-hexane:diethyl ether (EMD Chemicals, Gibbstown, NJ) and 1 mL of acetone (EMD Chemicals, Gibbstown, NJ) to wash out the fat and β-carotene. The norbixin was then eluted with 3 mL of 7:3 (v/v) methanol (EMD Chemicals, Gibbstown, NJ) : glacial acetic acid (99.5%, J.T.Baker, Phillipsburg, NJ) and collected. The absorbance of the sample was measured in duplicate using UV-Visible Spectrophotometer (UV-1700 PharmaSpec, Shimadzu Scientific Instruments, Columbia, MD) at 458 nm. The concentration of norbixin recovery was calculated based on the absorbance and standard curve.

**Descriptive Sensory Analysis**

Descriptive sensory analysis (n=10) was conducted in compliance with the North Carolina State University Institutional Review Board for human subjects approval. Samples were evaluated in duplicate by eight panelists (ages 22-46 y, 7 females, 1 male) who each had more than 150 h of experience with descriptive analysis of dried dairy
ingredients aroma and flavor, including WPC, using the Spectrum™ method (Drake and Civille, 2003; Meilgaard et al., 2007). The aroma and flavor attributes of reconstituted WPC34 were evaluated using a 0 to 15 point scale and an established lexicon for dried dairy ingredients (Drake et al., 2003; Carunchia-Whetstine et al., 2003, 2005; Russell, et al., 2006; Evans et al., 2009). Rehydrated WPC34 (10% w/v) was dispensed into 60 mL lidded soufflé cups (ca 30 mL) labeled with 3-digit random codes. These samples were tempered to 20°C and served at this temperature. Panelists were instructed to expectorate samples after evaluation. Deionized water and unsalted crackers were available to the panelists for palate cleansing. Each replicate was evaluated in duplicate by each panelist in a randomized block design using paper ballots or Compusense Five version 4.8 (Compusense, Guelph, Canada).

**Volatile Compound Analysis**

Volatile compounds were extracted by solid phase micro extraction (SPME) followed by gas chromatography mass spectrometry (GC-MS). This method was adapted from Croissant et al. (2009). Ten percent (w/w) sodium chloride (VWR International, West Chester, PA) was added to 20 mL SPME autosampler vials (MicroLiter Analytical Supplies, Inc., Sawanee, FL). Five grams of rehydrated sample (10% w/w) was then added to the vial, followed by 10μL of 8.1 mg/kg internal standard (2-methyl-3-heptanone (Sigma-Aldrich, St. Louis, MO) dissolved in methanol (EMD Chemicals, Gibbstown, NJ)). The vial was then capped with a screw top cap equipped with Teflon septa (MicroLiter Analytical Supplies, Inc., Sawanee, FL). The extracted volatiles were
separated by GC-MS (model 6890N, Agilent Technologies Inc., Cary, NC), equipped with 5973 mass selective detector and a DB-5 column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness, Restek U.S., Bellefonte, PA) equipped with a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland). Samples were maintained at 5°C prior to exposure with a DVB/Carboxen/PDMS StableFlex fiber (Supelco, Bellefonte, PA). Samples were equilibrated at 40°C for 25 min before 1 cm of fiber was exposed for 30 min at 31 mm. While the fiber was exposed to the sample, the vial was agitated at 250 rpm with 4 s agitation pulse. Then the fiber was inserted into the injector at 50 mm for 5 min at 250°C split/splitless injector. Helium gas was used as the carrier gas at a rate of 1 mL/min. The initial GC oven temperature was 40°C for 3 min. The temperature was then ramped to 90°C at a rate of 10°C/min and then to 200°C at 5°C/min. This temperature was held for 10 min. The temperature was then increased again to 250°C at 20°C/min and was held for 5 min. The total run time was 52 min per sample.

Retention indices (RI) were calculated (Van den Dool and Kratz, 1963) based on an alkane series (Sigma-Aldrich, St. Louis, MO). Scan mode and National Institute of Standards and Technology (NIST) 2005 mass spectral library were used to initially identify the volatile compounds of interest. Selective ion monitoring (SIM) mode was then used for higher sensitivity to pick out specific compounds of interest (Croissant et al., 2009; Campbell et al., 2011). In addition, authentic standards were injected under identical conditions to verify the identity of volatile compounds. Relative abundance of compounds was determined by comparing their area to the area of the known
concentration of the recovered internal standard. These analyses were conducted in triplicate.

**Benzoic Acid Extractions and Measurements**

Benzoic acid from all experimental and commercial dried whey proteins and also from liquid permeates from the experimental WPC34 and WPC80 manufacture was extracted in duplicate based on a method adapted from the ISO 9231 method (2008) and Qi et al. (2009). The sample (2.5 g) was weighed into a centrifuge tube (VWR International, West Chester, PA) and 15 mL of HPLC grade water at 40°C (Honeywell, Burdick & Jackson, Muskegon, MI) was added to it. The reconstituted sample was vortexed (Labnet International, Inc., Woodbridge, NJ) and transferred to a 50 mL volumetric flask (VWR International, West Chester, PA). Two more portions of 5 mL LC-MS grade water were added to the tube, vortexed, and transferred to the volumetric flask to rinse the rest of the sample from the tube. Five mL of 1M zinc acetate solution (Mallinckrodt Baker, Inc., Phillipsburg, NJ) followed by 5 mL of 0.25M potassium hexacyanoferrate(II) trihydrate (Alfa Aesar, Ward Hill, MA) in water solution were added to the sample to precipitate fat and protein. Zinc acetate solution was made by weighing 21.9 g of zinc acetate, adding 32 mL of acetic acid (99.5%, Mallinckrodt Baker, Inc., Phillipsburg, NJ), and diluting it with HPLC water to 100 mL total volume. Once the zinc acetate and potassium hexacyanoferrate solutions were added, the sample was diluted with LC-MS water to 50 mL and shaken manually for 1 min. The sample was then filtered using vacuum filtration through a 0.45 μm PES membrane (VWR
The filtrate was collected for analysis and diluted 1:1 with the HPLC mobile phase and placed into a 1.5 mL HPLC vial (Alltech Associates, Inc., Deerfield, IL).

The analysis was carried out by Dionex Summit HPLC system (Sunnyvale, CA) equipped with a P680 pump, ASI-100 autosampler, UVD340U diode array detector, and Phenomenex HyperClone ODS (C18) 120A column (4.0 x 250 mm, 5μm) with a C18 security guard (4 x 2.0 mm) (Phenomenex, Torrance, CA). The column oven temperature was kept at 30°C. The mobile phase used was 4% (v/v) methanol (Mallinckrodt Baker, Inc., Phillipsburg, NJ) and 96% (v/v) ammonium acetate buffer (0.02M) (Sigma Aldrich, St. Louis, MO) with the flow rate of 1.0 mL/min. The injection volume of the sample was 20 μL. The UV detector was set at a wavelength of 230 nm.

A 12 point standard curve for benzoic acid (EMD Chemicals Inc., Gibbstown, NJ) was constructed over the range of 0.050 to 50 mg/kg in mobile phase. The extracted samples were injected in duplicate. Benzoic acid concentrations were calculated based on the standard curve and dilution factor using Chromeleon 6.8 Chromatography Data System (Dionex, Sunnyvale, CA). In addition to this, some commercial and experimental WPC34 and WPC80 samples were rehydrated and spiked with benzoic acid standard (10 mg/kg), extracted and injected, and used to calculate benzoic acid recovery. Limit of detection (LOD) was calculated based on the blank sample signal plus three standard deviations of the blank. The spectra shown in the sample peak as well as retention time were also compared to those in the standard solutions. This method was used to extract and measure the benzoic acid residue in all of the commercial and experimental samples.
The concentration of benzoic acid was calculated based on the area of the peak and the standard curve generated. Dilution factors of the sample in the extraction procedure (2.5:50) and the extract injected (1:2), in addition to the recovery were taken into account in calculating the benzoic acid concentration.

**Statistical Analyses**

Data was analyzed using analysis of variance with means separation (ANOVA) (XLSTAT, version 2009.1.02, Addinsoft, New York, NY). Principal component analysis (PCA) was also conducted to visually illustrate volatile compounds differences in experimental WPC34.

**RESULTS AND DISCUSSION**

**Composition Analysis**

There were no differences in composition of the experimental WPC34 samples (p>0.05). Mean moisture, fat, and protein were 2.37 ± 0.66%, 1.64 ± 0.07% (dry wt), 1.48 ± 0.06% (wet wt), 33.7 ± 1.3% (dry wt), 30.4 ± 1.2% (wet wt), respectively. Lactose and mineral contents (combined) of WPC34 were 65.8 ± 1.4%. There was no differences (p>0.05) in mineral components tested, except for iron (Table 1). HP bleached WPC34 had lower iron content compared to the unbleached WPC34 (p<0.05). This result suggests that some reaction between HP and whey components occurs that releases iron or allows more iron to be removed during ultrafiltration.
**Color Analysis and Norbixin Extractions and Measurements**

Visually, there were no differences between WPC34 powders (Figure 1), however, significant differences in color were observed between rehydrated bleached and unbleached WPC34 samples (Figure 2). Similarly, the Hunter L*, a*, b* color values of bleached and unbleached WPC34 powders were not different, but differences (p<0.05) in b* values were observed between the reconstituted samples (Table 2). Reconstituted WPC34 (10% w/v) bleached with BP at either concentration (50 and 100 mg/kg) had lower reflectance (b*) values compared to the control unbleached WPC34 (p<0.05), while the HP bleached WPC34 was not different from the control unbleached WPC34 (p>0.05). WPC34 bleached with HP or BP had lower norbixin concentration compared to the unbleached WPC34 (p<0.05) (Figure 3) and bleaching with BP reduced the norbixin concentration of WPC34 more than HP bleaching (p<0.05). These results are consistent with those observed by Croissant et al. (2009) for WPC70.

**Descriptive Sensory Analysis**

Sensory profiles of rehydrated WPC34 (10% solids) were distinct (Table 3). These flavors have previously been documented in dried whey ingredients (Drake et al., 2003, 2009; Evans et al., 2009; Wright et al., 2009). The unbleached WPC34 had higher sweet aromatic and cooked/milky flavors compared to bleached WPC34 (p<0.05), and the HP bleached WPC34 had higher cardboard flavor compared to unbleached and BP bleached samples (Table 4). Higher cardboard flavor in HP bleached fluid whey and
WPC70 compared to control or BP bleached product has been documented previously (Croissant et al., 2009).

**Volatile Compound Analysis**

Twenty-five volatile compounds were selected for analysis based on previous studies with fluid whey and dried whey ingredients (Carunchia Whetstine et al., 2003, 2005; Wright et al., 2006; Croissant et al., 2009; Wright et al., 2009; Evans et al., 2009, 2010; Liaw et al., 2010; Leksrisompong et al., 2010; Campbell et al., 2011). These compounds included a range of fermentation flavor compounds (i.e., diacetyl, acetic acid, and butanoic acid) as well as protein oxidation (dimethyl disulfide and dimethyl trisulfide), Strecker degradation products (i.e., 3-methylbutanal, 2-methylbutanal), and lipid oxidation products (i.e, aldehydes, alcohols, and ketones). WPC34 bleached with HP had higher concentrations of lipid oxidation compounds, including pentanal, hexanal, heptanal, and octanal, compared to unbleached WPC34 and WPC34 bleached with 50 or 100 mg/kg BP (Table 4). WPC34 bleached with 100 mg/kg BP, in many cases, had higher concentrations of lipid oxidation compounds than WPC34 bleached with 50 mg/kg BP (Table 4). Whey bleached with HP 500 mg/kg and BP 100 mg/kg were more characterized by lipid oxidation compounds, while the unbleached whey and those bleached with BP at 50 mg/kg were characterized by lower concentrations of lipid oxidation compounds (Figure 4). These volatile compound results are consistent with sensory profiles which indicated higher cardboard flavors in HP bleached WPC34 compared to unbleached or BP bleached product. Cardboard flavor has been sourced to
lipid oxidation compounds in fluid whey and dried whey ingredients (Wright et al., 2009; Liaw et al., 2010; Whitson et al., 2010).

Lipid oxidation is initiated in the cheese making process (Tomaino et al., 2004). The unsaturated fatty acids, such as oleic, linoleic, and linolenic acids from the milk, can form unstable hydroperoxides, which decompose to carbonyl products, such as short chain fatty acids and aldehydes (Badings, 1991; O’Connor and O’Brien, 2006). In whey bleaching, HP reacts with UV light or transition metals in milk and creates hydroxyl radicals (McClements and Decker, 2008), whereas BP undergoes homolytic cleavage of the oxygen-oxygen bond, producing benzoyl radicals (Croissant et al., 2009). These peroxides can abstract hydrogen from carotenoid double bonds and thereby remove color or form alkyl radicals in lipids and promote oxidation. These alkyl radicals react with oxygen to form peroxyl radicals, which further abstract more hydrogen from another molecule to form hydroperoxides (Frankel, 1998). These hydroperoxides then decompose to volatile oxidation products (Nawar, 1996; Frankel, 1998). Overall, BP bleached WPC34 had lower norbixin concentration than HP bleached WPC34, and WPC34 bleached with HP resulted in higher lipid oxidation and cardboard flavor compared to unbleached and BP bleached samples.

**Benzoic Acid Analysis**

The instrumental limit of detection (LOD) for benzoic acid solution was 0.05 mg/kg. The LOD for benzoic acid was 12.5 mg/kg in WPC and 5.42 mg/kg in permeate. Bui and Cooper (1987) reported that the LOD for benzoic acid was 20 mg/kg in complex
foods such as dairy products and 5 mg/kg in less-complex matrixes such as soft drinks. Wen et al. (2007) reported that the LOD for benzoic acid was 1.2 ng/mL using an in-tube SPME-HPLC method. Qi et al. (2009) reported that the LOD for benzoic acid was 0.2 mg/kg from pasteurized and UHT milk and 2 mg/kg for milk powder and infant formula.

Experimental and commercial WPC34 that were bleached with BP had higher benzoic acid residue compared to the unbleached or HP bleached WPC34 (p<0.05) (Table 5). Benzoic acid content in both experimental and commercial WPC34 ranged from less than 12.5 to 634 mg/kg (Table 5). The unbleached and HP bleached WPC34 had lower benzoic acid contents than the range reported by Sieber et al. (1995): 12 – 13 mg/kg in whey and from 23 – 75 mg/kg in whey powder. The benzoic acid contents in commercial mozzarella WPC34 were within this range, whereas the BP bleached samples had higher benzoic acid contents than this range. The experimental BP (50 mg/kg) bleached WPC34 had a comparable amount of benzoic acid content to commercial BP bleached WPC34. However, the actual amount of BP added to the commercial sample was unknown. The experimental BP bleached WPC80 had higher benzoic acid content than unbleached and HP bleached WPC80 (p<0.05) (Table 6). The benzoic acid content in WPC80 that was not colored with annatto was lower than benzoic acid concentration from annatto colored WPC80 (p<0.05) (Table 6). This result may suggest that in annatto colored whey, benzoyl peroxide preferentially reacts with conjugated double bonds present in annatto as oxidizable substances which can be oxidized; and benzoyl peroxide decomposes to benzoic acid, resulting in higher benzoic acid content in the final product compared to product with no annatto. Alternatively, this result may reflect that BP is a
large and relatively nonpolar molecule. The presence of annatto with its nonpolar regions allows the BP to be more available for reaction.

Benzoic acid contents in commercial WPC80 were lower than the range of what was reported by Sieber et al. (1995), except for the experimental BP bleached WPC80 which were within this range. The values reported by Sieber et al. (1995) did not include whether the whey was bleached or unbleached and whether the whey came from Cheddar or Mozzarella cheese. Differences in benzoic acid contents found in this study and those found in Sieber et al. (1995) may possibly be due to different amounts of hippuric acid present in the milk as well as starter culture used and concentration of bleaching agent used. Qi et al. (2009) stated that benzoic acid content in fluid milk and cultured milk products could be influenced by various factors, including feeding practices (preservatives used in feeds), contamination, and storage conditions. In comparing WPC34 and WPC80, benzoic acid concentration in WPC34 was higher than in WPC80 even at the same concentration of BP applied (Table 5, 6). From benzoic acid extraction of WPC34 and WPC80 permeates, benzoic acid concentration in BP bleached WPC34 permeate was lower than in BP bleached WPC80 permeate (p<0.05) (Table 7). These benzoic acid contents in WPC34 and WPC80 permeate supported the results of higher benzoic acid concentrations in WPC34 compared to WPC80 powder and suggested that benzoic acid was removed by ultrafiltration and diafiltration. Benzoic acid contents in unbleached Mozzarella whey protein were generally higher than those in unbleached Cheddar whey protein possibly due to different starter cultures used. In Mozzarella cheese making, a thermophilic starter culture is used, while in Cheddar cheese making,
mesophilic starter culture is used. The unbleached and HP bleached WPC still contained some benzoic acid. This benzoic acid is likely from conversion of hippuric acid that is naturally present in the milk to benzoic acid by starter bacteria (Sieber et al., 1995).

**CONCLUSIONS**

These results demonstrate that benzoyl peroxide (BP) at both concentrations tested (50 and 100 mg/kg) is a more effective bleaching agent compared to hydrogen peroxide (HP) at its highest allowable concentration (500 mg/kg) for fluid whey. Descriptive sensory analysis demonstrated that HP bleached WPC34 had higher cardboard flavor followed by BP 100 mg/kg, BP 50 mg/kg, and unbleached WPC34. Consistent with sensory results, WPC34 bleached with HP at 500 mg/kg and BP at 100 mg/kg had more lipid oxidation products compared to the unbleached and BP 50 mg/kg bleached WPC34. Experimental and commercial WPC34 samples bleached with BP had higher benzoic acid residue compared to unbleached and HP bleached WPC34 (p<0.05). The annatto colored experimental WPC80 bleached with BP was higher in benzoic acid content compared to the unbleached and HP bleached WPC80 (p<0.05). Benzoic acid extraction from permeate demonstrated that WPC80 permeate contained more benzoic acid than WPC34 permeate (p<0.05). Overall, these results suggest that BP is a more effective bleaching agent compared to HP; whey bleaching with HP will result in increased lipid oxidation compared to BP; and that benzoic acid is removed by ultrafiltration and diafiltration.
ACKNOWLEDGEMENTS

Funding is provided by the Dairy Research Institute (DRI) managed by Dairy Management Inc. (Rosemont, IL). Special thanks to Dje Yobouet (USDA, Agricultural Marketing Service – Field Laboratory Services, Gastonia, NC) and Dr. Ping Qi (Institute of Food Industry Hygiene Inspection, Guangzhou, Guangdong Province, China) for their assistance with HPLC, and to Dr. David Barbano (Cornell University) for his assistance with composition analysis. Paper FSR XX 11 of the Department of Food, Bioprocessing and Nutritional Sciences, North Carolina State University. The use of trade names does not imply endorsement nor lack of endorsement by those not mentioned.
REFERENCES


TABLES AND FIGURES

Table 1. Mineral analysis of experimental WPC34

<table>
<thead>
<tr>
<th>Sample</th>
<th>%P</th>
<th>%Ca</th>
<th>%Mg</th>
<th>%K</th>
<th>%S</th>
<th>Fe (mg/kg)</th>
<th>Na (mg/kg)</th>
<th>Ash (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.601</td>
<td>0.740</td>
<td>0.113</td>
<td>1.87</td>
<td>0.440</td>
<td>17.4</td>
<td>5.51 x 10^3</td>
<td>0.175</td>
</tr>
<tr>
<td>HP 500</td>
<td>0.596</td>
<td>0.735</td>
<td>0.111</td>
<td>1.84</td>
<td>0.458</td>
<td>5.48</td>
<td>5.72 x 10^3</td>
<td>0.175</td>
</tr>
<tr>
<td>BP 50</td>
<td>0.597</td>
<td>0.751</td>
<td>0.112</td>
<td>1.80</td>
<td>0.446</td>
<td>7.99</td>
<td>5.26 x 10^3</td>
<td>0.131</td>
</tr>
<tr>
<td>BP 100</td>
<td>0.592</td>
<td>0.764</td>
<td>0.113</td>
<td>1.76</td>
<td>0.463</td>
<td>12.5</td>
<td>5.20 x 10^3</td>
<td>0.171</td>
</tr>
</tbody>
</table>

a,b Means in the same column not sharing a common superscript are significantly different (p<0.05)

1Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide
Table 2. Hunter L*, a*, b* color values of experimental bleached and unbleached Cheddar WPC34 as powders and reconstituted to 10% solids (w/v)

<table>
<thead>
<tr>
<th>Bleaching Treatment&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Powder</th>
<th>Reconstituted Powder (10% solids (w/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L*</td>
<td>a*</td>
</tr>
<tr>
<td>Control</td>
<td>89.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HP 500</td>
<td>91.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP 50</td>
<td>89.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.733&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP 100</td>
<td>90.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.473&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Means in the same column not sharing a common superscript are significantly different (<p<0.05)

<sup>1</sup> Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide. Numerals indicate bleach concentration in mg/kg
Table 3. Descriptive sensory profiles of bleached and unbleached Cheddar WPC34

<table>
<thead>
<tr>
<th>Bleaching Treatment</th>
<th>Aroma Intensity</th>
<th>Sweet Aromatic</th>
<th>Cardboard</th>
<th>Cooked Milky</th>
<th>Astringent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HP 500</td>
<td>2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP 50</td>
<td>1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP 100</td>
<td>1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Means in the same column not sharing a common superscript are significantly different (p<0.05)

<sup>1</sup>Intensities were scored based on a 0 to 15-point universal scale, with 0 being no intensity and 15 being the highest intensity. Most dried ingredient flavors fall between 0 and 4 on this scale (Evans et al., 2009; Wright et al., 2009; Liaw et al., 2010).

<sup>2</sup>Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide. Numerals indicate bleach concentration in mg/kg.

<sup>3</sup>ND = not detected
Table 4. Relative abundance (μg/kg) of selected volatile compounds in bleached and unbleached Cheddar WPC34

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Treatment¹</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HP 500</td>
<td>BP 50</td>
<td>BP 100</td>
<td></td>
</tr>
<tr>
<td>3-methyl butanal</td>
<td>0.159a</td>
<td>0.080b</td>
<td>0.024b</td>
<td>0.045b</td>
<td></td>
</tr>
<tr>
<td>2-methyl butanal</td>
<td>0.120a</td>
<td>0.065b</td>
<td>0.026b</td>
<td>0.037b</td>
<td></td>
</tr>
<tr>
<td>Pentanal</td>
<td>0.025c</td>
<td>0.182a</td>
<td>0.053bc</td>
<td>0.086b</td>
<td></td>
</tr>
<tr>
<td>Hexanal</td>
<td>4.11b</td>
<td>32.1a</td>
<td>18.8oab</td>
<td>32.6a</td>
<td></td>
</tr>
<tr>
<td>Z-4-heptenal</td>
<td>0.018a</td>
<td>0.015a</td>
<td>0.009a</td>
<td>0.013a</td>
<td></td>
</tr>
<tr>
<td>Heptanal</td>
<td>0.575b</td>
<td>2.87a</td>
<td>0.989b</td>
<td>2.69a</td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>0.242a</td>
<td>0.231a</td>
<td>0.075a</td>
<td>0.290a</td>
<td></td>
</tr>
<tr>
<td>Octanal</td>
<td>0.094b</td>
<td>0.934a</td>
<td>0.160b</td>
<td>0.472b</td>
<td></td>
</tr>
<tr>
<td>Phenylacetaldehyde</td>
<td>0.122a</td>
<td>0.063b</td>
<td>0.026b</td>
<td>0.067ab</td>
<td></td>
</tr>
<tr>
<td>2-octenal</td>
<td>0.013b</td>
<td>0.217a</td>
<td>0.096ab</td>
<td>0.197a</td>
<td></td>
</tr>
<tr>
<td>Nonanal</td>
<td>1.45a</td>
<td>1.98a</td>
<td>1.23a</td>
<td>2.56a</td>
<td></td>
</tr>
<tr>
<td>E-2-nonenal</td>
<td>0.030a</td>
<td>0.101a</td>
<td>0.052a</td>
<td>0.119a</td>
<td></td>
</tr>
<tr>
<td>Decanal</td>
<td>0.038a</td>
<td>0.053a</td>
<td>0.031a</td>
<td>0.056a</td>
<td></td>
</tr>
<tr>
<td>E,E-2,4-nonadienal</td>
<td>0.008b</td>
<td>0.092ab</td>
<td>0.051ab</td>
<td>0.127a</td>
<td></td>
</tr>
<tr>
<td>Diacetyl</td>
<td>0.064a</td>
<td>0.038a</td>
<td>0.038a</td>
<td>0.039a</td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.103a</td>
<td>0.096a</td>
<td>0.091a</td>
<td>0.106a</td>
<td></td>
</tr>
<tr>
<td>DMDS</td>
<td>0.045b</td>
<td>0.095a</td>
<td>0.008c</td>
<td>0.023bc</td>
<td></td>
</tr>
<tr>
<td>1-pentanol</td>
<td>0.076b</td>
<td>0.181b</td>
<td>0.256b</td>
<td>0.584a</td>
<td></td>
</tr>
<tr>
<td>Butanoic acid</td>
<td>0.026a</td>
<td>0.012a</td>
<td>0.015a</td>
<td>0.010a</td>
<td></td>
</tr>
<tr>
<td>2-heptanone</td>
<td>0.370a</td>
<td>0.373a</td>
<td>0.274a</td>
<td>0.357a</td>
<td></td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>0.065a</td>
<td>0.077a</td>
<td>0.070a</td>
<td>0.074a</td>
<td></td>
</tr>
<tr>
<td>1-octen-3-one</td>
<td>0.029b</td>
<td>0.178ab</td>
<td>0.116ab</td>
<td>0.185a</td>
<td></td>
</tr>
<tr>
<td>DMTS</td>
<td>0.085a</td>
<td>0.097a</td>
<td>0.092a</td>
<td>0.110a</td>
<td></td>
</tr>
<tr>
<td>2,5-octanedione</td>
<td>0.117b</td>
<td>0.340ab</td>
<td>0.197ab</td>
<td>0.460a</td>
<td></td>
</tr>
<tr>
<td>2-pentylfuran</td>
<td>0.559b</td>
<td>3.716a</td>
<td>1.446b</td>
<td>3.916a</td>
<td></td>
</tr>
</tbody>
</table>

a,b,cMeans in the same row not sharing a common superscript are significantly different (p<0.05)

¹Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide. Numerals indicate bleach concentration in mg/kg
Table 5. Benzoic acid concentration (mg/kg) in experimental and commercial WPC34

<table>
<thead>
<tr>
<th>Sample</th>
<th>Benzoic Acid Concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental</strong></td>
<td></td>
</tr>
<tr>
<td>Control (Cheddar no bleach)</td>
<td>&lt; 12.5</td>
</tr>
<tr>
<td>Cheddar HP 500 mg/kg bleached</td>
<td>&lt; 12.5</td>
</tr>
<tr>
<td>Cheddar BP 50 mg/kg bleached</td>
<td>272&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cheddar BP 100 mg/kg bleached</td>
<td>634&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Commercial</strong></td>
<td></td>
</tr>
<tr>
<td>Mozzarella no bleach</td>
<td>12.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mozzarella no bleach</td>
<td>27.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cheddar no bleach</td>
<td>&lt; 12.5</td>
</tr>
<tr>
<td>Cheddar BP bleached</td>
<td>278&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Means in the same sample category (experimental or commercial) not sharing a common superscript are significantly different (p<0.05)

<sup>1</sup>HP = hydrogen peroxide; BP = benzoyl peroxide
Table 6. Benzoic acid concentration (mg/kg) in experimental and commercial WPC80

<table>
<thead>
<tr>
<th>Sample</th>
<th>Benzoic Acid Concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental</strong></td>
<td></td>
</tr>
<tr>
<td>Cheddar NA no bleach</td>
<td>ND²</td>
</tr>
<tr>
<td>Cheddar NA HP 500 mg/kg bleached</td>
<td>ND</td>
</tr>
<tr>
<td>Cheddar NA BP 50 mg/kg bleached</td>
<td>30.6ᵇ</td>
</tr>
<tr>
<td>Cheddar A no bleach</td>
<td>ND</td>
</tr>
<tr>
<td>Cheddar A HP 500 mg/kg bleached</td>
<td>ND</td>
</tr>
<tr>
<td>Cheddar A BP 50 mg/kg bleached</td>
<td>60.0ᵃ</td>
</tr>
<tr>
<td><strong>Commercial</strong></td>
<td></td>
</tr>
<tr>
<td>Mozzarella no bleach</td>
<td>&lt; 12.5</td>
</tr>
<tr>
<td>Cheddar no bleach</td>
<td>&lt; 12.5</td>
</tr>
<tr>
<td>Cheddar no bleach</td>
<td>&lt; 12.5</td>
</tr>
<tr>
<td>Cheddar HP bleached</td>
<td>&lt; 12.5</td>
</tr>
<tr>
<td>Mixed HP bleached</td>
<td>&lt; 12.5</td>
</tr>
<tr>
<td>Mixed HP bleached</td>
<td>&lt; 12.5</td>
</tr>
</tbody>
</table>

ᵃᵇMeans in the same sample category (experimental or commercial) not sharing a common superscript are significantly different (p<0.05)

¹NA = no annatto; A = annatto; HP = hydrogen peroxide; BP = benzoyl peroxide

²ND = not detected
Table 7. Benzoic acid concentration (mg/kg) in experimental WPC34 and WPC80 permeates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Benzoic Acid Concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WPC34</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt; 5.42</td>
</tr>
<tr>
<td>HP 500</td>
<td>&lt; 5.42</td>
</tr>
<tr>
<td>BP 50</td>
<td>19.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP 100</td>
<td>20.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>WPC80</strong></td>
<td></td>
</tr>
<tr>
<td>(annatto)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>&lt; 5.42</td>
</tr>
<tr>
<td>HP 500</td>
<td>&lt; 5.42</td>
</tr>
<tr>
<td>BP 50</td>
<td>37.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Means not sharing a common superscript are significantly different (p<0.05)

<sup>1</sup> Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide
Control | HP 500 | BP 50 | BP 100

Figure 1. Experimental WPC34 powders. Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide. Numerals indicate bleach concentration in mg/kg.
Figure 2. Experimental reconstituted WPC34 at 10% solids (w/v).
Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide. Numerals indicate bleach concentration in mg/kg.
Figure 3. Norbixin recovery (mg/kg) from bleached or unbleached WPC34.

Means not sharing a common superscript are significantly different (p<0.05).

Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide. Numerals indicate bleach concentration in mg/kg.

1 Control = no bleach; HP = hydrogen peroxide; BP = benzoyl peroxide. Numerals indicate bleach concentration in mg/kg.
Figure 4. PCA biplot of volatile compounds in bleached and unbleached Cheddar WPC34.