ABSTRACT

QIU, YUTING. Investigation of Novel Methods for Removal of Norbixin from Liquid Whey and Effects on Flavor and Functionality of 80% of Whey Protein Concentrate. (Under the direction of Dr. MaryAnne Drake).

The residual annatto colorant (norbixin) in liquid whey should be removed to provide a desired neutral color in dry whey ingredients. Currently, hydrogen peroxide (HP) and benzoyl peroxide (BP) are utilized for bleaching liquid whey. However, chemical bleaching causes off-flavor formation mainly due to lipid oxidation and protein degradation. Previous studies have suggested a binding or interaction between norbixin and milk fat globule membrane (MFGM) or that norbixin exists as a micelle. As such, removal of milk fat globule membrane by microfiltration or precipitation of MFGM from liquid whey with hydrochloric acid at the isoelectric point might reduce residual annatto in liquid whey. The objective of first study was to determine the effect of pH induced MFGM precipitation (pH 4.2, 5.2) or microfiltration on norbixin levels in liquid Cheddar whey. Cheddar cheese whey was manufactured from colored, pasteurized milk. The fluid whey was pasteurized and fat separated. Liquid whey was subjected to 4 different treatments: Control (no treatment), pH 4.2 (acidification to 4.2 with hydrochloric acid, and centrifugation to remove precipitate), pH 5.2 (acidification to 5.2 with hydrochloric acid, and centrifugation to remove precipitate), or MF (microfiltration). The experiment was replicated 3 times. Proximate analysis of whey was conducted using standard methods. Norbixin content was determined by HPLC. The protein composition of liquid wheys was evaluated by polyacrylamide gel electrophoresis (SDS-PAGE). Norbixin was decreased by 49.3%, 40.0%, and 45.5% for pH 4.2 whey, pH 5.2 whey, and microfiltration treatments respectively. No differences were detected in solids and mineral content among liquid wheys ($P>0.05$). Fat and protein content for MF whey

were lower than other liquid wheys *(P*<0.05). SDS-PAGE gels showed no visible differences in main whey protein (alpha-lactalbumin and beta-lactoglobulin) bands between control whey and pH 4.2 and 5.2 and MF whey, but bovine serum albumin (67KDa) and immunoglobulin heavy chain (50-70KDa) bands were thinner in pH 4.2 and 5.2 wheys, suggesting that these proteins were removed by decreased pH. Based on color reduction and SDS-PAGE results, pH adjustment and/or MF could be used as novel methods to remove norbixin from Cheddar whey.

The objective of second study was to compare MF norbixin removal to chemical or enzymatic bleaching, hydrogen peroxide (HP) or lactoperoxidase (LP), and their effects on the flavor and functionality of 80% whey protein concentrate (WPC80). Cheddar cheese whey was manufactured from colored, pasteurized milk. The fluid whey was pasteurized and fat separated. Liquid whey was subjected to 4 different treatments: Control (no bleaching; 50°C, 1h), HP (250 mg hydrogen peroxide/kg; 50°C, 1h), LP (20 mg hydrogen peroxide/kg; 50°C, 1h), or MF (microfiltration; 50°C, 1h). The treated whey was then ultrafiltered, diafiltered, and spray-dried to 80% whey protein concentrate (WPC80). The entire experiment was replicated 3 times. Proximate analyses, color, functionality, descriptive sensory and instrumental volatile analysis were conducted on WPC80. MF, HP, and LP bleached WPC80 displayed a 39.5%, 40.9% and 92.8% norbixin decrease, respectively (*P*<0.05). The HP and LP WPC80 had higher cardboard flavors and distinct cabbage flavor compared with the unbleached and MF WPC80. Volatile compound results were consistent with sensory results. The HP and LP WPC80 were higher in lipid oxidation compounds (especially heptanal, hexanal, pentanal, 1-hexen-3-one, 2-pentylfuran, octanal) compared to unbleached and MF WPC80. All WPC80 had >85% solubility across the pH range of 3 to 7.

The microstructure of MF gels determined by confocal laser scanning showed an increased protein particle size in the gel network. MF WPC80 also had larger G' values indicating higher gel firmness. Based on bleaching efficacy, flavor and functionality results, MF may be a viable alternative to chemical or enzymatic bleaching of fluid whey.

© Copyright 2015 Yuting Qiu

All Rights Reserved

Investigation of Novel Methods for Removal of Norbixin from Liquid Whey and Effects on Flavor and Functionality of 80% of Whey Protein Concentrate

by
Yuting Qiu

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

2015

APPROVED BY:

_____	_____
Dr. Timothy Sanders	Dr. Allen Foegeding

_____
Dr. MaryAnne Drake

Committee Chair

DEDICATION

To my Mom and Dad, for believing in me and motivating me to do my best.

BIOGRAPHY

Yuting Qiu was born on February 1st, 1990 to Haijun Qiu and Xinyu Wang in Tai'an, China. She graduated from Tai'an No.1 middle school. After graduation, she started her Bachelor of Science degree in Food Safety and Quality in China Agricultural University, Beijing in the Fall of 2008. She received her B.S. degree in June 2012. She moved to the United States to continue her education. She started her Master's degree in Food Science in the Fall of 2012 as a thesis student under the direction of Dr. MaryAnne Drake at North Carolina State University.

ACKNOWLEDGMENTS

I would like to acknowledge my parents. Without their sacrifice and endless love, I could not have made it to this point in my life. To Dr. Drake, thank you for all that you have done for me, for believing in me and giving me this great opportunity. To all my committee members—Dr.Foegeding and Dr. Sanders, I was very lucky to have you all serve on my committee. To Evan Miracle and Michele Yates, thank you for helping solving every problem that I made. To Echo, thank you for being the best support I had here and thank you so much for the great friend I found in you. To Tucker, thank you for putting up with my questions and helping me in the best way possible. To every single member of MAD Lab, thanks for all of your help and support along the way. To NCSU Dairy staffs, thank you for helping me with the equipment in pilot plant. Lastly, to all my close friends, thank you for always being a great support in all my struggles and frustrations in my new life and studies in U.S and being great reliable people to whom I could always talk about my problems and excitements.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Food intake plays an important role in the life of humans, and it may be regarded as fuel for all the metabolic processes or the sources of nutrients to make life. Normally in most adults, body weight is constant despite big changes in daily food intake and energy expenditure. This balance of food intake and energy expenditure is controlled by a complex neural system. This neural system reflects the fundamental biological importance of adequate nutrient supply and energy balance. This system consists of many pathways which cooperate with each other in order to maintain the drive to eat or feel physically satisfied after eating. Thus, in the circulation, there are both hormones which act to initiate or terminate a meal and hormones which reflect energy balance. These signals are integrated by peripheral nerves and brain centers, such as the hypothalamus and brain stem. The integrated signals regulate central neuropeptides, which modulate hunger/eating and energy expenditure.

Much progress has been made in identifying these complex hormonal and neural mechanisms. The brain informs itself about the availability of ingested and stored nutrients, and in turn, generates behaviors like hormonal output in response to these signals. The hypothalamus and brainstem play important roles in this homeostatic function. Energy homeostasis, which ensures that the composition and properties of the human body's internal environment remain constant, in most cases, regulates body weight tightly and human beings can store a considerable amount of energy as fat for later use. However, this ability has been argued for becoming one of the major health risks for over-eating and the subsequent increasing prevalence of obesity. Numerous studies on smell and taste hedonics have revealed palatability (Sclafani et al., 1976) and variety (Rolls et al., 1981) as being two

important factors which increase food intake in both animals (Rolls et al., 1983) and humans (Louis-Sylvestre et al., 1981). On the other hand, neural and hormonal factors, as well as metabolic feedback-mechanisms, may be involved in mediating specific appetites (Tempel et al., 1993; York 1995) and specific satieties (de Graaf et al., 2004; Kamphuis et al., 2001).

SATIETY AND SENSORY-SPECIFIC SATIETY

Normally, hunger and satiety are the basic feelings which regulate food intake. They compensate each other and consequently, control eating. The feeling of hunger characterizes the mental state, which motivates food intake, whereas the feeling of satiety terminates intake of a meal. Satiation and satiety are part of the body's appetite control system and are involved in limiting energy intake. Definitions of satiation and satiety are shown in Figure 1 (Benelam, 2009). This satiating state may limit how much food and drink is consumed on one occasion and delays the next desired food consumption. The term satiety characterizes the period between two meals of about 4 h, where the feeling of satiety gradually is turned into the feeling of hunger (Blundell et al., 1991; Wardle 1987; Bellisle et al., 2012). Satiety is a central concept in the understanding of appetite control and has to do with inhibition of eating. Because of the rising prevalence of obesity, it is important to consider the impact of satiation and satiety on energy balance, and whether they can be enhanced in order to facilitate the reduction of energy intake, aiding weight control. Satiety represents the effect of inhibitory signals produced by the ingestion of food as the meal progresses. Such signals have many origins: sensory, cognitive, digestive, and hormonal. With the influence of these satiety signals, eating behavior ultimately comes to an end. The factors affecting satiation

and satiety from the start of eating to late satiety have been described by Blundell et al. (1987) in the satiety cascade shown in Figure 1.

Satiation	The process that leads to the termination of eating, which may be accompanied by a feeling of satisfaction
Satiety	The feeling of fullness that persists after eating, potentially suppressing further energy intake until hunger returns

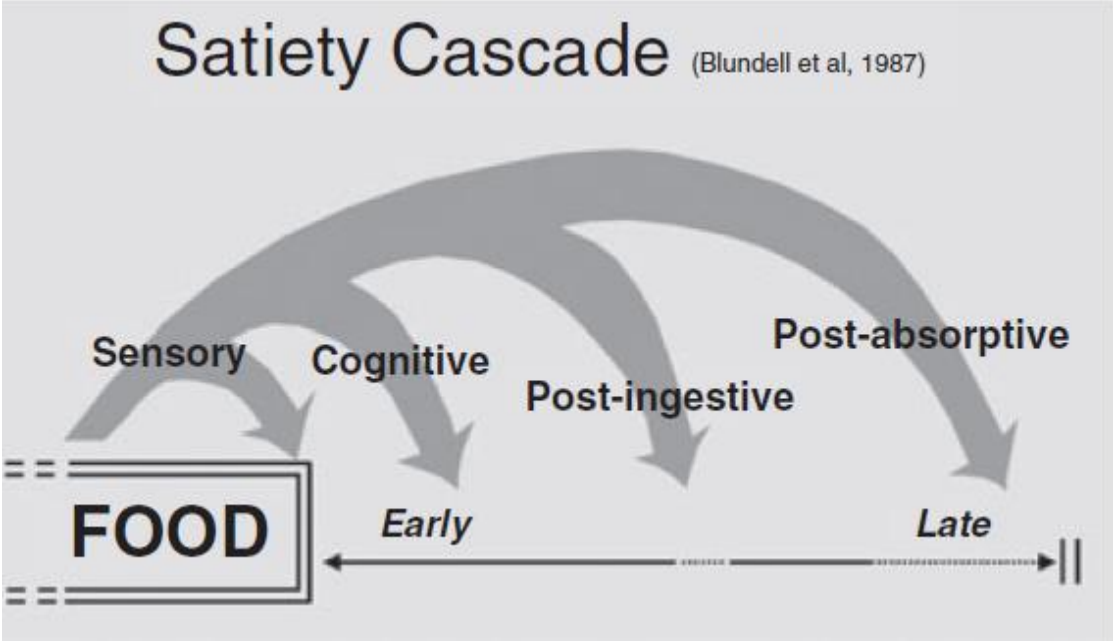


Figure 1. Definition of satiation and satiety and the satiety cascade showing the influences on satiation and satiety over time (Benelam, 2009; Blundell et al., 1987)

As this figure demonstrates, satiation and satiety are initially affected by sensory and cognitive factors including expectations about what is to be consumed, the taste, texture and smell of the food or drink and any associations with previous experience. Once the food or drink reaches the stomach, post-ingestive factors start to take effect. Initially, the distension of the stomach sends signals to the brain, initiating satiation. As digestion continues in the intestines, hormones that promote satiation and satiety are released from the gut. During satiety, sensory and cognitive processes interact with postingestive and postabsorptive peripheral and central mechanisms to inhibit further eating. Because satiety has to do with the inhibition of eating, process that control satiety can potentially affect total intake and facilitate body weight control. Scientific analysis of satiety requires identification of the influences from eating that reduce appetite. Satiety is not a response but the operation of a type of mechanism.

Satiety is essentially sensory: it is a tendency to reject foods or to accept them in limited amounts. It is conceivable that the strength of inhibition may vary with the sensory identity of the food. Such satiety with a degree of specificity to a food is called sensory-specific satiety (SSS) (Rolls et al., 1981). The term sensory-specific satiety was initiated by Le Magnen in 1956 in rats and confirmed by Rolls et al. in 1981 in people. It is defined as the decrease in pleasure aroused by a specific food which has just been eaten in contrast to other non-consumed foods (Rolls et al., 1981). Its specificity is related to the specific sensory properties of an ingested food and its pattern of stimulation on the "peripheral analyzer" (Rolls et al., 1986). Sensory-specific satiety occurs when foods that have been eaten decrease more in liking than foods that have not been eaten and leads to the termination of eating a

particular food (Rolls et al., 1981). For example, after eating sausages, the liking of the sausages strongly declined at the same time that liking of other savory products declined less and liking of sweet foods such as cookies increased (Rolls et al., 1981). Sensory-specific satiety occurs within 2 min after consumption, when there has been little opportunity for digestion and absorption, and it is specific for the sensory aspects of products (Rolls et al., 1986). Examples of sensory properties that can contribute to SSS are taste, smell, texture, color, size, and shape (Rolls et al., 1982). Thus, SSS is a sensory-based phenomenon, i.e. foods high in protein, sweet carbohydrates and fatty acids have been suggested to have relatively great sensory-specific satiety (Johnson et al., 1992; de Graaf et al., 1993; Kamphuis et al., 2001; Vandewater et al., 1996). Sensory factors such as similarity of color, shape, flavor, and texture are usually more important than metabolic equivalence in terms of protein, carbohydrate, and fat content in influencing how foods interact in this type of satiety (Rolls et al., 1982). For example, an important distinction regarding taste can be made between sweet and savory, which includes almost 90% of the food we eat (Mattes, 1985). Spiegel (2000) demonstrated that large foods were consumed with larger bites than small foods, and that large bites were chewed less than small bites, which reduced oral sensory stimulation. Sensory-specific satiety does not require food to enter the gastrointestinal tract, and is independent of the ingestion of calories. This is a mechanism which is strongest during the 2 h following consumption, and can affect liking ratings for over 24 h (Weenen et al., 2005). The amount of food consumed may have an important impact on SSS, whereas the roles of energy and macronutrient content remain controversial (Sorensen et al., 2003).

It has been suggested that SSS plays an important role in the termination of intake. This theory is supported by electrophysiological and functional imaging studies with humans (O'Doherty et al., 2000). In the previous study, SSS was observed for foods in their natural form. This original observation could explain how food variety can lead to over-consumption and in turn over-weight. SSS combined with other consumption strategies such as limiting variety, exercising portion control, and overall awareness of environmental eating cues, may be the recipe to counteract overeating (Wansink et al., 2007). Summarizing, SSS for a particular food during eating is supported by an increase in satiety, a decrease in hunger, a decrease in desire to eat, and expressed as a decrease in pleasantness of taste compared to uneaten foods. SSS during mere exposure without eating is supported by just a decrease in desire to eat and expressed as a decrease in pleasantness of taste compared to foods that a subject is not exposed to.

HORMONES IMPACT SATIETY

The past 20 years have seen significant advances in our understanding of the mechanisms of control of appetite and energy intake. The regulation of eating is complex. In particular, there is a growing awareness that hormonal signals released from the gastrointestinal (GI) tract interact with the central nervous system (CNS) to modulate appetite regulation. Digestion and nutrient absorption take place in the gastrointestinal (GI) tract, whereas sensations of hunger or satiety originate in the central nervous system (CNS).

Long-term and short-term hormonal signals from the periphery act on the central nervous system to influence eating behavior. The main regions involved are the hypothalamus, in particular the arcuate nucleus, and the dorsal vagal complex in the brain

stem (Kalra et al., 1999, Schwartz et al., 2000). The hypothalamus is located at the base of the brain, and is in evolutionary terms, one of the most ancient structures in the CNS. The hypothalamus plays a significant role in the regulation of a number of fundamental biological processes, including feeding. Ingested food evokes satiety in the GI tract primarily by two distinct ways: one by mechanical stimulation of the nerve endings, the other by humoral stimulation. When a hormone is released in response to a change in the blood or other body fluids, such as a change in the level of a mineral or a change in temperature, this is called a humoral stimulus (Karhunen et al., 2008). The latter factors seem to play a key role in satiety through secretion of various gut hormones by the wall of the small and large intestine in response to ingested food since gut hormones have long been known to optimize the processes of digestion and nutrient absorption through direct effects on the GI tract. Changes in the release of these gut hormones affect feeding behavior and energy expenditure, resulting in the maintenance of energy homeostasis.

The GI tract is the largest endocrine organ in the body. There are many receptors throughout the entire GI track for these GI hormones. However, the distribution of the receptors throughout the GI tract is not consistent. Some receptors are only located in the small intestine, whereas other receptors can be found throughout the entire GI tract (Lewin, 1992). We also have to take into consideration that the expression levels of the receptors may not directly reflect hormone secretion. It could be that some receptors are not highly expressed, but affect the release of satiety hormones stronger, compared with receptors that are more commonly found within the GI tract. The secretion of many GI hormones is influenced by gut contents, and so gut peptides act as signals to the CNS of current nutrient

status. Cholecystokinin (CCK) was the first hormone to be related in the short-term control of food intake (Gibbs et al., 1973), and other appetite-regulating hormones have subsequently been characterized. Of these, ghrelin is the only known orexigenic gut hormone (an orexigeni is a drug, hormone, or compound that increases appetite.), whereas a number of satiety factors exist, including glucagon-like peptide-1 (GLP-1), oxyntomodulin (OXM), peptide YY (PYY) and pancreatic polypeptide (PP). Regional differences exist in the release of gut peptides and the site of action of gut peptides (Figures 2 and 3). In the duodenum and jejunum, exposure of the gut wall to fat and protein results in the release of CCK, whereas in the distal small intestine, the presence of nutrients induces the release of GLP-1, oxyntomodulin and PYY (Cummings, 2007). Many of these gut peptides delay gastric emptying, contributing the stomach satiety and subsequently to meal termination (Table 1):

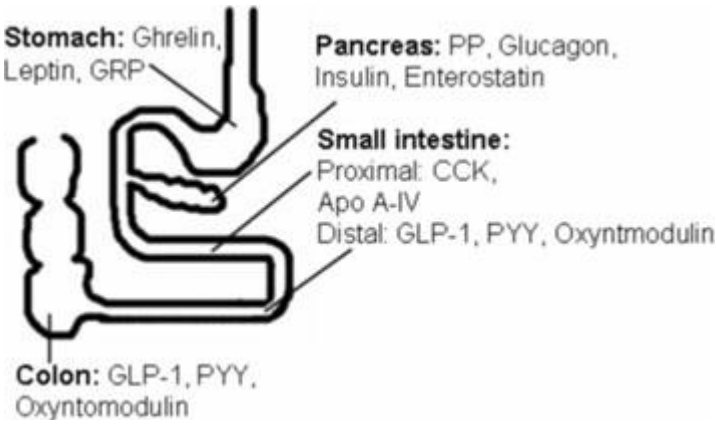


Figure 2. Principal site of secretion of gut peptides involved in regulation of eating behavior (Cummings, 2007).

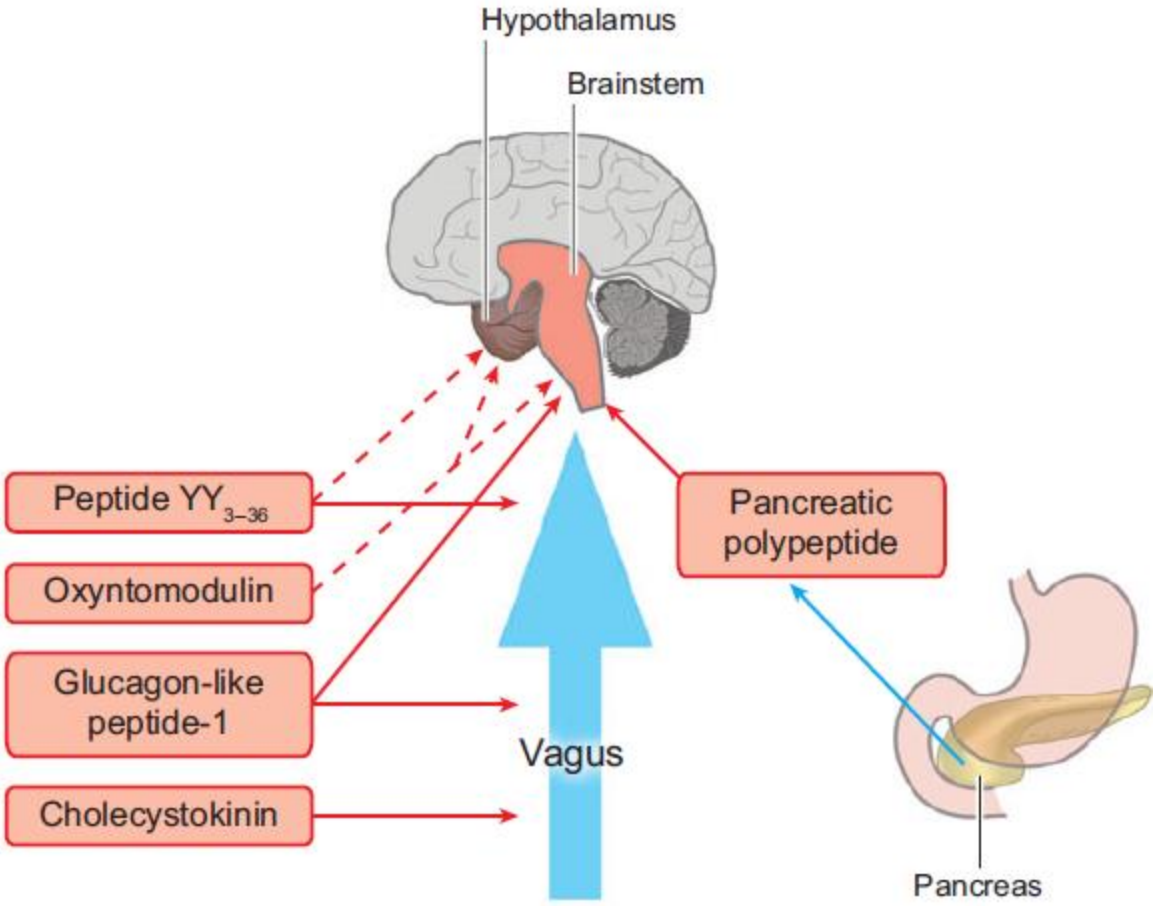


Figure 3. The site of production and principle site of action of the major anorexigenic (appetite-depressing) gut peptides (Cummings, 2007)

Table 1. Gut hormones and their actions (Benelam, 2009)

Name	Site of production	Effect on appetite	Mechanism	Additional effects
Ghrelin	Stomach	↑ Hunger	Via ghrelin receptors in the brain	Long-term effect on energy balance
Cholecystokinin (CCK)	Duodenum and jejunum	↑ Satiation	Via vagus nerve	Delays gastric emptying Stimulates pancreatic enzyme secretion Stimulates gall bladder contraction Acts as a neurotransmitter
Glucagon-like peptide-1 (GLP-1)	Intestine and brain	↑ Satiety	Via GLP-1R in brain	Incretin (stimulates insulin production) Slows gastric emptying
Oxyntomodulin (OXM)	Intestine and brain	↑ Satiety	Via GLP-1R in brain Via reductions in ghrelin	Slows gastric emptying
Peptide YY (3-36) (PYY 3-36)	Ileum, colon and rectum	↑ Satiety	Via Y2 receptors in brain	Slows gastric emptying and intestinal transport Reduces gastric secretions
Pancreatic polypeptide (PP)	Pancreas	↑ Satiety	Via Y5 receptors in brain Via vagus nerve	–

GLP-1

Glucagon-like peptide-1 is a product of proglucagon cleavage and is released from enteroendocrine L-cells in the distal small intestine, in response to exposure of the gut wall to fat and carbohydrates (Schirra et al., 2005). Secretion occurs not only when nutrients are present in the lumen of the distal small intestine, but also from the duodenum by an indirect neurohumoral pathway involving the autonomous nervous system (Brubaker et al., 2003). GLP-1 acts through specific GLP-1 receptors that are expressed in the stomach, peripheral nerves, and specific regions in the brain such as the hypothalamus, hindbrain, and amygdala. GLP-1 and longer-acting GLP-1 receptor agonists, such as exendin-4, reduce food intake in rodents when injected into the CNS (Turton et al., 1996) or peripherally (Baggio et al., 2004). Infusion of GLP-1 results in a reduction in food intake and elicits satiety in normal weight, obese and diabetic humans. Apart from the effects on food intake, GLP-1 plays an important role in glucose metabolism, stimulating insulin secretion and controlling other processes that

limit hyperglycemia. GLP-1 increases glucose-dependent insulin secretion, reduces glucagon-secretion and increases pancreatic b-cell growth. GLP-1 is therefore being developed for the treatment of diabetes. Degen et al. (2006) demonstrated that the anorexic effect of GLP-1 could be increased by a 400 mL (225 kcal) protein drink, but not by 400 mL water administered 20 min prior to a test meal. This study clearly demonstrated that GLP-1 interacts with gastric or duodenal satiety signals to increase satiety and reduce food intake. Central administration of GLP-1 causes anorexia, an effect mediated by GLP-1 action in the hypothalamus and hindbrain (Kinzig et al., 2002). Anorexia is an eating disorder characterized by immoderate food restriction, inappropriate eating habits or rituals, obsession with having a thin figure, and an irrational fear of weight gain, as well as a distorted body self-perception (Hockenbury, 2008). The mechanisms resulting in the anorexic effect of GLP-1 are not fully understood. A reduced secretion of GLP-1 could therefore contribute to the pathogenesis of obesity, and agonists (An agonist is a chemical that binds to a receptor and activates the receptor to produce a biological response) of the GLP-1 receptor are potential targets for treatment. The therapeutic potential of GLP-1 is limited by its rapid breakdown. Increasing endogenous GLP-1-release by specific food products is therefore an interesting target. Further understanding of the satiety effects of GLP-1 are of immediate clinical relevance.

OXM

OXM is co-secreted with GLP-1 into the circulation by intestinal L-cells following nutrient ingestion. OXM is a satiety signal and reduces energy intake in both rodents and humans (Dakin et al., 2004). This effect is inhibited by exendin 9−39 (the GLP-1 receptor

antagonist) and is lost in GLP-1R null mice, suggesting that OXM is regulated by the GLP-1 receptors (GLP-1R) (Dakin et al., 2001). OXM inhibits food intake in the rat with greater potency than GLP-1. OXM has a very different neuronal activation from that of GLP-1. OXM appears to act via a GLP-1-like receptor in oxynic glands which are responsible for forming or secreting acid, as the parietal cells of gastric glands. In humans, OXM reduced food intake at a free-choice buffet meal by 19.3%, with the total calorie intake remaining lower at 12 h after infusion compared to a control group (Cohen et al., 2003). OXM may play a role in the physiological regulation of appetite. Further evidence suggests that its role in the control of energy homeostasis also includes effects on energy expenditure (Wynne et al., 2007). Longer-term trials are now required to determine whether this unique dual effect will make it a useful treatment for obesity.

PYY

PYY is a peptide belonging to the neuropeptide Y (NPY) family, the major circulating form of PYY--PYY_{3-36}, and is co-secreted with GLP-1 by enteroendocrine L-cells in the distal small intestine (Adrian et al., 1985). Direct exposure of the distal small intestine to nutrients will release PYY, but an indirect neurohumoral pathway from the proximal small intestine to the distal small intestine also exists. After a meal, PYY is released shortly after food intake; this is likely to be under neural control because it occurs before ingested nutrients reach the distal small intestine and colon, where the greatest concentrations of PYY_{3-36} are found. It is further released in proportion to the amount of ingested calories, with fat being the most potent macronutrient, followed by carbohydrates, followed by proteins (Onaga et al., 2002). Peripheral administration of PYY was first reported in 1993 to decrease

appetite (Cummings et al., 2007). In addition, when PYY $_{3-36}$ was administered to a mouse, rat, or human, a marked inhibition of food intake was observed (Batterham et al., 2003). A number of research groups have demonstrated that peripheral PYY $_{3-36}$ inhibits food intake and reduces body weight gain in several species. In the first study of the effects of PYY $_{3-36}$ on food intake in humans, spontaneous food intake was reduced by 30% at plasma levels similar to those seen physiologically. A further recent study observed a dose-dependent reduction in appetite and food intake in response to intravenous PYY $_{3-36}$ administration in normal weight volunteers, although nausea occurred at higher doses (Degen et al., 2005). PYY $_{3-36}$ is likely to affect appetite via a direct central effect and also via its effects on gut motility; it acts as an "ileal brake" and so leads to a sensation of fullness and satiety (Batterham et al., 2003). However, the therapeutic potential of PYY $_{3-36}$ as an anti-obesity treatment is currently unknown, as there are limited data regarding the effect of repeated doses of PYY $_{3-36}$ on body weight in humans.

PP

PP is a 36-amino acid peptide from the NPY family. Sharing some common structural features with PYY 3-36, PP is secreted by a population of cells located at the periphery of the pancreatic islets. It is released into the circulation in response to nutrient ingestion and is controlled by the vagus nerve and a number of other factors. The amount of PP release is dependent on the digestive state: release is low when fasted and increased throughout all phases of digestion (Katsuura et al., 2002; Batterham et al., 2003). The role of PP in the regulation of energy balance is less controversial. In common with other appetite-regulating gut peptides, PP is released into the circulation in proportion to the calories ingested, and

levels remain elevated for up to 6 h after feeding (Adrian et al., 1976). As in mice, the anorexia effects of PP in humans persisted for 24 h after infusion (Batterham et al., 2003). It remains to be studied whether this effect is observed in obese humans. The long last of this effect suggests that PP may have a longer-term role in the regulation of appetite than simple meal-by-meal regulation of food intake and makes PP a potentially attractive target for weight therapy.

CCK

Cholecystokinin (CCK) is the prototypic GI satiety signal, an effect first described by Smith and Gibbs more than 30 years ago (Liddle et al., 1994). Finding CCK was an important step in our understanding of endocrine control of satiety. Research with GLP-1 and PYY today suggests that it plays an important role of gastric and intestinal signals to the brain in the regulation of hunger, satiety and eating behavior. CCK is widely distributed throughout the GI tract, but is most concentrated in the duodenum and the jejunum. CCK is released from intestinal I-cells in the duodenal and jejunal mucosa in response to the digestion products of fat and protein in the small intestine. Various biological forms of CCK exist, classified according to the number of amino acids they contain. Two receptors for CCK (the CCK-1 and CCK-2 receptors) have been identified. Although CCK can be measured in the circulating plasma, the mechanism by which it helps control food intake is dependent on signaling by the vagus nerve (Reidelberger et al., 2004). Gibbs et al. (1973) demonstrated in rats that administration of CCK-8 induced a dose-dependent reduction in both the size and duration of a meal. In humans, Kissileff et al. (1981) were the first to demonstrate that CCK-8 reduced food intake and hunger in humans. CCK plays a number of physiologic GI

functions, including the stimulation of pancreatic secretion, gallbladder contraction, intestinal motility, and the inhibition of gastric emptying, explaining the effect that CCK has on suppression of feeding (Hayes et al., 2004). The exploitation of the effects of CCK on appetite for therapeutic purposes has been explored. However, the effect of CCK-infusion on food intake is short-lasting: when the interval between the infusion and the start of the test meal is over 30 min, no anorexic effect will be observed (Cummings et al., 2007). As such, CCK may not be a promising therapeutic strategy.

The GI tract is the host to many signals that affect satiety and food intake. The role of gut hormones in the regulation of appetite involves both neurology and GI physiology. The characterization of the complex neuroendocrine interactions that regulate appetite has advanced significantly in the past two decades. These gut hormones act as meal motivators and terminators. Alterations in levels of gut hormones following bariatric surgery may contribute to the appetite suppression and sustained weight loss seen in patients undergoing this procedure and supports the development of these hormones as therapeutic targets (Korner et al., 2005; Le Roux et al., 2006). Delivery of exogenous peptide to animal models and human volunteers and measurement of their effect on food intake also displays the actions of these hormones on appetite. Although much of the physiology of appetite regulation remains unclear, gut hormones undoubtedly play a key role in the regulation of energy homeostasis. With increasing recognition of obesity as a significant public health and socioeconomic problem, the application of GI signals of satiety may yet prove to be an effective therapeutic strategy.

FOODS AND SATIETY

The macronutrient composition of the diet can influence hunger, satiety, food intake, and body weight and body composition. Carbohydrate, fat and protein are the macronutrients associated with overeating and obesity. With the current debate with nutrition and body weight, a critical question is whether changes in diet composition influence hunger, satiety, the amount of energy or macronutrients consumed, and body weight. Because food scientists can change the nutrient composition of foods while keeping palatability; there will be continuing growth in the variety of products in which sugar and fat substitutes are used to produce low energy food. Can such foods and other such changes in food composition be used to control satiety and to aid weight loss? Many such factors have been examined in both human and animal experiments to identify their potential impact on satiety. These factors are not totally independent of one another. Theoretically, all of them could be used, and some have indeed been used, to enhance satiety after a meal of fixed energy content is ingested.

Carbohydrates and Satiety

Evidence of satiety can come from the extent to which ingesting carbohydrate alters subsequent food intake, as well as from ratings or reports of hunger, fullness or appetite (Reid et al., 1997). Understanding the impact and the mechanisms that carbohydrates have on food intake is one of the major methods to the control of body weight, and the treatment of both malnutrition and diabetes. Potential mechanisms mediating the induction of satiety by carbohydrates include changes in the blood glucose concentration, hormones (including insulin, glucagon-like peptide-1, PYY, etc.), metabolic processes in the liver, and the resulting products of carbohydrate metabolism. Moreover, these various factors may exert

their effect at different times following meal ingestion. It has been suggested that certain monosaccharides, such as glucose, fructose, and malabsorbed sugars, may vary in their effects on food intake. The interaction of a nutrient with receptors in the gastrointestinal tract plays a major role in the induction of satiety by carbohydrates. Oral ingestion of carbohydrate decreases both the perception of appetite and intake at a subsequent meal (Blundell et al., 1994). For example, the addition of maltodextrin (a polysaccharide composed purely of glucose) into tomato soup was associated with a reduction in food intake at lunch 1 hour later when compared with the soup without adding maltodextrin by an amount that approximated the energy provided by the carbohydrate in the soup (Blundell et al., 1994). In addition, regional differences appear to exist in the effects of carbohydrate; for example, the effect of maltose in the distal small intestine or colon may be greater than in the proximal small intestine (Meyer et al., 1998).

Sweet taste detection via T1R2 +T1R3 (different taste receptors) is suggested to be one potential mechanism by which glucose is sensed in gut enteroendocrine cells to trigger peptide secretion (Jang et al., 2007). There is increasing interest in the potential impact of different carbohydrates on food intake, particularly sugars that are poorly absorbed and have the potential to substitute for glucose or other carbohydrates in the diet. There have been a number of studies lasting more than 24 hours focused on the effect of refined carbohydrates in the diet of obese or overweight people. A study (Robert et al., 2011) demonstrated that equisweet solutions of glucose, fructose or aspartame had different effects on gut peptide secretion: only glucose stimulated the secretion of GLP-1 and PYY and decreased ghrelin; in contrast, fructose was much less effective and aspartame had no effect (Born et al., 1994).

Glucose and fructose also differed in their mechanisms of absorption: glucose is absorbed via the sodium-dependent glucose transporter-1 (SGLT-1) and glucose transporter-2 (GLUT-2), whereas absorption of fructose occurs via a sodium-independent transporter and is relatively slow and limited in approximately 60% of adults. Other sugars and sugar alcohols, such as D-tagatose and xylitol, are present in the small intestinal lumen for a longer period of time owing to malabsorption, transported over a greater length of intestine (Meyer et al., 1998), and are therefore likely to interact with a greater number of intestinal receptors, thereby increasing feedback from the small intestine to reduce food intake. It is also possible that these sugars exert their effect through the release of gastrointestinal hormones. For example, D-tagatose increased plasma GLP-1 and CCK (Buemann et al., 2000). In addition, intake of liquid carbohydrates, particularly sugar-sweetened beverages, produced less satiety compared with solid carbohydrates (Pan, 2011).

Foods rich in dietary fiber tend to have a high volume and a low energy density, and should promote satiety, and play an important role in the control of energy balance. Short-term studies in which fiber was fed to subjects and food and energy intakes assessed at subsequent meals suggested that large amounts of short-chain fructooligosaccharides were most successful at reducing subsequent energy intake (Hess et al., 2011). Longer-term studies of fiber intake which examine the effects of both intrinsic and functional fibers on satiety are required. Additionally, more viscous fibers appeared most successful in promoting satiety, while soluble fibers, such as inulin, that are less viscous had minimal effects on satiety, even if consumed in very large doses (Archer et al., 2004).

Viscous polysaccharides, including food gums, slow down gastric emptying owing to their increased viscosity, and in turn lead to slower glucose absorption and increased small intestinal feedback (Bordoloi et al., 2012). Gums are all soluble dietary fibers that play an important part in developing foods with high satiating capacity. The effect of the food matrix and the rheology and composition of each gum on the physiological effect is complex. Furthermore, there are a group of non-viscous functional fibers including oligosaccharides, polydextrose, resistant starch, etc. Some recent studies have been published on the effects of these functional fibers and satiety (Slavin et al., 2007). In general, these fibers can be added to foods and drinks in fairly large amounts, more than 10 g/serving, with little effect on the food properties or consumer acceptability of the product. Overall, these new fibers have shown little effect on satiety (de Roos et al., 1995; Buckley et al., 2006).

Protein and Satiety

The World Health Organization (WHO) recommends that dietary protein should account for 10–15% of energy intake to keep energy balance and weight stable. Average daily protein intakes for various countries indicate that these recommendations reflect how proteins are consumed worldwide. Given the range of the normal protein intake, meals with on average 20% to 30% of energy from protein are representative for high protein diets. When subjects are not in energy balance, the relative percentages of protein intake may change, and also absolute amounts of protein intake should be considered. Eisenstein and Roberts (2002) published an extensive review on the efficacy and safety of high protein diets. Recent findings suggest that a relatively high protein intake seems to play a role during weight loss as well as during weight maintenance (Clifton et al., 2008; Lejeune et al., 2005).

Mechanisms that may contribute to protein-induced satiety are increases in concentrations of 'satiety' hormones, in energy expenditure, in concentrations of metabolites, i.e. amino acids, and the process of gluconeogenesis. It has been hypothesized that protein-induced satiety is somehow related to a relatively high increase in concentrations of anorexigenic hormones (Glucagon-like peptide-1, Cholecystokinine, PYY) or a larger decrease in 'orexigenic' (ghrelin) hormones (Smeets et al., 2008; Lejeune et al., 2006). In Smeets's study (Smeets et al., 2008), heathy volunteers with a body mass index (BMI) of 23.8±2.8kg/m^2 and a percentage body fat of 26±8.9 had no differences in ghrelin, GLP-1 or PYY responses after a high protein or protein condition lunch. The GLP-1 response was smaller following the high protein as compared to the adequate protein lunch. It was suggested that a high protein diet in the presence of carbohydrate stimulates GLP-1 release, since carbohydrate stimulates protein metabolism (van Loon et al., 2007). With respect to PYY responses, Karra et al. (2009) observed significantly higher plasma PYY responses to a high protein meal in both lean and obese subjects. Taken together, there is some evidence that a high protein meal in combination with carbohydrate stimulated GLP-1 release, yet this was dependent on the carbohydrate content. Little information is available on CCK, so a clear conclusion on its contribution to protein-induced satiety is not known.

Another the mechanism that has been suggested to explain protein-induced satiety is energy expenditure. The theoretical basis of this relationship may be that increased energy expenditure improved increased oxygen consumption and an increase in body temperature may lead to feeling deprived of oxygen and thus promote satiety (Westerterp-Plantenga et al., 2009). Energy expenditure is different due to different protein sources. The metabolisable

energy of protein, as defined is 17kJ/g. Amino acid oxidation may play a major role. Amino acid composition of the protein is a determinant of the metabolic efficacy of protein oxidation. Because large differences exist in the efficacy with which amino acids are oxidized. Protein metabolism and energy expenditure are dependent on the protein source. Different proteins may affect satiety differently (Veldhorst et al., 2007). Hall et al. (2003) reported a lower energy intake from consumption of whey protein compared to casein consumption and Anderson et al. (2004) reported subjects who consumed whey protein had enhanced satiety relative to other proteins (egg-albumin and soy protein) and carbohydrates.

Gluconeogenesis (GNG) is a metabolic pathway that results in the generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, glucogenic amino acids, and fatty acids (Glew,2010). The mechanism of GNG has been mentioned to contribute to satiety, or better food intake regulation, yet until now this only has been shown in the animal model. The satiating effect of high protein diets could be related to the improvement of glucose homeostasis through the modulation of hepatic gluconeogenesis and subsequent glucose metabolism (Westerterp-Plantenga et al., 2006).

Fat and Satiety

Proteins are more satiating than carbohydrates, which in turn are more satiating than fat (Skidmore, 2007). While lipids are commonly associated with poor satiety and weight gain, there is some evidence showing different points. Physicochemical properties such as fatty acid saturation, chain length, and lipid emulsification may all contribute to the relative role that fats may play in controlling food intake (Maljaars et al., 2009).

Fat in the gastrointestinal tract reduces hunger and impairs food intake by eliciting satiety signals (Maljaars et al., 2007). These signals are evoked by entry of triacylglycerols (after hydrolyzation to fatty acids) or fatty acids into the small intestine. Entry of fat into the small intestine induces the release of gut peptides [CCK and peptide YY (PYY)], and secretion of these peptides is partly responsible for the effects of fat on satiety and food intake. Duodenal fat induces the release of cholecystokinin (CCK) and other gastrointestinal peptides involved in the regulation of satiety and food intake (Maljaars et al., 2007). When infused into the ileum, fat also increases satiety and reduces food intake. Infusion of fat into the ileum activates the ileal brake mechanism, an inhibitory feedback mechanism that regulates the control of a meal through the digestive tract to optimize nutrient digestion and absorption (Van Citters et al., 2006). The satiating effect of fat is dependent on its physicochemical properties (Feltrin et al., 2004). For instance, reduction of hunger and food intake increases with increasing fatty acid chain length. Feltrin et al. (2004) showed that a reduction in energy intake and in hunger was stronger after isocaloric infusion of lauric acid (12:0) than of decanoic acid (10:0).

Another factor that can influence satiety is fat substitutes. Because high-fat diets are associated with obesity, in many situations, we may choose nonabsorbable fat substitutes in diets such as olestra. Olestra, which is formed from the reaction of fatty acids with sucrose, has similar properties to fat but it is not hydrolyzed by gastric or pancreatic lipase (Bray, 1995). As a result, olestra is not digested or absorbed and contributes no energy to the diet. Recently Swithers (2011) reported the results of a study of the use of olestra as part of a high-fat diet in rats. Rats that had olestra-based potato crisps added to their diet gained more

weight than those that consumed crisps prepared with conventional cooking oil (triacylglycerol). Although, fat may have a lower satiating power than carbohydrate and protein, partly because it is energy-dense and partly because of certain metabolic effects, the possible importance of dietary interactions has been recognized only recently, and this hypothesis has not been tested in many studies on human subjects.

Chromium Picolinate and satiety

Besides, carbohydrates, protein and fat, a recent study also studied the effects of chromium picolinate on food intake and satiety in both humans and animals (Stephen et al., 2008). Participants that received CrPic as a supplement reduced food intake and hunger levels compared to participants receiving a placebo. The human data were supported by animal studies demonstrating that CrPic suppressed food intake, particularly following central administration. If future studies confirm these results, then CrPic may be a useful alternative or adjunctive treatment for individuals desiring to reduce their food intake.

SATIETY MEASUREMENT

Food intake plays an important role in the life of humans. And normally, hunger and satiety are the basic feelings that regulate food intake. They regulate each other and control eating. The feeling of hunger is the mental state, which motivates the food intake, whereas the feeling of satiety terminates the intake of a meal. However, the reliable measurement of perceptions related to food intake is confronted by difficulties since the strength of perceptions shows individual differences and perceptions are influenced by several levels of response (Wardle, 1987). Presently, physiological parameters which are directly related to feelings of satiety are not available. Therefore, indirect methods have been applied to

estimate signals of satiety or hunger. The usual method of discovering the satiating potential of any component, macronutrient or food item is to conduct clinical trials. So the study design is important for correct interpretation of these results. We have to consider a number of individual and environmental influences such as variety of available foods (Brondel et al., 2009). Brondel et al. (2009) demonstrated that adding condiments to foods like in a 'fast food'-style meal increased food intake in the short term. This increase in food consumption may stem from disruption of sensory satiety for a given food by renewal of sensory stimulation; time of consumption. Chungchunlam et al. (2012) showed that the differences in the time interval between preload and test meal may account for the variation in the satiating effects of water and carbohydrate and protein-enriched food. Spill et al. (2011) showed that serving low-energy-dense, vegetable soup as a first course was an effective strategy to reduce children's intake of a more energy-dense main entree and increased vegetable consumption at the meal. Brunstrom et al. (2006) indicated that distraction had a potent effect on the changes in desire to eat that normally occur during a meal. Moreover, these effects appeared to operate in a food-specific manner. Expected satiety did not exclusively depend on the volume served, but participants were able to discriminate between soups with different sensory attributes and energy content (Hogenkamp et al., 2012).

Expectations may initially rely on sensory attributes and previous experiences, and expected satiety is assumed to be an important determinant in decisions on portion size; or dietary conditions (Mok, 2010). Consequently, it is necessary to take into consideration the many effects that can influence satiety. The measurement of satiety can be achieved through

methods that allow subjects to record feelings of satiety or hunger, and/or by measuring food intake directly. Some of the methods for studying satiety are outlined below.

Real world vs. laboratory studies

In attempting to measure satiety and eating behavior, there has to be a compromise between being able to measure these precisely and making the results applicable to the 'real world'. To control external conditions, studies are often conducted in a laboratory environment. However, sometimes laboratory studies cannot be related to free-living subjects, where conditions are not under the same control as laboratory conditions. For clinical studies, Jordan et al. (1966) measured food intake of a liquid meal to correlate amounts of food consumed using a modified feeding apparatus. Unfortunately, experimental parameters were not comparable to normal life conditions. Thus, although it is desirable to conduct studies whose results are relevant to free-living populations, in reality, it is extremely difficult to gain meaningful results in uncontrolled conditions. Therefore, the majority of studies on satiety have been conducted in the laboratory under controlled conditions.

Preload studies

A widely used method for investigating the acute effects of food and drink ingredients on satiety is to feed a fixed portion of a food or drink vehicle with and without the ingredient (the preload) and follow this with measurement of energy intake in a test meal (food served in excess of likely intake at a fixed interval after the preload). Pre-load experiments were used to determine the different satiating effects of macronutrients. The test and control preloads are matched for taste, appearance, texture and other sensory qualities that might

affect palatability. The subjects should be blinded to the differences between control and test preloads if the investigators wish to measure only the physiological effects of the treatments. If the purpose of the study is to measure both physiological and cognitive effects of the changes made, the subjects may be told how the preloads differ. As conditions before consumption need to be standardized, the usual procedure is to use a preload for breakfast after a night of fasting, or intervene later, after consumption of a controlled meal. The preloads can be consumed random or over a set time. Satiety is measured through the measurement of *random* food consumption of particular experimental foods (weight in grams or energy in kcal or kJ) under these standardized conditions. The random consumption of foods varies to a large extent. For example, a study on sensory specific satiety (Weenen et al., 2005) observed that people ate on average 70–80 g of savory cheese cookies, whereas they ate about five times as much of pears with light sugared syrup. This was not a result of differences in liking as the pears and biscuits were equally liked. Assessing subjective fullness, hunger, or desire-to-eat sensations (Blundell et al., 2010) before the preload, between the preload and the test meal and at fixed times after the meal; using validated visual analogue scales (VAS) can show the perceived degree of satiety. Self-reported measurements of appetite are used to monitor hunger, fullness and motivation to eat.

Self-reported measures of satiety

Self-report scales in common use include uni- and bipolar structured lines, verbal categories and numerical scoring (including magnitude estimation). The most common method is the unipolar unstructured line (visual analogue) (Table 1) (Blundell et al., 2010).

Table 2. Recommended primary scales for self-reported appetite in healthy adults (Blundell et al., 2010).

Scale	Question	Anchors	
		Low	High
Hunger	How hungry are you?	Not at all	Extremely As hungry as I have ever felt
Fullness	How full are you?	Not at all	Extremely As full as I have ever felt
Satiety	How satiated are you?	Not all	Extremely
Desire	How strong is your desire to eat?	Very weak Extremely low	Very strong Extremely high
Prospective consumption (quantity)	How much do you think you could (or would want to) eat right now?	Nothing at all	A very large amount

In general, these scales consist of a line, usually 100 or 150 mm, anchored by an extreme answer to the question posed at either end, for example 'How hungry are you?', 'Not at all hungry' *vs.* 'As hungry as I have ever felt' (Table 2) (Blundell et al., 2010). The feelings corresponding to food intake are measured by putting a mark on the unstructured scale. Statements of subjects studied showed a high variance, which required an appropriate statistical treatment of the data. A magnitude estimation method was first introduced by Tegtsoonian et al. (1981) to characterize the perception of satiety/satiety during food intake and just after food intake. On a standard meal, subjects described their feelings with the aid of a movable marker on a 150 mm-scale every 2 min indicating the perception related to stomach fullness. It was pointed out that first of all, the feeling of satiety continuously increased, but then after termination of eating, the perception slowly decreased. Category scales work on the same principle as VAS. However, instead of a continuous line, numbered

categories (usually from 1 to 9) are provided. These may go from *e.g.* 'How hungry are you?' 1 = not at all, 9 = extremely hungry) or represent the extremes of two variables, *e.g.* 1 = extremely hungry, 9 = extremely full. The *Satiety-labelled intensity magnitude (SLIM) scale* was developed by Cardello et al. (2005) in order to provide better quantitative data from self-reported measures of satiety, using a series of experiments to quantify the meaning of a number of statements on hunger and satiety. The responses were used to select and quantify a number of phrases to label a magnitude scale from +100 through zero to -100. When compared with VAS, the SLIM scale was more sensitive and reliable (Cardello et al., 2005).

Biomarkers studies

Some physiological indicators of satiety can also be measured. The possibility of the use of biomarkers to measure satiety is based on their potential to indicate putative mechanisms of action and their presumed lower susceptibility to subjectivity and modification by environmental factors (Graaf et al., 2004). Several changes in gastrointestinal hormones released during or after eating, such as cholecystokinin, salivary α-amylase, etc, could possibly be used. Freeman (2002) demonstrated that in women, the feeling of satiety caused by cholecystokinin release was enhanced by increasing either the fiber or fat content of a low-fat, low-fiber meal. Harthoorn (2008) reported that salivary α-amylase systematically increased upon food consumption and satiety, and served therefore as a potential measure of satiety and subsequent food intake and a high salivary α-amylase was a physiological measure associated with satiety in humans.

Measuring food intake

Measuring food intake is a common way to assess satiety, but eating behaviors may be affected by factors other than internal appetite signals. Thus, studies on satiety tend to be conducted in a laboratory study where the environment can be closely controlled. The subjects' eating behaviors can be monitored directly by keeping a record of foods consumed and using nutritional analysis software to calculate energy and nutrient intakes like using a universal eating monitor (UEM), which consists of a set of weighing scales concealed under a table cloth, connected to a computer that measures the amount of food consumed over time (Kissileff et al. 1980). In these studies, it is important to consider the kinds of foods offered and the way these are presented to subjects. Obviously, foods must be acceptable to subjects in the study. It is known that offering a wide variety of foods can increase energy intake through sensory specific satiety (Rolls, 1984).

Long term study

Long-term effects may differ from the short-term effects of a test meal, so short-term studies cannot necessarily be used to deduce long term effects. Some indicators of metabolic health, such as body fat distribution and parameters related to glucose and fat metabolism, are evaluated during this type of study. Longer-term studies may provide food to be consumed randomly and monitor the amount eaten and may also measure changes in bodyweight (Saquib, 2008). If it is assumed that subjects eat until they are full when eating randomly, energy intake and any changes in bodyweight can give an idea of the satiating properties of the diet as a whole. In these tests, additional considerations have to be taken

into account such as whether the subjects follow a normo- or hypocaloric diet, their sex differences, or their physical activity (Brandou, 2003).

These methods provide a way to compare the effects of different foods on satiety. However, it follows that all attempts for the evaluation of sensations related to food intake cannot determine the real perception of subjects exactly and simple methods for measuring interoceptive feelings (the sense of the physiological condition of the body), which are the less instinctive feelings such as vasomotor activity, hunger, thirst and internal sensations, are still not available.

CONCLUSIONS

Fundamental changes in our environment and behavior are needed to stop or reverse current trends in obesity prevalence. Knowledge about the effects of satiety on eating behavior and how these are manipulated can contribute to reverse these current trends, and translating this knowledge into practical advice can help people control their energy intake.

REFERENCES

Adrian TE, Ferri GL, Bacarese-Hamilton AJ, Fuessl HS, Polak JM, Bloom SR. 1985. Human distribution and release of a putative new gut hormone, peptide YY. Gastroenterology. 89:1070–1077.

Archer BJ, Johnson SK, Devereux HM. 2004. Effect of fat replacement by inulin or lupin-kernel fibre on sausage patty acceptability, post-meal perceptions of satiety and food intake in men. British Journal of Nutrition. 91: 591–599.

Adrian TE, Bloom SR, Bryant MG, Polak JM, Heitz PH, Barnes AJ. 1976. Distribution and release of human pancreatic polypeptide. Gut. 17:940–944.

Alfenas RC, Mattes RD. 2003. Effect of fat sources on satiety. Obes Res. 11:183–187.

Anderson, G. H., Tecimer, S. N., Shah, D., and Zafar, T. A. 2004. Protein source, quantity, and time of consumption determine the effect of proteins on short-term food intake in young men. Journal of Nutrition. 134:3011–3015.

Baggio LL, Huang Q, Brown TJ, Drucker DJ. 2004. Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure. Gastroenterology. 127: 546–558.

Batterham RL, Bloom SR. 2003. The gut hormone peptide YY regulates appetite. Ann NY Acad Sci. 994:162–168.

Batterham RL, Le Roux CW, Cohen MA, Park AJ, Ellis SM, Patterson M, Frost GS, Ghatei MA, Bloom SR. 2003. Pancreatic polypeptide reduces appetite and food intake in humans. J Clin Endocrinol Metab. 88:3989–3992.

Bellisle, F., Drewnowski, A., Anderson, G. H., Westerterp-Plantenga, M., and Martins, C. K.

2012. Sweetness, satiety, and satiety. Journal of Nutrition. 142: 1149-1154.

Blundell JE, Green S, Burley V. 1994. Carbohydrates and human appetite. Am J Clin Nutr. 59:728–734.

Blundell JE, Lawton CL, Hill AJ. 1993. Mechanisms of appetite control and their abnormalities in obese patients. Horm Res. 3:72–76.

Blundell, J. E., and P. J. Rogers. 1991. Encyclopedia of Human Biology. 6:723-733. Academic Press, Orlando 1991.

Blundell, J. E., de Graaf, C., Hulshof, T., Jebb, S., Livingstone, B., Lluch, A. 2010. Appetite control: methodological aspects of the evaluation of foods. Obesity Reviews. 11:251-270.

Bordoloi, A., Singh, J., and Kaur, L. 2012. In vitro digestibility of starch in cooked potatoes as affected by guar gum: microstructural and rheological characteristics. Food Chemistry, 133: 1206-1213.

Born P, Zech J, Stark M. 1994. Carbohydrate substitutes: comparative study of intestinal absorption of fructose, sorbitol and xylitol. Med Clin. 89:575–578.

Brandou F, Dumortier M, Garandeaub P and Merciera J. 2003. Effects of a two-month rehabilitation program on substrate utilization during exercise in obese adolescents. Diabetes & Metabolism. 29:20-27.

Bray G., Sparti A. 1995. Effect of Two Weeks Fat Replacement by Olestra on Food Intake and Energy Metabolism. FASEB J.9:A439

Brinkworth GD, Noakes M, Keogh JB. 2004. Long-term effects of a high-protein, low-carbohydrate diet on weight control and cardiovascular risk markers in obese

hyperinsulinemic subject. International Journal of Obesity 28: 661–670.

Britt Burton-Freeman, Paul A Davis, and Barbara O Schneeman. 2002. Plasma cholecystokinin is associated with subjective measures of satiety in women. Am J Clin Nutr. 76:659–667.

Brondel, L., Romer, M., Van Wymelbeke, V., Pineau, N., Jiang, T., Hanus, C. 2009. Variety enhances food intake in humans: role of sensory-specific satiety. Physiology & Behavior. 97:44-51.

Brubaker PL, Anini Y. 2003. Direct and indirect mechanisms regulating secretion of glucagon-like peptide-1 and glucagonlike peptide-2. Can J Physiol Pharmacol. 81: 1005–1012.

Brunstrom, J. M., and Mitchell, G. L. 2006. Effects of distraction on the development of satiety. British Journal of Nutition. 96:761-769.

Buckley JD, Thorp AA, Murphy KJ. 2006. Dose-dependent inhibition of the post-prandial glycaemic response to a standard carbohydrate meal following incorporation of alpha-cyclodextrin. Annals of Nutrition and Metabolism. 50: 108–114.

Buemann B, Toubro S, Holst JJ. 2000. D-tagatose, a stereoisomer of D-fructose, increases blood uric acid concentration. Metabolism. 49:969 –976.

Cardello AV, Schutz HG, Lesher LL et al. 2005. Development and testing of a labelled magnitude scale of perceived satiety. Appetite. 44: 1–13.

Chaudhri OB, Salem V, Murphy KG, Bloom SR. 2008. Gastrointestinal Satiety Signals. Annu. Rev. Physiol. 70:239–55.

Chungchunlam, S. M. S., Moughan, P. J., Henare, S. J., and Ganesh, S. 2012. Effect of time

of consumption of preloads on measures of satiety in healthy normal weight women. Appetite. 59: 281-288.

Clifton PM, Keogh JB, Noakes M. 2008. Long-term effects of a high-protein weight-loss diet. Am J Clin Nutr. 87:23– 29.

Cohen MA, Ellis SM, Le Roux CW, Batterham RL, Park A, Patterson M, Frost GS, Ghatei MA, and Bloom SR. 2003. Oxyntomodulin suppresses appetite and reduces food intake in humans. J Clin Endocrinol Metab. 88:4696–4701.

Cummings DE, Overduin J. 2007. Gastrointestinal regulation of food intake. J Clin Invest. 117: 13–23.

Dakin CL, Gunn I, Small CJ, Edwards CM, Hay DL. 2001. Oxyntomodulin inhibits food intake in the rat. Endocrinology. 142:4244–4250.

Dakin CL, Small CJ, Batterham RL, Neary NM, Cohen MA, and Patterson M. 2004. Peripheral oxyntomodulin reduces food intake and body weight gain in rats. Endocrinology. 145: 2687–2695.

de Graaf C, BlomWA, Smeets PA, Stafleu A, Hendriks HF. 2004. Biomarkers of satiety and satiety. Am J Clin Nutr. 79:946–961.

de Graaf C, Schreurs A, Blauw YH. 1993. Short-term effects of different amounts of sweet and nonsweet carbohydrates on satiety and energy intake. Physiol Behav. 54:833–843.

Degen L, Oesch S, Casanova M, Graf S, Ketterer S, Drewe J. 2005. Effect of peptide YY3-36 on food intake in humans. Gastroenterology. 129: 1430–1436.

Degen L, Oesch S, and Matzinger D. 2006. Effects of a preload on reduction of food intake by GLP-1 in healthy subjects. Digestion. 74:78–84.

de Roos N, deHeijnen ML, and Graaf C. 1995. Resistant starch has little effect on appetitive, food intake and insulin secretion of healthy young men. European Journal of Clinical Nutrition. 49: 532–541.

Efthimia Karra E, Chandarana K and Batterham RL. 2009. The role of peptide YY in appetite regulation and obesity. The Journal of Physiology. 587:19-25.

Eisenstein J, Roberts SB, Dallal G, Saltzman E. 2002. High protein weight loss diets: are they safe and do they work? A review of the experimental and epidemiologic data. Nutr Rev. 60:189–200.

Feltrin KL, Little TJ, Meyer JH. 2004. Effects of intraduodenal fatty acids on appetite, antropyloroduodenal motility, and plasma CCK and GLP-1 in humans vary with their chain length. Am J Physiol Regul Integr Comp Physiol. 287:524–533.

Gibbs J, Young RC, Smith GP. 1973. Cholecystokinin decreases food intake in rats. J Comp Physiol Psychol. 84: 488–495.

Glew, RH. 2010. You Can Get There From Here: Acetone, Anionic Ketones and Even-Carbon Fatty Acids can Provide Substrates for Gluconeogenesis. Nig. J. Physiol. Sci. 25:2-4.

Graaf, de, C; Stafleu, A; Heniks, H.F.J; Blom, W.A.M; Smeets, P.A.M. 2004. Biomarkers of satiety and satiety American Journal of Clinical Nutrition. 79(6):946 – 961.

Hall, W. L., Millward, D. J., Long, S. J., and Morgan, L. M. 2003. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. British Journal of Nutrition. 89:239–248.

Halton TL, Hu FB. 2004. The effects of high protein diets on thermogenesis, satiety and

weight loss: a critical review. J Am Coll Nutr. 23:373–385.

Hayes MR, Moore RL, Shah SM, Covasa M. 2004. 5-HT3 receptors participate in CCK induced suppression of food intake by delaying gastric emptying. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287:817–823.

Hess JR, Birkett AM, Thomas W and Slavin JL. 2011. Effects of short-chain fructooligosaccharides on satiety responses in healthy men and women. 56:128-134.

Hogenkamp, P. S., Brunstrom, J. M., Stafleu, A., Mars, M., and de Graaf, C. 2012. Expected satiety after repeated consumption of low- or high-energy-dense soup. British Journal of Nutrition. 108:182-190.

Jang H, Kokrashvili Z, and Theodorakis M. 2007 Gutexpressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1. Proc Natl Acad Sci. 104:15069–15074.

Johnson J, Vickers Z. 1992. Factors influencing sensory-specific satiety. Appetite. 19:15–31.

Jones KL, Doran S, and Hveem K. 1997. Relation between postprandial satiety and antral area in normal subjects. Am J Clin Nutr. 66:127–132.

Jordan, H. A., W. F. Wieland, S. P. Zebley, E. Stellar and A. J. Stunkard. 1966. Psychosomatic Medicine 28:836-842.

Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS. 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr Rev. 20:68-100.

Kamphuis MM, Westerterp-Plantenga MS, Saris WH. 2001. Fat-specific satiety in humans for fat high in linoleic acid vs fat high in oleic acid. Eur J Clin Nutr. 55:499–508.

Karhunen LJ, Juvonen KR, Huotari A, Purhonen AK, Herzig KH. 2008. Effect of protein,

fat, carbohydrate and fibre on gastrointestinal peptide release in humans. Regul Pept. 149: 70–78.

Katsuura G, Asakawa A, Inui A. 2002. Roles of pancreatic polypeptide in regulation of food intake. Peptides. 23:323–329.

Kinzig KP, D'Alessio DA, Seeley RJ. 2002. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci. 22:10470-10476.

Kissileff HR. 1984. Satiating efficiency and a strategy for conducting food loading experiments. Neuroscience and Behavior Reviews. 8:129–135.

Kissileff HR, Pi-Sunyer FX, Thornton J, Smith GP. 1981. C-terminal octapeptide of cholecystokinin decreases food intake in man. Am J Clin Nutr. 34: 154–160.

Korner J, Bessler M, Cirilo LJ, Conwell IM, Daud A, Restuccia NL. 2005. Effects of Roux-en-Y gastric bypass surgery on fasting and postprandial concentrations of plasma ghrelin, peptide YY, and insulin. J Clin Endocrinol Metab. 90: 359–365.

Le Magnen J. 1956. Induction of hyperphagia in white rats by changing the mechanism of peripheral satiety. CR Séances Soc Biol. 150:32–35.

Lejeune MP, Kovacs EM, Westerterp-Plantenga MS. 2005. Additional protein intake limits weight regain after weight loss in humans. Br J Nutr. 93:281–289.

Lejeune MP,Westerterp KR, Adam TC, Luscombe-Marsh ND,Westerterp-Plantenga MS. 2006. Ghrelin and glucagon-like peptide 1 concentrations, 24-h satiety, and energy and substrate metabolism during a high-protein diet and measured in a respiration chamber. Am J Clin Nutr. 83(1):89–94.

Levine AS, Tallman JR, and Grace MK. 1989. Effect of breakfast cereals on short-term food intake. American Journal of Clinical Nutrition. 50: 1303–1307

Le Roux CW, Aylwin SJ, Batterham RL, Borg CM, Coyle F, Prasad V. 2006. Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters. Ann Surg. 243:108–114.

Lewin MJM. 1992. The Somatostatin Receptor in the GI Tract. Annual Review of Physiology. 54: 455-466.

Liddle RA. 1994. Cholecystokinin. Gut Peptides. 1994:175-216.

Louis-Sylvestre J, Giachetti I, Le Magnen J. 1984. Sensory versus dietary factors in cafeteria induced overweight. Physiol Behav. 32:901–905.

Lucien F. Harthoorn. 2008. Salivary α-amylase: A measure associated with satiety and subsequent food intake in humans. International Dairy Journal 18:879–883.

Maljaars J, Peters HP, Masclee AM. 2007. Review article: the gastrointestinal tract: neuroendocrine regulation of satiety and food intake. Aliment Pharmacol Ther. 26:241–250.

Maljaars J, Romeyn EA, Haddeman E, Peters HPF, and Masclee AM. 2009. Effect of fat saturation on satiety, hormone release, and food intake. Am J Clin Nutr. 89:1019–1024.

Mattes RD. 1985. Gustation as a determinant of ingestion. Methodological issues. The American Journal of Clinical Nutrition, 41: 672-683.

Meyer JH, Gu YG, Jehn D, Taylor IL. 1988. Intragastric vs intraintestinal viscous polymers and glucose tolerance after liquid meals of glucose. Am J Clin Nutr. 48:260 –266.

Meyer JH, Hlinka M, Tabrizi Y. 1998. Chemical specities and intestinal distributions of nutrientdriven satiety. Am J Physiol. 275:1293–307.

Meyer JH, Tabrizi Y, DiMaso N. 1998. Length of intestinal contact on nutrient-driven satiety. Am J Physiol. 275:1308 –1319.

Mok, L. W. 2010. The effect of variety and dietary restraint on food intake in lean young women: a preliminary study. Journal of General Psychology. 137: 63-83.

O'Doherty J, Rolls ET, Francis S, Bowtell R, McGlone F, Kobal G. 2000. Sensory specific satiety-related olfactory activation of the human orbitofrontal cortex. Neuroreport. 11:893–897.

Onaga T, Zabielski R, Kato S. 2002. Multiple regulation of peptide YY secretion in the digestive tract. Peptides. 23: 279–290.

Pan A; Hu FB. 2011. Effects of carbohydrates on satiety: differences between liquid and solid food. Carbohydrates. 14:385-390.

Raben A, Macdonald I and Astrup A. 1997. Replacement of dietary fat by sucrose or starch: effects on 14 d ad libitum energy intake, energy expenditure and body weight in formerly obese and neverobese subjects. Journal of Obesity and Related Metabolic Disorder 21: 846–859.

Raben A, Vasilaras TH, Moller AC. 2002. Sucrose compared with artificial sweeteners: different effects on ad libitum food intake and body weight after 10 week of supplementation in overweight subjects. American Journal of Clinical Nutrition 76: 721–729.

Reid M., Hetherington M. 1997. The effects of carbohydrates and protein on satiety; a review

of methodology. Neuroscience and Biobehavioral Reviews 21: 295–308.

Reidelberger RD, Hernandez J, Fritzsch B, Hulce M. 2004. Abdominal vagal mediation of the satiety effects of CCK in rats. Am J Physiol Regul Integr Comp Physiol. 286:1005-1012.

Steinert RE. 2011. Effects of carbohydrate sugars and artificial sweeteners on appetite and the secretion of gastrointestinal satiety peptides. British Journal of Nutrition. 105:1320–1328.

Rolls BJ, Rolls ET, Rowe EA, Sweeney K. 1981. Sensory specific satiety in man. Physiol Behav. 27:137–142.

Rolls BJ, Rowe EA, Rolls ET, Kingston B, Megson A, Gunary R. 1981. Variety in a meal enhances food intake in man. Physiol Behav. 26:215–221.

Rolls BJ, Van Duijvenvoorde PM, Rowe EA. 1983. Variety in the diet enhances intake in a meal and contributes to the development of obesity in the rat. Physiol Behav. 31:21-27.

Rolls BJ. 1984. Sensory specific satiety. Nutrition Reviews. 44: 93–101.

Rolls, BJ., Rowe, E. A., and Rolls, E. T. 1982. How sensory properties of foods affect human feeding-behavior. Physiology & Behavior, 29:409–417.

Saquib N, Natarajan L and Rock CL. 2008. The impact of a longterm reduction in dietary energy density on body weight within a randomized diet trial. Nutrition and Cancer 60: 31–38.

Slavin J and Green H. 2007. Dietary fibre and satiety. Nutrition Bulletin. 32:32-42.

Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG. 2000. Central nervous system

control of food intake. Nature. 404:661–671.

Sclafani A, Berner CN. 1976. Influence of diet palatability on the meal taking behavior of hypothalamic hyperphagic and normal rats. Physiol Behav. 16:355–363.

Skidmore P. 2007 Macronutrient intakes and their role in obesity. Nutrition Bulletin. 32: 4–13.

Smeets, A.J., Soenen S, Luscombe-Marsh ND,Westerterp-Plantenga MS. 2008. The acute effects of a high protein lunch on energy and substrate utilization, ghrelin, glucagon-like peptide-1, PYY concentrations, and satiety. Physiology & Behavior. 94:300–307.

Sorensen LB, Moller P, Flint A, Martens M, Raben A. 2003. Effect of sensory perception of foods on appetite and food intake: a review of studies on humans. Int J Obes Relat Metab Disord. 27:1152–1166.

Spiegel TA. 2000. Rate of intake, bites, and chews—The interpretation of lean–obese differences. Neuroscience and Biobehavioral Reviews, 24: 229–237.

Spill, M. K., Birch, L. L., Roe, L. S., and Rolls, B. 2011. Serving large portions of vegetable soup at the start of a meal affected children's energy and vegetable intake. Appetite. 57:213-219.

Stephen D A. 2008. Effects of Chromium Picolinate on Food Intake and Satiety. Diabetes Technoligy & Therapeutics. 10(5):405-412.

Swithers SE, Ogden SB, Davidson TL. 2011. Fat substitutes promote weight gain in rats consuming high-fat diets. Behav Neurosci. 125:512–518.

Tegtsoonian, M., E. Becker and B. Edelman. 1981. Appetite: Journal for intake research.

217-229.

Tempel DL, Leibowitz SF. 1993. Glucocorticoid receptors in PVN: interactions with NE, NPY, and Gal in relation to feeding. Am J Physiol. 265:794–800.

Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K. 1996. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature. 379: 69–72.

Van Citters GW, Lin HC. 2006. Ileal brake: neuropeptidergic control of intestinal transit. Curr Gastroenterol Rep. 8:367–373.

Van Loon LJ, Saris WH, Verhagen H, Wagenmakers AJ. 2007. Plasma insulin responses after ingestion of different amino acid or protein mixtures with carbohydrate. Am J Clin Nutr. 72(1):96–105.

Vandewater K, Vickers Z. 1996. Higher-protein foods produce greater sensoryspecific satiety. Physiol Behav. 59:579–583.

Veldhorst MAB, Nieuwenhuizen A, Hochstenbach-Waelen A, Westerterp KR, Engelen MPKJ, Brummer RM. 2007. Effects of high or normal casein-soy-, or whey with or without GMP-protein breakfasts on satiety, 'satiety' hormones, and plasma amino acid responses. Appetite. 49(1):336.

Wansink, B., and Van Ittersum, K. 2007. Portion size me: downsizing our consumption norms. Journal of the American Dietetic Association. 107(7):1103-1106.

Weenen H, Stafleu A, de Graaf C. 2005. Dynamic aspects of liking: post-prandial persistence of sensory specific satiety. Food Qual Pref. 16: 528–535.

Westman EC, Yancy WS, Edman JS. 2002. Effect of 6 month adherence to a very low carbohydrate diet program. American Journal of Medicine 113: 30–36.

Westerterp-Plantenga MS, Luscombe-MarshND, Lejeune MPGM, Diepvens K, Nieuwenhuizen A, Engelen MPKJ. 2006. Dietary protein,metabolism, and body-weight regulation: dose-response effects. IJO. 30:16–23.

Westerterp-Plantenga MS, Nieuwenhuizen A, Tomé D, Soenen S and Westerterp KR. 2009. Dietary Protein, Weight Loss, and Weight Maintenance. Annual Review of Nutrition. 29: 21-41.

Wynne K, Park AJ, Small CJ, Meeran K, Ghatei MA. 2007. Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. Int. J. Obes. 30:1729–1736.

York DA. 1999. Peripheral and central mechanisms regulating food intake and macronutrient selection. Obes Surg. 9:471–479.

CHAPTER 2: EFFECT OF PH AND MICROFILTRATION ON NORBIXIN LEVELS IN CHEDDAR LIQUID WHEY

Effect of pH and microfiltration on norbixin levels in Cheddar liquid whey

Y. Qiu,* T.J. Smith,* and M.A. Drake[1]

[*]Department of Food, Bioprocessing & Nutrition Sciences, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh 27695

[1]Corresponding Author:

MaryAnne Drake

Box 7624, Department of Food Science

North Carolina State University Raleigh, NC 27695-7624

Phone: 919-513-4598

Fax: 919-513-0014

E-mail: maryanne_drake@ncsu.edu

ABSTRACT

The residual annatto colorant (norbixin) in liquid whey should be removed to provide a desired neutral color in dry whey ingredients. Previous studies have suggested a binding or interaction between norbixin and milk fat globule membrane (MFGM). As such, removal of milk fat globule membrane by microfiltration or precipitation of MFGM from liquid whey with hydrochloric acid at the isoelectric point might reduce residual annatto in liquid whey. The objective of this study was to determine the effect of pH induced MFGM precipitation (pH 4.2, 5.2) or microfiltration on norbixin levels in liquid Cheddar whey. Cheddar cheese whey was manufactured from colored, pasteurized milk. The fluid whey was pasteurized and fat separated. Liquid whey was subjected to 4 different treatments: Control (no treatment), pH 4.2 (acidification to 4.2 with hydrochloric acid, and centrifugation to remove precipitate), pH 5.2 (acidification to 5.2 with hydrochloric acid, and centrifugation to remove precipitate), or MF (microfiltration). The experiment was replicated 3 times. Proximate analysis of whey was conducted using standard methods. Norbixin content was determined by HPLC. The protein composition of liquid wheys was evaluated by polyacrylamide gel electrophoresis (SDS-PAGE). Norbixin was decreased by 49.3%, 40.0%, and 45.5% for pH 4.2 whey, pH 5.2 whey, and microfiltration treatments respectively. No differences were detected in solids and mineral content among liquid wheys ($P>0.05$). Fat and protein content for MF whey were lower than other liquid wheys ($P<0.05$). SDS-PAGE gels showed no visible differences in main whey protein (alpha-lactalbumin and beta-lactoglobulin) bands between control whey and pH 4.2 and 5.2 and MF whey, but bovine serum albumin (67KDa) and immunoglobulin heavy chain (50-70KDa) bands were thinner in pH 4.2 and 5.2 wheys,

suggesting that these proteins were removed by decreased pH. Based on color reduction and SDS-PAGE results, pH adjustment and/or MF could be used as novel methods to remove norbixin from Cheddar whey.

Key words: annatto, liquid whey, acidification, microfiltration

INTRODUCTION

The U.S. is the largest cheese producer in the world, at 4,570,000 metric tons in 2012 (USDEC 2012) and therefore is naturally the largest whey producer worldwide. The functional and nutritional properties of whey protein are valued by food manufacturers and in recent years its applications have expanded tremendously (Smithers, 2008). Cheddar cheese is colored with annatto, giving the cheese an orange color. Annatto colorant, of which norbixin is the primary carotenoid, is added to Cheddar cheese and is also present in the whey (ca 10% of what was added to cheese milk) (Smith et al., 2014). Whey proteins are used in many foods and beverages, and bland and colorless whey ingredients are desirable. Therefore, bleaching is a common and necessary whey processing step (Kang et al., 2010). Bleaching is conducted chemically with hydrogen peroxide (HP) or benzoyl peroxide (BP) or enzymatically with native lactoperoxidase or added peroxidase (Kang et al., 2010; Campbell et al., 2012, 2013). However, bleaching causes lipid oxidation, which results in formation of volatile off-flavors that carry through to final whey protein products (Croissant et al., 2009; Listiyani et al., 2011; Jervis et al., 2012). In order to facilitate bleaching with minimal or no off-flavors, it is beneficial to understand the interactions between norbixin and whey.

Norbixin is a polar water soluble carotenoid, derived from the saponification of bixin, a hydrophobic carotenoid naturally found in the seeds of the *Bixa orellana* tree. Norbixin has a

carboxylic acid group on each end of an eighteen carbon backbone and contains a series of nine conjugated double bonds. There are few publications that have investigated the interactions between norbixin and fluid whey. Previous research suggested that norbixin may have the ability to bind with whey proteins (Cho et al., 1994; Govindarajan and Morris, 1973; Hammond et al., 1975; Zhu and Damodoran, 2012). Recent research has also suggested that norbixin may exist in the form of a micelle dispersed in whey rather than in a "soluble" state due to its hydrophilic and hydrophobic characteristics. Micellar norbixin would more likely be associated with the milk fat globule membrane (MFGM) than with globular whey proteins (Zhu and Damodaran, 2012). It is also possible that micellar norbixin can be removed along with the removal of MFGM through microfiltration (MF). The milk fat globule membrane (MFGM) that surrounds milk fat droplets is derived from the apical plasma membrane of secretory cells in the lactating mammary gland (Keenan, 2001). The membrane itself consists of a complex mixture of proteins, glycoproteins, enzymes, phospholipids, triacylglycerols, cholesterol, glycolipids, and other minor components (Singh, 2006). In milk, MFGM enables the fat to remain dispersed in the aqueous phase, acting as a natural emulsifying agent. It prevents flocculation and coalescence of fat globules in milk and protects the fat against enzyme action. Addition of acid to liquid whey appears to disturb the electrostatic balance of the MFGM system (Fong, 2007). Thus, MFGM can be released from liquid whey by lowering the pH to the isoelectric point of 4.2 (Damodaran, 2010). The objective of this study was to determine the effect of pH induced MFGM precipitation (pH 4.2, 5.2) and microfiltration (MF) on norbixin levels in Cheddar liquid whey. A better understanding of norbixin interaction with whey components or whey processing will facilitate the

development of novel methods to remove norbixin with little impact on flavor/functionality of the whey protein.

MATERIALS AND METHODS

Experimental Design

Colored fluid Cheddar whey was manufactured, fat separated and pasteurized. Whey was then subjected to no treatment (control), MF or pH adjustment (pH 4.2 or 5.2). The experiment was replicated three times. Norbixin was measured and proximate analyses and SDS-PAGE were conducted.

Liquid whey production

Whole, raw bovine milk (50 kg) was obtained from the North Carolina State University Dairy Research and Education Unit. The milk was HTST pasteurized (720 kg/h) with a plate heat exchanger (model T4 RGS-16/2, SPX Flow Technology, Greensboro, NC) at 72°C with a hold time of 15 sec. The milk was then cooled to 31°C and transferred to a cheese vat (Kusel Equipment, Watertown, WI). Mesophilic starter culture containing Lactococcus lactis ssp. *lactis* and Lactococcus lactis ssp. *cremoris* (Danisco Choozit MA11 LYO, Dairy Connection Inc., Madison, WI) was added at the rate of 50 DCU/454 kg of milk. A calcium chloride solution (50% w/v, Dairy Connection Inc.) was also added at the rate of 0.39 ml/kg of milk. The milk was then allowed to ripen for 60 min. After 30 min of ripening, 1:20 diluted double strength norbixin color (Cheese Color DS Double Strength, Dairy Connection Inc.) was added (0.066 mL/kg of milk). Next, the milk was coagulated with 1:80 diluted double strength recombinant rennet (Dairy Connection Inc.) for 30 min at a rate of 0.09 mL/kg of milk at the end of the ripening period. The coagulum was then cut with a 0.95 cm

knives and the curd was allowed to heal for 5 min. The curd was stirred gently for 30 min while the temperature was increased gradually to 39°C. The pH was monitored and liquid whey was drained at pH 6.4. The drained whey was fat separated using a hot bowl centrifugal separator (Model SI600E, Agri-Lac, Miami, FL). After fat separation, the whey was HTST pasteurized (750 kg/h) at 72°C for 15 sec. One portion (about 5kg) of pasteurized liquid whey was transferred into a bench top MF system (Tetra Alcross M7, TetraPak Filtration Systems, Aarhus, Denmark) at 21°C. The MF system was equipped with PVDF (polyvinylidene fluoride) graded permeability membranes (Pall Corp., Cortland, NY, nominal pore diameter of 0.1µm, surface area of 1.7 m^2) and a feed/retentate recirculation pump (type LKH 10/110 SSS, 1.75kW) from Alfa Laval (Kansas City, MO). Permeate from microfiltration was collected for analysis. The run time was approximately 1h. Two other portions of fluid whey were adjusted to pH 4.2 or 5.2 with 1M HCl (37% v/v Sigma-Aldrich, St. Louis, MO), respectively, and all held for 1h at 21°C. One portion of acidified whey was transferred into a 50 mL centrifuge tube (Nalgene, Rochester, NY) and centrifuged for 20 min at 25,000×*g* at 4°C in a centrifuge (Model RC5B, Thermo Scientific, Waltham, MA). The supernatant was removed to a separate centrifuge tube for analysis. The other portion of acidified whey was kept without centrifugation for color analysis to confirm that adjustment of pH alone does not affect norbixin content. A control whey (no treatment) was held at 21°C for 1h and a sample of control whey was also centrifuged for 20 min at 25,000×*g* at 4°C to confirm that centrifugation alone had no effect on norbixin concentration.

Composition Analysis

Percent solids of liquid whey were measured using a vacuum oven (AOAC, 2000; method number 990.20: 33.2.44). The total fat content of liquid whey was measured using a modified Mojonnier ether extraction (AOAC, 2000; method number 932.06; 33.5.08), total protein was measured using the Kjeldahl method (AOAC, 2000; method number 991.20; 33.2.11) using a nitrogen multiplication factor of 6.38. Mineral analysis [P, Mg, Fe, Ca, K, S, and Na] were conducted by the North Carolina State Univ. Analytical Services Laboratory (Raleigh, N.C., U.S.A.) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy. All samples were measured in triplicate.

Norbixin Measurement

Norbixin extractions and measurement were conducted in triplicate on experimental liquid wheys as described by Campbell et al. (2014). 200µL of liquid whey was dissolved in extraction solution (80% v/v acetonitrile (99.9% w/v, ACROS, Fair Lawn, NJ) and 20% v/v HPLC grade water (Honeywell, Burdick & Jackson, Muskegon, MI)) and vortexed for 30 sec and centrifuged at 14,000×*g* for 5 min in a Microfuge 18 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA). 50µL of the supernatant was injected in duplicate onto a reverse phase HPLC column (Waters 2707 Autosampler, Waters, Milford, MA) (Phenomenex Kinetex 2.6µ 100×4.6mm, 40°C). The auto sampler was set at 4°C throughout the injections. An isocratic mobile phase (70% v/v acetonitrile (99.9% w/v, ACROS, Fair Lawn, NJ), 29.9% v/v HPLC grade water (Honeywell, Burdick & Jackson, Muskegon, MI) and 0.1% v/v formic acid (98-100% w/v Sigma-Aldrich, St. Louis, MO)) was used at a flow rate of 1mL/min (Waters 1525 Binary Pump, Waters, Milford, MA). The injector temperature was at 4°C and a photodiode

array detector (Waters 2998) at 482 nm was used. The norbixin content of liquid wheys was quantified using an external calibration curve [content norbixin (0-0.2mg/L) (45%w/w, Chr. Hasen, Milwaukee, WI)] and calculated as percentage norbixin decrease compared to liquid control wheys.

SDS-PAGE (polyacrylamide gel electrophoresis)

Control whey (Con), pH 4.2 uncentrifuged whey (4.2 uncent), pH 4.2 centrifuged whey (4.2 cent), pH 5.2 uncentrifuged whey (5.2 uncent), pH 5.2 centrifuged whey (5.2 uncent), and microfiltration whey (MF) were subjected to SDS-PAGE. A NuPAGE Bis-Tris electrophoresis system (Invitrogen, Carlsbad, CA) was used to characterize the protein composition under reduced conditions. The protein concentrations of liquid whey with different treatments were standardized to the same protein concentration prior to loading on to the gel (1mg/ml). Each standardized whey (50µl) was dissolved in NuPAGE LDS sample buffer (25µl) with NuPAGE reducing agent (10µl). Final protein concentrations were 2.0mg/ml for standardized whey. Samples were then heated at 70°C for 10 min and a 20uL aliquot of each sample was loaded per well. Proteins were separated on precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) using NuPAGE MES SDS running buffer (Invitrogen) at a constant 200 volts for 50 min. After the electrophoresis was complete, the gel was removed from the cast and soaked in 50% methanol (EMD Chemicals Inc., Gibbstown, NJ) and 10% acetic acid (99.5%; J.T. Baker Chemicals, Phillipsburg, NJ) solution for 10 min. Then the gel was transferred and stained with colloidal blue staining solution (Invitrogen). After 3 h, the gel was destained in DI water overnight. Novex sharp protein standard (Invitrogen) was used

to identify components in wheys. A gel-dry drying solution (Invitrogen) was used to preserve the gel. Each experimental replication was evaluated by SDS-PAGE in duplicate.

Densitometry

Densitometric analysis of SDS-PAGE protein bands density was performed with a Biorad Molecular imager gel doc XR+ imaging system (Biorad, Hercules, CA).

Statistical Analysis

Proximate analysis and instrumental results were analyzed by one-way ANOVA with means separation (XLSTAT, version 2009. 1.02; Addinsoft, New York, N.Y., U.S.A).

RESULTS AND DISCUSSION

No significant differences ($P>0.05$) were detected in the solids (6.4 ± 0.0008%) or mineral content [P, Mg, Fe, Ca, K, S, and Na] of fluid wheys (results not shown). Fat content for MF whey was lower than Con whey or other liquid wheys due to fat removal by the MF process, (MF: not detected vs. other treatments: 0.017 ± 0.0005%, $P<0.05$). Protein content of MF whey was also lower than Con whey or other liquid whey which was probably due to inability of some of the larger whey protein constituents (i.e. immunoglobulin) or protein associated with fat droplets to pass through the microfiltration membrane (0.84% vs. 0.92%, $P<0.05$). SDS-PAGE gels (Figure not shown) and desitometry results (Table 1) showed no differences in the main whey protein (alpha-lactalbumin and beta-lactoglobulin) bands among control and pH 4.2 and 5.2 and MF wheys, but bovine serum albumin (67KDa) and immunoglobulin heavy chain (50-70KDa) bands were thinner in pH 4.2 and 5.2 wheys than Con and MF liquid whey, suggesting that these proteins were removed by decreased pH.

MF, pH 4.2, and 5.2 liquid whey displayed a 45.5%, 49.0% and 38.0% norbixin decrease, respectively (Figure 1). Based on percent norbixin decrease, pH 4.2 whey was more effective ($P<0.05$) than MF or pH 5.2 whey. Centrifuged control whey or acidified whey without centrifugation showed no differences ($P>0.05$) in norbixin content compared to control whey (results not shown). These results confirm that centrifugation or adjustment of pH alone does not affect norbixin content. Previous studies using hydrogen peroxide (HP) to bleach fluid whey have shown a norbixin destruction of 39.1% to 47.8% depending on HP concentration and the time/ temperature applied (Listiyani et al., 2012). Listiyani and others (2012) and Fox and others (2013) reported that benzoyl peroxide (BP) bleaching (25mg BP/kg) resulted in higher norbixin destruction than HP (73% vs 47%; 79% vs 60%). Compared to these current chemical bleaching methods, MF or pH 4.2 is comparable to HP in bleaching efficacy. Chemical bleaching processes are non-specific oxidation processes that induce lipid oxidation and off-flavors (Croissant et al., 2009; Jervis et al., 2012). We did not investigate volatile compounds or sensory properties in the current study, but previous studies with norbixin removal processes rather than norbixin destruction have demonstrated that elimination of an oxidative bleaching step improve improves flavor of dried whey ingredients (Kang et al., 2012).

Norbixin has a carboxylic acid group on each end of an eighteen carbon backbone and contains a series of nine conjugated double bonds. Zhu and Damodaran (2012) suggested that due to the hydrophobicity and hydrophilicity characteristics of norbixin, norbixin might exist in the form of micelles dispersed in the aqueous medium, rather than in a “soluble” state. Recently, it has been shown that the MFGM separated from cheese whey were

predominantly in the form of bilayer lamellar sheets (Damodaran, 2011). Thus, micellar norbixin would more likely be associated with the milk fat globule membrane (MFGM) via adsorption to the lamellar surface or via incorporation into the bilayer structure of the MFGM. However, Smith (2014) found no difference between the norbixin partition levels of full-fat and fat-free cheese and whey. This result suggested that fat content had no effect on norbixin binding or entrapment in Cheddar cheese. Thus, norbixin might exist in the form of micelles dispersed in the aqueous medium and nonspecifically be associated with MFGM. MF might physically remove large norbixin micelles directly as it removes milk fat globules from fluid wheys. These microfiltration-based techniques have shown the potential to produce MFGM fractions from buttermilk at commercial scale (Morin et al., 2004).

It is known that the MFGM particles remain stable in cheese whey due to electrostatic repulsion from the negatively charged phosphate groups of membrane (Singh, 2006). Adding acid to liquid whey appears to disturb the electrostatic balance of the MFGM system (Fong, 2007). Thus, MFGM can be separated from liquid whey by lowering the pH to the pH 4.2 where the maximum precipitation of MFGM occurred (Damodaran, 2011). If the hypothesis that norbixin exists as a micelle and/ or is associated with the MFGM is valid, micellar norbixin should be removed from liquid whey as MFGM are precipitated. The efficiency of precipitation would be expected to be a function of the pH of the whey (Kanno and Kim, 1990). Thus, the difference ($P<0.05$) in norbixin decrease between pH 4.2 and pH 5.2 acidified whey can be explained by the efficiency of MFGM precipitation. pH 4.2 is nearer to the MFGM isoelectric point (4.2-4.5), more norbixin micelles were precipitated out along with MFGM at pH 4.2. Alternatively, if norbixin is not specifically associated with MFGM,

the decrease in pH also can destroy the electrostatic balance of the negatively charged carboxylic acid group of norbixin and precipitate micellar norbixin out of liquid whey.

CONCLUSIONS

Norbixin was decreased by 49.3%, 40.0%, and 45.5% for pH 4.2 whey, pH 5.2 whey, and MF treatments respectively ($P<0.05$). Based on color reduction, pH reduction of norbixin and/or microfiltration could be used as novel alternative methods to remove annatto from Cheddar whey.

REFERENCES

Campbell, R. E., Boogers, I. A. L. A., & Drake, M. A. (2014a). Development of a novel method for the extraction of norbixin from whey and its subsequent quantifi-cation via high performance liquid chromatography. Short communication Journal of Dairy Science. In press.

Cho Y., Batt CA, Sawyer L. 1994. Probing the retinol-binding site of bovine β-lactoglobulin. J. Biol. Chem. 269:11102-11107.

Croissant AE., Kang EJ, Campbell RE, Bastian E, Drake MA. 2009. The effect of bleaching agent on the flavor of liquid whey and whey protein concentrate. J. Dairy Sci. 92:5917–27.

Damodaran S. 2010. Zinc-Induced precipitation of milk fat globule membranes: A simple method for the precipitation of fat-free whey protein isolate. J. Agric. Food Chem. 58:11052- 11057.

Damodaran S. 2011. Straightforward process for removal of milk fat globule membranes and production of fat-free whey protein concentration from cheese whey. J. Agric. Food Chem. 59:10271- 10276.

Federal Register National Archives and Records Administration, Washington, DC. USDEC. 2010. Products: cheese and whey.

Fong BY., Norris CS and MacGibbon KH. 2007. Protein and lipid composition of bovine milk fat globule membrane. Int. Dairy J. 17:275-288.

Fox AJ, Tucker TJ, Gerard PD, Drake MA, 2013. The influence of bleaching agent and temperature on bleaching efficacy and volatile components of fluid whey and whey

retentate. . J. Food Sci. 78:1535- 1542.

Govindarajan S., H.A. Morris. 1973. Pink discoloration in Cheddar cheese. J. Food Sci. 28:675-678.

Hammond, EG., Chang J and Reinbold GW. 1975. Colorimetric method for residual annatto in dry whey. J. Dairy Sci. 58:1365-1366.

Jervis S., Campbell RE, Wojciechowski K, Drake MA, Barbano DM. 2012. Effect of bleaching whey on sensory and functional properties of 80% whey protein concentrate. J. Dairy Sci. 95:2848–62.

Kang EJ, Campbell RE, Bastian E, Drake MA. 2010. Annatto usage and bleaching in dairy foods. J. Dairy Sci. 93:3891–3901.

Kang EJ, Smith TJ and Drake MA. 2012. Alternative bleaching methods for Cheddar cheese whey. J. Food Sci. 77:818–823.

Kanno C and Kim D. 1990. A simple procedure for the precipitation of bovine milk fat globule membrane and a comparison of its comparison, enzymatic activities and electrophoretic properties with those prepared by other methods. Agric. Biol Chem. 54 (11): 2845-2854.

Listiyani MAD., Campbell RE, Miracle RE, Barbano DM, Gerard PD, Drake MA. 2012. Effect of temperature and bleaching agent on bleaching of liquid Cheddar whey. J. Dairy Sci. 95:36–49.

Listiyani MAD., Campbell RE, Miracle RE, Dean LO, Drake MA. 2011. Influence of bleaching on flavor of 34% whey protein concentrate and residual benzoic acid concentration in dried whey proteins. J. Dairy Sci. 94:4347–59.

Keenan TW., 2001. Milk lipid globules and their surrounding membrane: a brief history and perspectives for future research. J. Mammary Gland Biol Neoplasia. 6:365-371.

Morin P, Jimenez-Flores R, Pouliot Y. 2004. Effect of temperature and pore size on the fractionation of fresh and reconstituted buttermilk by microfiltration. J. Dairy Sci. 87:267-273.

Singh H. 2006. The milk fat globule membrane –A biophysical system for food applications. Current Opinion in Colloid & Interface Science. 11: 154-163.

Smith TJ and Li XE, Drake MA. 2014. Short communication: Norbixin and bixin partitioning in Cheddar cheese and whey. J. Dairy Sci. 95:614-617.

Smithers GW. 2008. Whey and whey proteins: from 'gutter-to-gold'. Int. Dairy J. 18:695–704.

Zhu D and Damodaran S. 2012. Short communication: Annatto in Cheddar cheese-derived Whey protein concentrate is primarily associated with milk fat globule membrane. J. Dairy Sci. 95:614-617.

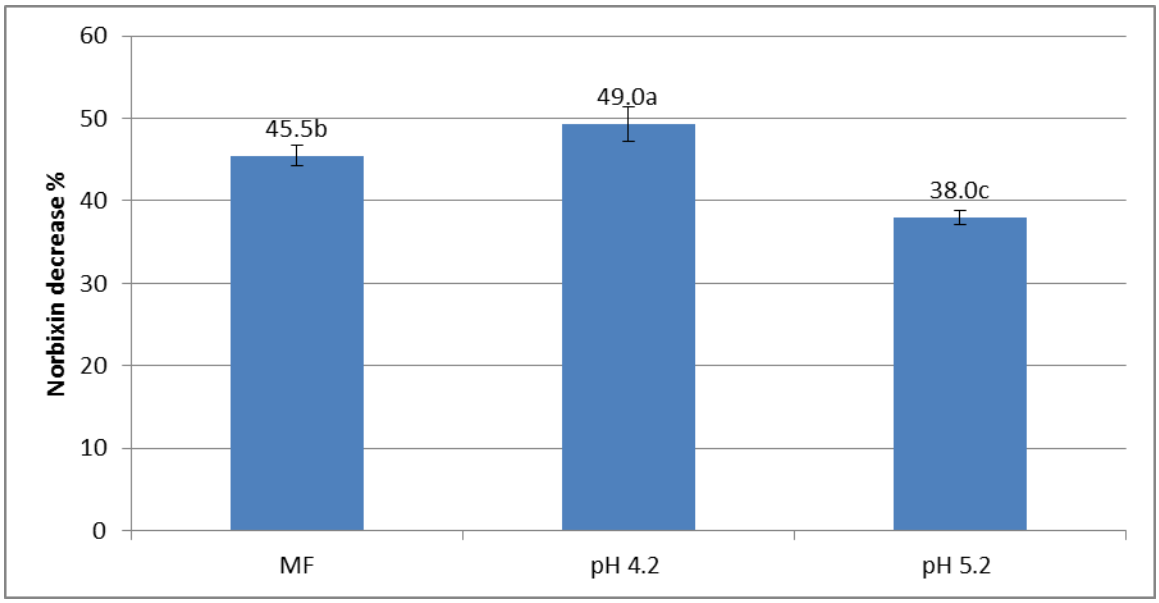


Figure 1: The percent norbixin decrease compared to control liquid whey. MF= microfiltration; pH 4.2= acidification to 4.2 with hydrochloric acid, and centrifugation to remove precipitate; pH 5.2= acidification to 5.2 with hydrochloric acid, and centrifugation to remove precipitate.

Table 1: Densitonetry results (based on volume intensity values) of SDS-PAGE protein bands.

	Treatment			
Category	**Con**	**MF**	**pH 4.2**	**pH 5.2**
Alpha-lactalbumin	1.4×10^{6a}	1.4×10^{6a}	1.4×10^{6a}	1.4×10^{6a}
Beta-lactoglobulin	7.4×10^{6a}	7.4×10^{6a}	7.4×10^{6a}	7.4×10^{6a}
Bovine serum albumin	4.6×10^{5a}	4.6×10^{5a}	1.5×10^{5b}	1.5×10^{5b}
Immunoglobulin heavy chain	2.3×10^{5a}	2.3×10^{5a}	ND	ND

Con= no treatment;

MF= microfiltration;

pH 4.2= acidification to 4.2 with hydrochloric acid, and centrifugation to remove precipitate;

pH 5.2= acidification to 5.2 with hydrochloric acid, and centrifugation to remove precipitate.

CHAPTER 3: EFFECT OF BLEACHING WHEY ON FLAVOR AND FUNCTIONALITY OF 80% WHEY PROTEIN CONCENTRATE

The impact of microfiltration on color, flavor and functionality of 80% whey protein concentrate

Y. Qiu,* T.J. Smith,* E.A. Foegeding* and M.A. Drake[1]

[*]Department of Food, Bioprocessing & Nutrition Sciences, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh 27695

[1]Corresponding Author:

MaryAnne Drake

Box 7624, Department of Food Science

North Carolina State University Raleigh, NC 27695-7624

Phone: 919-513-4598

Fax: 919-513-0014

E-mail: maryanne_drake@ncsu.edu

ABSTRACT

The residual annatto colorant in fluid Cheddar cheese whey is bleached to provide a neutral-colored final product. Currently, hydrogen peroxide (HP) and benzoyl peroxide (BP) are utilized for bleaching liquid whey. However, previous studies have shown that chemical bleaching causes off-flavor formation, mainly due to lipid oxidation and protein degradation. The objective of this study was to evaluate the efficacy of microfiltration (MF) on norbixin removal and to compare flavor and functionality of WPC80 from MF whey to WPC80 from whey bleached with hydrogen peroxide (HP) or lactoperoxidase (LP). Cheddar cheese whey was manufactured from colored, pasteurized milk. The fluid whey was pasteurized and fat separated. Liquid whey was subjected to 4 different treatments: Control (no bleaching; 50°C, 1h), HP (250 mg hydrogen peroxide/kg; 50°C, 1h), and LP (20 mg hydrogen peroxide/kg; 50°C, 1h), or MF (microfiltration; 50°C, 1h). The treated whey was then ultrafiltered, diafiltered, and spray-dried to 80% whey protein concentrate (WPC80). The entire experiment was replicated 3 times. Proximate analyses, color, functionality, descriptive sensory and instrumental volatile analysis were conducted on WPC80. MF, HP, and LP bleached WPC80 displayed a 39.5%, 40.9% and 92.8% norbixin decrease, respectively ($P<0.05$). The HP and LP WPC80 had higher cardboard flavors and distinct cabbage flavor compared with the unbleached and MF WPC80. Volatile compound results were consistent with sensory results. The HP and LP WPC80 were higher in lipid oxidation compounds (especially heptanal, hexanal, pentanal, 1-hexen-3-one, 2-pentylfuran, octanal) compared to unbleached and MF WPC80. All WPC80 had >85% solubility across the pH range of 3 to 7. The microstructure of MF gels determined by confocal laser scanning showed an increased

protein particle size in the gel network. MF WPC80 also had larger G' values indicating higher gel firmness. Based on bleaching efficacy, flavor and functionality results, MF is a viable alternative to chemical or enzymatic bleaching of fluid whey.

Key words: bleaching, whey protein, flavor, functionality

INTRODUCTION

Whey is a by-product of cheese making, and has become a source for high-value ingredients because of its nutritional and functional attributes in recent years (Smithers, 2008). Liquid whey can be dried and further processed into whey protein concentrates (WPC; 34-89% protein) and whey protein isolates (WPI; >90% protein). Whey proteins are used in many foods and beverages and bland and colorless whey ingredients are desirable. Therefore, bleaching is a common and necessary whey processing step (Kang et al., 2010). Hydrogen peroxide (maximum usage rate at 500mg/kg; US FDA, 2009b) and benzoyl peroxide (regulated by good manufacturing process, GMP5) (US FDA, 2009b) are the 2 approved chemical bleaching agents in United States. Chemical bleaching causes lipid oxidation, which results in formation of volatile off-flavors that carry through to final whey protein products (Croissant et al., 2009; Listiyani et al., 2011; Jervis et al., 2012). In addition, it is likely that these oxidants cause oxidation of some amino acid constituents of protein which may influence nutritive value and functional properties of whey proteins (Shacter, 2000; Jervis et al., 2012). Thus, alternative bleaching methods are desirable for whey bleaching and were the focus of this study.

Lactoperoxidase (LP) is an oxidoreductase enzyme and is found in a wide range of mammalian milks. The LP system consists of 3 components: LP, thiocyanate (SCN^-), and

hydrogen peroxide (H_2O_2). The system will not be active unless all 3 components are present in sufficient amounts. The major intermediate oxidation product of the LP-catalyzed oxidation of SCN^- is the hypothiocyanate ion ($OSCN^-$), which inhibits microbial growth (Seifu et al., 2005). In addition, $OSCN^-$ also has bleaching capabilities. The strong oxidizing capacity allows $OSCN^-$ to react with carotenoids, leading to color loss of norbixin. Campbell and others (2012) demonstrated that the LP system with an optimal concentration of 20 mg of H_2O_2 /kg was more effective than HP alone in norbixin destruction (bleaching >99%) but lipid oxidation products were also higher in concentration in LP bleached whey compared to unbleached whey.

Microfiltration has been used widely in separating fine particles (in the range of 0.1 to 10μm) from suspension in biological products. In the dairy industry, microfiltration is used for bacteria removal, fat removal and fractionation of milk proteins and separation of casein micelles and whey proteins (Ye et al., 2011). Zhu and others (2012) have hypothesized that norbixin is more likely in the form of micelles than a soluble molecules. They further suggested that the micelle associated with the milk fat globule membrane particles in Cheddar cheese whey due to its hydrophilic and hydrophobic characteristics. Therefore, fat removal through microfiltration may also remove associated norbixin. The effect of MF bleaching on the flavor and functional properties of WPC80 has not been determined. The objective of this study was to characterize and compare the composition, processing, sensory, and functional properties of WPC80 from bleached (HP, LP, and MF) and unbleached Cheddar whey.

MATERIALS AND METHODS

Experimental Design

Two experiments (experiment I and II) were included in this study. The purpose of experiment I was to compare the influence of 3 different bleaching methods/norbixin removal (HP, LP, and MF) on color of liquid whey (proof of concept). Cheddar cheese whey with no bleach, HP bleach, LP bleach and MF were manufactured in triplicate. Norbixin analysis was then conducted to determine the bleaching efficacy of different bleaching methods.

The purpose of experiment II was to compare the influence of 3 different bleaching/norbixin removal methods on flavor and functional properties of WPC80. Again, Cheddar cheese whey was manufactured. After pasteurization and fat separation of whey, aliquots of liquid whey were taken for bleaching. After bleaching/norbixin removal, liquid whey was ultrafiltered and diafiltered to 12% solids (w/w) and 80% (w/w) protein and spray dried to produce WPC80. Sensory and volatile analysis, foam stability, protein solubility, microstructure and small strain rheological properties were conducted to determine the influence of different bleaching/norbixin removal methods on flavor and functionality of WPC80.

Liquid Whey Production

Whole, raw bovine milk (1188 kg) was obtained from the North Carolina State University Dairy Research and Education Unit. The milk was HTST pasteurized (720 kg/h) with a plate heat exchanger (model T4 RGS-16/2, SPX Flow Technology, Greensboro, NC) at 72°C with a hold time of 15 sec. The milk was then cooled to 31°C and transferred to a

cheese vat (Kusel Equipment, Watertown, WI). Mesophilic starter culture containing Lactococcus lactis ssp. *lactis* and Lactococcus lactis ssp. *cremoris* (Danisco Choozit MA11 LYO, Dairy Connection Inc., Madison, WI) was added at the rate of 50 DCU/454 kg of milk. A calcium chloride solution (50% w/v, Dairy Connection Inc.) was also added at the rate of 0.39 ml/Kg of milk. The milk was then allowed to ripen for 60 min. After 30 min of ripening, 1:20 diluted double strength annatto color (Cheese Color DS Double Strength, Dairy Connection Inc.) was added (0.066mL/kg of milk). Next, the milk was coagulated with 1:80 diluted double strength recombinant rennet (Dairy Connection Inc.) for 30 min at a rate of 0.09 mL/kg of milk at the end of the ripening period. The coagulum was then cut with a 0.95 cm knives and the curd was allowed to heal for 5 min. The curd was stirred gently for 30 min while the temperature was increased gradually to 39°C. The pH was monitored and liquid whey was drained at pH 6.4. The drained whey was fat separated using a hot bowl centrifugal separator (Model SI600E, Agri-Lac, Miami, FL). After fat separation, the whey was HTST pasteurized (750 kg/h) at 72°C for 15 sec. The whey was then portioned into batches of 272 kg each. Each portion was transferred into a stainless vat equipped with a coil heater. The whey was heated to 50°C and 1 of 4 treatments was conducted: no bleach for the control whey (50°C, 1h), 250 mg hydrogen peroxide/kg (35% w/v, VWR International, Westchester, PA) for 1 h, 20 mg hydrogen peroxide/kg (35% w/v, VWR International, Westchester, PA) for 15 min (LP bleach), hydrogen peroxide was then removed using 20 ppm catalase (FoodPro CAT, Danisco, New Century, NJ), followed by 50°C for 45 min. The absence of HP was confirmed by testing with a peroxide strip (EMD Chemicals Inc.; VWR International, West Chester, PA). For the fourth treatment, liquid whey was microfiltered in a

pilot scale microfiltration unit (Synder Filtration, Vacaville, CA; nominal cutoff: 100,000 Da, surface area 5.0 m^2). Two spiral wound MF membranes were used. The temperature for MF was 50°C and the run time was approximately 1h. Heat treatment was consistent across all four treatments.

WPC80 Production

The liquid wheys were ultrafiltered using a pilot scale ultrafiltration unit (Synder Filtration, Vacaville, CA; nominal cutoff: 10,000 Da, surface area 5.0 m^2). Two spiral wound UF membranes were used. The temperature for UF was 50°C. Diafiltration water was added as 40% of the original weight of whey. This process continued until the retentate reached 78 to 80% protein (wt/vol) content confirmed by a Sprint rapid protein analyzer (CEM USA, Matthews, NC). Retentates were then collected and spray dried (Model Lab 1; Anhydro Inc., Søborg, Denmark). The inlet temperature was 195°C and the outlet temperature was at 90°C. The total spray-drying time was approximate 1 h for each treatment. The powder was collected and stored in Mylar bags (TF-4000, Impak Corp., Central City, SD) and stored at -80°C until subsequent analyses. Production of the liquid whey and the 4 spray-dried WPC80 treatments were completed in 2 days. All treatments were manufactured from the same lot of milk and the experiment was carried out in triplicate.

Composition Analysis

Percent moisture of powders was measured using a vacuum oven (AOAC, 2000; method number 990.20: 33.2.44). The total fat content of powders was measured using a Modified Mojonnier ether extraction (AOAC, 2000; method number 932.06; 33.5.08). Total protein of powders was measured using the Kjeldahl method (AOAC, 2000; method number

991.20; 33.2.11) by multiplying total nitrogen by a factor of 6.38. Mineral analysis (phosphorus, calcium, magnesium, potassium, sulfur, sodium, and iron) was done by the North Carolina State Univ. Analytical Services Laboratory (Raleigh, N.C., U.S.A.) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy. All samples were measured in triplicate.

Hunter L*a*b* Values

The Hunter CIE Lab L (lightness), a (red-green), and b (yellow-blue) values for the WPC80, and 10% solids (w/v) rehydrated WPC80 were determined in triplicate using a Minolta Chroma meter (CR-410; Ramsey, N.J., U.S.A.). Ten milliliters of each sample were placed into a 60 mm × 15 mm polystyrene petri dish in triplicate (Beckton Dickinson, Franklin Lakes, N.J., U.S.A.), each petri dish was measured in triplicate as well.

Norbixin Measurement

Norbixin extractions and measurement were conducted in triplicate on liquid whey and WPC80 as described by Campbell et al (2012). To extract powders, samples were first reconstituted to 10% solids using DI water. Then, 200µL of liquid whey was dissolved in acetonitrile (99.9% w/v, ACROS, Fair Lawn, NJ) and vortexed for 30 sec and centrifuged at 14000 × g for 5 min in a Microfuge 18 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA). 50µL of the supernatant was injected in duplicate onto an reverse phase HPLC column (Waters 2707 Auto sampler, Waters, Milford, MA) (Phenomenex Kinetex 2.6µ 100×4.6mm, 40°C). The auto sampler was set at 4°C throughout the injections. An isocratic mobile phase (70% v/v acetonitrile (99.9% w/v, ACROS, Fair Lawn, NJ), 29.9% v/v HPLC grade water (Honeywell, Burdick & Jackson, Muskegon, MI) and 0.1% v/v formic acid (98-

100% w/v Sigma-Aldrich, St. Louis, MO)) was used at a flow rate of 1mL/min (Waters 1525 Binary Pump, Waters, Milford, MA). The injector temperature was at 4°C and a photodiode array detector (Waters 2998) at 482 nm was used. The norbixin recovery of bleached samples were measured and calculated as percentage norbixin decrease compared to liquid unbleached control samples.

Descriptive Analysis

Descriptive sensory analysis (n=8) was performed in compliance with the North Carolina State University Institutional Review Board for Human Subjects Approval. Spray dried WPC80 powders were rehydrated to 10% solids (wt/vol) in deionized (DI) water. The rehydrated WPC80 were dispensed into lidded 3-digit coded soufflé cups (Solo Cup, Highland Park, IL). These samples were tempered to 20°C and served at this temperature. Eight trained panelists (23-45 y, each with over 150 h of experience with descriptive analysis of dried dairy ingredient aroma and flavor), evaluated the rehydrated powders using a 0 to 15 point intensity scale and the Spectrum^{TM} method (Meilgaard et al., 2007) and an established sensory lexicon (Wright et al., 2009; Jevis et al., 2012; Campbell et al., 2012). Panelists were instructed to expectorate samples after evaluation. DI water and unsalted crackers were available to the panelists for palate cleansing. Each replicate was evaluated in triplicate by each panelist using Compusense Five version 4.8 (Compusense, Guelph, Canada).

Volatile Compound Analysis

Volatile compounds in WPC80 were extracted by solid-phase microextraction (SPME). Compounds were then separated and identified by gas chromatography-mass spectrometry (GC-MS) using the method of Listiyani and others (2011). WPC80 powders

were reconstituted at 10% solids (wt/vol). All samples contained 10% (wt/vol) sodium chloride (Fisher Scientific, Pittsburgh, PA), and 10 μL of internal standard solution (2-methyl-3-heptanone in methanol at 81 mg/kg; Sigma-Aldrich Co. LLC) in 20mL autosampler vials. Each vial was sealed airtight with a teflon-sided silicon septum and a steel screw cap (MicroLiter Analytical Supplies Inc., Sawanee, Fla., U.S.A.). Samples were injected using a Combi PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) attached to an Agilent 6890N gas chromatograph with 5973 inert mass selective detector (MSD; Agilent Technologies Inc., Santa Clara, CA). Samples were maintained at 5°C before fiber exposure. Samples were equilibrated at 40°C for 25 min before 1 cm of fiber was exposed for 30min at 31mm. While the fiber was exposed to the sample, the vial was agitated at 250 rpm with 4 s of pulsed agitation. Then, fibers were injected for 5 min at a depth of 50 mm. The GC method used an initial temperature of 40 °C for 3 min with a ramp rate of 10 °C /min to 250 °C held for 5 min. SPME fibers were introduced into the split/splitless injector at 250 °C. A Zb-5ms column (Zb-5ms, 30 m length × 0.25 mm inner dia × 0.25 um film thickness; Phenomenex, Torrance, CA) was used for all analysis at a constant flow rate of 1 mL/min in SIM mode. Purge time was set at 1 min. The MS transfer line was maintained at 250°C with the Quad at 150°C and source at 250°C. Compounds were identified using the Natl. Inst. of Standards and Technology (2002) mass spectral database, retention indexes (RI) based on an alkane series (Sigma Aldrich), and comparison of spectra of authentic standards injected under identical conditions. Relative abundance for each compound was determined by comparing their area to the area of the known concentration of the recovered internal standard. These analyses were conducted in triplicate.

Functional Properties

Foam Generation. The method for foam measurement was previously described by Jervis et al. (2012). Briefly, an Artisan KitchenAid mixer (KitchenAid, St. Joseph, MI) with a 4.5-quart stationary bowl and a rotating wire beater was used for foam formation. Solutions of protein (10% wt/vol of protein) were rehydrated for 6 h at room temperature (20 to 24°C) on a stir plate set to 200 rpm. Solutions were refrigerated overnight at 4°C for equilibration, and brought to room temperature before whipping. Solutions were then adjusted to pH 7 using 1 M NaOH (VWR International) and prepared in triplicate. A 200mL sample of solution was whipped at speed 10 (beater rpm of 752) for 19 min and 36 s (Davis and Foegeding, 2007).

Yield Stress. Yield stress was conducted using vane rheometry as described by Jervis et al. (2012). Maximum torque response (M_0) was recorded for each foam in triplicate.

Overrun. Overrun measurements were conducted following yield stress measurements. Foam was removed from the bowl using a rubber spatula in a circular pattern, gently placed in a weighing dish (100 mL), and then weighed. The mean value was used to calculate overrun and the air phase fraction using the following equations:

$$\text{Overrun} = \frac{(\text{wt. of 100 mL of solution}) - (\text{wt. of 100 mL of foam})}{(\text{wt. of 100 mL of foam})},$$

$$\text{Air phase fraction } (\varphi) = \frac{\%\ \text{overrun}}{(\%\ \text{overrun} + 100)}.$$

Foam measurements were done in triplicate (Dickinson, 1999; Wilde, 2000; Davis and Foegeding, 2007).

Stability. Foam drainage was measured using the methods of Phillips et al. (1990) and Luck et al. (2001). Foam stability was measured by the time it took for half of the prefoam mass to drain through a 6-mm hole in a whipping bowl. The starting time for these measurements was immediately after foam formation (Davis and Foegeding, 2007).

Solubility. Solubility was measured as described by Jervis et al. (2012). The WPC80 was rehydrated to 10% protein (wt/vol) as described for foam generation. Solutions were then adjusted using 1 M HCl or 1 M NaOH to pH 3, 4, 5, 6, or 7 and brought to a total volume of 100 mL with deionized water, resulting in 10% (wt/vol) protein solutions. Turbidity and solubility were measured and recorded on samples both before and after centrifugation.

Gel formation. Solutions for gels were prepared as described by Cakir et al. (2012). Solutions were poured into glass tubes (19mm internal diameter×180mm long), which were coated with silicone (Sigma-Aldrich, St. Louis, MO) and closed with a rubber stopper at the bottom. Tubes were covered with aluminum foil to prevent evaporation. The solutions were heated to 80°C in a water bath for 30 min, followed by cooling at room temperature (22±2 °C) for 1h and overnight refrigeration at 4°C. Gels were allowed to equilibrate at room temperature (22±2 °C) for at least 1h before microstructure imaging.

Microstructure. The method for confocal laser scanning microscopy (CLSM) was previously described by Cakir et al. (2012). Microstructure images of gels were recorded on a Zeiss LSM 710 confocal laser scanning microscope. The gels were stained by an aqueous solution of Rhodamine B (0.2%, w/w), which binds to the protein network. The gel pieces were allowed 10 min to absorb the dye before images were taken. The light source was a multiline argon laser with an excitation wavelength at 514nm. The emission of Rhodamine B

was recorded between 531 and 703nm. Digital images were recorded using a 40× objective lens (LD C- Apochromat 40×/1.1 W Korr M27).

Small strain rheological properties. Small strain experiments were conducted as described by Cakir and Foegeding (2011) with some modifications. A controlled stress rheometer (StressTech, Rheologica Instruments AB, Lund, Sweden) in a strain control mode was used to determine rheological properties. The measuring system consisted of a cup and a bob. The WPC80 solutions were prepared as previously described, poured into the cup at 25 °C, and covered with a thin layer of mineral oil to minimize evaporation. Solutions were heated from 25 to 80 °C at a constant rate of 2 °C /min, held at 80 °C for 30 min, then cooled to 25 °C at a constant rate of 2 °C /min and kept for 15 min at 25 °C. The storage modulus (G'), loss modulus (G") and phase angle (δ) were measured continuously during the entire thermal treatment at a frequency of 1 Hz and a strain of 0.5%. The strain level and the frequency were selected based on strain and frequency sweeps of thermally formed gels to ensure that rheological measurements were within the linear viscoelastic region.

Statistical Analysis

Proximate analysis, sensory, and instrumental results were analyzed by one-way ANOVA with means separation (XLSTAT, version 2009. 1.02; Addinsoft, New York, N.Y., U.S.A). Principal component analysis (PCA) was also conducted to visually illustrate volatile compounds differences in experimental WPC80.

RESULTS AND DISCUSSTION

Composition Analysis

No differences (P>0.05) were detected in the moisture (3.20 ± 0.02%), protein (79.40 ± 0.92%) or mineral content [P, Mg, Fe, Ca, K, S, and Na] (P>0.05) of bleached and unbleached WPC80, respectively. Fat content of MF WPC80 was lower (P<0.05) than Con WPC80 or other WPC80 due to fat removed by the microfiltration procedure, (MF: 0.11% vs. other treatments: 4.47 ± 0.06%, P<0.05).

Color

MF, HP, and LP bleached liquid whey displayed a 45.5%, 45.9% and 94.8% norbixin decrease, respectively (Figure 1a). MF, HP, and LP bleached WPC80 displayed a 39.5%, 40.9% and 92.8% norbixin decrease, respectively (Figure 1b). Based on norbixin decrease, LP was more effective (P<0.05) than MF or HP bleaching. This is consistent with previous studies and can be explained by the LP system which generates $OSCN^-$ and ferrous iron, not only allowing for bleaching efficacy from $OSCN^-$, but also from Fenton-type reactions that may further participate in bleaching efficacy and possible side reactions such as lipid oxidation (Campbell et al., 2012; Jervis and Drake, 2013). HP bleaches through the formation of radicals, which attack conjugated double bonds of norbixin, while LP is an enzymatic bleaching based on LP-catalyzed oxidation of SCN^-, the hypothiocyanate ion ($OSCN^-$), which is a stronger oxidizing agent. The strong oxidizing capacity of hypothiocyanate makes it capable of reacting with carotenoids leading to destruction of conjugation and subsequent color loss of norbixin (Jervis and Drake, 2013). Zhu and Damodaran (2012) suggested that due to the hydrophobicity and hydrophilicity

characteristics of the norbixin molecule, norbixin might be in the form of micelles dispersed in the aqueous medium, rather than in a "soluble" state. Thus, MF might physically remove norbixin micelles by the microfiltration membrane. This hypothesis was consistent with results from the current study. The pore size of MF membrane is between 0.1μm-10 μm. Bacteria, fat, casein and the larger whey protein constituents (i.e. immunoglobulin) cannot pass through the membrane and are kept in the retentate. Whey proteins (i.e. alpha-lactalbumin and beta-lactoglobulin), lactose, minerals and water can pass through the membrane and are in the permeate.

Visible appearance and Hunter values for both liquids and powders were consistent with norbixin decrease values (Figure 2a and 2b; Table 1): Differences in color were observed between bleached and unbleached WPC80 powders (Figure 2a) and in rehydrated bleached and unbleached WPC80 (Figure 2b). MF treatment of fluid whey improved clarity as well as color of WPC80, due to fat removal. LP bleached better than HP and MF, corresponding to lower b-values ($P<0.05$) (a more positive b-value is more associated with yellow). The L-values for MF samples were higher than other WPC80 ($P<0.05$), MF created a brighter whey product compared to others.

Descriptive Sensory Analysis

Sensory profiles of rehydrated WPC80 were distinct based on treatment (Table 2). Consistent with oxidative reactions, HP bleaching and LP bleaching decreased cooked/milky flavor and increased cardboard flavor in WPC80, consistent with previous studies (Jervis et al., 2012; Campbell et al., 2012). Both bleached WPC80 displayed cabbage flavor which was not detected in control and MF WPC80. MF WPC80 displayed a distinct increase in

sweet aromatic flavor compared to control WPC80 ($P<0.05$). The elevated fat content might have obscured the sweet aromatic flavor and without this obscuring factor, sweet aromatic flavor might be more discernible in MF WPC80 than in control WPC80.

Volatile Compound Analysis

Selected volatile compounds (15) were identified and quantified (Table 2). Compounds were selected based on their previous relevance to whey off-flavor (Croissant et al., 2009; Listiyani et al., 2011; Jervis et al., 2012). Consistent with sensory results, HP, LP bleached WPC80 were more characterized by lipid oxidation compounds, whereas the unbleached and MF WPC80 were characterized by lower concentration of lipid oxidation compounds (Figure 3; Table 2). Each bleaching method forms different lipid oxidation compounds based on different pathways. HP reacts with UV light or transition metals in milk and creates hydroxyl radicals (McClements and Decker, 2008). These free radicals can be scavenged by carotenoid double bonds and thus remove color, or attack unsaturated fatty acids to form hydroperoxides (Frankel, 1998), then decompose to volatile oxidation products. HP bleached WPC80 was higher in heptanal, hexanal, pentanal, 1-hexen-3-one, 2-pentylfuran, and octanal compared to other WPC80. Bleaching with LP resulted in more norbixin decrease than chemical bleaching (HP), but also resulted in increased oxidation volatiles compared with HP WPC80. $OSCN^-$ is produced when LP reacts with SCN^- and it has strong oxidizing capacity which allows $OSCN^-$ to react with carotenoids, leading to color loss of norbixin, but also react with unsaturated fatty acids to produce volatile oxidation products. Ferrous iron is also produced by LP system, thus Fenton-type reactions that may also further participate in possible side reactions such as lipid oxidation (Campbell et al., 2012; Jervis and Drake, 2013). HP and LP

bleached WPC80 were also higher in sulfur compounds, which may be due to protein degradation and account for the distinct cabbage flavor detected by trained panelists (Wright et al., 2006; Jervis et al., 2012).

Microfiltration physically removes norbixin through the microfiltration membrane, not related to any oxidative mechanism and should not initiate or promote lipid oxidation. This result was observed. Volatile lipid oxidation compounds were generally lower than in MF WPC80 than other bleached WPC80, and the sensory profile of MF WPC80 was most similar to unbleached WPC80. Unfortunately, lipid oxidation is initiated in cheese manufacture (Tomaino et al., 2004; Liaw et al., 2010; Campbell et al., 2011). Thus, lipid oxidation compounds were also found in MF WPC80 and control WPC80.

Functionality

Foam Stability. Only MF WPC80 foamed. Therefore, yield stress, overrun, and stability could not be measured on Con, HP and LP WPC80. Lack of foaming can be due to a variety of factors, including fat content, or other antifoaming agents in whey or are created as a result of milk or whey processing or cheese manufacture (de La Fuente et al., 2002; Jevis et al., 2012). Since all WPC80 were manufactured from the same whey and used similar processes, the presence of fat is the most likely source of lack of foaming.

Protein Solubility. No significant differences in protein solubility among samples at various pH values were observed in WPC80 with different treatment ($P>0.05$). All WPC80 had >85% solubility across the pH range tested. This result suggests that HP and LP bleaching and MF were not damaging protein functionality since the unbleached WPC80 showed a similar behavior. This result was consistent with previous studies (Jevis et al.,

2012). Different bleach/norbixin removal treatments may damage proteins in WPC80 differently. HP and LP are oxidative mechanisms, whereas, MF depends on the pore size of the microfiltration membrane. When liquid whey is forced through the MF membrane under pressure, some proteins with larger particle size may be destroyed physically. Oxidation of protein leads to cleavage of peptide bonds, producing free amino acids and amino acids residues which are highly susceptible to oxidation (Stadtman and Levine, 2003). If sulfur-containing amino acids are being damaged, this could also affect disulfide bond formation and flavor development.

Microstructure. To have an understanding the changes in the structure of the protein gel matrix in control, MF, HP and LP WPC80, confocal laser scanning microscopy (CLSM) was applied on WPC80 gels. Changes in the microstructure of whey protein concentrate (WPC) gels as a function of ionic strength are shown in Figure 4. The bright areas indicate the protein network while the dark areas correspond to lack of protein. At 50mM, 100mM and 250mM NaCl, all gels were particulate: a clear phase separation with protein rich phases (bright areas) and serum phases (black areas). Gels from control, HP and LP WPC80 exhibited very similar microstructure at all ionic strengths, which suggested that bleaching did not damage the proteins. However, different from the other WPC80 gels, MF gels showed a high density of protein matrix with an increasing protein particle size in gel network. This could be explained by fat globules exposed on the surface of some hydrophobic portions of proteins in control, HP and LP WPC80, therefore, decreasing the interactions between whey proteins in the gel matrix. Previous studies have demonstrated that increased protein concentration in the gel phase as the amount of fat decreased led to an

increase in G' and cheese firmness was also related to decreasing the amount of fat and increasing protein concentration in the gel phase (Ustunol et al., 1995; Rogers et al., 2010). Thus, decreasing the amounts of fat in gels caused a general trend of increasing the size of protein particles in the gel network and improved the gel firmness.

Small Strain Rheological Properties. When heated in aqueous solutions above their denaturation temperature, globular proteins, such as whey protein, will partially unfold and bind to each other leading to aggregation and at higher concentration, gelation. Gelation characterized by the change in storage modulus (G') during heating (at 2 °C /min) and holding for 30min at 80 °C, then cooling to 25 °C is presented in Figure 6. An increase in G' during heating indicated a detectable gel network. Elasticity continued to develop during the heating and holding time. Upon cooling, an additional increase appeared in G' due to the formation of additional non-covalent interactions among denatured proteins (Cakir and Foegeding, 2011).

From 50 to 100 mM NaCl, gel storage modulus (G') increased, however, above 200mM NaCl, the storage modulus decreased. At pH 7.0, above the isoelectric point of whey protein (pI~5.2, Swaisgood, 1982), whey proteins are negatively charged. As the salt concentration increased, electrostatic repulsion between protein molecules decreased, facilitating protein-protein interactions. MF WPC80 had larger G' values of gels at these salt concentrations than other WPC80 ($P<0.05$) (Figure 5), indicating higher gel firmness. This result was consistent with microstructure results. Residual milk fat may inhibit the ability of protein molecules to form a continuous gel structure by competing with hydrophobic protein interaction sites

(Karleskind, 1995). Lack of fat interference, increasing interactions between whey proteins led to a firmer gel structure in MF WPC80 gels.

CONCLUSIONS

MF, HP, and LP bleached WPC80 displayed a 39.5%, 40.9% and 92.8% norbixin decrease, respectively ($P<0.05$). Distinct sensory differences among WPC80 were detected. HP and LP WPC80 had higher cardboard flavors and distinct cabbage flavor compared with the unbleached and MF WPC8, and were also higher in volatile lipid oxidation compounds compared with MF WPC80 or control WPC80. All WPC80 had >85% solubility across the pH range. Different from the other WPC80 gels, MF gels showed an increased protein particle size in the gel network and had higher gel firmness. Based on bleaching efficacy, flavor and functionality results, MF is a viable alternative to chemical or enzymatic bleaching of fluid whey.

Acknowledgements

Funding provided in part by Dairy Research Institute (Rosemont, IL) and Agropur, Inc. The use of tradenames does not imply endorsement nor lack of endorsement by those not mentioned.

REFERENCES

Cakir, E., and E. A. Feogeding. 2011. Combining protein micro-phase separation and protein-polysaccharide segregative phase separation to produce gel structures. Food Hydrocolloids, 25:1538–1546.

Cakir, E., C. J. Vinyrad, G. Essick, C.R. Daubert, M. A. Drake and E. A. Foegeding .2012. Interrelations among physical characteristics, sensory perception and oral processing of protein- based soft-solid structures. Food Hydrocolloids. 29: 234-245.

Campbell, R.E., E. J. Kang, E. Bastian and M. A. Drake. 2012. The use of lactoperoxidase for the bleaching of fluid whey. J. Dairy Sci. 95:2882-2890.

Campbell, R.E., R. E. Miracle and M. A. Drake. 2011. The impact of starter culture and annatto on the flavor and functionality of whey protein concentrate. J. Dairy Sci.94:1185-1193.

Croissant, A. E., E. J. Kang EJ, R. E. Campbell, E. Bastian and M. A. Drake. 2009. The effect of bleaching agent on the flavor of liquid whey and whey protein concentrate. J. Dairy Sci. 92:5917–27.

Davis, J.P., and E. A. Foegeding. 2007. Comparisons of the foaming and interfacial properties of whey protein isolate and egg white proteins colloids and surfaces. Colloids Surf. B Biointerfaces 54:200–210.

de La Fuente, M. A., Y. Hemar, M. Tamehana, P.A. Munro and H. Singh. 2002. Process induced changes in whey proteins during the manufacture of whey protein concentrates. Int. Dairy J. 12:361-369.

Dickinson, E., 1999. Adsorbed protein layers at fluid interfaces: Interactions, structure and surface rheology. Colloid Surf. B Biointerfaces 15:161–176.

Dickinson, E., and M. Akhtar. 2003. Emulsifying properties of whey protein- dextran conjugated at low pH and different salt concentrations. Colloids and Surf. 31: 125-132.

Dickinson, E., 2003. Flocculation of protein-stabilized oil-in-water emulsions. Colloids and Surf. 31:125-132.

Frankel, E.N., 1998. Hydroperoxide decomposition. Lipid Oxidation. The Oily Press, Dundee, Scotland. 55-77.

Jervis, S., R. Campbell, K. Wojciechowski, M.A. Drake and D.M. Barbano. 2012. Effect of bleaching whey on sensory and functional properties of 80% whey protein concentrate. J. Dairy Sci. 95:2848–62.

Jervis, S.M., and M.A. Drake. 2013. The impact of iron on the bleaching efficacy of hydrogen peroxide in liquid whey systems. J. Food Sci.78: 129-137.

Kang, E. J., R. E. Campbell, E. Bastian and M. A. Drake. 2010. Annatto usage and bleaching in Dairy foods. J. Dairy Sci. 93:3891–3901.

Liaw, I., H. Eshpari, P. S. Tong, and M. A. Drake. 2010. The impact of antioxidant addition on flavor of Cheddar and Mozzarella whey and Cheddar whey protein concentrate. J. Food Sci. 75:559-569.

Listiyani, M. A. D., R. E. Campbell, R. E. Miracle, L.O. Dean and M. A. Drake. 2011. Influence of bleaching on flavor of 34% whey protein concentrate and residual benzoic acid concentration in dried whey proteins. J. Dairy Sci. 94:4347–59.

Luck, P. J., N. Bray, and E. A. Foegeding. 2001. Factors determining yield stress and overrun of whey protein foams. J. Food Sci. 67:1677–1681.

McClements, D. J., and E. A. Decker. 2008. Lipids. Fennema's Food Chemistry. 4th ed. S.

Damodaran, K. L. Parkin, and O. R. Fennema, ed. CRC Press, Boca Raton, FL. 155-216.

Meilgaard, M. M., G. V. Civille and B. T. Carr. 1999. The Spectrum descriptive analysis method. Sensory Evaluation Techniques. 4th ed. CRC Press, Boca Raton, FL. 189-254.

Karleskind, D., I. Laye, F. I. Mei and C. V. Morr. 1995. Gelation properties of lipid-reduced, and Calcium-reduced whey protein concentrates. J. Food. Sci. 60:731–737.

Phillips, L. G., J. B. German, T. E. Oneill, E. A. Foegeding, V. R. Harwalkar, A. Kilara, B. A. Lewis, M. E. Mangino, C. V. Morr, J. M. Regenstein, D. M. Smith and J. E. Kinsella. 1990. Standardized procedure for measuring foaming properties of three proteins, a collaborative study. J. Food Sci. 55:1441–1444.

Rogers, N. R., D. J. McMahon, C. R. Daubert, T. K. Berry and E. A. Foegeding. 2010. Rheological properties and microstructure of cheddar cheese made with different fat contents. J. Dairy Sci. 93:4565-4576.

Seifu, E., E. M. Buys, and E. F. Donkin. 2005. Significance of the lactoperoxidase system in the dairy industry and its potential applications: A review. Trends Food Sci. Technol. 16:137–154.

Shacter, E., 2000. Quantification and significance of protein oxidation in biological samples. Drug Metab. Rev. 32:307-326.

Smithers, G. W., 2008. Whey and whey proteins: from ‘gutter-to-gold’. Int. Dairy J 18:695–704.

Stadtman, E. R., and Levine RL. 2003. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids. 25:207-218.

Swaisgood, H. E., 1982. Chemistry of milk proteins. In P. F. Fox, & J.J. Condon (Eds.),

Developments in dairy chemistry. London: Elsevier Applied Science.

Tomaino, R. M., Turner, L. G., and Larick DK. 2004. The effect of Lactococcus lactis starter cultures on the oxidative stability of liquid whey. J. Dairy Sci. 87:303-307.

Ustunol, Z., K. Kawachi and J. Steffe. 1995.Rheological properties of cheddar cheese as influenced by fat reduction and ripening time. J. Food Sci. 60:1208-1210.

Van Scheppingen, W. B., I. A. Boogers, A. L. Duc hateau. 2012. Study on decomposition products of norbixin during bleaching with hydrogen peroxide and a peroxidase by means of UPLCUV and mass spectrometry. Food Chem. 132: 1354-1359.

Whitson, M. E., R. E. Miracle, M. A. Drake. 2010. Sensory characterization of chemical components responsible for cardboard flavor in whey protein. J Sensory Stud 25:616–636.

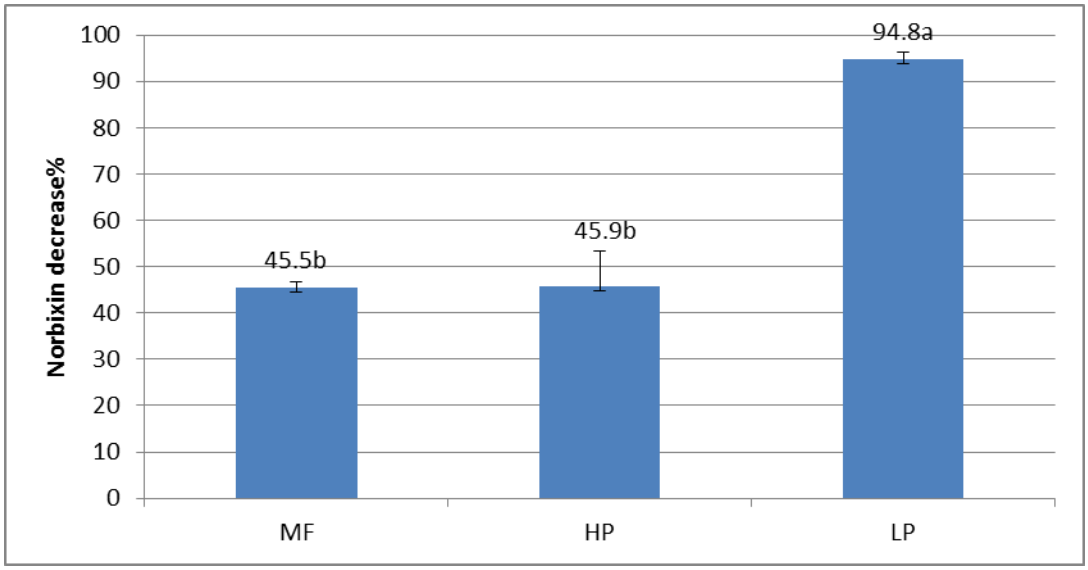
Wilde, P. J., 2000. Interfaces: Their role in foam and emulsion behavior. Curr. Opin. Colloid Interface Sci. 5:176–181.

Wright, B. J., S. E. Zevchak, J. M. Wright and M. A. Drake. 2009. The impact of Agglomeration and storage on flavor and flavor stability of whey protein concentrate 80 and whey protein isolate. J. Food Sci. 74:17-29.

Ye, H., J. J. Lei, Y. Z. Zhang, H. Li, G. F. Li, Z. P. Cao and L. P. Xie. 2011. Investigation of microfiltration for pretreatment of whey concentration. Desalination and Water treatment. 34:173-178.

Zhu, D., and S. Damodaran. 2012. Short communication: Annatto in Cheddar cheese-derived whey protein concentrate is primarily associated with milk fat globule membrane. J. Dairy Sci. 95:614-617.

a)



b)

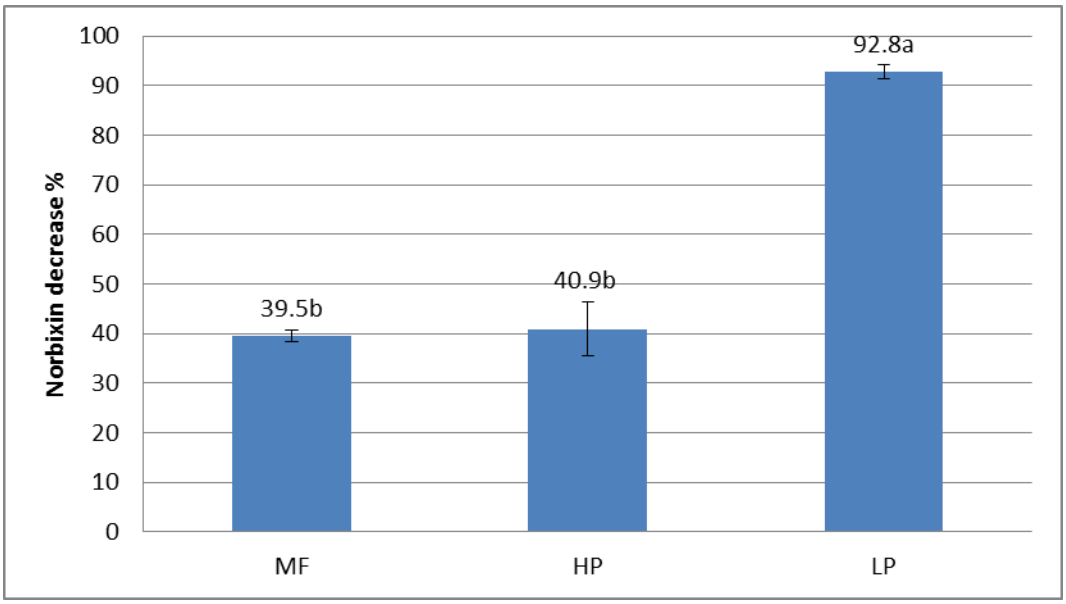
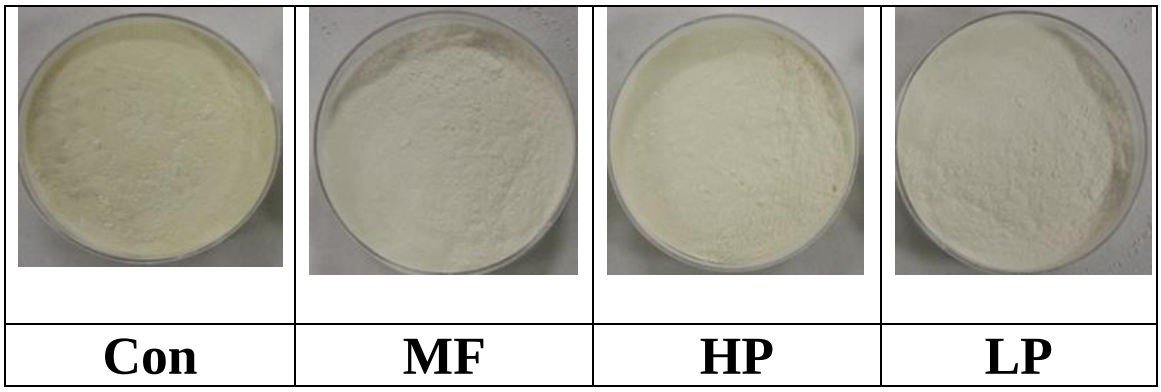


Figure 1. The percent norbixin decrease compared to unbleached control liquid whey (a) and unbleached control WPC80 (b). MF= microfiltration; HP= hydrogen peroxide; LP= lactoperoxidase. [a,b] Means in the same row with different letters are significantly different ($P < 0.05$).

a)



b)

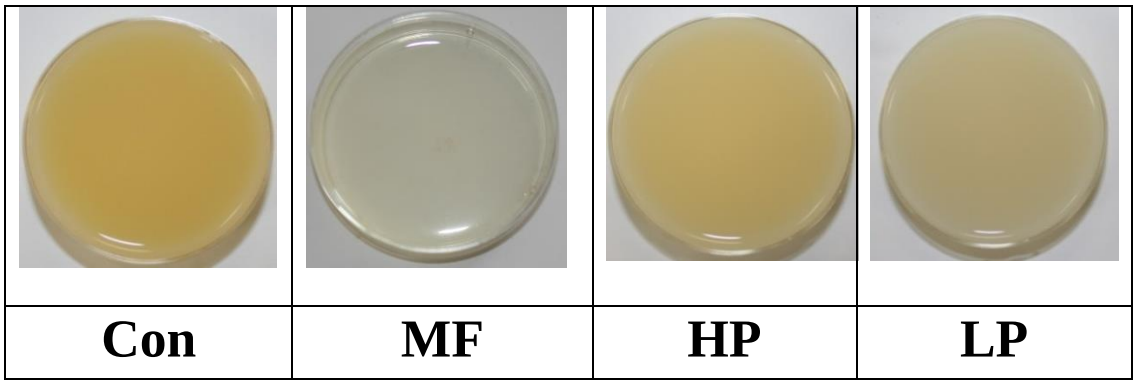


Figure 2. WPC80 powders (a) and Rehydrated WPC80 (b) at 10% solids (wt/vol). Con=no bleach; MF= microfiltration; HP= hydrogen peroxide; LP= lactoperoxidase.

Table 1. Hunter color values for dried and rehydrated WPC 80% (10% wt/vol)

	L-value		a-value		b-value	
Treatment	Liquid	Powder	Liquid	Powder	Liquid	Powder
con	61.9[b]	83.1[d]	1.27[a]	-0.80[a]	33.5[a]	20.9[a]
MF	71.1[a]	89.8[a]	-1.59[c]	-1.17[b]	21.2[b]	12.7[b]
hp bleaching	66.3[ab]	86.1[b]	-0.75[b]	-0.64[a]	21.8[b]	12.1[b]
lp bleaching	69.4[a]	84.9[c]	-0.65[b]	-0.71[a]	19.9[c]	9.8[c]

L-value= luminosity (degree of lightness from black to white);
a-value= the degree of redness or greenness;
b-value= the degree of yellowness or blueness.
Con= no bleach treatment; MF= microfiltration; HP= hydrogen peroxide;
LP= lactoperoxidase.
[a,b] Means in the same row with different letters are significantly different ($P < 0.05$).

Table 2. Descriptive analysis results of bleached and unbleached Cheddar WPC80.

	Treatment			
Category	**Con**	**MF**	**HP**	**LP**
Aroma Intensity	2.0[b]	2.2[b]	2.6[a]	2.1[b]
Sweet aromatic	0.9[b]	1.9[a]	0.8[b]	ND
Milky	1.9[a]	1.4[ab]	1.0[b]	1.2[b]
Cardboard	1.5[b]	1.3[b]	2.3[ab]	2.5[a]
Cabbage /brothy	ND	ND	0.8[b]	1.4[a]
Astringency	2.0[a]	2.0[a]	2.0[a]	2.1[a]

[a,b] Means in the same row with different letters are significantly different ($P < 0.05$).

Intensities were scored using a 0 to 15 point universal scale, with 0 being no intensity and 15 the highest intensity. Most dried dairy ingredient flavors fall between 0 and 4 on this scale (Wright et al., 2009; Whitson et al., 2010).

ND = not detected; Con = control; MF = microfiltration;

HP = hydrogen peroxide; LP = lactoperoxidase.

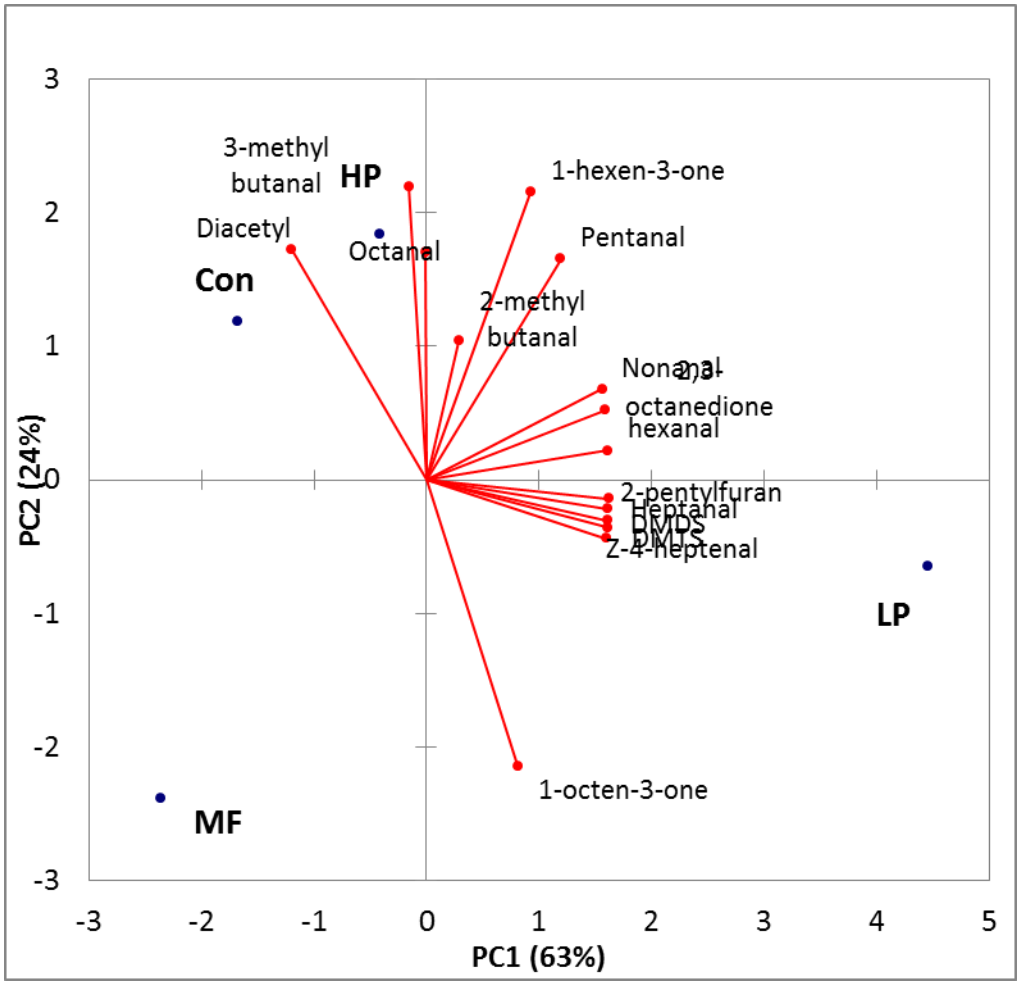


Figure 3. Principal component analysis (PCA) biplot of volatile compounds in bleached and unbleached WPC80. Con= no bleach treatment; MF= microfiltration; HP= hydrogenperoxide; LP= lactoperoxidase. PC1=principal component 1; PC2=principal component 2.

Table 3. Relative abundance (µg/kg) of selected volatile compounds in bleached and unbleached WPC80

No.	Compounds	Con	MF	HP	LP
1	Heptanal	0.493[c]	0.296[c]	1.346[b]	5.462[a]
2	DMTS	ND	ND	0.049[b]	0.333[a]
3	DMDS	0.180[b]	0.130[b]	0.573[b]	2.809[a]
4	1-octen-3-one	0.308[b]	0.401[a]	0.237[b]	0.449[a]
5	Octanal	0.424[b]	0.409[b]	1.563[a]	0.516[b]
6	Nonanal	1.054[c]	0.170[d]	2.088[b]	3.748[a]
7	Z-4-heptenal	0.053[c]	0.039[c]	0.404[b]	3.888[a]
8	Diacetyl	0.764[a]	0.555[b]	0.691[a]	0.404[c]
9	Pentanal	2.245[b]	0.302[c]	4.519[a]	4.527[a]
10	2-methyl butanal	0.139[a]	0.112[a]	0.115[a]	0.127[a]
11	3-methyl butanal	0.075[a]	0.052[a]	0.065[a]	0.060[a]
12	hexanal	8.091[c]	0.600[d]	30.92[b]	75.93[a]
13	2-pentylfuran	4.102[c]	0.542[d]	11.02[b]	47.17[a]
14	1-hexen-3-one	0.338[a]	0.046[b]	0.424[a]	0.397[a]
15	2,3-octanedione	0.315[b]	0.229[b]	0.356[b]	0.528[a]

[a,b,c] Means in the same row with different letters are significantly different ($P < 0.05$).
Mean concentration of selected compounds in WPC80 by SPME GC-MS using SIM mode quantified using single point response factors.
ND = not detected; Con = control; MF = microfiltration;
HP = hydrogen peroxide; LP = lactoperoxidase.

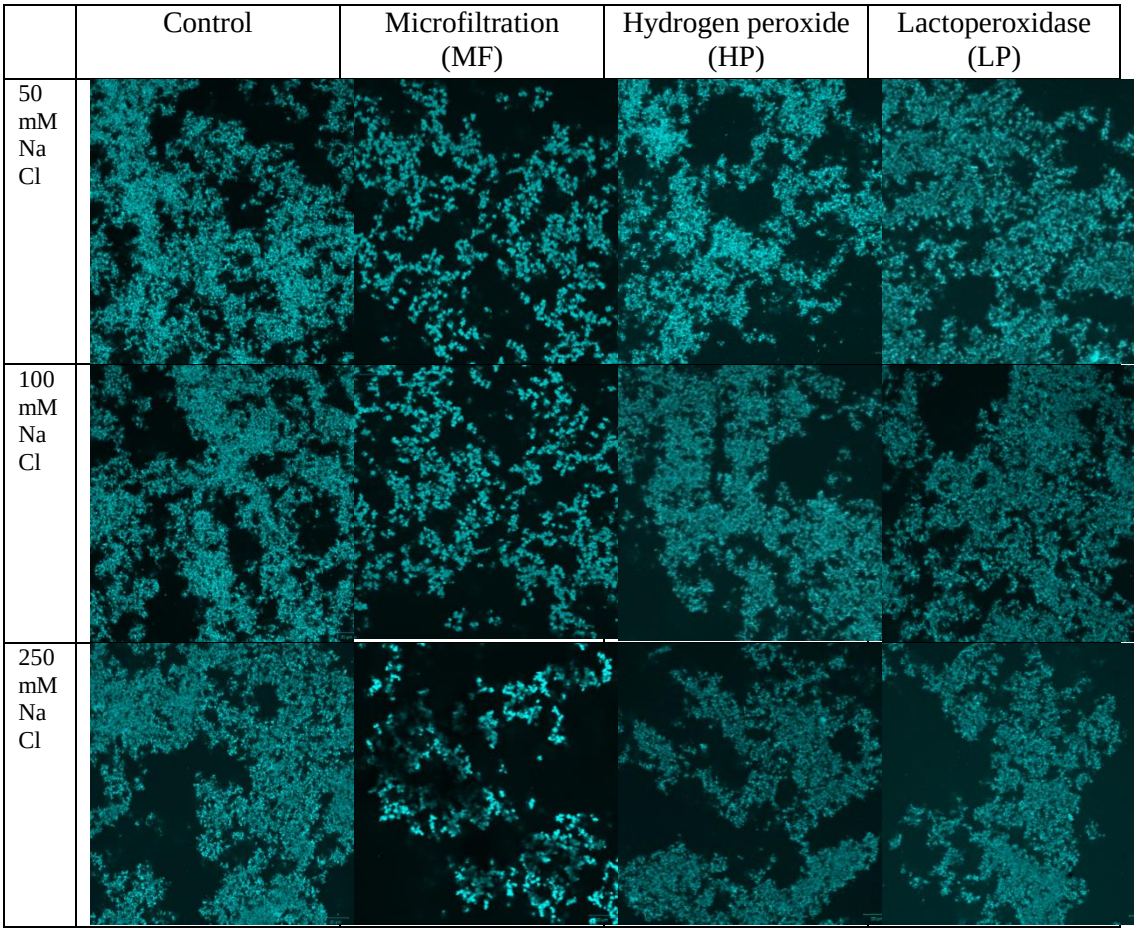
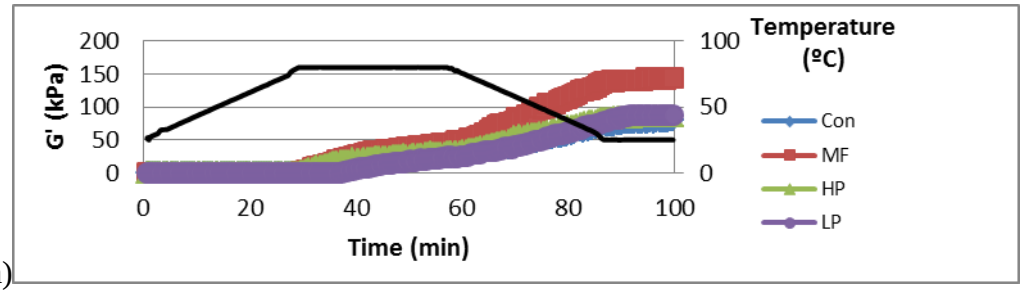
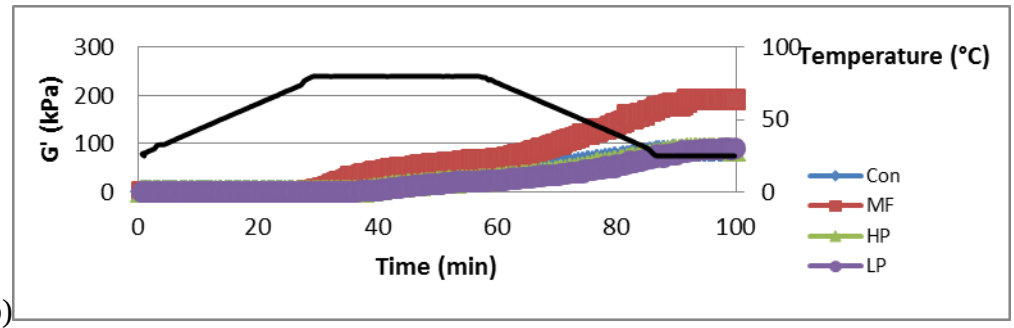


Figure 4. Microstructures of WPC gels at three different ionic strengths as determined by confocal laser scanning microscopy (CLSM). Field size is 250 μm × 250 μm for each image.

a)



b)



c)

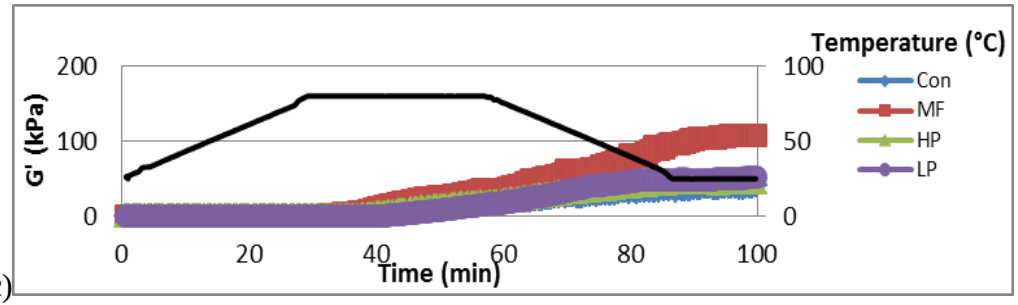


Figure 5. Changes in G' during gel formation of WPC80 (13%w/v) at 50mM (a), 100mM (b) and 250mM (c) NaCl. The straight line indicates the temperature profile where samples were heated from 25 to 80 °C at 2°C/min, held for 30min, then cooled to 25°C at the same scan rate and held for 15min.