ABSTRACT

FOX, KATHRYN MICHELLE. The Impact of Heating and Acidification on the Flavor of Whey Protein Isolate. (Under the direction of Dr. MaryAnne Drake.)

Previous studies have established that whey protein manufacturing processes greatly influence the flavor of dried whey protein isolate (≥ 90% protein) (WPI). WPI is often subjected to additional processing steps in ingredient applications including acidification and heat treatment. These post-processing treatments may further influence formation or release of flavors. The objective of this study was to characterize the effect of two processing steps inherent to manufacture of acidic protein beverages, acidification and heat treatment, on the flavor of WPI. Duplicate samples of five commercial WPI (non-instantized) were rehydrated at 10% solids (w/v) and evaluated as-is (no treatment, NT), acidified to pH 3.2 (A), heated to 85°C for 5 min (H), or acidified to 3.2 and heated to 85°C (AH). The samples were evaluated by descriptive sensory analysis, solid phase microextraction (SPME) gas chromatography mass spectrometry (GC-MS) and SPME gas chromatography-olfactometry (GC-O). The five WPI were distinct in sensory and volatile compound profiles. Acidification of the WPI produced higher concentrations of aldehydes and sulfur compounds and increased cardboard and soapy flavors, while heating increased sulfur compounds and cooked/sulfur flavors. Acidification and heating increased cardboard, potato/brothy, and soapy flavors and produced higher concentrations of aldehydes and sulfur compounds. These results demonstrate that further processing of WPI in food applications has negative effects on WPI flavor, which can carry over into the finished product.

The Impact of Heating and Acidification on the Flavor of Whey Protein Isolate

by
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DEDICATION

To my parents: Michael and Janet Fox. Thank you for always believing in me, even when I didn’t believe in myself.

BIOGRAPHY

Kathryn (Katie) Michelle Fox was born on November 27, 1985, in Memphis, Tennessee, to Marianne Fox-Pagington and Michael Fox. She has one sister, Mary Margaret Pagington, who resides in Berryville, Virginia. Her mother and stepfather, Ralph Pagington, currently resides in Berryville, Virginia, and her father and stepmother, Janet Fox, reside in Cary, North Carolina.

Katie graduated from Clarke County High School in June 2004, and spent her summer in Yachiyo and Nagareyama, Japan, where she learned about Japanese cuisine. Upon returning, she began her Bachelor of Science degree at the College of William and Mary in Williamsburg, VA. In the summer of 2004, she participated in a summer undergraduate research program. There she learned about ligand synthesis and crystallography, and how it can affect our food chain. In May 2008, Katie graduated with a Bachelor of Science degree in Chemistry with a minor in East Asian Studies. She also became certified in biochemistry with the American Chemical Society.

Katie began her graduate program with the Department of Food, Bioprocessing, and Nutrition Science at North Carolina State University in 2008, majoring in Food Science. She served as the historian of the Food Science Club for 2009-2010. She will graduate with her Master of Science in May 2011, under the direction of Dr. MaryAnne Drake.

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

The market for whey protein is a rapidly growing section of the food industry. With new process technology enabling manufacturers to increase protein concentration levels, multiple whey protein products have emerged, increasing both functionality and the application of whey protein as an ingredient. Whey protein is expected to have a bland flavor, making it an ideal addition to functional foods. However, whey protein is often characterized with undesirable flavors (Morr et al, 1991; Carunchia Whetsine et al., 2005a and b; Wright et al., 2006), which often originate from the milk or result from whey protein processing (Mahajan et al., 2004; Carunchia Whetsine et al., 2005b; Gallardo-Escamilla et al., 2005; Wright et al., 2006, 2009). The formation of off-flavors is one of the major limitations that prevents widespread use of whey protein in food applications. A great deal of research has been conducted concerning the formation of off-flavors in whey protein as well of the effect of processing on the functionality of whey protein (Mangino et al., 1987; Morr, 1987; Haque et al., 2003; El-Salam et el., 2009). During ingredient applications such as protein beverages, however, whey proteins are often subjected to additional processing steps, such as acidification and heating. These post-processing steps may also cause the formation of off-flavors, which carry over into the final product, affecting its success. It is therefore necessary to discover how these additional processing steps affect the flavor of whey protein.

WHEY AND ITS MANUFACTURE

Whey is the green liquid that is separated from the curd of milk, skim milk, or cream during coagulation in the cheesemaking process (CFR 21 Section 184.1979). Whey protein is globular in nature and is more water-soluble than casein, which is the primary protein found

in milk (Yalcin, 2006). Whey proteins make up about 50% of the milk solids and 20% proteins (Smithers, 1996 and 2008). Liquid whey is the product removed from the curd and has been clarified. It contains about 93% water and 0.6% protein (Huffman, 1996). Liquid whey, with its high water content, is assumed to have an ideal application in beverages; however, there are problems with expensive transportation cost, high susceptibility to deterioration in storage, as well as some undesired sensory characteristics (Gallardo-Escamilla et al., 2005).

Acid whey is created during the production of cottage cheese and ricotta, where significant levels of lactose are converted into lactic acid, or during the formation of curd from direct acidification of milk. Acid whey has a pH of less than 5.1 and a higher mineral content than sweet whey. This type of whey often requires additional treatment, as it can taste bitter and metallic (Canning, 2004). Sweet whey is derived from hard cheeses, such as Cheddar cheese and mozzarella, and has a pH of greater than 5.6. Most of the whey powders produced in the United States are made from sweet whey. Both sweet and acid whey powder are considered low sources of protein (Yalcin, 2006). Another type of whey, called salted whey, is mainly produced from the soft Domiati cheese in Egypt (El-Salam et al., 2009). These different whey types may be further processed to create different functional products.

Originally, whey was seen as a waste product that was added to feed stock (Matthews, 1984). With its high biological oxygen demands, whey was considered a disposal problem (Regester et al., 1996). World production of cheese was estimated to be 16.47 million tons in 2005, thus whey production in 2005 was around 150 million tons (El-Salam et al., 2009). With its high production rate, it was necessary to find other uses for whey rather

than waste and animal feed. However, as processing technology improved, products could be manufactured that allowed for applications of dairy products with increased nutritional benefits for consumers and application-specific functionality (Henning et al. 2006). Whey protein is now considered a value-added product, and the price of whey products increases with protein content (Russell et al., 2006).

Once the whey is removed from the curd, it is centrifuged (clarified) to remove low levels of curd that are present. These small particles of cheese can ruin membranes during later processing (Mulvihill and Grufferty, 1997). The whey is then pasteurized to halt the cheese culture microorganisms that are present to convert lactose into lactic acid, as well as to prevent the growth of any potential pathogens. The liquid whey is then passed through a separator to remove the whey cream, or fat, from the whey. The whey is then further processed into the desired whey products (Smith, 2004). During this processing, it is essential to create a consistent product for the consumer (Matthews, 1984).

The use of membrane technology has allowed for the concentration of whey proteins and lactose, as well as the separation of whey into multiple fractions. These fractions can be further used for both food and pharmaceutical applications (Henning et al, 2006). The membranes differ in pore size, thereby varying the degree of separation. Microfiltration separates out suspended particles, ultrafiltration removes macromolecules, nanofiltration takes out sugars, divalent salts, and dissociated salts, while reverse osmosis separates out monovalent salts and undissociated acids. In the United States dairy industry, ultrafiltration is mostly used for the production of whey protein concentrate and filtering out water, lactose, nonprotein nitrogen, and soluble minerals (Nelson and Barbano, 2005). Ultrafiltration

isolates whey proteins, retaining their solubility and functionality (Swartz, 1995). Not as widely used, microfiltration has been used to remove bacteria, somatic cells, and fat from milk and to remove casein from whey (Nelson and Barbano, 2005).

However, Nelson and Barbano (2005) believe that the use of microfiltration to the cheese milk before the cheese making process may improve the quality of whey protein, as the permeate (containing the "whey" protein) from the microfiltration is sterile and the proteins are in their native form. The filtration can occur before the cheese making process without altering the cheese composition. Another important benefit is that the filtered fluid whey contains little to no fat, which often has a negative effect on flavor, thereby eliminating the defects that are often associated with the storage of whey. Sweet whey made from Cheddar cheese can contain as much as 0.23 to 0.49% fat, which is much higher than the whey produced through microfiltration. The whey also lacks residual coagulant, starter culture, lactic acid, or color that usually arises from the cheese making process. Evans and others (2009) compared the volatile and sensory characteristics of serum protein concentrate (SPC), the soluble proteins separated by caseins and skim milk by microfiltration, and WPC at 34% protein. While the sensory profiles of both the SPC and WPC were very similar, the composition and the physical property differences were different from one another, which may have an impact on functionality. A similar study was conducted using SPC and WPC at 80% protein (Evans et al., 2010). The pilot plant-produced WPC had higher concentrations of lipid oxidation products, which may result from its higher fat content.

Liquid whey products are often dried to increase ease of transportation. There are several options in drying, including spray and drum drying. In spray drying, the liquid

product is sprayed in combination with hot air into a drying chamber and collected. In roller drying, the concentrated whey is dried on a hot drum and is removed by a scraper (Henning et al, 2006). Spray dying is the most common process applied to whey today. Whey processing has a major impact on final powder attributes in relation to functionality (Ji and Haque, 2003). Each product mainly differs based on their concentrations of protein, lactose, fat, and ash (El-Salam et al., 2009). Because high ash content can negatively affect the flavor and nutritional quality of whey protein products, whey protein is sometimes demineralized by electrodialysis, ion exchange, and nanofiltration or "loose" reverse osmosis, enabling the manufacturer to remove up to 70% of the ash. These products are often used in baby foods and special diet foods (Mulvihill and Grufferty, 1997). Whey protein products can also be delactosed through concentration and crystallization of the lactose, which can then be recovered through centrifugation. These products are often demineralized as well, as they can have a high mineral content.

Advances in filtration has allowed for the creation of whey protein products with varying protein concentrations and functionalities. Whey protein concentrate (WPC) is a dry whey protein product that must contain no less that 25 % protein (CFR 21 Section 184.1979c). WPC products have a protein concentration that usually ranges from 35-80% (El-Salam et al., 2009). The protein concentration of a WPC product is commonly specified in the name, for example, WPC 80 has a protein concentration of 80% (CFR 21 Section 184.1979c). WPC have functional properties, such as heat-gelation, foaming, and the ability to act as an emulsifier (Barbut and Foegeding, 1993; Fachin and Viooto, 2005; Sodini et al., 2006). WPC can therefore be used in products as a cheaper replacement of skim milk

powder, egg substitutions, ice-cream mixes, processed cheese foods, and whipped toppings (Mathur and Shahani, 1979). Factors, such as pH, temperature, and mineral content must be controlled in order to optimize WPC functionality (Canning, 2004; Sodini et al., 2006).

Whey protein isolate (WPI), has a minimum protein concentration of 90% and is manufactured by ion exchange chromatography or microfiltration. The ion exchange process was developed in the early 1980's, enabling WPI to be used as an egg albumin substitute. In the 1990's, body builders discovered the benefits of whey protein products, and whey protein was seen as a convenient and portable delivery system of nutrition during the nutritional bar revolution (Neville et al., 2001). In ion exchange chromatography, major whey proteins become bound to specially developed resins in the ion exchanger and are eluted by changes in pH. This process creates chemical modifications in the protein and calcium is replaced with sodium during the binding/release process (Neville et al., 2001). During manufacture, there is a degree of protein denaturation, which results in an increase in functional properties, such as gelling, whipping, and foaming (Neville et al., 2001). In microfiltration, lipids and protein aggregates as well as microbial debris are removed by pores < 1μm. The clarified material is filtered by ultrafiltration to reach the desired concentration, removing salts and lactose from the whey protein isolate (Neville et al., 2001). The microfiltrated isolate lacks the protein denaturation of ion exchange isolate, and if there is no change in pH and the processing is conducted under mild temperatures, microfiltrated whey protein isolate can be nearly free of denatured protein (Neville et al., 2001). There is less glycomacropeptide content in WPI that is processed by ion exchange than by microfiltration (El-Salam et al., 2009).

Glycomacropeptide (GMP), a glycophosphopeptide, is cleaved from bovine κ-casein with the addition of chymosin (rennin) during the cheesemaking process. GMP, the hydrophillic portion of the casein micelle in milk, is removed with the whey, leaving para-κ-casein with the curd. Making up only 15-20% of the protein found in renneted cheese whey, GMP contains no aromatic amino acids. Currently, GMP is used to facilitate the diet of phenylketonuria patients, who are unable to metabolize phenylalanine. GMP may also have other abilities, such as the ability to bind cholera and *Escherichia coli* enterotoxins, promote bifidobacterial growth, and modulate immune system responses (Damodaran et al., 1996; Brody, 2000).

Whey protein hydrolysates (WPH) are produced through enzymatic processes, such a fungal protease and papain, acids, or alkali where concentrated whey proteins are broken down into peptides of varying sizes and free amino acids. Hydrolysis through chemical means is difficult to control, possibly producing products with reduced nutritional value or creating toxic substances like lysino-alanine (Sinha et al., 2006). Hydrolysis by enzymatic means, however, under mild conditions can develop biologically active nutritional components, increasing functional food opportunities (Sinha et al., 2006). The degree of hydrolysis is the term is used to describe the extent in which a protein has been hydrolyzed by proteases and it plays a role in the functionality of whey protein hydrolysates, (West and Gallagher, 2007). Most hydrolysates have a degree of hydrolysis of 4-40%. Sinha et al. (2006) found that as the level of hydrolysis increased, the water absorption capacity of WPH increases. This increase in water absorption is most likely from the break of proteins into smaller peptides, which increases the number of available water binding sites. A small degree

of hydrolysis improves the foaming and emulsification properties; the hydrolysis by alkaline bacterial protease (6%) produced a hydrolysate with the best foaming properties (West and Gallagher, 2007). Hydrolysates are also better absorbed than whole proteins. In *in vitro* digestibility tests, hydrolysates had a threefold increase in digestion compared to the control (Sinha et al, 2006). Whey protein hydrolysates, with their variable functionality and high nutritional value, have been used for a number of products including acid beverages/fruit juices, nutrition bars, tablets and supplements, pet food, yogurt drinks and smoothies, infant milk formula, convalescence foods, meal replacement products, and weight management products (West and Gallagher, 2007). Although intact proteins are usually recommended for consumers with a normally functioning digestion system, hydrolysates are used in infants and those with an impaired gastrointestinal tract. This is because hydrolysates are absorbed by the body better than intact proteins and are better tolerated, thus there is less prevalence of diarrhea (Swartz, 1995). Enzymatic hydrolysis also reduces the allergenicity of whey protein; manufacturers often use the proteases that are involved in human digestion (trypsin, chymotrypsin, and pepsin) in combination with UF membrane treatment, resulting in a reduction of allergenicity of almost two orders of magnitude (West and Gallagher, 2007). A higher degree of hydrolysis is recommended for allergy treatments, while a milder treatment is used for gastrointestinal and metabolic disorders (Swartz, 1995). Besides their variability in hydrolysis, WPH products are limited by their bitter taste; however, this development can be controlled by controlling hydrolysis through the use of endo-proteases and exo-proteases (West and Gallagher, 2007).

FUNCTIONALITY

Functionality can be defined as “any property of a food or food ingredients, except its nutritional ones that affects its utilization;” these include water binding, gelation, viscosity, emulsification, thermal extrusion, and flavor (Mangino, 1984). Whey proteins are amphiphilic, thus allowing them to form cohesive viscoelastic films between oil and water interfaces. This ability makes whey proteins an ideal choice as an emulsifier as compared to low molecular surfactants in foods. However, many factors influence the functionality of protein emulsions, including amino acid composition, pH, temperature, protein conformation, storage conditions, surface hydrophobicity, protein flexibility, electrostatic interactions and steric effects, as well as ionic strength and the concentration of the protein (Fachin and Viotto, 2004; Mangino, 1984; Schmidt et al., 1984). While proteins will exist in the lowest kinetically attainable state of free energy, environmental factors, such as pH and temperature, air, fat, and denaturants can cause the conformation to change and thus cause the denaturation of the protein (Mangino, 1984). Heat and pH can greatly affect the behavior of whey protein emulsions prior to ultrafiltration, most likely due to the degree of denaturation. Schmidt and others (1984) believe that heating during ingredient applications have a direct effect on functionality, while direct addition of acid in process modification has an indirect effect on functionality.

Whey protein products from different manufacturers often vary in functionality due to many differences, such as the original milk quality, the type of cheese produced, handling practices, and different processing equipment (Banavara et al., 2003). High turbidity values result from excessive heat, acid, and storage treatments, which cause proteins to denature and

consequently produce colloidal protein aggregates large enough to refract light (Banavara et al., 2003). Foaming properties are positively correlated to particle size and negatively correlated to lipid content, and protein solubility is positively correlated to with protein content and negatively correlated with turbidity (Banavara et al., 2003). Sweet whey powder may become hygroscopic or nonhygroscopic based on the difference in manufacturing processing and the consequential state of the lactose remaining is the powder (Banavara et al., 2003). It is important to understand the effects of processing in the functionality of whey protein in order to create products specific to applications and to create a more uniform product.

WPC is sometimes limited in its applications due to its variability in functionality, especially between manufacturers. This limits food processors to one whey manufacturer or forces them to personally make a whey combination blended from whey products from several manufacturers. Particulate size is a major factor in determining the functionality of WPC. Larger particulate size is often associated with higher fat content, which results in poorer solubility and foam stability. One solution is to reduce particle size by passing the WPC through a sieve. Particles with a size of less than 100 microns have the highest solubility. Creating a WPC with uniformly sized particles significantly reduces variability between products (Onwulata et al., 2004).

There are numerous experiments on the effect of processing on the functionality of whey proteins. Morr (1987) tested the effect of high temperature-short time (HTST), 72° C for 15 seconds, on whey. HTST is often the pasteurization method preferred for milk and milk derived products to ensure microbiological safety and shelf stability, and is thought to

affect the functionality on whey. While HTST pasteurization on milk and whey had no effect on resulting WPC solubility, pasteurization of the UF retentate resulted in a significant loss in solubility, most likely due to its high protein content. Also, pasteurization of whey and UF retentate had no effect on the functionality of maximum foam expansion, but HTST pasteurization of milk caused a significant reduction in foaming ability. The composition of whey was also not significantly altered, except for a reduction in ash and lactose content. Because pasteurization affected different stages of whey production, Morr believed that only minor changes well required, in either protein composition or conformational state, to greatly affect the functionality of whey. However, Mangino et al. (1987) found that only the initial milk pasteurization affected the functionality of whey, with a positive correlation with foam and foam stability, and a negative correlation with gel strength and alkane binding. This might be due to the reduction of neutral lipids and the decrease protein hydrophobicity in WPC that has been pasteurized.

Heating also affects certain components of whey protein, such as total solids, pH, lactose concentration, and mineral components (Schmidt, 1984). By increasing the amount of total solids, one can reduce the rate of protein denaturation, however, this decrease is in relation with the whey protein fraction β - lactoglobulin; α-lactalbumin denaturation is actually increased with an increase in total solids (Schmidt, 1984). When raising the pH from 6 to 7, it is thought that the free thiol group of β - lactoglobulin becomes more reactive, causing more dissociation of the β - lactoglobulin dimer to the monomer form (Fachin and Viotto, 2004). There is some conflict in the literature on the effect of pH on denaturation. Some researchers have found that minimal denaturation occurs at the isoelectric point, while

other believe that the lowest rate occurs near pH 6 to 7 (Schmidt, 1984). Heat treatment in an acidic pH causes high protein viscosity, lowers gel temperature, and causes high water absorption (Schmidt, 1984). The protein β-lactoglobulin has reversible unfolding at low pH, but at high temperature, a single thiol group causes irreversible denaturation at high temperature, resulting in gelation or disulphide cross-linking with κ-casein (Creamer and MacGibbon, 1996). Therefore care must be taken during the processing of whey to reduce variability in whey functionality.

Along with its functional uses, whey protein has been shown to have numerous health benefits. Whey proteins make up about 20% of the proteins originally found in milk, which have been found to have putative biological and physiological effects. Some proteins, such as α-lactalbumin, β-lactoglobulins, lactoferrin, lactoperoxidase, immunoglobulins, glycomacropeptide, and some growth factors have been identified in whey that can evoke health benefits to consumers. Whey proteins have a high biological value of 104, as compared to eggs (100), soy protein (74), and wheat (54) (Haines, 2005). Biological value refers to the measure of the percentage of a specified nutrient and how it is used in the body, in other words, the rate and ease of protein metabolism. With whey's high biological value, it is ideal for body builders, elite athletes, and whose who health is compromised (Smithers, 2008). The benefits of consuming total whey protein include improved digestive function; improve stimulation of the immune system, hypocholesterolaemic effects, and anti-cancer activity. The proposed mechanism of whey protein effectiveness is by the intracellular conversion of the amino acid cysteine to glutathione (Marshall, 2004). Moreover, as these proteins are digested, they can provide the human body with bioactive peptides that

supplement physiological activity (Etzel, 2004; McIntosh et al., 1998). Further separation of these peptides would enable processors to customize special individual diets to meet specific needs for the consumer.

Whey fractions have multiple health benefits. Lactoferrin and lactoperoxidase have antimicrobial and antibacterial properties, immunoglobulins affect passive immunity, and growth factors promote cell growth and differentiation, as well as aid in wound repair (McIntosh et al., 1998). Whey proteins have been shown to be effective against the organisms *Escherichia coli, Salmonella typhimurium, Shigella dysenteriae, Listeria monocytogenes, Bacillus stearothermophilus, Bacillus subtilis, and Micrococcus luteus* (Marshall, 2004). β - lactoglobulin makes up about half of the total whey proteins that comes from milk, and it binds to calcium and zinc and contains multiple binding sites for minerals, fat-soluble vitamins, and lipids (Yalcin, 2006). α - lactabumin makes up about 25% of whey proteins, and is one of the few proteins that survive pasteurization without denaturation. This protein provides good sources of lysine, leucine, threonin, trytophan, and cystine, and it also enhances calcium absorption. Glycomacropeptide (GMP), along with α - lactabumin, is suspected to improve bone health by enhancing calcium retention and decreasing bone resorption. While one study found that this is more of an acute effect rather than chronic, the enhancement of absorption of calcium by whey protein is significant enough to increase bone mineral content (Zhao et al., 2005). The fractionation of bioactive compounds can allow consumers to modify the health benefits they wish to receive from the consumption of whey proteins.

In addition to bioactive compounds, whey protein also contains high levels of amino acids, including the essential amino acids, which are necessary for the continued growth and health of the human body. The concentration of amino acids found in whey protein are higher than vegetable protein sources, like soy, corn, and wheat gluten, and they are efficiently absorbed and utilized, as compared to free amino acid solutions (Marshall, 2004). Free amino acids are found in higher levels in acid whey powders (0.59%) than in sweet whey powders (0.34%) (Mavropoulou and Kosikowski, 1973). Whey proteins contain around 26 grams of branched chain amino acids per 100 grams of protein (Haines, 2005). Some branched chain amino acids (BCAA) have other roles in metabolism, beyond the function of providing substrate for protein synthesis. For example, leucine, (Leu) can trigger protein synthesis in muscles, where it is sensed by an insulin-signaling pathway (Etzel, 2004). Whey proteins can also be considered as "fast proteins," because they reach the jejunum very quickly and then their hydrolysis, digestion, and absorption occur over a lengthy period in the intestine, longer than most other proteins (Yalcin, 2006). This increases the therapeutic worth of whey proteins, and applications range from infant formula to clinical protein supplements.

One health benefit that is currently being investigated is the effect of whey protein on cancer growth. Colorectal/bowel cancer is greatly influenced by the diet; the risk of developing colorectal cancer is increased with the consumption of red meat and animal fat. However, whey proteins have been shown to have anti-growth activity against the development of dimethylhydrazine-induced colon tumors in rat models, as well as having an effect in reducing the development of gastrointestinal tumors, as compared to animal and soy proteins in animal models (McIntosh et al, 1998). Phenol and *p*-cresol, metabolites that are

suspected to initiate colorectal cancer growth, had less of an influence on whey protein – fed rats. It is suspected that the sulfur-containing amino acids proteins found in whey protein enhance the methylation status of DNA, thereby preventing cancerous mutations. The addition of a methyl group on the cytosine or adenosine in DNA allows for the silencing of mutant DNA strands, which may produce cancerous cells (Jaenisch and Bird, 2003). In another study, whey protein concentrate made tumor cells more vulnerable to chemotherapy by depleting sources of glutathione (Yalcin, 2006). Marshall (2004) believes that the amino acids that are capable of converting to glutathione might be able to increase glutathione concentration in relevant tissues, stimulate immunity, and to detoxify potential carcinogens. However, further trials are necessary before whey protein is used as a form of treatment or prevention of cancer.

Whey Protein Flavor

Flavor plays a critical role in the acceptance and success of whey protein with consumers (Russell et al., 2006). Understanding which volatile compounds are responsible for the desirable and undesirable flavors aid in the identification of the sources of flavors, such as raw product stream and processing and storage, which will eventually allow for an effective way for manipulating whey flavor (Drake, 2006). Volatile compounds are responsible for the aroma of a product, while nonvolatile compounds affect the taste (sweet, sour, salty, bitter, and umami) as well as mouthfeel and astringency (Kinsella, 1991). Aroma is very complex and can be composed of thousands of different sensations. Compounds that make up flavor in whey exist in several environments, including in the vapor phase, free in the liquid phase, or bound to protein (Mills, 1986). Flavor compounds can arise from

chemical pathways such a Maillard browning, lipid oxidation, thermal degradation of β-keto fatty acids, lipolysis, and fermentation (Mills, 1993). Flavors tend to be dissolved in the lipid phase of a product, while the flavor binding in proteins is dependent on the total surface area of the protein that is available for flavor absorption (Kinsella, 1991). In order to understand flavor, the sensory perception of flavor and its volatile chemical components must be combined. Therefore both sensory (usually in the form of descriptive analysis) and instrumental analysis is required (Drake et al., 2007).

Instrumental analysis without sensory analysis has little to no relevance to flavor. However, instruments used must be very sensitive, as some key volatiles can be present in foods in very low concentrations, such as parts per billion or trillion (ppb and ppt). Gas chromatography-olfactometry is also used to identify key flavor-contributing compounds (Drake et al., 2007). In headspace sampling, volatile compounds from whey proteins are trapped on a porous polymer, which is then desorbed onto the injector port of a gas chromatograph, which allows the researcher to then identify compounds by mass spectrometry. This system is ideal for monitoring flavor deterioration in products, such as whey protein (Mills, 1986).

Sensory analysis is a scientific discipline than includes the depth and breadth of all properties of a food that are perceived by the human senses (Drake et al., 2009). Two basic groups of tests, analytical and affective, are used in sensory analysis. Analytical testing is objective and uses trained panels, such as descriptive analysis, while affective tests are subjective and use consumers to measure data either qualitatively or quantitatively. It is essential to have clearly defined terms for sensory attributes (Drake, 2009). Descriptive

sensory languages can aid in the documentation of product flavor, as well as act as an interface between instrumental results and consumer responses.

In order to most effectively link sensory and instrumental analysis, several steps must be followed. First, products with the desired or targeted flavors must be selected. This is most often conducted through descriptive analysis. Next, the volatile flavor compounds are identified through instrumental means. Finally, key aroma-active compounds are confirmed by quantification, threshold analysis, and descriptive sensory analysis with model systems (Drake et al., 2007). This also can allow researchers to possibly trace flavors during ingredient applications. Flavor analysis is also a method for defining the quality of a product (Drake, 2004). Expected flavors also vary with different consumer groups, and understanding flavor will aid in the delivery of said flavors. As consumption and competition increase for these dairy products, flavor continues to become an essential component in market research and product development.

Research results show that about fifteen compound types (aliaphatic hydrocarbons, aromatic hydrocarbons, aldehydes, methyl ketones, esters, furans, alkyl pyrazines, pyrroles, saturated fatty acids, carboxylic acids, alcohols, pyridines, imidazoles and sulfur-containing compounds) make up the volatile fraction of WPC (Quach, 1999). Carunchia Whetstine and others (2005 b) reported that the key volatile flavor compounds of both WPC 80 and WPI were butanoic acid (cheesy), 2-acetyl-1-pyrroline (popcorn), 2-methyl-3furanthiol (brothy/burnt), 2,5 –dimethyl-4-hydroxy-3-(2H)-furanone (maple/spicy), 2-nonenal (fatty/old books), (E,Z)-2,6-nonadienal (cucumber), and (E,Z)-2,4-decadienal (fatty/oxidized). Saturated aldehydes of $C_5 – C_9$ are the resulting products from the oxidation of unsaturated

fatty acids, and 2-nonenal and 2,4-heptadienal are secondary reaction products (Mills, 1993). When β-keto fatty acids are heated in the presence of water, methyl ketones can form (Mills, 1993). Branched fatty acids that have 8-10 carbon atoms, such as 4-methyloctanoic and 4-methylnonanoic acid attribute the undesirable cooked mutton flavor, while low molecular weight volatile branched chain fatty acids can contribute significantly to the flavor of some cheeses, which can carry over to the resulting whey (Brennand et al., 1989). Brennand and others (1989) found that n-chain fatty acids thresholds increased from butanoic (C_4) to octanoic (C_8) acid. This was once thought to be due to increasing vapor pressures and decreasing water solubility. However, C_9 through C_{11} have decreased thresholds, while fatty acids beyond C_{12} had higher thresholds. This is assumed to be due to correspondingly higher vapor pressures.

Whey protein products are expected to have a bland and delicate flavor, however, whey protein is often associated with aged and stale undesirable, flavors with consumers (Carunchia Whetstine et al., 2003 b). Reconstituted whey is expected to have the same flavor as fresh liquid whey; however, this is usually not what occurs (Drake, 2006). The off-flavors brothy, diacetyl, sourness, and bitterness are some the major limiting factors that prevents wider application use of whey protein in products that are bland (Carunchia Whetstine et al., 1005 b). Aged and stale off-flavor was previously reported as the single most important flavor criticism of dried whey and WPC products (Morr and Ha, 1991). Now, consumer acceptance of dried whey products is largely affected by both flavor and flavor variability (Caudle et al., 2005). Both the flavor and off flavor in whey products are the products of multiple factors: Tomaino and others (2004) found that whey flavor is based on the type of

cheese produced, while Carunchia Whetstine and others (2003 b) found differences in flavor based on the varying manufacture processes.

Off-flavor production, such as heat-induced, oxidized, and rancid flavors, are greatly impacted by minor milk components and processing conditions. Major components of milk, like fat, lactose, proteins, and salts are influenced by breed of the cow, health of the animal, stage of lactation, season of the year, feed, and individuality of the cow. For example, fat content greatly affects the flavor milk and its components, and while Jersey cows produce milk with 6% fat, Holstein cow milk contains 3.5% milk (Tobias, 1990). Feeding by drylot, with the absence of pasture, often contributes to oxidation flavors in milk, mostly due to compositional changes, that have not been currently identified, which makes the milk more susceptible to oxidation (Tobias, 1990). Weeds and feed of the dairy cows may contribute to sulfur-containing volatile compounds that are found in milk that is used in cheese and whey production (Lee et al., 1996). The contamination of copper also leads to the development of oxidation. Hydrolytic rancidity can be caused by the enzyme lipase, which is generally in raw milk (Tobias, 1990). Croissant and others (2007) studied the effects of pastured based systems versus the conventional total mixed ration on the flavor of fluid milk. Pasture based milks had greater concentration of unsaturated fatty acids and lower saturated fatty acids than total mixed ration milks. Although a train panel detected higher levels of grassy and cowy/barny flavor in pasture based milks, solid-phase microextraction and gas chromatography-mass spectrometry, no unique compounds were found in either milk type. Through consumer testing it was discovered that while there were differences in descriptive sensory analysis, proximate analysis, and volatile compound profiles, the two different

feeding regimens did not affect consumer acceptance of the fluid milk flavor (Croissant et al., 2007).

There are some compounds that are found in whey that are not in the original milk, including 1-propanol, hexanal, nonanal, and 2-nonanone; these compounds are considered volatile lipid oxidation products. Hexanal, often found in whey products, originates from linoleic acid. It is assumed that these compounds developed during the cheese-making process and the pasteurization of the whey. An increase in aldehydes, such as hexanal, methyl ketones, and pentane in stored whey verifies that oxidation had accelerated during storage (Tomaino et al., 2004). Starter culture can also play a role in flavor development in whey. Tomaino and others (2001) found that the starter culture *Lactococcus lactis* subsp. *lactis* produced a whey product with the highest level of total free fatty acids, with significantly higher levels of lauric, myristic, and palmitic acid. Short and medium chain fatty acids, such as acetic, butanoic, hexanoic, and octanoic acids play a role in flavor (Carunchia Whetstine et al., 2003). Sable and Cottenceau (1999) found that shorter chain fatty acids have lower thresholds than longer chain fatty acids and therefore have a greater impact on flavor. Lactic acid bacteria are thought to be slightly lipolytic and tend to generally hydrolyze triacylglycerides in a nonspecific manner (Meyers et al., 1996). This type of bacteria can also produce phenylacetaldehyde and 2-phenethanol, compounds which produce an undesirable or “unclean” rosy aroma, through the catabolism of aromatic amino acids, especially phenylalanine (Carunchia Whestine, 2005 a). Starter cultures used in the production of Mozzarella and Quarq cheese produce ethanal (acetaldehyde) and 2,3 butadione (diacetyl), both of which contribute to the flavor of the resulting whey (Gallardo-

Escamilla et al., 2005). Dimethyl trisulfide (DMTS) is often associated with a cabbage off-flavor in WPI, however, concentrations present of DMTS must be above threshold concentration before they are detected (Wright et al., 2006).

Bleaching, the process of removing the color of residual annatto from the cheesemaking process, can also affect the flavor of whey protein products. Barnicoat (1950) estimates that about 20% of the annatto that is added to the cheesemilk, and this residual color can negatively impact consumer acceptance of food with whey as an ingredient. Before the spray-drying process whey is currently bleached using either hydrogen peroxide or benzoyl peroxide (Croissant et al., 2009). Croissant and others (2009) found that liquid whey and WPC that was bleached with hydrogen peroxide had higher levels of oxidation reaction products, such as heptanal, hexanal, and octanal. These hydrogen peroxide bleached whey products also had higher intensities of fatty and cardboard flavors, as compared to unbleached and benzoyl peroxide flavors. However, benzoyl peroxide also has a negative connotation in regarding health concerns. Morstenson and others (2008) found that levels of benzoic acid in bleached WPC and WPI samples. Benzoic acid is a by-product of the bleaching process by benzoyl peroxide, and several European and Asian countries consider it to be harmful and therefore do not allow the use of benzoyl peroxide in bleaching for whey (Kang et al. in press).

Liquid whey is also expected to have a different flavor profile than whey powder, due to the additional processing steps involved in producing the powdered product, like concentrating and spray-drying (Mahajan et al., 2004). Tomaino and others (2004) found that many off flavors that developed in milk appeared in liquid whey in lower concentration,

probably because of dilution or the compounds are retained in the curd. These off-flavors that remain from milk can become concentrated, especially in spray-drying (Quach et al., 199). Thijssen's selective diffusion theory, where a film of high solid-containing material is formed on the droplet that is dried explains this mechanism (Quach et al., 1999). A semi-dried crust around the liquid particle forms in the beginning of spray drying, preventing certain volatile from escaping, while still allowing water to pass through. Volatiles that are trapped are larger than water and are usually organic flavor volatiles (Stevenson and Chen, 1996).

With the influence of both lipid oxidation and Maillard reaction in the flavor profile of whey protein products, residual lactose and lipids play a crucial role in flavor development (Morr and Ha, 1991; Tomaino et al., 2004). Mortenson and others (2008) found that WPC is higher in attributes of milky, sweet, and caramel flavors, as well as more volatiles, than WPI, this is most likely due to WPC's higher concentrations of residual lactose and lipids. Mills (1993) reported that the major difference in volatile profiles among WPC was due to the difference in the method of manufacture. It is also known that several chemicals can create joint effects, thereby generated other attributes that would not be present if these compounds were present alone (Karagul-Yuceer, 2002). Evans and others (2010) found 2-isobutyl-3-methoxy pyrazine, 2-acetylpyridine, 2-acetyl-1-pyrroline, 2-methyl-3-furanthiol, and sotolon, which as compounds created through the Maillard reaction and caramelization. These compounds are often found in WPC and WPI because these whey products are usually spray dried (Friedman, 1996; Mahajan et al., 2004). Pyrrolines, pyrazines, and furanones give whey

products flavors such as cooked/milky and sweet aromatic (Carunchia Whetstine et al., 2005b).

Even through whey contains only a small amount of lipids, volatile lipid oxidation products are the main source of off-flavors (Carunchia Whestine et al., 2005 b).
Since WPI has a lower lipid content that WPC 80, there is usually less lipid oxidation compounds associated with the volatile compound profile of WPI. Volatile lipid oxidation products and hydroperoxides, developed through lipid oxidation, are associated with whey protein ingredients by entrapment, within the matrix, or through hydrophobic interactions. It is also thought that covalent linkages with ε- and α-amino groups of protein could occur. Through these interactions it is possible for the carryover of these undesirable compounds into whey products (Tomaino et al., 2004). There was a positive linear relationship of increasing protein concentration with free fatty acids, with the exception of WPI, which had the lowest concentration in free fatty acids (Tomaino et al., 2004). This supports the fact that Tomaino and others (2001) found that WPC at 86.5% protein had much higher concentrations of free fatty acids compared to WPI.

Proteolysis is another important reaction that affects the flavor profile of whey protein, and chymosin, a proteolytic enzyme, can degrade amino acids that can carry over into the whey, resulting in undesirable flavors, such as animal/wet dog flavor, which is associated with the degradation of caseins and caseinates. WPI, with its high protein content, can result in the undesirable animal/wet dog flavor. Proteolysis has also been associated with the bitterness and astringency.

Astringency is another factor that affects the acceptance of whey protein products. It has been described as a complex group of sensation, which includes dryness, roughness of oral surfaces, and tightening, drawing, or puckering of the mucosa and muscles around the mouth (Lee and Lawless, 1991). High levels of astringency can affect consumer liking of whey protein food and beverages, thus limiting acceptance and the success of the whey products (Beecher et al., 2008). Beecher and others (2008) found that in whey protein solutions, astringency decreased between pH of 3.4 and 3.2, which coincides with a decrease in protein aggregation (as seen through changes in turbidity). Thus Beecher and others propose that astringency is related to the interactions between the positively charges whey proteins and the negatively charged saliva proteins that are found in your mouth. At these low pHs, the saliva proteins have a decrease in negative charge, thereby decreasing the protein-protein interactions with the whey protein. Another study compared the astringency of a whey protein, β-lactoglobulin, to phosphate buffers at similar pHs. The β-lactoglobulin samples were more astringent, which signifies that acid alone does not cause astringency (Vardhanabhuti et al., 2010). Vardhanabhuti and other's results support the theory that protein-protein interactions between whey proteins and saliva proteins may cause astringency proposed by Beecher and others (2008). Kelly and others (2010) studied the effects of whey protein concentration on astringency and the interactions between whey and salivary proteins. As the concentration of protein increase, astringency increased; astringency leveled out at higher concentrations.

The Maillard reaction, which causes nonenzymatic browning, also plays an important role in the flavor profile of whey protein products, especially in dried products. This reaction

can cause off-flavors that can limit the shelf-life of the whey powders (Sithole et al., 2005). The Maillard reaction is affected by the composition of the powder, such as the initial reactant species, pH, water content, and the presence of humectants and bisulfate, as well as by physical conditions, such as the temperatures of processing and storage, atmospheric oxygen, and packaging (Sithole et al., 2005). Higher concentrations of water as well as certain salts and buffers can accelerate browning. Dattatreya and Rankin (2005) found that pH can have a significant effect on the rate of Maillard browning, by catalyzing the pathway involving the 3-deoxyosone intermediate. The rate of browning in the acidic environment accelerated with increasing temperatures. Diacetyl is also a product that is formed from the intermediate methyl alpha-dicabonyl (Scanlan et al., 1968). Beyond the affect on flavor, the Maillard reaction in whey can cause protein loss, undesirable color, decreased solubility, texture changes, destruction of vitamins, and increase acidity (Sithole et al., 2005).

Agglomeration, the process where a small amount of liquid is added back into the product thereby making small clumps of several whey particles, thereby increasing disperability, also plays a role in flavor stability. Wright and others (2009) found that "instantized" whey products (products that have been agglomerated, oftentimes with lecithin as a binder) have an increased rate of lipid oxidation in storage as compared to nonagglomerated product. During storage, flavors such as cardboard, cucumber, and fatty developed, which suggests that lipid oxidation has occurred. Trained panelists were also able to detect these off-flavors faster in ingredient applications made with agglomerated whey products as opposed to applications with nonagglomerated products. Pasteurizing milk can also develop off flavors that can potentially carry over into whey products. Carunchia

Whestine and others (2003a) stated that there are several aroma-active compounds that are thermally generated during the pasteurization process, such as methyl ketones, 2-nonanone (vitamin), 2-undecanone (floral), vanillin (vanilla), δ-decalactone (peach), and δ-dedecalacone (coconut). Diacetyl is also thought to contribute to the rich or heated note (Scalan et al., 1968).

Off-flavors in dried ingredients can carry-through in ingredient applications. Whey proteins are supposed to be bland and flavorless (Drake, 2005). This is usually not the case, however, as whey proteins so have flavor that vary greatly. Therefore, these flavors are considered to be off-flavors. Caudle and others (2005) investigated the influence of the variability of flavor in skim milk powder on consumer acceptability in ingredient applications. In some products, off-flavors can be successfully masked, and the degree of impact of off-flavors in products is dependent on the type/intensity of the given off-flavor as well as the type of application. The off flavors that were documented by trained panelists were also detected by untrained consumers in ingredient applications; the carry over negatively impacted the acceptability of the products. Because whey protein is a dairy product produced from milk, it makes sense that these findings can be applied to whey protein and their ingredient applications. Low pH beverages made with off-flavored WPI had lower consumer acceptance scores compared to similar beverages prepared with fresh WPI (Drake, 2005). Branger and others (1999) created prototype grapefruit juice blends with cottage cheese whey. A low pH is required to maintain optical clarity and stability in beverages as WPC and WPI are least stable (soluble) at pH 4.5-4.7, however, this low pH is often associated with increased sensations of astringency (Beecher et al., 2006).

APPLICATIONS IN WHEY PROTEIN

With its numerous functional properties and health benefits, whey protein can be used for a wide variety of food products. With health and convenience becoming two major factors affecting the development of new foods (Burrington, 2004), whey proteins can play a critical role in functional foods. A food that offers health benefits to the consumer is often referred to as a functional food. The Japanese Ministry of Health and Welfare defines functional foods as having been derived from natural substances, meant to be part of a person's daily diet, and has a specific targeted physiological function (Smithers et al., 1996). The functional food category is rapidly growing in both Asia and North America, and whey protein is an ideal ingredient for food formulations. In 2005, Mintel International's Global New Products Database reported that 1,763 products in the United States and 6,435 products worldwide containing whey proteins were introduced in the market (Gottschalk, 2006). Beyond the health and functional benefits that can be added to a product, whey protein ingredients can reduce the need for additional emulsifiers, stabilizers, or structure-forming ingredients, thereby reducing cost and imparting a cleaner ingredient label (Gottschalk, 2006). Table 1 shows the functional properties of whey protein and the corresponding ingredient application (de Wit, 2001).

Table 1: Functional Properties of Whey and Food Ingredient Applications (de Wit, 2001)

Functional Property	Mode of Action	Food System
Solubility	dissolvable	beverages
Water absorption	water binding	meat/bakery items
Viscosity	thickening	soups/gravy
Gelation	structure-forming	meat/fish
Emulsion properties	emulsifying	infant formula
Fat absorption	binding free fat	sausages
Foaming Properties	aeration	whipping topping
Flavor binding	binding/release	formulated foods
Mineral binding (Ca, Zn, Fe)	specific adsorption	nutritional foods

One such application is in the beverage industry. While fluid milk sales are stagnant at around $12 billion in sales a year, carbonated soft drinks are the highest ($63 billion/year), followed by juice and juice drinks ($20 billion/year), functional food beverages ($11 billion/year), and bottled water ($8 billion/year) (Etzel, 2004). The use of whey in therapeutic drinks can be found back as far as Hippocrates in 460 B.C., who prescribed whey for a variety of conditions. Its use reached a high point in the Middle Ages, so much so that over 400 whey houses were established in Western Europe. Even in the 1940's, whey was prescribed (up to 1500 g) in Central Europe for dyspepsia, uremia, arthritis, gout, liver diseases, anemia, and tuberculosis (Holsinger et al, 1974). In the 1980s, the theme of "better for you" beverages equated with removing negative ingredients, with claims such as "all natural, no additive/preservations, and no artificial ingredients."

An alternative to the low-protein, high-sugar drinks that are currently produced would be a soft drink with high-protein and low sugar with a slightly higher pH that the traditional

soft drink. This can be done by using certain fruits, which have a higher pH and also have a higher corresponding sweetness, thereby eliminating the need for a high sugar content. Thus the balance of sweetness and acidity is key in developing a healthier soft drink (Etzel, 2004). High acid drinks have a pH range of 2.8-3.5 and are usually processed with mostly hot/ambient-fill or ultra-high-temperature (UHT) procedures. Soft and energy drinks are considered high acid beverages. Low acid drinks have a pH range of 3.5-4.5 and are mostly hot-fill or UHT processed; these are often juice drinks and cultured smoothies. Neutral pH beverages (6.5-7.0) are usually UHT or retort processed and usually thick and shake-like (Miller, 2005). Beaucler and others (2005) found that consumers experienced more thirst quenching from the clear whey protein beverages than from the opaque. Whey protein additions would increase the protein levels as well as health benefits, and calories from the whey protein can be compensated through the reduction of sugar. A protein concentration of 25 g/L qualifies for the claim label "good source of protein" (CFR 21 2008). Acid foods, such as soft drinks, are defined by the FDA to have a $pH \leq 4.6$. While most current soft drinks have a $pH \approx 2.5$, creating a soft drink with a pH closer to 4.6 could increase health claims such as "high protein," "reduced sugar," and "reduced calorie" (Etzel, 2004). WPI is favored in the production of clear sports nutrition drinks, as it contains less than 1% fat, which is the main source of cloudiness (Haines, 2005). In shake-type drinks, whey proteins can improve stability and mouthfeel (Gottschalk, 2006). Whey proteins can also be used as a thickener, replacing starches and other carbohydrates, lowering the carbohydrate levels in drinks (Gottschalk, 2006). Some whey protein beverages have calcium and riboflavin levels

similar to milk, thus these beverages would be advantageous to consumers that do not drink the recommended levels of milk (Jelen et al., 1987).

One challenge of creating a whey protein beverage is that any heat treatment causes denaturation and aggregation of the protein, increasing the turbidity of the beverage. Laclair and Etzel (2009) explored varying methods to reduce the turbidity of heated whey protein beverges. When beverages at a pH of 3.6 or lower were heated, the samples remained clear regardless of the ingredients used. Any pH above 3.8 became turbid, requiring careful ingredient selection. Only glycerol (20% v/v) and urea (4M) were successful in keeping the beverage clear. Small sugar alcohols, xylitol, sorbitol erythriol, and mannitol, reduced the turbidiy by 50% or more, but salts increased the turbidity of the samples. Laclair and Etzel also found that the removal of damaged proteins by centrifugation can keep the beverages clear.

Flavor is an essential component in the acceptance of whey protein beverages in consumers, and complimentary flavors should be used. Because whey proteins do not coagulate in high acid environments, they can be used to make fruit-flavored beverages (Mathur and Shahani, 1979). Childs et al. (2007) developed meal replacement beverages containing 100% soy, 50% whey/50% soy, and 100% whey protein. The beverages with whey protein had sweet aromatic and vanillin flavors and were preferred over the soy beverages. However, when both prototype and commercial beverages were compared, both had low liking scores revealing that these types of beverages are not preferred. It is therefore necessary to continue to investigate flavor of whey protein in order to increase liking with consumers.

Baked goods are also gaining benefits from whey proteins. Whey protein addition may reduce baking time and improve yield. Whey proteins can increase both moisture and water retention, thereby increasing the shelf life of the products, as well as improving mouthfeel and texture. The Dairy Products Technology Center at California Polytechnic State University has been able to substitute as much as 25% of carbohydrates in bread products by using sucralose for sugar and whey protein for flour (Haines, 2005). In low-carbohydrate products, the substitution of fiber often leads to an over absorption of water, while the sugar alcohols can make products develop an undesirable sweetness or off-flavors (Burrington, 2004). Whey protein concentrates with high levels of phospholipids are effective emulsifiers and impart a softer texture in baked snacks and bars (Burrington, 2004). WPC 60 is often used to partly replace egg proteins in baking products (de Wit, 2001). Yang and Foegeding (2009) examined the effects of sucrose on wet and dry foam made with egg white protein and WPI. Wet foams that were created using WPI lacked stability were they were baked as a cake (transitioning to a dry foam). The cakes developed large bubbles even at room temperature, which may be due to destabilization, which includes disproportionation and coalescence in addition to drainage of the thin film in between the bubbles (Yang and Foegeding, 2010). The addition of sucrose to the cakes increased stability of the WPI foams, but it also decreased stability through a loss of interfacial elasticity. A follow-up study with angel food cakes made with WPI and egg white proteins concluded that the addition of sucrose had little to no effect on the stability of WPI wet foam. However, WPI foams began to destabilized before heating and continued during the baking process, which causes the

cakes to collapse (Berry et al., 2009). This trend also occurred in angel food cakes made with a combination of WPI and egg white protein.

Nutrition bars are a fast growing market that can also benefit from whey protein. Nutritional bars fall under the category of meal replacement products, which should provide 100% of the United States Recommended Daily Intake (RDI) of at least 12 essential vitamins and minerals, 8 to 10 grams of proteins, and have about 300 calories per serving (Anonymous, 2002). From 1999 to 2004, the market for nutrition and energy bars experienced a 169% increase in sales (Gottschalk, 2006). Bars can have up to 30% protein without hardening (Haines, 2005). Some nutrition bars contain high concentrations of glycerin or other humectants, resulting in a chewy or sticky texture, while the proteins can lend to a hardening effect, limiting the shelf life of products with their firm and dry texture (Burrington, 2004). Whey proteins that have been textured (textured whey proteins) can increase the crispiness of nutrition bars, while hydrolyzed whey proteins can enhance pliability (Burrington, 2004). Thus a combination of whey protein concentrates, isolates, and hydrolysates are often used in bars, thereby increasing the shelf life of the nutritional bars (Gottschalk, 2006). Liu and others (2009) tested the effect of four major polyol (sugar alcohols: glycerol, propylene glycol, maltitol, and sorbitol) on the stability of nutritional bars containing WPI. Glycerol was the most effective in lowering the water activity of the prototype bars, and glycerol also supplied the softest textures. By lowering the water activity, the polyols increased the stability of the nutritional bar by decreasing the moisture-induced protein aggregation, which may be a cause for hardening during storage (Liu et al., 2009). McMahon and other (2009) suggest that bar hardening is induced by a phase separation of

proteins and carbohydrates, leading to protein aggregation. The prevention of bar hardening during storage depends on the cosolvent properties of the sugar towards the protein. Childs and others (2007) developed a prototype meal replacement bar with whey protein, and it was characterized by sweet aromatic and vanillin flavor notes and a texture of adhesiveness and cohesiveness. The 100% whey and 50% whey, 50% soy bars were significantly higher in overall acceptance, appearance liking, aroma liking, and flavor liking and were preferred over 100% soy bars.

Whey proteins are also being used in meat products, such as surimi-type products and luncheon meats. It can be added to sausages in order to create a low-fat, high-protein product. A breakfast patty with WPC80 created at Ohio State University contained 20% of the fat and a third of the calories that a normal patty, and consumer panelists considered the taste and texture as good if not better than the traditional (Haines, 2005). Whey protein's ability to retain water also aids in the reduction of shrinking during cooking, thereby increasing the consumer perception of a better value. The Maillard reaction with whey protein adds to the flavor and color of gravy (Haines, 2005). Singh and others developed a mushroom soup with concentrated whey (45% solids) added to improve the protein quality of the soup. Consumer testing found that even after 8 months of storage, the samples were considered acceptable.

Whey proteins are also applied to other products, such as infant formulas, specialized and clinical supplements, sports nutrition products, weight management products, and mood control (Yalcin, 2006). As more whey fractions are becoming isolated, the protein composition for infant formulas resembles the protein composition of human milk (Regester et al., 1996). Whey is also easy to digest and contains essential amino acids, making it one of

the most economical sources of proteins (Regester et al., 1996). Hydrolyzed whey proteins are often used for infant formulas. In one study, it was found that infants on a whey protein enhanced formula had significantly more *Bifidobacteria*, which improves gastrointestinal immunity, than infants fed normal formula (Marshall, 2004). A Japanese study revealed that a humanized infant formula with a casein to whey protein ratio of 60:40 (similar to that of human milk) caused infants to have normal physiological functions (Mathur and Shahani, 1979). In a survey of pediatricians, infant formulas with whey protein hydrolysates were most recommended for chronic diarrhea (59%), allergy (26%), and colic (16%). Nutritional advancement of infant formulas has decreased the mortality level due to chronic diarrhea from 45% to 7% (Swartz, 1995).

New technologies also allow manufacturers to manipulate whey proteins for a broader range of applications. Whey solids, with a small amount of gelatin, is capable of holding twice its own weight of oils, fats, and flavors, and may be a new kind of flow agent (Mathur and Shahani, 1979). A combination of whey protein and an edible polysaccharide, like cornstarch, forms a crunchy, textured whey product through twin-screw extrusion. With a protein content of 50% as well as a clean dairy flavor, this new product from Utah State University may soon be found in cereal, nutrition bars, trail mixes, frozen desert toppings, and crunchy snacks (Haines, 2005). Whey protein's ability to form edible films is currently being investigated for the use of coatings for products such as nuts, produce, and confections. These films may be capable of preventing oxygen, aromas, and oils to come into contact with foods, thus foods resistant to spoilage, making whey protein is an ideal substitute for guar gum or additional packaging (Haines, 2005).

Whey applications may expand beyond commercial products and into fortified blended foods (FBF) for providing aid to malnourished individuals and vulnerable groups (HIV/AIDS) in low income populations. The addition of whey to FBF increases the quality of the proteins, resulting in the reduction of proteins in FBF, possibly creating metabolic advantages (Hoppe et al., 2008). Whey protein can also allow for the reduction of soy and cereal in FBF. Soy and cereal contain probable antinutrients, which can prevent the uptake and utilization of food components. Some proposed antinutrients include lectin, hemagglutinins, saponins, antitrypsin, antichymotrypsin, thiaminase, and phytate (Hoppe, 2008). Whey FBF may also improve the immune system and muscle synthesis of vulnerable groups, but more evidence is needed.

Because of its wide range of functionality and health benefits, whey protein has the potential to be used in a wide range of ingredient applications, such as beverages. However, whey protein's off flavors, produced during manufacture, currently limits its use in food products. These off-flavors are compounded when whey protein is subjected to additional processing during ingredient applications, such as acidification and heating. If is therefore imperative to discover what causes these post-manufactured off-flavors. The objective of this study is to examine the processing effects, notably pH and temperature (in pasteurization) on the flavor of several whey protein products as an ingredient in beverages.

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CHAPTER 2: IMPACT OF HEATING AND ACIDIFICATION ON THE FLAVOR OF WHEY PROTEIN ISOLATE

The Impact of Heating and Acidification on the Flavor of Whey Protein Isolate

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Abstract

Previous studies have established that whey protein manufacturing processes greatly influence the flavor of dried whey protein isolate (≥ 90% protein) (WPI). WPI is often subjected to additional processing steps in ingredient applications including acidification and heat treatment. These post-processing treatments may further influence formation or release of flavors. The objective of this study was to characterize the effect of two processing steps inherent to manufacture of acidic protein beverages, acidification and heat treatment, on the flavor of WPI. Duplicate samples of five commercial WPI (non-instantized) were rehydrated at 10% solids (w/v) and evaluated as-is (no treatment, NT), acidified to pH 3.2 (A), heated to 85°C for 5 min (H), or acidified to 3.2 and heated to 85°C (AH). The samples were evaluated by descriptive sensory analysis, solid phase microextraction (SPME) gas chromatography mass spectrometry (GC-MS) and SPME gas chromatography-olfactometry (GC-O). The five WPI were distinct in sensory and volatile compound profiles. Acidification of the WPI produced higher concentrations of aldehydes and sulfur compounds and increased cardboard and soapy flavors, while heating increased sulfur compounds and cooked/sulfur flavors. Acidification and heating increased cardboard, potato/brothy, and soapy flavors and produced higher concentrations of aldehydes and sulfur compounds. These results demonstrate that further processing of WPI in food applications has negative effects on WPI flavor, which can carry over into the finished product.

Key words: Whey protein isolate; acidification; heat treatment; whey protein flavor; ingredient applications

Introduction

Whey protein and its derivatives have been added to a growing number of functional foods. In ingredient applications, whey protein is expected to have a bland flavor; however, current whey protein ingredients usually contain off-flavors (Drake 2006; Wright and others 2006; Wright and others, 2009). Past research has associated these undesirable flavors with multiple variables along the whey manufacturing process, including milk type, cheese starter culture, processing conditions, and storage conditions (Mahajan and others 2004; Gallardo-Escamilla and others 2005; Carunchia Whetsine and others 2003b, 2005; Wright and others 2006; Croissant and others 2009; Wright and others 2009). The flavor variability of dried whey protein products impacts the flavor profile and consumer acceptance of products containing whey protein (Childs and others 2007; Wright and others 2009; Evans and others 2010). The presence of these off-flavors limits the widespread use of whey protein products.

Whey protein isolate (WPI) is a value-added dried whey product containing at least 90% protein. WPI is used in many applications but one of the most value-added and growing categories is the protein beverage market (Anonymous 2010). WPI are typically added to ready to drink (RTD) beverages at levels of 3 – 8% protein (3 – 8 grams per 100 mL serving) (Hazen 2003). Beverages with higher levels of protein contain 25-40 g of protein per 100 mL serving (25-40% protein) and are marketed towards body builders (Anonymous 2010). Cardboard, soapy, cabbage, and potato flavors are flavors associated with whey protein that have been documented in whey protein beverages (Childs and others 2007; Wright and others 2009; Evans and others 2010). In beverage manufacture, dried whey protein is subjected to additional post-processing steps. These steps include acidification and heat

treatment which can further impact the flavor of the WPI and the beverage. A better understanding of how these post processing steps affect the flavor of WPI may lead to the identification of methods to reduce or minimize WPI flavor contributions to beverages or may lead to the identification of key compounds which may mask WPI-related off-flavors to produce a more palatable product. The objective of this study was to understand the effects of two beverage processing steps, acidification and heating, on the flavor of WPI. Sensory and instrumental analyses of five WPI from different manufacturers under various treatments (control, acidified, heated, and acidified and heated) were conducted to achieve this objective.

Materials and Methods

Sample Acquisition

Commercial whey protein isolates (WPI) were received from five different US manufacturers, with 2 lots acquired from each manufacturer. Samples were stored at -20°C upon receipt and throughout the experiments.

Chemicals

Chemical standards (Table 11) were obtained from Aldrich (St. Louis, MO) with some exceptions: dimethyl sulfide, Z-4-heptenal, phenylacetaldehyde, octanal, and *o*-aminoacetophenone were obtained from Acros Organic (Morris Plains, NJ); 1-hexen-3-one, 2-pentyl furan, δ-decalactone, and δ-dodecalactone was obtained from Alfa Aesar (Ward Hill, MA). 2-acetyl-2-thiazoline was obtained from Astatech, Inc (Bristol, PA). 2-acetyl-thiazole, 2-nonanone, and ethyl octanoate were obtained from SAFC Supply Solutions (St. Louis, MO). P-cresol was obtained from Fluka (Buchs, Switzerland).

Proximate Analysis

Total protein of each WPI was determined using the Kjeldahl method (AOAC, 2000: method number 991.20; 33.2.11) followed by multiplying the total nitrogen of each sample by a conversion factor of 6.38. Total moisture of WPI was determined by vacuum oven drying (AOAC, 2000; method number 990.20: 33.2.44), and total lipid concentration was determined by Mojonnier ether extraction (AOAC, 2000: method number 989.05; 33.2.26). Phosphorus, calcium, magnesium, potassium, sulfur, and sodium analysis was conducted by the North Carolina State University Analytical Services Laboratory (Raleigh, NC) using a standard dry ash method with inductively coupled plasma optical emission spectroscopy (Lloyd and others 2009). Both replications of each of the five WPI were measured in duplicate.

Preparation of WPI Samples

No Treatment (NT) Sample Preparation

Each WPI sample was rehydrated to 10% solids (w/v) in deionized (DI) water using a hand-held blender and the pH was recorded using the SevenEasy pH meter (Mettler Toledo, Inc., Columbus, OH).

Acidified (A) Sample Preparation

Rehydrated WPI samples at 10% solids (w/v) were acidified to pH 3.2 using a 1:1 2N mixture of citric acid (J.T. Baker, Mallinkrodt Baker, Phillipsburg, NJ) and phosphoric acid (Mallinkrodt Chemical, Phillipsburg, NJ). This combination of acids was used since commercial acidified protein beverages commonly use a combination of these two acids. Preliminary measurements were obtained to determine the volume of acid that was necessary

to lower the pH of each 10% WPI solution. This volume was then subtracted from the volume of water used to rehydrate the samples. Total percent solids was verified using the Smart System 5 moisture/solids analyzer with Smart Trac rapid fat analysis (CEM, Matthews, NC).

Heated (H) Sample Preparation

During preliminary testing, three out of the five WPI coagulated when heated. Therefore, the heated samples could not be prepared using a benchtop high temperature short time (HTST) coil. Samples were rehydrated to 10% solids (w/v) and blended with a hand-held immersion blender. Samples were heated in a 99.9°C digital shaker water bath (VWR apparatus, West Chester, PA) with an Arrow 1750 stirrer placed inside the sample (Arrow Engineering, Inc., Hillside, NJ) along with a thermometer (VWR apparatus, West Chester, PA). The water bath and sample were covered in foil to maintain the temperature. Once the samples reached a temperature of 85°C, the samples were held in the water bath for 5 min, and then removed to an ice bath. The samples were held in the ice bath, under constant stirring, until samples reached 4°C (ca 15 min). To validate the method in which WPI samples were heated at their natural pH, a crossover test was conducted. The sample with the highest pH (WPI 2) was acidified with the 1:1 2N solution of phosphoric acid and citric acid to the lowest pH of the samples (WPI 1). Conversely, the sample with the lowest pH (WPI 1) was raised to the highest pH of the samples (WPI 2) with 2N sodium hydroxide (EMD Chemicals, Gibbstown, NJ). The pH-modified samples were then processed and analyzed in the same manner as the other WPI samples.

Acidified and Heated (AH) Sample Preparation

Prior to heating, the samples were rehydrated and acidified as previously described (See Acidified (A) Samples Preparation). Samples were then heated by pumping using a peristaltic pump (Masterflex L/S, Cole Parmer, Vernon Hills, Il) through a benchtop HTST coil immersed in a 95°C digital water bath (VWR apparatus, West Chester, PA) with 1/8" Stainless Steel tubing (Cole Parmer, Vernon Hill, Il). The apparatus was similar to that described by Cole and Jones (1990). When samples reached a temperature of 85°C, they were held for 30 sec. The flow rate was 170 ml/min. Samples were then cooled in an ice bath with agitation until 4°C was reached (ca 15 min). Total percent solids was verified using the Smart System 5 moisture/solids analyzer with Smart Trac rapid fat analysis (CEM, Matthews, NC).

Descriptive Sensory Analysis

WPI samples (NT, A, H, AH) were dispensed into 3-digit coded soufflé cups (Solo Cup, Highland Park, Il) and lidded. Samples were held overnight at 4°C and brought to room temperature (22°C) prior to evaluation. Aromatics and basic tastes intensities were evaluated in duplicate by trained panelists (n = 10) using an established sensory language for dried dairy ingredients and a 0 to 15 point Spectrum^{TM} intensity scale (Wright and others 2009). Panelists were between the ages of 23 and 45 y and each had over 150 h of experience with descriptive analysis of dried dairy ingredients. Panelists expectorated samples and were provided with room temperature DI water for palate cleansing. Each sample was evaluated in duplicate by each panelist.

Solid Phase Microextraction: Gas Chromatography Olfactometry (SPME GC-O)

All WPI samples were subjected to SPME GC-O to evaluate the aroma active compounds and to document compounds that may be unique to an individual WPI or treatment. WPI samples, previously rehydrated to 10% solids (w/v) with the addition of 10% sodium chloride (w/v), were distributed in aliquots of 20 ml in to four 40 ml amber vials (28 × 98 mm, Supelco, Bellefonte, PA) with a PTFE/Silicone septum (Supelco, Bellefonte, PA) and a stir bar. The vials were heated at 40°C for 30 min under constant stirring. A SPME fiber (DVB/CAR/PDMS) (Supelco, Bellefonte, PA) was exposed in each sample at 2 cm for another 30 min. The fiber was then injected onto an Agilent 6850 gas chromatograph–flame ionization detector (GC-FID) equipped with an olfactometer port (Agilent Technologies, Santa Clara, CA) at 3 cm. The WPI samples were evaluated using two different columns: a polar ZB-WAX (30 –m length × 0.25-mm i.d. ×0.25-µm df) and a nonpolar ZB-5 ms (30 –m length × 0.25-mm i.d. ×0.25-µm df) (Phenomenex Zebron, Torrance, CA). The GC method was programmed with an initial temperature of 40°C for 3 min, then the temperature increased at a rate of 10°C/min until 150°C was reached, then the rate changed to 30°C/min to 200°C, which was then held for 5 min. Column effluent was split 1:1 between the FID and the sniffing port using deactivated fused silica capillaries (1 m length × 0.25-mm i.d.) (Phenomenex Zebron, Torrance, CA). The FID sniffing port held a temperature of 300°C with a helium carrier gas flow of 1018.6 cm s^{-1}, and the port was supplied with humidified air at 30 mL/min. Each WPI treatment was evaluated for aroma character, retention time, and maximum perceived aroma intensity (0 to 5 point scale) by two highly experienced sniffers

each with > 60 h of experience with GC-O of dairy products on each column (Van Ruth, 2001).

Solid Phase Microextraction: Gas Chromatography-Mass Spectrometry (SPME GC-MS) for Volatile Compound Analysis

All WPI samples were subjected to SPME GC-MS for extraction and identification of volatile compounds. The sample preparation and SPME GC-MS method were modified from the method used by Wright and others (2006). An aliquot (5 mL) of prepared samples were placed in 20 ml autosampler vials with steel screw tops containing silicon septa face in Teflon (Microliter Analytical, Suwanee, GA). Internal standard solution (10 µl of 2-methyl-3-heptanone in methanol at 81 ppm (Sigma)) and 10% (w/v) sodium chloride (Fischer Scientific) were added to the samples. Samples were injected using a CTC Analytics CombiPal autosampler (Leap Technologies, Carrboro, NC) attached to an Agilent 6890N GC (Agilent Technologies, Santa Clara, CA). Samples were held at 5°C prior to fiber exposure. Samples were equilibrated at 40°C for 25 min before exposure to a 3-phase fiber DVB/CAR/PDMS (Supelco, Bellefonte, PA) at 31 mm for 30 min, with 4 s pulsed agitation at 250 rpm. Fibers were injected for 5 min at a depth of 50 mm. The initial temperature was set at 40° for 3 min with a ramp rate of 10°/min until 90°C was reached, then increased at the rate of 5°C/min to 200°C, held for 10 min, and then finally increased at the rate of 20°C/min to 250°C and held for 5 min. SPME fibers were then introduced into the split/splitless injector at 250°C at a pressure of 7.06 psi with helium as the carrier gas with a purge flow of 169737 cm s^{-1}. A ZB-5 ms column (30-m length × 0.25-mm i.d. × 0.25 µm df) (Phenomenex Zebron) was used for all analyses with a constant flow rate of 34 cm s^{-1}. Purge time was set

at 1 min. The MS transfer line was maintained at 250°C with the quad at 150°C and the source at 250°C. All samples were analyzed in triplicate.

Compound Recovery Utilizing Single Ion Monitoring Mode (SIM)

Following SPME GC-O and initial SPME GC-MS, all WPI samples were re-evaluated by SPME GC-MS using the MS set in single ion monitoring (SIM) mode. The SIM mode allowed for more sensitive detection by focusing on the unique ion(s) at a certain retention time for a compound of interest while ignoring all other ions. Sensory results, GC-O, and initial SPME GC-MS results as well as previous studies (Carunchia Whetstine and others 2005; Wright and others 2006; Evans and others 2010; Leksrisompong and others 2010) were used to select compounds for analysis by SIM. Exact retention time on the GC-MS for each compound was determined by injection of authentic standards in scan and SIM mode or converted from retention indices (for compounds with no authentic standard commercially available). The unique ions for each compound were selected using spectra acquired from NIST Mass Spectral Search Program 2.0 and the authentic standards. The most abundant ion with *m/z* greater than 50 was then selected. The data was analyzed using MS ChemStation software (Agilent Technologies, Durham, NC). Relative abundance for each compound was calculated using the calculated recovery of the internal standard concentration to determine relative concentrations of each compound.

Identification of Odorants

Tentative identification of compounds were based on a comparison of odor properties, retention indices (RI), and/or mass spectra of the unknowns against authentic standards and/or based on literature evaluation. 2-octyl furan, a compound not commercially

available, was tentatively identified based on odor properties and retention index from the literature. RI were calculated with the use of an *n*- alkane series (Fluka, Buchs, Switzerland) (Van den Dool and Kratz 1963). For the identification of aroma active compounds, authentic standards were used to compare the odor properties, retention indices (RI) on both GC columns, and spectra of unknown compounds.

Quantification of Odorants

Preliminary analysis of the volatile composition of the WPI treatments suggested that the matrix pH was influencing the relative recovery of volatiles and hindering comparison of volatile compound relative abundance between treatments. Single point external standards of selected compounds at a concentration of 200 ppb were evaluated by SPME GC-MS in SIM mode in both dried egg white powder (EWP) (Michael Foods Inc, Minnetonka, MN) hydrated in HPLC grade water (EMD Chemicals Inc., Gibbstown, NJ) and EWP hydrated HPLC grade water acidified to pH 3.2 with 1:1 2 M citric acid and phosphoric acid (see Acidified (A) Sample Preparation). EWP is considered flavorless and has a similar composition to the WPI. Response factors (the area response on the GC-MS of a known concentration) relative to the internal standard (81 ppm of 2-methyl-3-heptanone in methanol) of these compounds were obtained and the concentration of each compound were calculated for all WPI treatments.

Volatile Free Fatty Acid Analysis

Volatile free fatty acids in rehydrated (NT) WPI were extracted and evaluated by SPME GC FID using a method modified from Tomaino and others (2004). WPI samples were rehydrated at 10% solids (w/v) in HPLC grade water. The pH of the samples were

adjusted to 2.0 using 3.3M hydrochloric acid (20% v/v, VWR). Aliquots of 5 grams of the pH adjusted samples were added to 20 ml autosampler vials with steel screw tops containing silicone septa faced in Teflon (Microliter Analytical). 100µl of an internal standard, heptadecanoic acid (1 ppm in ether (EMD Chemicals)), was added to the samples. The WPI samples were injected using a CombiPal autosampler (CTC Analytics) attached to a Varian CP-3380 gas chromatograph (Agilent Technologies). Samples were held at 10°C prior to fiber exposure. Samples were then brought to 110°C and held for 10 min and followed by a 40 min fiber exposure (22 mm vial penetration) of a 30µm polydimethylsiloxane fiber (PDMS) (Supelco) with 4 sec pulsed agitation at 250 rpm. Fibers were injected at a depth of 50 mm. WPI samples were run in triplicate. The initial temperature for the GC method of 100°C was held for 2 min with a ramp rate of 10°C.min to 245°C and the held for 13.5 min. The helium flow rate was 1ml/min and the SPME fibers were introduced in the split/splitless injector at 250°C. A ZB-FFAP column (30-m length × 0.25-mm i.d. × 0.25-µm df (Phenomenex Zebron)) was used for all analyses. Compounds were identified using authentic standards injected under identical conditions. Relative abundance for each free fatty acid was calculated based on the internal standard calculated recovery.

Statistical Analysis

Proximate analyses, sensory, and instrumental results were analyzed using XLSTAT statistical software (Addinsoft, New York, NY). Analysis of variance (ANOVA) with Fisher's least significant difference was conducted to test significance at $p \leq 0.05$ significance level. Principal component analysis was also conducted to determine how the

different treatments (no treatment, acidified, heated, and acidified and heated) were differentiated across sensory and instrumental measurements.

Results and Discussion

Proximate Analysis

There were compositional differences among the different WPI ($p \leq 0.05$; Table 1): protein content ranged from 90.3 and 95.2%, fat content between 0.2 – 0.7%, and ash between 2.0 – 2.9%. There was no statistical difference in moisture content (4.1 ± 0.67%) ($p>0.05$). Phosphate content ranged from 2808.8 – 649.6 ppm, calcium content between 6328.5 – 134.3 ppm, magnesium content between 1204.4 – 17.0 ppm, potassium content between 4509.3 – 241.3 ppm, sulfur content between 13843.1 – 11039.7 ppm, and sodium content between 9067.3 – 1373.9 ppm. The differences in proximate analysis of WPI were likely due to different processing parameters at each manufacturing facility (Carunchia Whetstine and others 2005) and all values were within expected ranges (Holt and others 1999).

Descriptive Analysis

WPI samples from different manufacturers varied in sensory properties initially and in all treatments (Tables 2-5). These results of initial flavor variability are consistent with previous research that also reported flavor variability in WPI from different manufacturers (Carunchia Whetstine and other 2005). Some flavors were associated with specific WPI. WPI 1 and 2 consistently had soapy flavor in all treatments. WPI 3 was higher in aroma intensity in the NT and A treatments. WPI 3 was also consistently higher in cardboard in the NT and A treatments and cabbage brothy flavors in all treatments except for in the H treatment. WPI

4 was consistently higher in potato/brothy flavor in the NT, H, and AH treatments. WPI 5 was the only sample with oatmeal flavor. Many of these flavors, such as cardboard and brothy flavors, are common in whey proteins (Russell and others 2006; Wright and others 2009; Evans and others 2010). There were also trends that were consistent in all treatments throughout all five WPI samples (Tables 6-10). Acidified samples were characterized by higher intensities of soapy and cardboard flavors, as well as higher astringency and sour taste. Heated samples were consistently higher in cardboard flavor and had flavors not present in rehydrated untreated samples, such as animal/tortilla and cooked/sulfur flavors. Acidified and heated samples were characterized by higher intensities of cardboard, potato brothy, and soapy flavors compared to the other WPI treatments. AH WPI were also astringent and sour and had the highest aroma intensity of the WPI treatments evaluated.

Principle component analysis biplots of NT vs. A, NT vs. H, and NT vs. AH WPI samples were generated to illustrate the effects of each treatment on the flavor of WPI (Figures 1 – 3). Arrows were included to show the trends of treatments across the PCA biplot. Acidification of WPI resulted in a general loss of sweet aromatic/oatmeal flavor, and increases in cardboard, malty, potato/brothy, cabbage/brothy, and animal/tortilla flavors (Figure 1). Heat treatment of the WPI samples resulted in lower intensities in sweet aromatic/oatmeal and hay flavors and increased cardboard, animal/tortilla, and cooked/sulfur flavors (Figure 2). The combination of heating and acidification of the WPI resulted in a reduction in sweet aromatic/oatmeal notes and higher intensities of cardboard, potato/brothy, and soapy flavors as well as astringency and sour taste (Figure 3). The crossover study samples (see Heated (H) Sample Preparation) were not significantly different from their

native pH sample counterparts (results not shown), indicating that the initial pH range of 6.02 – 6.84 did not influence the effect of heating or acidification.

Gas Chromatography – Olfactometry (GC-O)

Fifty-eight aroma-active compounds were detected by solid phase microextraction (SPME) GC-O of all WPI (Table 11). The compounds identified were aldehydes, ketones, esters, sulfur-containing compounds, lactones, and free fatty acids. Of these compounds, 32 were positively identified (detected by MS, RI, and odor compared to authentic standards), 25 compounds were tentatively identified (detected by RI and odor compared to authentic standard or RI and odor compared with published literature), and 1 compound remained unknown. Thirteen of the 58 aroma active compounds detected by SPME GC-O were present in all WPI. All of these compounds have previously been documented in dairy products (Majahan and others 2004; Carunchia Whetstine and others 2005; Gallardo-Escamila and others 2005; Wright and others 2006; Wright and others 2009; Leksrisompong and others 2010), except *o*-cresol.

A comparison of the perceived aroma intensity of the aldehydes and sulfur compounds found in the WPI allowed for further analysis of the WPI (Table 12). Four of the sulfur compounds: dimethyl sulfide, dimethyl disulfide, 2-methyl-3-furanthiol, and dimethyl tetrasulfide had the highest aroma intensity in at least three of the five heated (H) treated WPI, suggesting that heating results in release of sulfur compounds. The two main protein strands in whey, β-lactoglobulin and α-lactalbumin, denature when heated, releasing sulfur-containing amino acids to form volatile sulfur compounds (Linden and others 1999). Trained panel profiles also confirmed increased cooked/eggy/sulfur flavors upon heating of WPI; all

5 of the H WPI had the highest intensity of cooked/eggy/sulfur flavor compared to all the other treatments. Acidification also increased both sensory perception and volatile concentration of sulfur compounds. Four of the 5 acidified (A) WPI had higher aroma intensities of 2-methyl-3-furanthiol compared to the corresponding NT treatment. Four out of the five A WPI had the highest aroma intensity for dimethyl trisulfide. Six of the aldehydes: 3-methyl butanal, nonanal, *E,Z*-2,6-nonadienal, *E*-2-nonenal, *E,E*-2,4-nonadienal, and 2,4-decadienal, had the highest aroma intensity in at least three of the five acidified (A) WPI. Nonanal is a compound that contributes to cardboard flavor in whey (Whitson and others 2010). 4 out of the 5 A WPI had higher intensities in cardboard flavor compared to the corresponding NT WPI. 2- and 3-methyl butanal both contribute to potato/brothy flavors, and 3 out of the 5 A WPI had higher intensities of potato/brothy flavor compared to the corresponding NT WPI. The acidified and heated (AH) WPI had the highest intensities in the compounds 2-methyl butanal, heptanal, and 2-octenal. 3 out of the 5 AH WPI had the highest intensity of potato/brothy flavor compared to all other treatments. 4 out of the 5 AH WPI had higher cardboard flavor intensity compared to the corresponding NT WPI.

Instrumental Volatile Analysis

Tables 13-15 contain the concentrations (μg/kg) of the selected volatile compounds in each WPI (all treatments). Increases in certain volatile compounds were associated with higher intensities of flavors by descriptive analysis. Overall, the A WPI had higher intensities of cardboard flavor by descriptive analysis compared to the other treatments. The compounds heptanal, nonanal, 1-octen-3-one, and DMTS have been documented as components of cardboard flavor (Whitson and others 2010). In volatile analysis, 3 of the 5 A WPI had higher

concentrations of DMTS compared to other treatments. The H WPI had higher intensities of cooked/sulfur flavors; four out the five H WPI had significantly ($p \leq 0.05$) higher concentrations of DMS, and four of the five H WPI had significantly higher concentrations of DMTS compared to the respective NT treatments. The AH WPI had high intensities of cardboard and potato/brothy flavors. By volatile compound analysis, 3 out of the 5 AH WPI had higher concentrations of DMTS compared to the corresponding NT WPI. A number of compounds contribute to potato flavor, including 2- and 3-methyl butanal, decanal, phenylacetaldehyde, methoxy pyrazines, methional, DMDS, and DMTS (Jansky 2010). Four out of the five AH WPI had higher concentrations of both 2- and 3-methyl butanal compared to the corresponding NT WPI, which can contribute to potato/brothy flavors.

Each of the WPI from the different manufacturers also showed similar relationships between descriptive sensory analysis and instrumental volatile analysis. The A WPI 1 was highest in potato/brothy flavor compared to other WPI 1 treatments (Table 6). Benzaldehyde was significantly higher in concentration in the A WPI 1 compared to the NT treatment (Table 13). It may be synergistically increasing potato/brothy flavor with methional. The A WPI 1 also had higher concentrations of 2- and 3-methyl butanal compared to the NT treatment.

The H WPI 2 was significantly higher in cooked/sulfur flavor compared to all the other treatments, and it also had significantly higher DMDS concentrations compared to the NT treatment. The AH WPI 2 was higher in potato/brothy flavors and garbage/eggy/sulfur flavors compared to all the treatments. In volatile analysis, the AH WPI 2 was higher in 1-octen-3-one than the NT treatment, and this compound contributes to cardboard flavor. AH

WPI 2 had the highest concentrations of 2- and 3-methyl butanal and DMDS compared to all treatments. All these compounds also contribute to potato/brothy flavors.

The H WPI 3 was the only sample among the WPI3 treatment to have cooked/sulfur flavor (Table 8); in the volatile analysis, the H WPI 3 was higher in DMDS and DMTS compared to the NT treatment (Table 15). DMTS is responsible for cabbage off-flavors in WPI (Wright and others 2006). The AH WPI 4 was higher in malty flavor compared to all treatments (Table 9). AH WPI 4 was higher in both 2- and 3-methyl butanal compared to the NT treatment, which are responsible for malty flavors (Avsar and others 2004) (Table 16).

Both the A and AH treatments of WPI 5 were higher in potato/brothy flavors than the NT and H WPI 5, and AH WPI 5 was highest in cardboard flavor compared to all the other treatments (Table 10). Both the A and AH WPI 5 were significantly higher in methional than the other two treatments, and both A and AH WPI 5 were higher in heptanal concentration overall (Table 17). AH WPI 5 had the highest concentration of 3-methyl butanal. H WPI 5 was higher in cooked/sulfur flavors than all treatments, while in the GC-MS results the H WPI 5 was higher in DMS and DMTS than the NT treatment.

These changes in compound concentration and flavor in the different treatments may be because of conformational changes in the two main proteins in WPI, β-lactoglobulin and α-lactalbumin. Conformational changes in these proteins, in relation to changes in the surrounding environment, may expose residues which can then participate in reactions with other compounds to produce higher concentrations of volatiles and off-flavors in the post-processing treatments. Hunt and Dalgleish (1994) have reported that α-lactalbumin transforms from its native (N) state to an acidic (A) conformation or molten globular state at

pH 3. At neutral pH, α-lactalbumin contains Ca^{2+} in a high affinity binding site, however, as the pH lowers, the calcium becomes dissociated as the β-carboxyl groups become protonated and about half of the hydrophobic domains become exposed (Permyakov and others 1985; Hunt and Dalgleish 1994). As new groups are exposed, parts of the protein originally unavailable are now able to interact with other residues, lipids, and sugars to produce volatile compounds. Yasosky and others (1984) theorized that when histidine residues are ionized (pKa < 6.0) at a low pH, a protein's net charge and/or tertiary structure is altered and reduces the protein's ability to sequester catalytic ions, including Mn, Cu, Co, and Fe. The metal ions can then participate in lipid oxidation, as they are considered to be proxidants. The metal ions can activate oxygen and initiate lipid oxidation with the unsaturated fatty acids found in the WPI (Frankel 1998).

A heat treatment, as well as the combination of acidification and heat, can also denature whey proteins. Both β-lactoglobulin and α-lactalbumin are stabilized by intramolecular disulfide bonds in their native globular state, two and four bonds, respectively (Monahan and others 1995). The protein β-lactoglobulin also has a free sulfhydryl group. When the native whey protein structure is destroyed, the unfolding protein creates interactions between proteins, including thiol/thiol oxidation reactions and thiol/disulfide interchanges. Ledl and Schleicher (1990) reported that when heated, the cysteine residues in proteins release hydrogen sulfide, which can then react with Maillard browning products to create sulfur-containing products. Many of these compounds, some of which have been found to be important in meat flavor, have very low thresholds, including 2-methyl-2-thiazoline, 2-acetyl-2-thiazoline (Leod and others 1986), and dimethyl tetrasulfide (Barbieri

and others 1992). In heating, β-lactoglobulin forms sulfhydryl reducing compounds, which contribute to a cooked flavor in dairy products (Frankel 1998). These sulfhydryl reducing compounds may be responsible for the cooked/sulfur flavor found in the H WPI. β-lactoglobulin does not denature even at a pH of 2 (Casal and others 1988). However, the combination of low pH and thermal treatment can affect the native conformation of the globular protein. Beginning at 60°C, the α-helices and exposed β-sheets begin to unfold in the first stage of heat denaturation; the second stage begins around 90°C, in which the rest of the strands unfold (Casal and others 1988). When α-lactalbumin is heated at neutral pH, high temperatures are necessary for denaturation to occur in which the protein completely unfolds. However, at low pH, lower temperatures are needed, and the protein enters a more intermediate compact denatured state (the molten globule state). Between pH 3.5-3.0, between 80-100% of the protein immediately transforms into the compact intermediate state upon heat denaturation (Griko and others 1994).

Although all the WPI contained less than 1% fat, this small amount can still participate in lipid oxidation and produce undesirable off-flavors. Lipid degradation can be catalyzed by both acidic conditions and thermal treatment, although their pathways are different (Frankel 1998). The acid-catalyzed decomposition produces simpler products than thermal decomposition, mostly containing aldehydes. The A WPI contained higher concentrations of several aldehydes, including hexanal, heptanal, benzaldehyde, phenylacetaldehyde, nonanal, and *E,Z*-2,6-nonadienal compared to the NT treatment. The AH WPI also contained higher concentrations of aldehydes, including hexanal, heptanal, and *E,Z*-2,6-nonadienal compared to the NT treatment. Lipids can also interact with proteins to

create more compounds. Farmer and Mottram (1990) reported that a model system of ribose and cysteine in the presence of phospholipids and triacylglcerols produced multiple sulfur-containing volatile compounds, including 2-methyl-3-furanthiol. As the whey proteins become denatured and cysteine bonds are broken, new compounds can be formed from the now-exposed cysteine residues.

The low pH of the acidified WPI can also affect ionisable compounds, such as butyric acid. The pKa of butyric acid is 4.8, and when the pH is below 4.8, the compound is in its molecular form and is therefore more volatile (Spinnler 2003). Thus butyric acid may be detectable by sensory analysis in the A and AH WPI due to the decrease in pH. Other ionisable compounds, such as 2 and 3-methyl butyric acid, as well as other free fatty acids, which contribute to soapy flavors, may also be affected in the same manner.

Volatile Free Fatty Acid Analysis

The relative abundance of free fatty acids in WPI from different manufacturers was variable (Table 18). WPI 1 had greater ($p \leq 0.05$) levels of butyric (C4), dodecanoic (C12), tetradecanoic (C14), and hexdecanoic acid (C16) than the other WPI. WPI 5 had significantly higher levels of hexanoic (C6), octanoic (C8), octadecanoic (C18), oleic (C18:1), and linoleic acid (C18:2) as compared to the other WPI. WPI 4 also had higher levels of octanoic acid (C8) than the other WPI. There were no differences in concentrations of decanoic (C10) and linolenic acid (C18:3) among the WPI. Previous studies have shown that the shorter chain fatty acids (C4-8) have lower sensory thresholds than free fatty acids longer than C10, and therefore have more influence on WPI flavor (Karagul-Yuceer and others 2003). Butyric (C4) and hexanoic acid (C6) impart a cheesey/rancid aroma, octanoic (C8) an animal-like

and waxy aroma, while both decanoic (C10) and dodecanoic (C12) acid have soapy aromas. Carunchia and others (2005) reported that higher concentrations of octanoic (C8) and decanoic (C10) acid contributed to waxy/soapy flavors in WPI. Free fatty acids C10-C18:2 have been previously found in whey (Tomaino and others 2001). Milk or bacterial lipase reacting with triglycerides and phospholipids in the milk results in the formation of free fatty acids (Ferretti and Flanagan 1971; Kim and others 2003). Not only can the individual free fatty acids impart flavor, they are also available to participate in lipid oxidation. Previously, Carunchia Whetstine and others (2005) found no association between higher fat concentrations and higher intensities of flavors generated by lipid oxidation products, such as cardboards, doughy/fatty, and cucumber. However, additional processing (acidification and heating, or a combination of the two) of WPI with higher free fatty acids did have more lipid oxidation flavors and contained higher concentrations of lipid oxidation products. Two of the WPI (1 and 5), which had high levels of free fatty acids, had higher intensities of cardboard flavors when acidified and heated. When acidified, both WPI 1, 4, and 5 contained higher concentrations of 2- and 3- methyl butanal and *E*-2-decenal compared to the corresponding NT treatment. When acidified and heated, WPI 1, 4, and 5 had higher concentrations of 2 and 3-methyl butanal, benzaldehyde, and *E*-2-decenal (except WPI 1) compared to the corresponding NT treatment.

Conclusions

The post-processing treatments of acidification and heating negatively affect the flavor of WPI. Acidification increases concentrations of aldehydes and sulfur compounds while enhancing cardboard and soapy flavors. Heating the WPI increased sulfur compounds

and cooked/sulfur flavors. The combination of acidification and heating created higher concentrations of aldehydes and sulfur compounds and higher intensities of cardboard, potato/brothy, and soapy flavors, as well as higher overall aroma intensities and sour taste and astringency. These undesirable flavors can carry over into ingredient applications, presenting a challenge to manufacturers. Further research is necessary to discover alternative processing treatments or complementary ingredients in order to remove, lessen, or mask the off-flavors of the treated WPI.

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Tables and Figures

Table 1: Proximate Analysis of WPI from Different Manufacturers

	WPI				
Manufacturer	1	2	3	4	5
% Protein (dry weight basis)	90.3^{b}	95.2^{a}	92.8^{ab}	94.3^{a}	92.2^{ab}
% Fat	0.7^{a}	0.3^{ab}	0.2^{b}	0.3^{ab}	0.4^{ab}
% Moisture	4.5^{a}	3.9^{a}	4.0^{a}	3.2^{a}	5.0^{a}
% Ash	2.7^{ab}	2.0^{d}	2.9^{a}	2.4^{bc}	2.3^{c}
Phosphorous (mg/kg)	2350^{b}	649.3^{e}	2809^{a}	1518^{d}	2111^{c}
Calcium (mg/kg)	6329^{a}	903.5^{d}	5350^{b}	134.3^{e}	4405^{c}
Magnesium (mg/kg)	8344^{b}	119.6^{d}	1204^{a}	16.95^{d}	671.3^{c}
Potassium (mg/kg)	4476^{ab}	241.3^{d}	4272^{b}	1035^{c}	4509^{a}
Sulfur (mg/kg)	11040^{c}	13843^{a}	11505^{bc}	11619^{bc}	11674^{b}
Sodium (mg/kg)	1374^{e}	7092^{b}	2086^{c}	9067^{a}	1893^{d}

Statistical analysis with means separations using Fisher's (LSD) with 95% confidence interval.
Means in a row followed by a different letter are different ($p \leq 0.05$).
Each sample represents duplicate analyses of 2 separate lots from a manufacturer.

Table 2: Mean Descriptive Sensory Profiles of NT WPI from Different Manufacturers[1]

Sample ID	Aroma intensity	Cardboard	Potato/brothy	Cabbage/brothy	Soapy	Oatmeal	Hay	Bitter	Astringency
1NT	1.8^{ab}	1.4^{ab}	ND[2]	ND	2.3^{a}	ND	2.1	0.5^{d}	2.1^{a}
2NT	1.2^{b}	1.3^{b}	ND	ND	2.6^{a}	ND	ND	1.2^{a}	2.1^{a}
3NT	2.4^{a}	2.3^{a}	0.5^{a}	1.5	ND	ND	ND	ND	2.0^{a}
4NT	1.9^{ab}	1.6^{ab}	0.6^{a}	ND	1.9^{a}	ND	ND	1.1^{b}	2.0^{a}
5NT	1.4^{b}	1.6^{ab}	ND	ND	ND	1.1	ND	0.8^{c}	2.2^{a}

[1] Descriptive sensory profiles of no treatment (NT) WPI using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD). Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

Table 3: Mean Descriptive Sensory Profiles of A WPI from Different Manufacturers[1]

Sample ID	Aroma intensity	Cardboard	Potato/ brothy	Cabbage/ brothy	Soapy	Hay	Malty	Fatty	Animal/ tortilla	Sour	Bitter	Astringency
1A	2.8[a]	1.4[b]	ND[2]	ND	2.5[a]	2.1	ND	1.1	1.2[a]	4.5[a]	ND	4.4[a]
2A	2.6[a]	2.1[ab]	ND	ND	2.7[a]	ND	ND	ND	0.8[a]	4.5[a]	0.9[a]	4.4[a]
3A	3.0[a]	2.8[a]	1.1[b]	1.6	ND	ND	ND	ND	ND	4.5[a]	ND	4.5[a]
4A	2.7[a]	2.3[ab]	1.7[a]	ND	1.6[b]	ND	1.2	ND	ND	4.3[a]	0.9[a]	4.3[a]
5A	1.8[a]	1.9[ab]	1.9[a]	ND	1.3[b]	ND	ND	ND	ND	4.6[a]	1.1[a]	4.4[a]

[1] Descriptive sensory profiles of acidified (A) WPI using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD). Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

Table 4: Mean Descriptive Sensory Profiles of H WPI from Different Manufacturers[1]

Sample ID	Aroma intensity	Cardboard	Potato/ brothy	Soapy	Animal/ tortilla	Sweet aromatic	Cooked/ Sulfur	Bitter	Astringency
1H	3.3[ab]	2.1[a]	1.1[b]	1.3[a]	1.5[a]	ND	2.1[a]	0.5[ab]	2.8[b]
2H	4.6[a]	2.5[a]	ND[2]	1.3[a]	ND	ND	3.0[a]	1.0[a]	2.0[c]
3H	3.5[ab]	2.4[a]	ND	ND	ND	ND	2.1[a]	0.5[ab]	2.8[b]
4H	2.8[b]	2.4[a]	2.0[a]	0.7[b]	1.3[a]	ND	2.2[a]	0.8[ab]	2.0[c]
5H	2.6[b]	2.0[a]	ND	ND	0.6[ab]	0.5	2.1[a]	ND	4.8[a]

[1] Descriptive sensory profiles of heated (H) WPI using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD). Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

Table 5: Mean Descriptive Sensory Profiles of AH WPI from Different Manufacturers[1]

Sample ID	Aroma intensity	Cardboard	Potato/ brothy	Cabbage /brothy	Soapy	Hay	Garbage/eggy /sulfur	Malty	Sour	Bitter	Astringency
1AH	2.6[bc]	1.0[a]	ND[2]	ND	4.3[a]	1.5	ND	ND	4.8[a]	1.3[ab]	4.4[a]
2AH	2.1[c]	2.3[a]	1.3[bc]	ND	3.5[a]	ND	0.6[a]	ND	4.5[a]	1.2[ab]	4.6[a]
3AH	4.0[ab]	2.9[a]	1.0[bc]	2.1	1.5[b]	ND	1.7[a]	ND	4.5[a]	0.8[b]	4.7[a]
4AH	4.4[a]	3.4[a]	3.6[a]	ND	0.5[b]	ND	ND	2.3	4.7[a]	1.0[ab]	4.8[a]
5AH	3.8[abc]	3.1[a]	2.6[ab]	ND	1.3[b]	ND	ND	ND	4.8[a]	1.6[a]	4.7[a]

[1] Descriptive sensory profiles of acidified and heated (AH) WPI using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD). Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

Table 6: Mean Descriptive Sensory Profiles of WPI 1 (All Treatments)[1]

Sample ID	Aroma intensity	Cardboard	Potato/ brothy	Soapy	Hay	Fatty	Animal/ tortilla	Cooked/ sulfur	Sour	Astringency
1NT	1.8[a]	1.4[a]	ND[2]	2.3[b]	2.1[a]	ND	ND	ND	ND	2.1[b]
1A	2.8[a]	1.4[a]	ND	2.5[b]	2.1[a]	1.1	1.2[a]	ND	4.5[a]	4.4[a]
1H	3.3[a]	2.1[a]	1.1	1.3[b]	ND	ND	1.5[a]	2.1	ND	2.8[b]
1AH	2.6[a]	1.0[a]	ND	4.3[a]	1.5[a]	ND	ND	ND	4.8[a]	4.4[a]

[1] Descriptive sensory profiles of WPI 1 using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD) with 95% confidence interval. Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

Table 7: Mean Descriptive Sensory Profiles of WPI 2 (All Treatments)[1]

Sample ID	Aroma intensity	Cardboard	Potato/ brothy	Soapy	Animal/ Tortilla	Cooked /Sulfur	Garbage/eggy /sulfur	Sour	Bitter	Astringency
2NT	1.2[b]	1.3[a]	ND[2]	2.6[a]	ND	ND	ND	ND	1.2[a]	2.1[b]
2A	2.6[b]	2.1[a]	ND	2.7[a]	0.8	ND	ND	4.5[a]	0.9[a]	4.4[a]
2H	4.6[a]	2.5[a]	ND	1.3[b]	ND	3.0[a]	ND	ND	1.0[a]	2.0[b]
2AH	2.1[b]	2.3[a]	1.3	3.5[a]	ND	0.7[ab]	0.6	4.5[a]	1.2[a]	4.6[a]

[1] Descriptive sensory profiles of WPI 2 using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD) with 95% confidence interval. Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

Table 8: Mean Descriptive Sensory Profiles of WPI 3 (All Treatments)[1]

Sample ID	Aroma intensity	Cardboard	Potato/ brothy	Cabbage/ brothy	Soapy	Cooked/ Sulfur	Garbage/eggy /sulfur	Sour	Bitter	Astringency
3NT	2.4[b]	2.3[b]	0.5[a]	1.5[ab]	ND	ND	ND	ND	ND	2.0[b]
3A	3.0[ab]	2.8[ab]	1.1[a]	1.6[a]	ND	ND	ND	4.5[a]	ND	4.5[a]
3H	3.5[ab]	2.4[ab]	ND[2]	ND	ND	2.1	ND	ND	0.5[a]	2.8[b]
3AH	4.0[a]	2.9[a]	1.0[a]	2.1[a]	1.5	ND	1.7	4.5[a]	0.8[a]	4.7[a]

[1] Descriptive sensory profiles of WPI 3 using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD) with 95% confidence interval. Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

Table 9: Mean Descriptive Sensory Profiles of WPI 4 (All Treatments)[1]

Sample ID	Aroma intensity	Cardboard	Potato/ brothy	Soapy	Malty	Cooked /sulfur	Animal/ Tortilla	Sour	Bitter	Astringency
4NT	1.9[b]	1.6[b]	0.6[c]	1.9[a]	ND	ND	ND	ND	1.1[a]	ND[2]
4A	2.7[b]	2.3[b]	1.7[bc]	1.6[ab]	1.2[b]	ND	ND	4.3[a]	0.9[a]	4.3[a]
4H	2.8[b]	2.4[ab]	2.0[b]	0.7[ab]	ND	2.2	1.3	ND	0.8[a]	2.0[b]
4AH	4.4[a]	3.4[a]	3.6[a]	0.5[b]	2.3[a]	ND	ND	4.7[a]	1.0[a]	4.8[a]

[1] Descriptive sensory profiles of WPI 4 using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD) with 95% confidence interval. Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

Table 10: Mean Descriptive Profiles of WPI 5 (All Treatments)[1]

Sample ID	Aroma intensity	Cardboard	Potato/ brothy	Soapy	Cooked/ sulfur	Animal/ Tortilla	Oatmeal	Sour	Bitter	Astringency
5NT	1.4[b]	1.6[b]	ND[2]	ND	ND	ND	1.1	ND	0.8[a]	2.15[a]
5A	1.8[b]	1.9[ab]	1.9[ab]	1.3[a]	ND	ND	ND	4.6[a]	1.1[a]	4.4[a]
5H	2.5[ab]	2.1[ab]	0.9[ab]	ND	2.1	1.1	ND	ND	0.5[a]	3.5[a]
5AH	3.8[a]	3.1[a]	2.6[a]	1.3[a]	ND	ND	ND	4.8[a]	1.6[a]	4.7[a]

[1] Descriptive sensory profiles of WPI 5 using 0 – 15 Spectrum scale where 0 = absence of attribute and 15 = extremely high intensity. Statistical analysis with means separations using Fisher's (LSD) with 95% confidence interval. Means in a column followed by a different letter are different ($p \leq 0.05$). [2] ND: not detected.

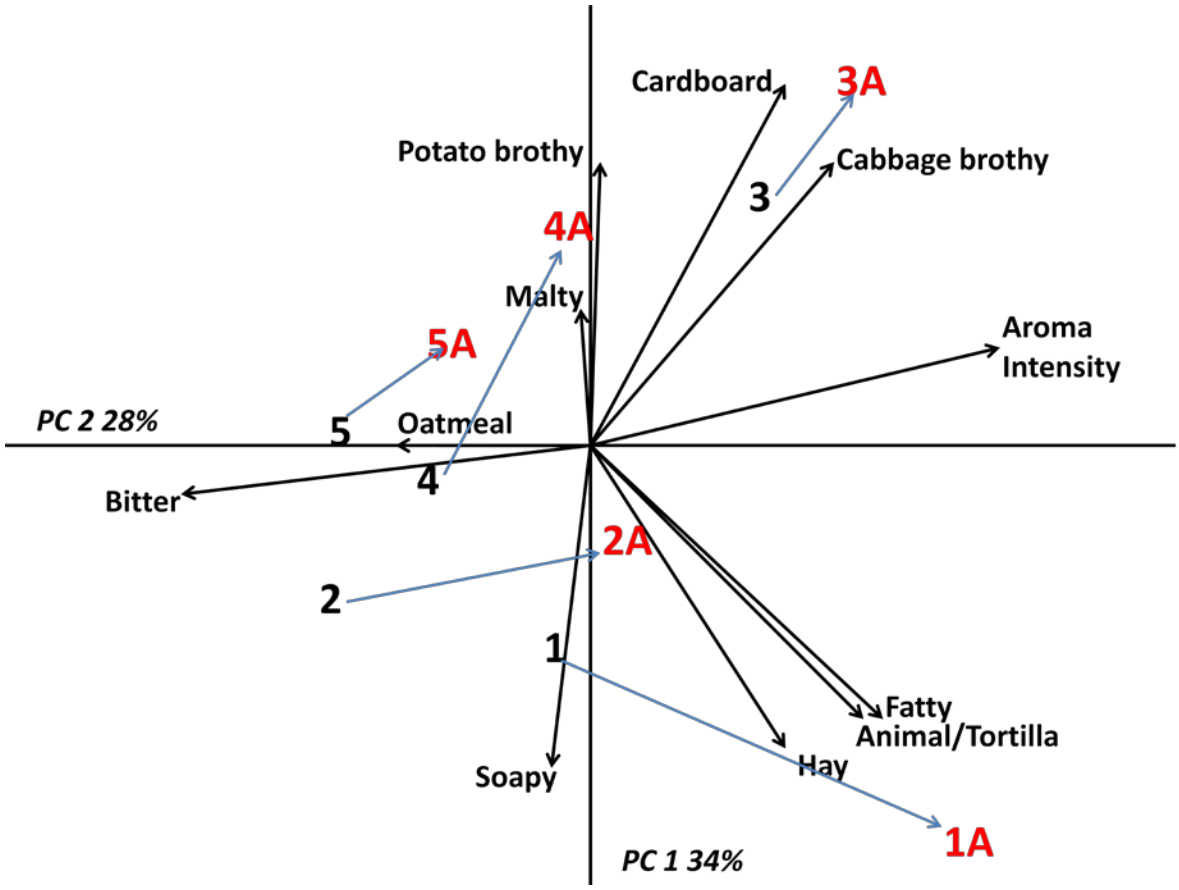

Figure 1: Principle component biplot of descriptive sensory analysis results of NT vs A WPI without astringency and sour taste. Trendlines on graph illustrate similar movement of samples. 1-5 = NT WPI from different manufacturers, 1A-5A = A WPI from different manufacturers. NT and A samples with the same number represent WPI from the same manufacturer.

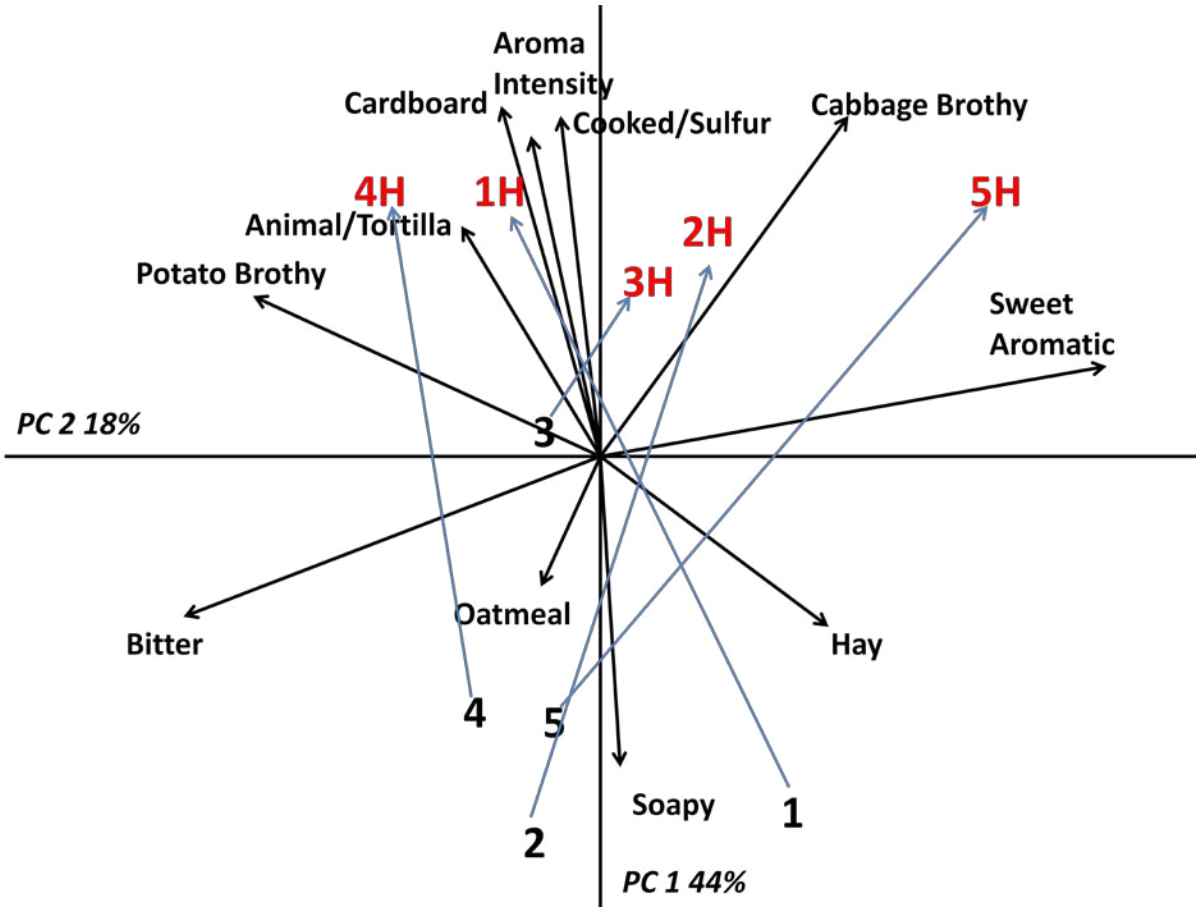

Figure 2: Principle component biplot of descriptive sensory analysis results of NT vs H WPI without astringency. Trendlines on graph illustrate similar movement of samples. 1-5 = NT WPI from different manufacturers, 1H-5H = H WPI from different manufacturers. NT and H samples with the same number represent WPI from the same manufacturer.

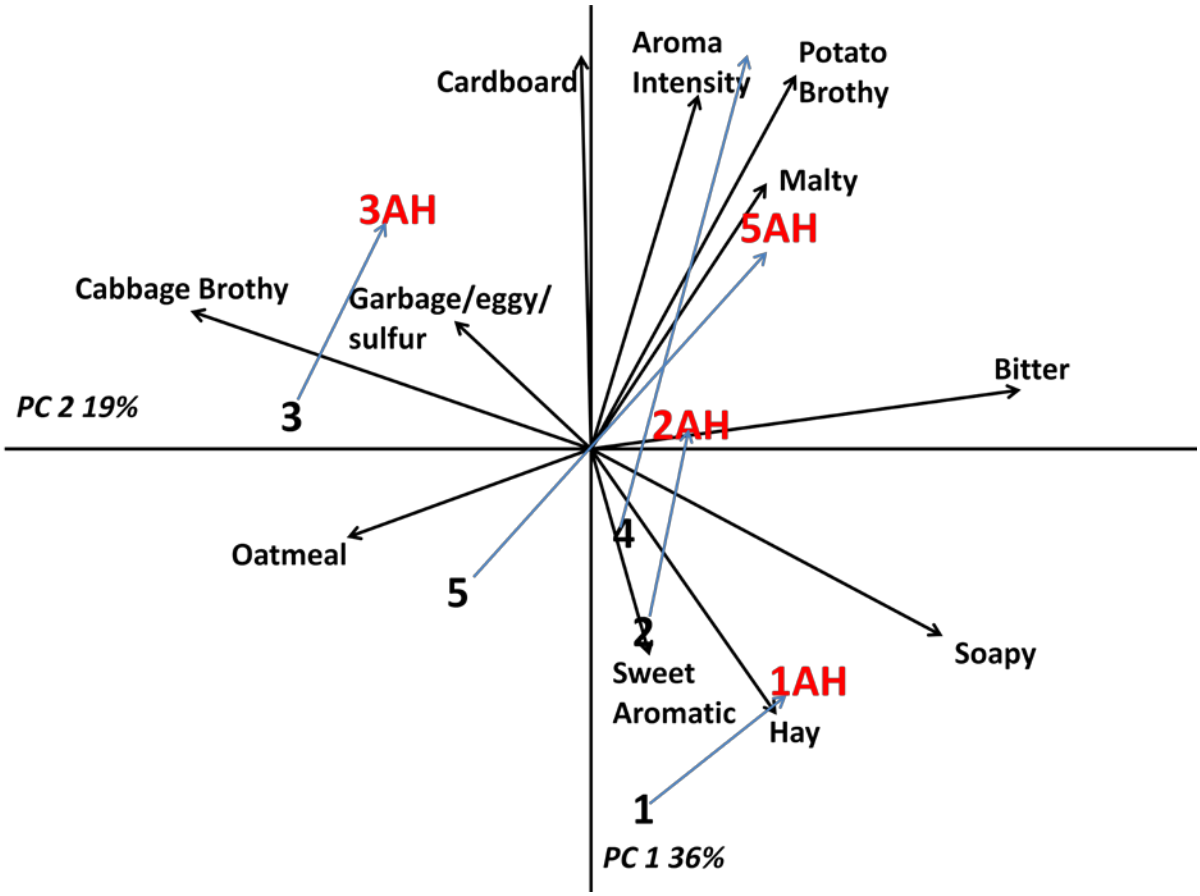

Figure 3: Principle component biplot of descriptive sensory analysis results of NT vs AH WPI without astringency and sour taste. Trendlines on graph illustrate similar movement of samples. 1-5 = NT WPI from different manufacturers, 1AH-5AH = AH WPI from different manufacturers. NT and AH samples with the same number represent WPI from the same manufacturer.

Table 11: Aroma-Active Compounds in all WPI from Different Manufacturers by SPME GC-O

Description	Possible Compound	RI (ZB5) [a]	RI (WAX) [b]	method of ID [c]	1 NT [d]	1 A	1 H	1 AH	2 NT	2 A	2 H	2 AH	3 NT	3 A	3 H	3 AH	4 NT	4 A	4 H	4 AH	5 NT	5 A	5 H	5 AH
sulfur	dimethyl sulfide	<600	<600	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
butter	diacetyl	<600	972	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ether	2-butanone	<600	<600	RI, O	+		+	+				+	+		+	+	+		+	+	+		+	+
sour, vinegar	acetic acid	617	1340	RI, O														+						
malt	3-methyl butanal	618	<600	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cocoa, almond	2-methyl butanal	621	910	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
fruity	ethyl propionate	664	948	RI, O, MS											+						+			
onion, cabbage	dimethyl disulfide	703	1073	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
rubber	1-hexen-3-one	754	1012	RI, O	+	+	+	+		+		+	+	+	+	+	+	+	+		+	+	+	+
cheesey, rancid	butyric acid	768	1611	RI, O		+	+	+				+		+				+	+					+
grass, tallow, fat	Hexanal	795	1082	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cheese/dried fruit	3-methylbutyric acid	843	1777	RI, O	+	+	+	+		+	+	+		+	+	+	+	+		+	+	+		+
fruity	isopropyl butanoate	843	1162	RI, O, MS			+			+		+	+		+	+		+		+			+	+
cheese/dried fruit	2-methylbutyric acid	847	1669	RI, O	+	+	+	+			+	+		+	+	+	+	+		+		+		+
meaty, sulfur	2-methyl-2-thiazoline	863	1326	RI, O		+	+	+		+	+	+									+	+	+	
cooked nutty	2-methyl-3-furanthiol	865	1317	RI, O	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
fatty, fishy	*Z*-4 heptenal	896	1191	RI, O	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+		+
fat, citrus, rancid	heptanal	900	1185	RI, O, MS		+	+	+		+		+			+	+	+			+				+
potato	methional	906	1468	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sweet popcorn	2-acetyl pyrroline	919	1343	RI, O	+		+		+	+	+	+	+	+	+	+	+		+		+	+	+	
garlic, cabbage	dimethyl trisulfide	966	1382	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 11 Continued

Description	Possible Compound	RI (ZB5) [a]	RI (WAX) [b]	method of ID[c]	1 NT [d]	1 A	1 H	1 AH	2 NT	2 A	2 H	2 AH	3 NT	3 A	3 H	3 AH	4 NT	4 A	4 H	4 AH	5 NT	5 A	5 H	5 AH
sweaty	hexanoic acid	1014	1826	RI, O						+				+			+	+				+		
popcorn	2-acetyl pyridine	1036	1649	RI, O, MS			+	+					+		+									
rosey/fatty/ honey	phenylacetaldehyde	1042	1647	RI, O, MS		+			+	+				+		+	+			+	+			
green, nut, fat	2-octenal	1046	1350	RI, O, MS								+	+			+				+				
caramel	furaneol	1050	1451	RI, O										+			+				+		+	
bell pepper	2-ethyl-3,5-dimethyl pyrazine	1074		RI, O				+			+					+			+	+				+
burnt, smokey	guaiacol	1081	1524	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sweet, waxy, soap	2-nonanone	1092	1382	RI, O, MS											+									
phenol, bandaid	p-cresol	1099		RI, O																	+		+	
toasted, nutty	2 acetyl thiazoline	1104	1737	RI, O										+			+		+			+		+
citrus, fatty, sweet	nonanal	1104	1376	RI, O, MS	+	+	+	+	+	+	+		+	+	+		+	+	+	+	+	+	+	+
curry, maple	sotolone	1107		RI, O	+	+	+	+		+	+		+	+	+		+	+	+		+	+	+	
rosey, fruity	2-phenethanol	1144	1926	RI, O, MS	+	+	+		+	+		+	+				+	+	+		+	+		+
fatty, doughy	z-2-nonenal	1144	1570	RI, O, MS	+	+	+	+		+	+	+	+	+		+	+	+	+	+	+	+	+	+
cucumbers	E,Z - 2,6-nonadienal	1153	1558	RI, O, MS	+	+	+	+		+	+	+		+	+	+	+	+		+	+	+	+	+
carpet/ cucumbers	E,2-nonenal	1156	1527	RI, O, MS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
bell pepper	2-isobutyl-3-methoxypyrazine	1164	1690	RI, O, MS		+		+		+	+	+		+		+		+						+
brothy/cook ed nutty	methyl-2-methyl-3-furyl disulfide	1173	1669	RI, O	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sweet/fatty/ citrus	ethyl octanoate	1175	1450	RI, O, MS				+			+					+		+	+					

Table 11 Continued

Description	Possible Compound	RI (ZB5) [a]	RI (WAX) [b]	method of ID[c]	1 NT [d]	1 A	1 H	1 AH	2 NT	2 A	2 H	2 AH	3 NT	3 A	3 H	3 AH	4 NT	4 A	4 H	4 AH	5 NT	5 A	5 H	5 AH
fatty, hay, sweet	E,E - 2,4-nonadienal	1215	1716	RI, O		+			+	+				+			+			+	+	+		
garlic, cabbage	dimethyl tetra sulfide	1215		RI, O			+		+		+	+										+	+	
minty, hay	2-octyl furan[e]	1237		RI, O	+	+	+	+	+															
hay	E-2-decenal	1254	1642	RI, O, MS	+		+	+	+				+				+							+
coconut	gamma-octalactone	1278	1933	RI, O, MS											+		+	+						
fatty, frying oil	2,4-decadienal	1310		RI, O		+			+	+	+		+	+			+	+			+	+	+	
grapy, tortilla	o-aminoacetophenone	1338	> 2000	RI, O		+	+			+	+										+			
cilantro/ waxy	γ nonalactone	1358		RI, O, MS			+		+	+			+				+	+						
mothball, fecal	Skatole	1429		RI, O							+													
peach	γ-decalactone	1484		RI, O													+				+			
coconut	δ-decalactone	1495		RI, O, MS														+						
fruity/floral	β-ionone	1498	1951	RI, O, MS	+			+	+				+			+					+			+
peach	γ-dodecalactone	1681		RI, O					+		+	+	+				+				+			

[a] Retention indices of the aroma event on the ZB-5 column. [b] Retention indices of the aroma event on the ZB-WAX column. [c] Method of identification by RI (retention indices), O (olfactometry), and MS (mass spectrometry) compared with authentic standards. [d] Sample identification. [e] Compound identified by RI and aroma from Greenberg and others (1981). (+) indicates the presence of the compound detected by two experienced sniffers, ( ) blank-indicates the absence of compound.

Table 12: Mean Aroma Intensities of Selected Aldehydes and Sulfur Compounds of All WPI by Different Manufacturers by SPME GC-O

Possible Compound	RI[a] (ZB5)	RI[b] (WAX)	Method of ID[c]	1NT	1A	1H	1AH	2NT	2A	2H	2AH	3NT	3A	3H	3AH	4NT	4A	4H	4AH	5NT	5A	5H	5AH
dimethyl sulfide	<600	<600	RI, O, MS	1.5	3	3	2	1.8	1.5	2	1.5	3	3	2.8	2	3	1.5	2	3	2.3	1	1.5	3
3 methyl butanal	618	<600	RI, O, MS	1.8	1	1.5	2	1	1	2	1.5	1	3	2.3	2.5	2.5	3	3	2.5	1.5	2.2	2	2
2 methyl butanal	621	910	RI, O, MS	1.5	2.2	2.5	2.5	2	2.5	3	2.5	2.7	2.3	2.5	3.5	3	3	3	3	2.2	2	1.5	2.5
Dimethyl Disulfide	703	1073	RI, O, MS	3	1	2.8	2.3	1.5	2.5	3.5	1.5	3	3.2	4	3.5	1.5	3	3.5	3.5	1.7	2.5	4	1
Hexanal	795	1082	RI, O, MS	2.3	1.5	2	1	3	3	2	1.5	2.5	2	1.5	2	2	2.2	2	3	1.9	2	2.3	2
2-methyl-2-thiazoline	863	1326	RI, O		1.5	1	1.5		1	1.8	2									2.5	3	1.2	
2-methyl-3-furanthiol	865	1317	RI, O	3	2.5	2.5	3	2.2	3	3.5	1	2.3	3.2	3.3	1.3	2.5	2.8	2.5	3	1.3	3	2.8	1.5
Z-4 Heptenal	896	1191	RI, O	2.5	1.7	2.5		1.5	3	2.3	2	2	1.5	2.5	1.5	2.7	2	2.5	2.5	2	1		2
Heptanal	900	1185	RI, O, MS		1.5	1.5	3		1.5		2				1	1.5			2				2
Methional	906	1468	RI, O, MS	2	3.5	2.5	2.5	1.5	1.5	2	1	1.5	3.2	2.8	2.5	2.2	3	3	3.5	1.8	3	3	3.5
Dimethyl Trisulfide	966	1382	RI, O, MS	3.5	1.5	2.8	1.5	1	2.5	1.5	2	3	3.5	3	3.5	3	3	3	3	3.2	4	2.5	3
Octanal	1001	1294	RI, O, MS	2	1.5	2.5	2.5	1.7	1.5	2	2	1.5	2	1.5	2	3	1.5	2	2.5	1.5	2	2.5	1
phenylacetaldehyde	1042	1647	RI, O, MS		1.5			1.5	1				1		1	1.5			2	2			
2-octenal	1046	1350	RI, O, MS								1	1			1.5				1				
2 acetyl thiazoline	1104	1737	RI, O										4			2					1.5		1.5
Nonanal	1104	1376	RI, O, MS	3.5	3	3	1.5	2	4	2.8		2	2	2		3.5	2.5	2.3	3.5	2	4	2	3
z-2-nonenal	1144	1570	RI, O, MS	1.5	3	3	2.5		3	2	2	3	1.8		2.5	2	2	1.5	3.6	1	2	1.7	3
E,Z - 2,6-Nonadienal	1153	1558	RI, O, MS	2.3	2.5	3	2.5		2	1.5	1.5		1.8	1.5	2	2.5	3		2	2	1.5	2	1.5
E,2-nonenal	1156	1527	RI, O, MS	2	2.5	2	1.8	2.3	2	2	2	2	3	2	3	3	3	2	3	1.5	3	1.5	3
methyl-2-methyl-3-furyl disulfide	1173	1669	RI, O	2.5	3	3	1.5	2.5	2	1.5	3	3.3	2.8	2	2.5	3	2	3	2	1.5	2	2.7	2
Decanal	1194	1498	RI, O, MS	1			2		3	2	2	2		1.5	2	2	2.5	2	2				
E,E - 2,4-Nonadienal	1215	1716	RI, O		2.3			1.7	2.5				2			2.3			2	1.5	2		

Table 12 Continued

Possible Compound	RI[a] (ZB5)	RI[b] (WAX)	Method of ID[c]	1NT	1A	1H	1AH	2NT	2A	2H	2AH	3NT	3A	3H	3AH	4NT	4A	4H	4AH	5NT	5A	5H	5AH
E-2-decenal	1254	1642	RI, O, MS	1		1.5	1.3	1			2.5	1				1.5					1.5		1
2,4-decadienal	1310		RI, O		2			1.5	2.5	2		1.5	1.5			2	1.5			1	2	2	

[a] Retention indices of the aroma event on the ZB-5 column. [b] Retention indices of the aroma event on the ZB-WAX column. [c] Method of identification by RI (retention indices), O (olfactometry), and MS (mass spectrometry) compared with authentic standards. [d] Sample identification. (#) indicates the average aroma intensity of the compound detected by two experienced sniffers, ( ) blank-indicates the absence of compound.

Table 1 3: Relative Abundance of Selected Volatile Compounds (in µg/kg) in WPI 1 (All Treatments) by SPME GC-MS[a]

Compound	1NT	1A	1H	1HC	1AH
DMS	0.346^{bc}	1.15^{a}	0.816^{ab}	ND	0.904^{a}
diacetyl	1.13^{ab}	4.43^{a}	3.50^{a}	ND	4.38^{a}
3 methyl butanal	ND	2.30^{b}	0.799^{c}	ND	3.38^{a}
2 methyl butanal	ND	4.89^{ab}	0.525^{b}	ND	11.1^{a}
ethyl propionate	0.860^{a}	0.948^{a}	ND	0.881^{a}	0.234^{b}
DMDS	4.28^{cd}	69.7^{a}	12.2^{c}	ND	30.9^{b}
hexanal	318^{a}	251^{a}	40.5^{b}	47.9^{b}	336^{a}
isopropyl butanoate	38.4^{a}	15.5^{b}	1.59^{b}	2.69^{b}	7.84^{b}
heptanal	329^{a}	187^{ab}	44.3^{b}	44.2^{b}	200^{a}
methional	24.1^{a}	40.1^{a}	45.2^{a}	25.2^{a}	37.7^{a}
benzaldehyde	30.5^{b}	64.7^{ab}	171^{a}	108^{ab}	84.0^{ab}
DMTS	0.760^{c}	8.79^{a}	4.08^{b}	ND	2.71^{b}
1-octen-3-one	3.01^{ab}	5.24^{a}	1.43^{b}	4.07^{ab}	3.31^{ab}
2-pental furan	65.0^{a}	47.3^{a}	12.3^{b}	13.1^{b}	51.2^{a}
octanal	48.4^{a}	36.9^{a}	30.5^{ab}	17.4^{b}	30.7^{ab}
2-acetyl pyridine	8.77^{a}	7.83^{ab}	3.67^{c}	2.46^{c}	5.23^{bc}
phenylacetaldehyde	4.29^{ab}	5.82^{a}	3.54^{bc}	1.90^{c}	4.63^{ab}
o-cresol	50.3^{a}	30.0^{bc}	19.4^{c}	17.6^{c}	35.7^{ab}
E-2-octenal	548^{a}	326^{bc}	203^{c}	183^{c}	362^{b}
2-nonanone	57.0^{a}	29.0^{b}	6.61^{c}	6.54^{c}	31.8^{b}
guaiacol	0.714^{a}	0.794^{a}	0.471^{ab}	0.277^{b}	0.552^{ab}
nonanal	824^{a}	628^{a}	295^{bc}	146^{c}	449^{bc}
2-phenethanol	1.42^{ab}	2.90^{a}	0.876^{bc}	0.543^{c}	1.63^{ab}
E,Z-2,6-nonadienal	1.94^{a}	1.98^{a}	1.01^{bc}	0.681^{c}	1.56^{ab}
E/Z-2-nonenal	12.8^{a}	13.3^{a}	2.39^{c}	1.38^{c}	7.27^{b}
2-isobutyl-3-methoxy pyrazine	0.468^{a}	0.499^{a}	0.169^{b}	0.130^{b}	0.507^{a}
ethyl octanoate	11.4^{a}	8.59^{a}	3.71^{b}	3.04^{b}	8.58^{a}
decanal	40.3^{a}	32.8^{a}	115^{a}	4.85^{a}	15.2^{a}
γ-octalactone	2.57^{a}	0.407^{b}	0.196^{b}	0.0971^{b}	0.254^{b}
E-2-decenal	9.50^{a}	11.1^{a}	0.787^{b}	0.897^{b}	7.12^{a}
γ-nonalactone	1.97^{a}	2.01^{a}	0.783^{b}	0.486^{b}	1.32^{ab}
2-dodecanone	78.4^{a}	58.9^{ab}	14.7^{b}	12.5^{b}	57.9^{ab}
β-ionone	1.04^{a}	0.478^{b}	0.167^{c}	0.218^{c}	0.577^{b}
δ-decalactone	1.30^{a}	0.651^{a}	1.30^{a}	0.449^{a}	0.881^{a}

[a] Mean concentration of selected compounds in WPI by SPME GC-MS using the SIM mode. Means in a row not followed by a common letter are statistically different ($p < 0.05$).

Table 14: Relative Abundance of Selected Volatile Compounds (in µg/kg) in WPI 2 (All Treatments) by SPME GC-MS[a]

Category	2NT	2A	2H	2HC	2AH
DMS	0.0700^{a}	0.280^{a}	0.258^{a}	ND	0.194^{a}
diacetyl	1.59^{c}	8.98^{ab}	5.54^{b}	ND	9.33^{a}
3 methyl butanal	ND	2.07^{ab}	1.69^{b}	ND	2.40^{a}
2 methyl butanal	ND	ND	6.73^{a}	ND	4.61^{b}
ethyl propionate	3.80^{a}	0.341^{b}	1.30^{b}	0.497^{b}	0.311^{b}
DMDS	0.311^{b}	2.22^{ab}	2.95^{a}	ND	4.24^{a}
hexanal	871^{a}	583^{b}	62.4^{c}	28.9^{c}	833^{ab}
isopropyl butanoate	1.27^{a}	0.632^{b}	0.466^{bc}	0.274^{c}	0.582^{bc}
heptanal	169^{a}	177^{a}	15.5^{b}	11.1^{b}	174^{a}
methional	25.4^{a}	25.2^{a}	22.9^{a}	24.5^{a}	34.8^{a}
benzaldehyde	37.3^{c}	138^{ab}	79.6^{bc}	80.5^{bc}	161^{a}
1-octen-3-one	2.78^{bc}	4.78^{ab}	1.18^{cd}	0.571^{d}	5.70^{a}
2-pental furan	387^{a}	240^{b}	11.4^{c}	13.3^{c}	317^{ab}
octanal	61.3^{a}	52.2^{a}	16.3^{b}	19.9^{b}	54.4^{a}
2-acetyl pyridine	28.8^{a}	9.93^{b}	2.55^{b}	2.97^{b}	15.6^{ab}
phenylacetaldehyde	2.36^{ab}	2.85^{a}	0.950^{c}	1.11^{bc}	2.40^{ab}
o-cresol	29.4^{a}	24.9^{ab}	13.1^{c}	13.8^{c}	20.3^{bc}
E-2-octenal	287^{a}	173^{b}	118^{b}	120^{b}	194^{ab}
2-nonanone	106^{a}	76.4^{a}	3.89^{b}	5.88^{b}	80.7^{a}
guaiacol	2.58^{a}	2.26^{a}	0.248^{a}	0.392^{b}	1.91^{a}
nonanal	440^{a}	376^{a}	49.3^{b}	78.5^{b}	386^{a}
2-phenethanol	4.64^{a}	1.85^{bc}	0.592^{c}	0.782^{c}	2.85^{ab}
E,Z-2,6-nonadienal	13.6^{a}	1.72^{b}	0.943^{b}	1.20^{b}	5.87^{ab}
E/Z-2-nonenal	34.7^{a}	16.6^{ab}	1.89^{b}	2.28^{b}	25.5^{a}
2-isobutyl-3-methoxy pyrazine	1.66^{a}	0.545^{b}	0.0525^{b}	0.187^{b}	0.884^{ab}
ethyl octanoate	42.6^{a}	15.4^{bc}	2.01^{c}	3.06^{c}	21.6^{b}
decanal	17.8^{ab}	8.88^{bc}	9.27^{bc}	21.1^{a}	4.97^{c}
γ-octalactone	61.5^{a}	20.3^{ab}	1.38^{b}	2.31^{b}	35.1^{ab}
E-2-decenal	27.1^{b}	266^{a}	0.717^{b}	1.18^{b}	308^{a}
γ-nonalactone	5.75^{a}	6.14^{a}	0.494^{b}	0.394^{b}	4.99^{a}
2-dodecanone	11.4^{c}	26.4^{a}	1.06^{d}	1.53^{d}	18.7^{b}
β-ionone	0.409^{a}	0.311^{b}	0.0070^{c}	0.0565^{c}	0.260^{b}
δ-decalactone	17.3^{a}	9.29^{b}	0.486^{c}	0.682^{c}	9.08^{b}

[a] Mean concentration of selected compounds in WPI by SPME GC-MS using the SIM mode. Means in a row not followed by a common letter are statistically different ($p < 0.05$).

Table 15: Relative Abundance of Selected Volatile Compounds (in µg/kg) in WPI 3 (All Treatments) by SPME GC-MS[a]

Compound	3NT	3A	3H	3AH
DMS	0.124^{b}	10.4^{ab}	1.32^{ab}	1.54^{a}
diacetyl	0.586^{c}	15.2^{ab}	8.37^{b}	18.2^{a}
3 methyl butanal	0.0928^{b}	6.16^{a}	0.893^{b}	5.65^{a}
2 methyl butanal	ND	11.3^{a}	2.97^{b}	9.20^{a}
ethyl propionate	1.81^{b}	1.50^{b}	7.76^{a}	0.476^{b}
DMDS	0.596^{c}	184^{a}	39.4^{b}	71.3^{b}
hexanal	463^{ab}	363^{b}	179^{c}	511^{a}
isopropyl butanoate	1.52^{a}	0.807^{b}	0.384^{c}	0.652^{bc}
heptanal	74.2^{a}	62.3^{a}	28.9^{b}	60.3^{a}
methional	18.6^{b}	32.3^{a}	20.5^{b}	32.4^{a}
benzaldehyde	75.3^{a}	198^{a}	164^{a}	141^{a}
DMTS	1.30^{c}	79.5^{a}	67.1^{a}	25.3^{b}
1-octen-3-one	2.89^{a}	1.89^{c}	2.49^{ab}	2.29^{bc}
2-pental furan	338^{a}	182^{b}	48.0^{c}	191^{b}
octanal	32.0^{a}	31.1^{a}	18.3^{b}	18.9^{b}
2-acetyl pyridine	20.1^{a}	10.2^{b}	2.28^{c}	7.97^{bc}
phenylacetaldehyde	2.89^{ab}	3.87^{a}	1.64^{c}	2.51^{bc}
o-cresol	77.3^{a}	49.8^{b}	13.7^{c}	52.1^{b}
E-2-octenal	853^{a}	562^{b}	130^{c}	568^{b}
2-nonanone	100^{a}	55.2^{b}	5.81^{c}	43.6^{b}
guaiacol	0.639^{b}	0.483^{b}	0.259^{b}	1.55^{a}
nonanal	164^{a}	176^{a}	63.2^{b}	85.5^{b}
2-phenethanol	2.04^{a}	1.39^{b}	0.656^{c}	1.06b^{c}
E,Z-2,6-nonadienal	2.88^{a}	1.95^{ab}	0.877^{b}	1.47^{b}
E/Z-2-nonenal	18.3^{a}	7.17^{b}	1.48^{b}	6.95^{b}
2-isobutyl-3-methoxy pyrazine	0.548^{a}	0.370^{b}	0.129^{c}	0.409^{b}
ethyl octanoate	15.8^{a}	8.26^{b}	1.47^{c}	6.42^{b}
decanal	10.7^{a}	10.5^{a}	7.38^{ab}	2.25^{b}
γ-octalactone	10.3^{a}	2.10^{b}	0.678^{b}	3.12^{b}
E-2-decenal	7.82^{a}	4.63^{b}	0.461^{d}	2.19^{c}
γ-nonalactone	3.85^{a}	1.96^{b}	0.528^{c}	1.88^{b}
2-dodecanone	3.21^{ab}	3.27^{a}	0.379^{c}	2.24^{b}
β-ionone	1.48^{a}	0.580^{b}	0.134^{c}	0.524^{b}
δ-decalactone	1.24^{a}	0.878^{b}	0.172^{c}	0.472^{c}

[a] Mean concentration of selected compounds in WPI by SPME GC-MS using the SIM mode. Means in a row not followed by a common letter are statistically different ($p < 0.05$).

Table 16: Relative Abundance of Selected Volatile Compounds (in µg/kg) in WPI 4 (All Treatments) by SPME GC-MS[a]

Compound	4NT	4A	4H	4AH
DMS	0.0205^{b}	0.0815^{ab}	0.0893^{a}	0.0864^{ab}
diacetyl	2.37^{c}	22.1^{ab}	15.3^{b}	28.8^{a}
3 methyl butanal	1.64^{c}	26.6^{a}	12.3^{b}	26.4^{a}
2 methyl butanal	1.68^{c}	25.6^{a}	11.0^{b}	22.0^{a}
ethyl propionate	0.924^{b}	1.08^{ab}	0.436^{b}	2.03^{a}
DMDS	0.663^{b}	39.5^{ab}	59.9^{a}	23.7ab
hexanal	1457^{a}	1125^{a}	107^{b}	1297^{a}
isopropyl butanoate	2.26^{a}	1.23^{b}	0.382^{c}	0.721^{c}
heptanal	256^{a}	188^{ab}	16.9^{c}	175^{b}
methional	35.8^{a}	36.6^{a}	15.5^{c}	30.6^{b}
benzaldehyde	123^{c}	236^{b}	83.6^{c}	373^{a}
DMTS	3.02^{b}	7.38^{a}	9.05^{a}	3.32^{b}
1-octen-3-one	7.28^{a}	3.96^{c}	6.03^{b}	4.92^{bc}
2-pental furan	1951^{a}	1197^{b}	58.7^{c}	874^{b}
octanal	84.3^{a}	65.8^{ab}	20.8^{c}	51.0^{b}
2-acetyl pyridine	8.12^{a}	6.38^{b}	2.52^{d}	4.00^{c}
phenylacetaldehyde	13.4^{b}	18.36^{a}	3.22^{c}	15.4^{b}
o-cresol	59.1^{a}	37.4^{b}	11.5^{c}	37.7^{b}
E-2-octenal	649^{a}	402^{b}	101^{c}	389^{b}
2-nonanone	539^{a}	368^{b}	14.7^{c}	284^{b}
guaiacol	1.77^{a}	1.69^{a}	0.412^{b}	1.26^{a}
nonanal	351^{a}	329^{a}	53.7^{c}	23.4^{b}
2-phenethanol	1.13^{a}	0.877^{ab}	0.608^{b}	0.831^{ab}
E,Z-2,6-nonadienal	1.03^{a}	1.05^{a}	0.551^{b}	1.21^{a}
E/Z-2-nonenal	9.96^{a}	6.23^{b}	1.02^{c}	6.52^{b}
2-isobutyl-3-methoxy pyrazine	0.775^{a}	0.644^{ab}	0.135^{c}	0.591^{b}
ethyl octanoate	8.65^{a}	4.11^{bc}	0.845^{c}	5.84^{ab}
decanal	4.75^{a}	6.65^{a}	6.55^{a}	4.24^{a}
γ-octalactone	3.61^{a}	2.01^{ab}	0.0940^{b}	0.370^{b}
E-2-decenal	2.60^{b}	7.87^{a}	0.136^{c}	6.72^{a}
γ-nonalactone	2.31^{a}	1.70^{b}	0.189^{d}	1.09^{c}
2-dodecanone	5.92^{b}	7.78^{a}	0.520^{c}	5.60^{b}
β-ionone	2.14^{a}	1.19^{b}	0.114^{d}	0.744^{c}
δ-decalactone	3.89^{a}	2.78^{b}	0.148^{d}	1.51^{c}

[a] Mean concentration of selected compounds in WPI by SPME GC-MS using the SIM mode. Means in a row not followed by a common letter are statistically different ($p < 0.05$).

Table 17: Relative Abundance of Selected Volatile Compounds (in µg/kg) WPI 5 (All Treatments) by SPME GC-MS[a]

Compound	5NT	5A	5H	5AH
DMS	0.0209^{b}	1.24^{ab}	1.63^{a}	1.27^{a}
diacetyl	1.54^{a}	12.9^{a}	9.59^{a}	12.8^{a}
3 methyl butanal	ND	4.09^{b}	1.76^{c}	5.80^{a}
2 methyl butanal	ND	9.17^{a}	5.72^{b}	7.62^{ab}
ethyl propionate	111^{a}	43.4^{b}	6.26^{b}	28.1^{b}
DMDS	0.0751^{c}	12.4^{a}	3.45^{bc}	4.70^{b}
hexanal	228^{bc}	500^{ab}	88.4^{c}	607^{a}
isopropyl butanoate	4.49^{a}	2.62^{b}	0.365^{d}	1.44^{c}
heptanal	84.6^{ab}	144^{a}	29.0^{b}	144^{a}
methional	14.6^{b}	34.2^{a}	17.1^{b}	30.0^{a}
benzaldehyde	17.5^{c}	179^{ab}	117^{b}	262^{a}
DMTS	ND	13.2^{a}	6.18^{ab}	4.47^{ab}
1-octen-3-one	7.67^{a}	5.15^{a}	1.51^{b}	7.22^{a}
2-pental furan	155^{ab}	193^{a}	23.7^{b}	220^{a}
octanal	22.4^{a}	25.3^{a}	17.1^{a}	25.9^{a}
2-acetyl pyridine	10.8^{a}	8.98^{a}	2.07^{c}	5.84^{b}
phenylacetaldehyde	5.92^{ab}	6.73^{a}	1.66^{c}	4.53^{b}
o-cresol	21.9^{a}	27.8^{a}	4.87^{b}	30.2^{a}
E-2-octenal	2.59^{ab}	5.71^{a}	0.116^{b}	4.81^{a}
2-nonanone	64.3^{a}	48.8^{ab}	5.37^{c}	39.5^{b}
guaiacol	1.14^{a}	0.922^{ab}	0.208^{c}	0.565^{bc}
nonanal	212^{a}	192^{ab}	95.0^{b}	148^{ab}
2-phenethanol	1.66^{a}	1.06^{ab}	0.620^{b}	0.918^{ab}
E,Z-2,6-nonadienal	1.08^{a}	1.17^{a}	0.376^{b}	1.47^{a}
E/Z-2-nonenal	8.08^{a}	5.57^{b}	1.22^{c}	4.94^{b}
2-isobutyl-3-methoxy pyrazine	0.688^{a}	0.573^{a}	0.294^{b}	0.606^{a}
ethyl octanoate	4.19^{ab}	5.31^{a}	0.855^{b}	4.40^{ab}
decanal	7.78^{a}	5.47^{a}	6.22^{a}	2.61^{a}
γ-octalactone	0.280^{b}	0.960^{a}	0.0730^{b}	0.211^{b}
E-2-decenal	2.59a^{b}	5.71^{a}	0.116^{b}	4.81^{a}
γ-nonalactone	0.565^{c}	2.30^{a}	0.176^{c}	1.37^{b}
2-dodecanone	2.01^{a}	1.70^{a}	0.282^{c}	1.23^{b}
β-ionone	0.954^{a}	0.429^{b}	0.102^{c}	0.374^{bc}
δ-decalactone	0.373^{a}	0.391^{a}	0.0446^{c}	0.208^{b}

[a] Mean concentration of selected compounds in WPI by SPME GC-MS using the SIM mode. Means in a row not followed by a common letter are statistically different ($p < 0.05$).

Table 18: Relative Abundance (mg/kg) of Volatile Free Fatty Acids in WPI from Different Manufacturers

Manufacturer	C4	C6	C8	C10	C12	C14	C16	C18	C18:1	C18:2	C18:3
1	0.742^{a}	0.0339^{ab}	0.0697^{ab}	0.361^{a}	2.11^{a}	9.31^{a}	1.45^{a}	1.14^{ab}	4.27^{ab}	0.220^{b}	0.0982^{a}
2	0.303^{c}	0.0205^{b}	0.0131^{c}	0.376^{a}	0.582^{c}	0.841^{d}	2.89^{c}	0.711^{b}	1.80^{c}	0.332^{b}	0.0319^{a}
3	0.336^{bc}	0.0204^{b}	0.0307^{bc}	0.152^{a}	0.870^{bc}	3.94^{bc}	7.63^{b}	0.897^{ab}	2.48^{bc}	0.468^{b}	0.0399^{a}
4	0.617^{ab}	0.0232^{ab}	0.0909^{a}	0.164^{a}	0.723^{bc}	3.16^{c}	3.93^{c}	0.633^{b}	1.31^{c}	0.316^{b}	0.0160^{a}
5	0.622^{ab}	0.0458^{a}	0.0824^{a}	0.345^{a}	1.15^{b}	5.10^{b}	8.69^{b}	1.43^{a}	4.70^{a}	2.09^{a}	0.374^{a}

Statistical analysis with means separations using Fisher's (LSD) with 95% confidence interval.
Means in a column followed by a different letter are different ($p \leq 0.05$).