EFFECTS OF MILK-FLAVORING CONSTITUENTS ON THE FLUOROMETRIC ASSAY OF BOVINE ALKALINE PHOSPHATASE

By

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ABSTRACT

EFFECTS OF MILK-FLAVORING CONSTITUENTS ON THE FLUOROMETRIC ASSAY OF BOVINE ALKALINE PHOSPHATASE

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Determining the activity of residual alkaline phosphatase (ALP, EC 3.1.3.1) in milk validates adequate pasteurization and confirms that cross-contamination of raw milk has not occurred, thus ensuring product safety. However, flavored milks have been shown to yield false positive or false negative ALP activities, which inaccurately represent the microbiological safety of the milk sample. The objectives of this study were to determine the effects of 1) homogenization, 2) pasteurization conditions, and 3) ingredient inclusion on residual ALP activity using the fluorometric assay for analyses. Preliminary work determined no significant differences ($P > 0.05$) in ALP activity as a function of dual-stage homogenization (1,500 and 500 psi) or differing pasteurization methods including high temperature, short time (HTST, 72°C for 15 s) and low temperature, long time (LTLT, 63°C for 30 min). Pure vanilla extract, cocoa powder, or sucrose was added to standardized 3.25% fat raw milk at 0.1, 1.5, and 8%, respectively. A control (no ingredients) was included as the fourth group. All four treatments were pasteurized at HTST conditions. ALP activity did not significantly differ among treatment groups ($P > 0.05$) and was adequately inactivated (< 350 mU/L). However, the treatment containing cocoa powder had the highest ALP activity at 106.70 mU/L in comparison to the control at 19.32 mU/L, suggesting the primary polyphenols of the ingredient are inducing a conformational change of the enzyme or interacting with the quantified fluorescence produced. With flavored milk sales rising in recent years, ensuring product safety and understanding potential interactions are imperative in the dairy industry.

This thesis is dedicated to my parents, Ann and Chris Brock. Thank you for your love and support.

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KEY TO ABBREVIATIONS

ALP	Alkaline Phosphatase
LTLT	Low Temperature, Long Time
HTST	High Temperature, Short Time
MFGM	Milkfat Globule Membrane
PMO	Pasteurized Milk Ordinance
FDA	Food and Drug Administration
CDC	Centers for Disease Control

CHAPTER 1

INTRODUCTION

1.1 Background

From novel ice cream treats to artisanal cheeses, milk boasts a wide variety of applications and is consumed in a myriad of forms. Milk, per the United States Food and Drug Administration (FDA), "is the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows. Milk that is in final package form for beverage use shall have been pasteurized or ultrapasteurized, and shall contain not less than 8 ¼ percent milk solids not fat and not less than 3 ¼ percent milkfat. Milk may have been adjusted by separating part of the milkfat therefrom, or by adding thereto cream, concentrated milk, dry whole milk, skim milk, concentrated skim milk, or nonfat dry milk. Milk may be homogenized" (FDA 2015a). The majority of dairy products consumed in the United States are derived from female bovine. In the United States, consumption of dairy products recently increased to roughly 614 lbs per person per year, evidencing the shear demand for milk production (USDA 2016).

Aside from its versatility and unique functionality, milk is also consumed for its health properties. Milk provides the consumer with a variety of micro and macronutrients, which have been shown to increase bone health, reduce risk of cardiovascular disease, lower blood pressure, and reduce the risk of hypertension (Rice and others 2013). Many consumers perceive milk to be a fresher, more affordable, and more nutritious compared to other popular beverage counterparts, such as sweetened non-dairy alternatives (Mintel 2016). These consumer perceptions can be deemed true based on the unique and wholesome nutritional composition of milk. Bovine milk is protein-rich and contains a variety of vitamins and minerals (Claeys and

others 2002). Milk is also a complete protein, meaning it provides all nine essential amino acids in quantities meeting recommended intake levels (Rice and others 2013). Milk contains, on average, 3% protein, 3% fat, and nearly 5% lactose, 87% water, with the remaining 2% being minerals (Linn 1988). **Table 1.1.1** contains the average composition of various dairy products. The complex biochemical matrix comprising milk products is quite functional and allows milk applications to be extensive in the food industry.

Fluid milk is the basis for many other frequently consumed dairy products, as it is used in the production of cheese, yogurt, or simply added as a functional ingredient in a recipe or addition to a beverage. It is imperative for milk producers to ensure the safety of fluid milk. Fluid milk, if consumed without heat treatment, could contain pathogenic organisms detrimental to one's health. To eliminate the pathogens in raw milk, such as *Coxiella burnetii*, *Salmonella* spp, *Listeria monocytogenes*, and *Campylobacter jejuni*, milk is heated to at least 72°C (161°F) for 15 seconds (IDFA 2016). This heat treatment is referred to as high temperature, short time pasteurization, or HTST. Fluid milk can also be subjected to a lower temperature for a longer time interval (LTLT) at 63°C (145°F) for 30 minutes, or a higher temperature for shorter time (UP) at 138°C (280°F) for 2 seconds. However, HTST pasteurization is more common in the dairy industry and all fluid milk available for commercial retail is legally required to be pasteurized, with some exceptions (IDFA 2016). **Table 1.1.2** shows the more common methods of pasteurization. The Center for Disease Control in the United States has confirmed over 1,000 illnesses from raw milk consumption since 2007, evidencing the need for strict regulations regarding pasteurization (CDC 2016).

Table 1.1.1 Average nutritional properties of various dairy products adapted from Parsons and others (1992).

	Moisture	**Protein**	**Total Fat**	**Total Carbohydrate**	**Total Ash**	**Calcium**	**Phosphorous**	**Sodium**
Whole Milk	88	3.3	3.3	4.7	0.7	0.12	0.09	0.05
Skim Milk	90.8	3.4	0.2	4.9	0.8	0.12	0.10	0.05
Half and Half	80.6	3.0	11.5	4.3	0.7	0.10	0.09	0.05
Yogurt (Plain)	87.9	3.5	3.3	4.7	0.7	0.12	0.10	0.05
Dried Sweet Whey	3.2	12.9	1.1	74.5	8.3	0.80	0.93	1.08
Cheddar Cheese	36.7	24.9	33.1	1.3	3.9	0.72	0.51	0.62
Butter	15.9	0.9	81.1	0.1	2.1	-	-	-

Table 1.1.2. Various milk pasteurization conditions (IDFA 2016).

	Temperature	**Time**
Low Temperature, Long Time (LTLT)	63°C (145°F)	30 minutes
High Temperature, Short Time (HTST)	72°C (161°F)	15 seconds
Higher Heat, Shorter Time (HHST)	89°C (191°F)	1.0 seconds
	90°C (194°F)	0.5 seconds
	94°C (201°F)	0.1 seconds
	96°C (204°F)	0.05 seconds
	100°C (212°F)	0.01 seconds
Ultra Pasteurization (UP)	138°C (280°F)	2 seconds
Ultra High Temperature (UHT, Aseptic)[a]	138°C (280°F)	2 seconds

[a]UHT milk is pasteurized in a sterile environment, while UP occurs in a nearly sterile environment. UHT is shelf-stable, UP requires refrigeration (IDFA 2016).

Commercial dairy processing plants produce large quantities of milk per day. Maintaining sanitary processing conditions and ensuring a safe, final product is vital in the food industry to protect consumers and the business. Although nearly all plants utilize hazard analysis critical control points (HACCP) and good manufacturing plans (GMPs), end product testing is still utilized and must be conducted efficiently and accurately. The unique composition of milk allows scientists to create faster, yet accurate, methods of testing. For example, scientists study the thermal stability of an enzyme largely concentrated on the fat globule of milk that becomes deactivated at temperatures slightly above the temperature at which the most thermostable pathogenic microorganism in milk is destroyed (Rankin and others 2010). This enzyme studied to ensure adequate pasteurization of milk is alkaline phosphatase (ALP). ALP's unique thermal stability allows it be assayed in order to determine that a dairy product was adequately heat-treated, thus meeting the temperatures required to eliminate harmful pathogens. According to the FDA (2015b), ALP is expressed in milliUnits of enzyme activity per liter of sample (mU/L), and a test result of 350 mU/L or above indicates that pasteurization was insufficient or contamination of raw milk had occurred during processing, which is correlated with the incomplete destruction of pathogens. The FDA's Pasteurized Milk Ordinance (2015b) instructs producers to immediately inspect pasteurization equipment when a positive (> 350 mU/L) ALP test result arises.

In 2016, a Wisconsin dairy processing facility, which routinely utilized ALP testing as a means of rapid product safety verification, found levels of alkaline phosphatase above the allowable limit (Food Safety News 2016). The facility administered a voluntary recall of the product and were able to investigate the cause of the positive tests immediately, which was determined to be a pasteurization failure (Food Safety News 2016). In 2011, a Texas dairy

processing facility recalled nearly 64,000 units of chocolate milk due to ALP tests yielding positive results (> 350 mU/L) (Food Safety News 2011). Interestingly, the cause of the positive test results was never determined. The investigation and plant inspection completed by Texas Department of State Health Services found the pasteurizer and other processing equipment to be functioning properly and in sanitary conditions without opportunity for contamination of raw milk to occur (Food Safety News 2011). From this information, it can be speculated that chocolate-flavored milk may yield false positive ALP test results.

This present research explores the effects of 1) the subjection of milk to homogenization, 2) various methods of pasteurization, and 3) the inclusion of milk-flavoring constituents (cocoa powder, vanilla extract, and sucrose) on ALP activity measured by the fluorometric assay. Identifying causes of false positive and false negative ALP test results will provide the dairy industry with practical information that can be utilized when completing end-product testing and safety verification. Knowing possible interactions between milk-flavoring ingredients, ALP activity, and the fluorometric assay will help guide further research into developing more specified safety parameters for specific dairy foods. This research will also provide insight on changes in ALP activity as a result of varying physical and thermal processing.

1.2 Hypotheses and Objectives

1.2.1 Hypotheses

This study hypothesizes that there will be significant differences in residual bovine ALP activity in 3.25% fat milk determined using the Fluorophos® (fluorometric) ALP when milk is subjected to a shear physical force of 2,000 psi (dual-stage homogenization; 1,500 and 500 psi), due to the force applied to the milkfat globule where ALP is localized, which can ultimately disrupt the ALP structure. It is also expected that there will be differences in ALP activity in

3.25% fat milk subjected to varying pasteurization conditions (HTST and LTLT), as differing time and temperature conditions may impact the denaturation process of ALP. Additionally, this study hypothesizes that there will be significant differences in ALP activity in 3.25% milk containing either pure vanilla extract, cocoa powder, or sucrose, based on the ability of common ingredients to potentially interact with the ALP enzyme or assay reagents.

1.2.2 Objectives

1) Determine the effects of dual-stage homogenization (1,500 and 500 psi) on residual bovine ALP activity in 3.25% fat milk.
2) Determine the effects of different methods of pasteurization (LTLT or HTST) on residual bovine ALP activity in 3.25% fat milk.
3) Determine the effects of pure vanilla extract, cocoa powder, and sucrose on residual bovine ALP activity in 3.25% fat milk.

CHAPTER 2

LITERATURE REVIEW

2.1 Milk Composition and Processing

Dairy milk holds a place in the diets of nearly six billion people worldwide (Visioli and Strata 2014). Milk contains a wide variety of micro- and macronutrients, including, but not limited to, high quality complete proteins, carbohydrates, vitamins D, E, and B_{12}, calcium, potassium, magnesium, zinc, niacin, and phosphorus (Visioli and Strata 2014). Prior to commercialization and consumption, milk is processed to ensure safety and quality. Raw milk has the potential to cause severe illnesses due to its compositional capacity to harbor pathogens, which are shown in **Table 2.1**. To destroy these pathogens, milk is heat-treated, which is also known as pasteurization. Louis Pasteur developed pasteurization in the late 1800's. Pasteurization conditions do not disrupt the nutritional integrity of milk (Steele 2000). There is strict monitoring, regulating, and analytical testing in dairy processing facilities to ensure that the milk was subjected to an adequate heat treatment to eliminate the harmful pathogens.

2.2 Milk Enzymes

Milk enzymes have been studied considerably for over 130 years, and 70 enzymes endogenous to healthy bovine milk have been discovered (Fox and others 2003). Many of the endogenous enzymes in bovine milk have undetermined roles and functions in milk production and secretion. Only two enzymes have reported roles in the biosynthesis and physiological activity of milk; all other enzymes present in milk are typically a result of the mechanism by which secretion occurs (O'Mahony and others 2013). Furthermore, these endogenous enzymes

are either inactive due to the absence of substrates, or inactivated by environmental or processing conditions. Although they have a very small role in the nutritional and sensorial properties of milk, enzymes play a vital role in providing industry professionals, milk producers, and food scientists with valuable information regarding the composition, quality, and safety of fluid milk.

Moreover, researchers have set standards for enzyme concentrations and developed standardized assays for the assessment of fluid milk. For example, the presence of the enzyme N-acetyl-beta-D-glucosaminidase is an indicator that the milk was sourced from a cow suffering from mastitis, which is the inflammation of a cow's udder (O'Mahony and others 2013). Detection of such an enzyme allows for milk producers to utilize precautionary procedures to avoid the sale of potentially harmful milk to the public. Another important endogenous enzyme, known as alkaline phosphatase (ALP), is used to evaluate the sufficiency of pasteurization or to detect possible contamination of raw milk. Based on the thermal stability of ALP, its presence in pasteurized milk is an indicator of either incomplete elimination of the pathogenic organisms that would otherwise be destroyed during HTST pasteurization or that there was contamination of raw milk with the pasteurized milk (IDFA 2016).

Table 2.1. Harmful pathogens that can be found in raw milk (CDC 2015, CDC 2017).

Pathogen	Symptoms
Listeria monocytogenes	Fever, muscle aches, convulsions, miscarriage (in pregnant women), diarrhea, fever
Coxiella burnetii	Fever, chills, fatigue, and muscle pain
Campylobacter jejuni	Fever, nausea, vomiting, abdominal discomfort, diarrhea
Brucella abortus	Fever, headache, sweating, chills, joint pains, weight loss, fatigue
Mycobacterium bovis	Fever, chills, fatigue, weight loss
Salmonella	Diarrhea, fever, chills, headache, bloody stools, abdominal pain, nausea, vomiting
Escherichia coli O157	Abdominal pain and cramping, watery diarrhea, gas, loss of appetite, fatigue, fever
Shigella	Watery diarrhea, abdominal cramping, nausea, vomiting
Yersinia	Abdominal pain, joint pain, diarrhea
Giardia	Bloating, gas, abdominal discomfort, nausea, fatigue
Norovirus	Vomiting, nausea, diarrhea, abdominal pain

2.3 Alkaline Phosphatase

2.3.1 History

In 1925, Demuth first discovered the presence of phosphatase in bovine milk. Kay and Graham, in 1933, later characterized the enzyme as an alkaline phosphatase and researched the thermal stability of this endogenous enzyme (ALP, EC 3.1.3.1) (O'Mahony and others 2013). Kay and Graham (1933) determined that ALP is inactivated as a result of the temperatures and time exposure similar to that used in HTST and LTLT pasteurization. Thus, ALP detection was the first laboratory test used to ensure effective pasteurization or determine if contamination with raw milk had occurred (**Figure 2.3.1**). ALP is a hydrolase enzyme, with three known isoforms, which cleaves a monophosphoric ester bond of substrates to release phosphate radicals (Rankin and others 2010).

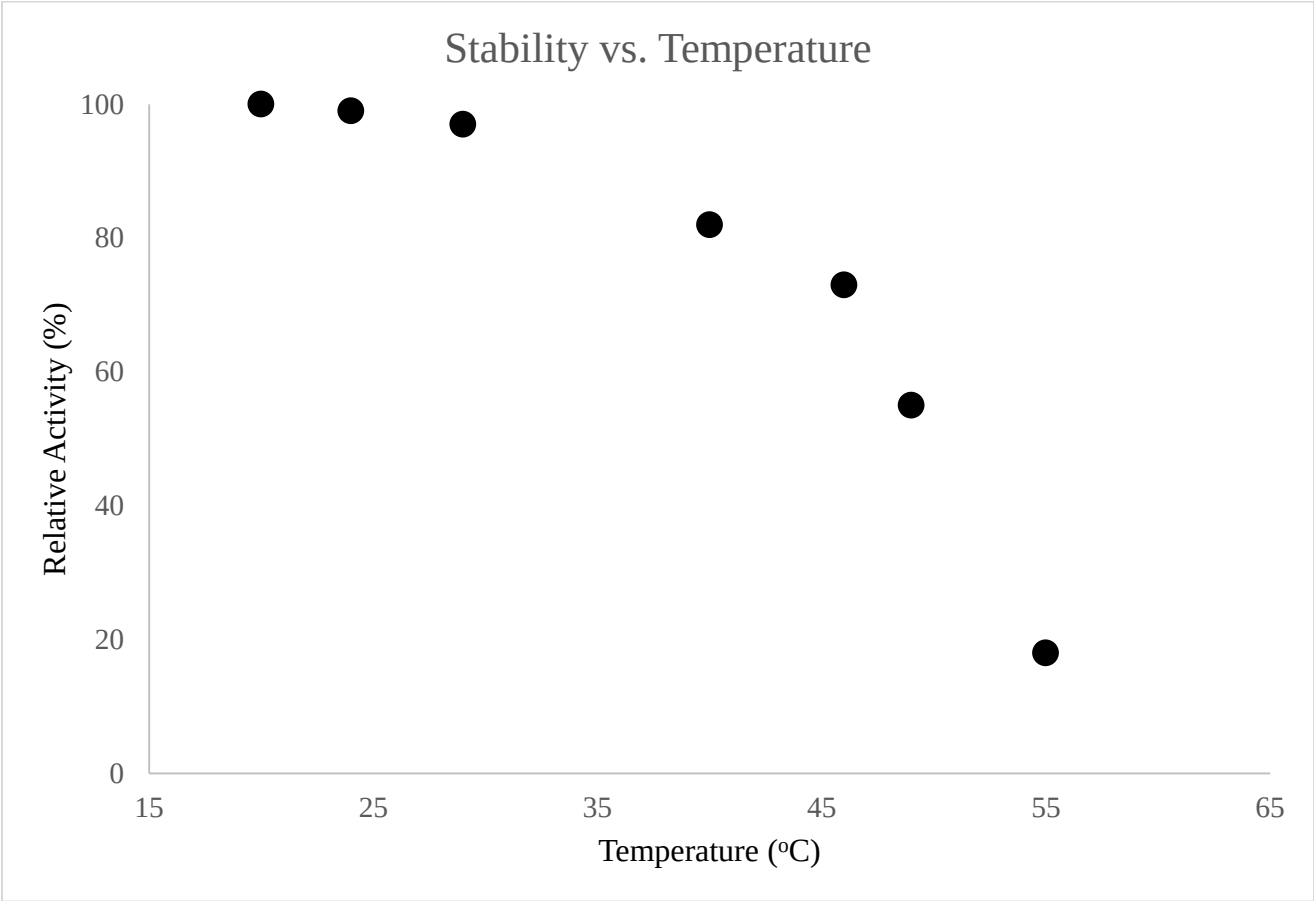

Figure 2.3.1. Purified bovine intestinal alkaline phosphatase stability at various temperatures adapted from Sigma-Aldrich (2017).

2.3.2 Characterization

As mentioned, Kay and Graham (1933) were one of the first researchers to characterize the phosphatase endogenous to milk as an alkaline phosphatase, with the highest activity around the pH of 10.5 and most stable around pH of 7.5-9.5. ALP is a homodimer glycoprotein with a molecular weight from 140-180 kDa. ALP has an isoelectric point in the pH range of 5.4-6.0 (Vega-Warner and others 1991). Currently, only three known isoforms of ALP exist and all of which require four atoms of zinc and two atoms of magnesium (or calcium in some instances), to become active (Wright and Tramer 1956). In the presence of metal chelators, ALP becomes inactivated. However, ALP can become reactivated when metal ions become present. ALP is mainly active in the cream portion of milk and can be isolated through an extraction accompanied with a form of separation chromatography or salting-in and salting-out (O'Mahony and others 2013; Dumitrascu and others 2014).

The complete amino acid sequence of the ALP present in milk has not been reported and although there are some similarities between microbial ALP and native bovine ALP, it is not to be assumed that they are identical, and it has been shown that microbial ALP can be more heat resistant (Painter and Bradley 1997). Additionally, the amino acid sequence for ALP extracted from kidney and intestinal tissue of bovine has been determined, but it is important to note that the ALP present in milk does not have the same amino acid sequence due to differences in functionality and activity (Morton 1954). Morton (1954) studied the differences in cow intestinal ALP and ALP present in milk. In **Table 2.3.2**, some differences in amino acid residues are displayed. In milk, ALP is primarily attached to the milkfat globule membrane by a glycosylphosphatidylinositol moiety (Bortolato and others 1999). It is rarely found in the aqueous phase. The inactivation temperatures of ALP in the aqueous phase and the ALP

associated with the milkfat globule membrane are very similar at roughly 58°C (Bortolato and others 1999).

Table 2.3.2. Differences in various amino acid residues in bovine intestinal ALP and ALP present in raw, fluid bovine milk adapted from Morton (1954).

Alkaline Phosphatase Enzymes		
	Moles/10^5 g	
	Tyrosine	**Tryptophan**
Milk	27	13
Bovine Intestinal	26	11

2.3.3 Assays

Various methods of testing have been developed for the detection of ALP and can be utilized on a multitude of dairy products from fluid milk to cheese. Many of the earlier developed quantitative methods for ALP detection required lengthy incubation times and extractions. Alternative methods were later developed to increase specificity and efficiency. Rankin and others (2010) classified the ALP assays into four differentiating group: colorimetric, chemiluminescent, fluorometric, and immunochemical.

2.4 Fluorometric Analysis

Testing for levels of ALP in milk is an integral part of quality control in the dairy industry, in that it ensures the safety and integrity of dairy products intended for the consumption by humans. Within the dairy industry, ensuring safe products warrants the need for quick, efficient, and accurate laboratory tests with highly sensitive modes of detection and quantification. The Fluorophos® fluorometric analysis was developed by Advanced Instruments Inc. to provide industry professionals with an objective laboratory test that provides quantitative results in minutes. The Fluorophos® ALP test is approved by Interstate Milk Shippers, the US Food and Drug Administration, the Association of Official Analytical Chemists, the International Dairy Federation, and the International Organization for Standardization; with the set legal upper limit of ALP at 350 mU/L (ISO 11816-1; IDF 155-1).

Due to the subjectivity and non-specificity of the colorimetric methods previously employed as standardized methods for ALP detection, newer and more efficient methods for ALP quantification were researched. In 1990a, Rocco published his work on the newly developed, more efficient, and sensitive method of laboratory testing for insufficiently

pasteurized or contaminated fluid bovine milk. The fluorescence testing has been shown to detect levels of around 0.003% raw milk (25 mU/L of ALP), which is highly sensitive when compared to the colorimetric tests with a reported sensitivity to 0.1-0.5% (Rocco 1990a; Claeys and others 2002).

The fluorometric assay functions under the principle described in **Figure 2.4**. The fluorometer internally calibrates using prepared calibration standards with the specific product standard that is being tested (i.e. 3.25% fat whole milk). The fluorometer also internally calculates mU/L of ALP activity and provides a printout of the values. The calculations can be done manually as well. One unit of ALP (U) is the amount of enzyme that catalyzes the conversion of one μmol of the Fluorophos® substrate (monophosphorylated benzothiazole derivative diluted in diethanolamine) to the highly fluorescent, Fluoroyellow®, molecule per minute, per liter of sample (mU/L). Typically, 0.05% raw milk (v/v) in whole milk is correlated with 250 mU/L (Rocco 1990b). If a sample produces a reading above 350 mU/L, the sample is deemed "insufficiently pasteurized or contaminated," which is associated with higher prevalence of harmful pathogens.

In comparison to the colorimetric methods of detection, the Fluorophos® ALP test has a myriad of positive attributes that make it the preferred method of pasteurization validation by dairy industry professionals. However, there are some limitations to the fluorescence testing that are vital for discussion and warrant further research. It is important to consider interactions of additional food constituents in specialized dairy products with the reagents in the Fluorophos® ALP test, the resulting fluorescence from the test, or with the ALP enzyme, itself. The complex food matrix that comprises flavored milks has shown to contribute to inaccurate results of the fluorometric testing for ALP in milk.

Table 2.4. Common assays for alkaline phosphatase in bovine dairy products (Rocco 2004; Rankin and others 2010)[a].

	Type	Scope
Scharer Rapid Visual ALP Method	Colorimetric	The substrate, disodium phenyl phosphate, in the presence of ALP, has a phosphate group cleaved off. The phenol group is extracted, reacted with 2,6-dichloroquinone-chlorimide, forms an indophenol, which is blue.
Scharer Spectrophotometric ALP Method	Colorimetric	Same experimental scope as described in the "Scharer Rapid Visual ALP Method," but utilizes spectrophotometric readings to quantitatively assess ALP levels.
Rutger's Visual ALP Method	Colorimetric	Phenolphthalein monophosphate is used the substrate for the reaction to expunge the need for a butanol extraction. A titration with sodium hydroxide determines level of ALP.
Fluorophos® Fluorometric ALP Method	Fluorometric	Reaction of monophosphorylated benzothiazole derivative substrate with ALP creates fluorescent compound with excitation of 440 nm and an emission of 560 nm, which can be detected using a fluorometer
CHARM Chemiluminescent ALP Method	Chemiluminescent	Reaction of ALP with substrate, an ortho phosphoric monoester dioxetane, yields a phenoxide that causes the transformed substrate to produce a luminescence that can be read using a luminometer.

[a] Immunochemical methods are less common in dairy manufacturing setting.

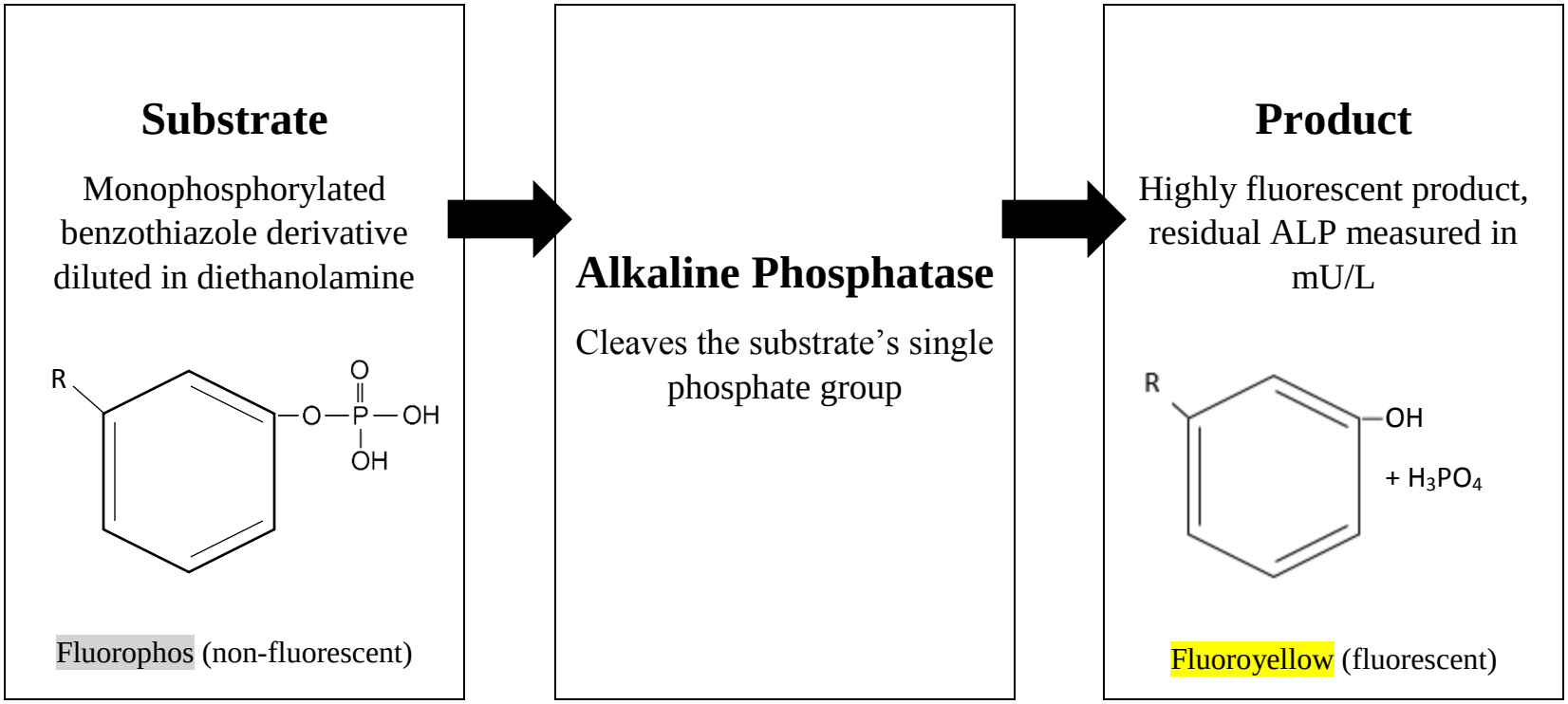

Figure 2.4. Simplified depiction of the reaction between ALP and the Fluorophos® substrate.

2.4.1 Effect of Homogenization

Prior to exploring the effects of milk-flavoring constituents on the ALP fluorometric assay, the possible effects of homogenization were researched. Milk producers are not required to homogenize milk, which is the process involving shear force applied to milk that reduces the size of the milk fat globule so an even dispersion is created among the product (Picart and others 2006). However, there is potential for homogenization to impact ALP activity. Picart and others (2006) found that solely higher pressure homogenization (above 14,000 psi) caused ALP activity, measured by the Fluorophos® test, to be decreased significantly. The researchers attributed this finding to the force of the homogenizer significantly disrupting the integrity of the fat globule, particular the membrane, where ALP is concentrated but not structurally bound to, thus inhibiting the activity of ALP through possible unfolding of the enzyme (Picart and others 2006). In this present study, this phenomenon was further researched in regards to standard homogenization conditions (dual-stage; 1,500 and 500 psi) for 3.25% fat milk to determine if homogenization significantly affects ALP activity. Applied physical force does have potential to disrupt the native structure of proteins, thus it is theorized that dual-stage homogenization may affect ALP structure and activity.

2.4.2 Effect of Pasteurization Conditions

Additionally, the effect of pasteurization conditions on the fluorometric assay for bovine ALP was explored prior to investigation of the effect of milk-flavoring constituents. When subjected to various levels of heat for a specified time interval, inactivation kinetics tend to vary from enzyme to enzyme. Due to the complex biochemical matrix of milk, there are a multitude of opportunities for additional milk constituents to contribute to the stabilization or destabilization of ALP when subjected to various thermal conditions (Wilinska and others 2007).

Generally, ALP adheres to first-order inactivation kinetics at higher temperatures (Wilinska and others 2007). The inactivation of bovine ALP was explored as preliminary work to determine if there is a significant difference in ALP activities between HTST (72°C for 15 seconds) and LTLT conditions (63°C for 30 minutes).

2.4.3 Effect of Milk-Flavoring Constituents

There is very little published literature that explores further into the effects of added food constituents on Fluorophos® ALP test results, which is the focus of this present study. Rocco (1990a) observed a lowered detection of ALP in spiked chocolate and eggnog milk samples in comparison to non-flavored milk with the same spiked levels of ALP. In his work, Rocco (1990a) explained that the results were attributed to the flavoring agents' ability to quench the fluorescence, thus producing an inaccurate lower reading. Additionally, there are compounds in chocolate milk, which remain undetermined, that have been shown to inhibit milk ALP activity. In colorimetric testing for ALP, chocolate milk has shown to create a false-negative caused by an undetermined constituent of the flavored milk's food matrix that interacts with the activity of ALP (Kosikowsky 1949). From a food safety standpoint, this seemingly minor room for error can be highly detrimental to consumer health. Inaccurate ALP test results can also be financially costing as well, due to inaccurate readings causing an unwarranted corrective action, such as disposal of thousands of gallons of fluid bovine milk.

The same result seen in chocolate milk in Rocco's (1990a) work was also found in eggnog. ALP spiked eggnog samples revealed a lower detection of ALP than regular milk spiked with the same amount (Rocco 1990a). The explanation for these findings can be based on an undetermined flavoring agent or compound in eggnog reacting with the Fluorophos® substrate or ALP directly. It has also been found that vanillin (4-hydroxy-3-

methoxybenzaldehyde), which is the primary chemical constituent of vanilla, can oxidize to form vanillic acid and react with formed complexes in colorimetric testing that can yield a false positive result (Anklam and others 1997; Rocco 2004). Additionally, sucrose has exhibited the ability to increase the heat stability of ALP, thus creating skewed ALP test readings if in combination with chocolate constituents (Caulfield and Martin 1939; Sanders and Sager 1948; Wang 1999). A common list of ingredients found in chocolate milk, strawberry milk, eggnog, and vanilla milk are listed below (Table 3).

2.5 Significance

As a means of quality and safety assurance in the dairy industry, the Fluorophos® ALP test remains highly utilized to validate pasteurization and ensure the contamination of raw milk has not occurred. Therefore, it is imperative that potential extrinsic interactions between the ALP enzyme, the Fluorophos® ALP test reagents, and resulting fluorescent products of the test with flavored milk constituents are researched in more detail. With flavored milk sales on the rise, any possible inaccurate ALP test results can cost consumers their health or cost the producers billions of dollars or much worse, their business. Flavored milk sales increased by 5.1% in 2015, with the leading manufactures continuously developing innovative new flavors and milk-based beverages such as mocha milk-based coffee drinks, vanilla chai latte, chocolate truffle milk, and salted caramel milk (Mintel 2016). Interfering substance control tests can be performed; however, pinpointing the exact constituent that can contribute to false-negative or false-positive test results takes time and money. Further research into the interactions between the ALP enzyme in milk, the reagents and products of the Fluorophos® ALP test, and the ingredients in flavored milk is necessary.

The objective of this study was to determine the effect of sugar, vanilla extract, and cocoa on the Fluorophos® ALP test results. Determining the potential interactions between common flavored milk constituents may help regulatory groups and agencies make proper adjustments to the upper limit (350 mu/L) of ALP. Cocoa, typically in a powdered form, is a main constituent of chocolate milk, and it is added around 1.5% in a commercial process. In the literature regarding ALP testing of chocolate milk, the constituents of said flavored milk were not disclosed nor were proximate analyses completed; however, it was reported that undetermined constituents in chocolate milk do have an inhibitory effect on ALP or can quench the fluorescence produced (Murthy and Peeler 1979; Rocco 1990a; Painter and Bradley 1997). Additionally, the polyphenolic compounds comprising cocoa powder have potential to form polyphenolic-protein complexes, thus inhibiting its activity and quenching fluorescence (Skrt and others 2012).

Additional interactions are seen with other flavored milk ingredients. When sucrose is added, an increase in the heat stability of ALP was observed (Caulfield and Martin 1939; Sanders and Sager 1948). Although high fructose corn syrup (HFCS) sometimes appears as a replacement for sugar in flavored milks, the negative consumer perceptions and the key structural differences of it from sucrose are the reasons for it not being included as a treatment in this present study. A reported quenching effect of fluorescence was also seen in egg nog, as well as chocolate milk, this phenome may be due to the addition of vanilla extract, which is a similar ingredient in both flavored milks (Rocco 1990a). Furthermore, it is important to understand the organic compounds that comprise cocoa, sugar, and vanilla extract (**Table 2.5**) and assess all possible interactions with the fluorometric analysis and the enzyme, ALP, itself.

Table 2.4.3. Common ingredients found in commercial flavored bovine milk products.

Chocolate Milk	**Strawberry Milk**	**Vanilla Milk**	**Egg Nog**
Milk	Milk	Milk	Milk
Cocoa	Sugar	Sugar	Sugar
Sugar	Carrageenan	Carrageenan	Eggs
Carrageenan	Strawberry Flavor*	Vanilla Flavor*	Nutmeg
Vanilla Extract*	Red #40	Vanilla Extract*	Cinnamon
Salt	Blue #1	-	Vanilla Extract*
All milks are then homogenized and pasteurized once all ingredients are combined.			
Other common ingredients include Vitamin A Palmitate and Vitamin D_3 for fortification.			

* Denotes either artificial or natural flavoring or extract.

Table 2.5. Key organic compounds present in cocoa, sugar, and vanilla extract.

Cocoa[a]	**Sugar**	**Vanilla Extract**[b]
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	sucrose	vanillin
2- and 3-methylbutanoic acid		*p*-hydroxybenzaldehyde
2-phenylacetic acid		vanillic acid
dimethyl trisulfide		*p*-hydroxybenzylmethyl ether
2-ethyl-3,5-dimethylprazine		acetic acid
(+)-catechin (polyphenol)		
(-)-epicatechin (polyphenol)		

[a]Six most abundant compounds in cocoa powder (Frauendorfer and Schieberle 2006).

[b]All compounds listed are present in vanilla extract $\geq$0.02% (Anklam and others 1997).

As of recent, there has not been further research into the effects of flavored milk constituents on ALP activity and the fluorometric assay, particularly looking at the interactions between milk ALP and the ingredients, rather than extracted and purified bovine kidney or intestinal ALP, which have differences in structure and functionality. Due to increased precautions regarding food safety and regulatory changes, narrowing and specifying the upper limits of ALP for flavored milks will help to allow industry professionals continue to use the rapid and inexpensive ALP testing without further scrutiny and refrain from having to engage in costly and time-consuming pathogen testing for ensuring product safety. Understanding common ingredient interactions with the widely accepted and utilized Fluorophos® ALP test in the dairy industry is the first step in narrowing and specifying food safety regulations and saving time and costs in production facilities. The main objective of this present study is to determine the effects of cocoa, sugar, and vanilla extract on the Fluorophos® ALP test results for whole milk. Additionally, this study proposed to observe any differentiation between resulting ALP readings as a function of varying pasteurization conditions (LTLT or HTST) and homogenization.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Milk samples were collected fresh (Michigan Milk Producers Association, Ovid, MI, USA) at the Michigan State University Dairy Plant (East Lansing, MI, USA) on a week-to-week basis. The milk was separated using a CENTRIWESTA LWA-205 multi-purpose centrifugal laboratory liquid-liquid separator (Centrico Inc, Englewood, NJ, USA). Collected cream and skim samples were analyzed for fat content using the Babcock method described in **Figure 3.2.1**. Sulfuric acid (Sigma-Aldrich, St. Louis, MO, USA), a centrifuge, heated water bath (Precision Scientific, Chicago, IL, USA), and Babcock bottles with 8 and 50% maximum were used in the analysis of the fat levels of the milk. Milk samples were homogenized using an APV SPX dual-stage homogenizer (SPX Flow Technology, Delavan, WI, USA). LTLT pasteurized samples and HTST pasteurized samples were heat-treated using a water bath (Precision Scientific, Chicago, IL, USA). Lastly, the Fluorophos® ALP testing was completed using an advanced filter fluorometer with excitation at 440 nm and emission 560 nm (Advanced Instruments, Inc., Norwood, MA, USA). This present study followed standard procedure for the Fluorophos® ALP and utilize all reagents and all apparatuses described (ISO 11816-1; IDF 155-1).

3.2 Methods

Preliminary data was collected regarding the effects of pasteurization temperatures and homogenization on the enzymatic activity of ALP and the fluorescence produced. The preliminary work was divided into two objectives:

1. Exploring the effects of homogenization
2. Exploring the effects of pasteurization temperatures (LTLT or HTST)

The experimental procedure for the preliminary objectives is outlined in **Figure 3.2**.

3.2.1. Separation and Standardization

For the preliminary objectives, the effects of different pasteurization methods and homogenization were explored. Percent of fat in the raw milk received was determined by the Michigan Milk Producers Association (Ovid, MI, USA) and ranged from 3.7% milk fat to 3.9% milk fat. The raw milk was warmed to 38°C (104°F) prior to separation. The warmed, raw milk was poured into the separator bowl. The centrifugal separator (2,500 rpm), separated the warmed raw milk into a cream and skim portion. Once separated, the fat levels of the cream and skim portion were tested using the Babcock method for cream and skim in duplicates as described in **Figure 3.2.1**. For the Babcock method, the milk was warmed to 38°C in a heated water bath and then portioned into respective Babcock bottles. Sulfuric acid (17.6 mL at 20°C) was added to two 8% maximum Babcock bottle with 17.6 mL (18g) of the skim portion and two 50% maximum Babcock bottle with 8 g of the cream portion and 9 mL of deionized water. The bottles were shaken to digest the curd and then placed in a heated centrifuge (60°C) for 5 minutes at 800 rpm. The bottles were removed, filled to the bottom of the neck with warmed (60°C) deionized water, centrifuged for 2 minutes, removed and filled with more warm water to displace the fat into the neck, centrifuged for 2 more minutes, and then removed and tempered in a warm

water bath (60°C) for 3 minutes. The milk was standardized to reach the target fat level of 3.25%. Calculations for amounts of skim and cream to mix were done algebraically. A confirmatory Babcock test was done in duplicate to ensure target fat level was reached and there was uniformity among experimental replications.

3.2.2 Methods for Homogenization and Pasteurization Objectives

The standardized, raw milk was then divided into two separate portions. One portion was homogenized at 2,000 psi (dual-stage; 1,500 and 500 psi), the other was left unhomogenized. Then, the raw milk portions were either LTLT or HTST pasteurized. To HTST pasteurize the samples, a water bath set to 75°C was used to heat the milk to 72°C (162°F) for 15 seconds. LTLT pasteurization was done using a hot water bath set to 65°C. The temperatures of the LTLT milk samples were carefully monitored to ensure that the milk was heated to 63°C (145°F) for 30 minutes. If the temperature of the milk exceeded $\pm$ 3°C, the sample was disposed of properly. The samples were then analyzed to determine ALP levels using the Fluorophos® ALP test. **Figure 3.2.2** describes the ALP test procedure using whole milk calibrations. A positive and negative control was also tested to ensure the fluorometer and reagents were functioning properly (**Table 3.2.2**).

For the Fluorophos® ALP test, 2.0 mL of working Fluorophos substrate was warmed to 38°C in labeled cuvettes for 20 min in dry incubator. Then 75 µL of sample was added to the 2.0 mL of substrate, mixed using a vortex mixer, wiped down, and the cuvette was placed in the fluorometer. After 1 minute, the measured rate of increase of fluorescence (F/minute) over 1-2 min in mU/L of ALP is displayed. Calibrations were conducted before objectives 1 and 2 and then before objective 3. Whole milk calibrations were used for all samples aside from the cocoa powder treatment in objective 3, which needed to correct for the turbidity. Calibrations were

done by dispensing 2.0 mL of A, B, and C calibrators to two cuvettes each and then warmed in the incubator for 5 min at 38°C. Then, 75 µL of the sample (3.25% fat milk) was added into each tube, mixed using a vortex mixer, and returned to incubator. With A calibrator, set fluorometer to zero then take a reading and then take readings for B and C. Each day of experimentation, a positive and negative control, which remained the same for the duration of data collection, was tested to ensure the fluorometer was working properly and did not need recalibration.

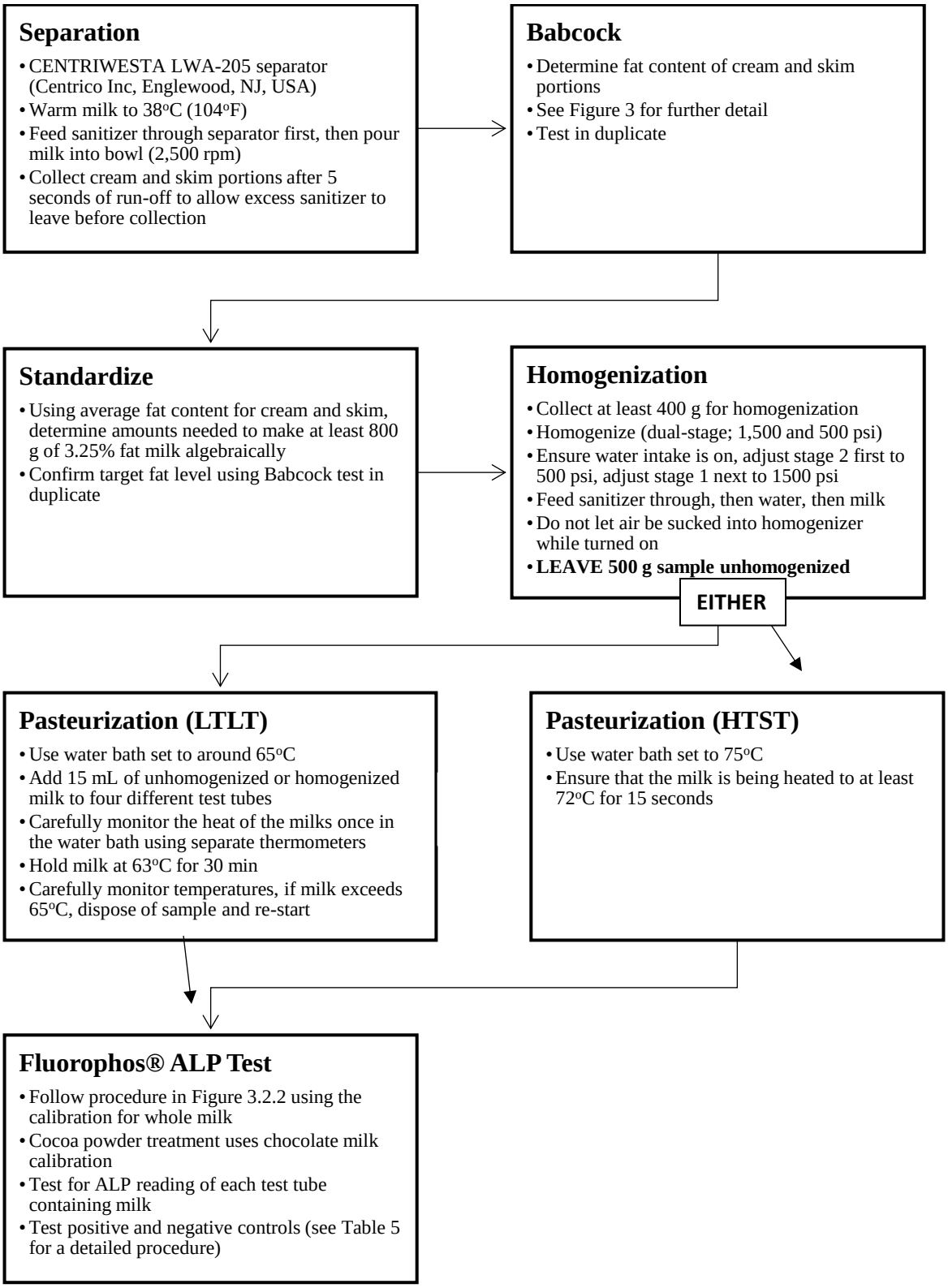

Figure 3.2. Outline of preliminary and main experimentation regarding the effects of homogenization and pasteurization on ALP activity determined through produced fluorescence.

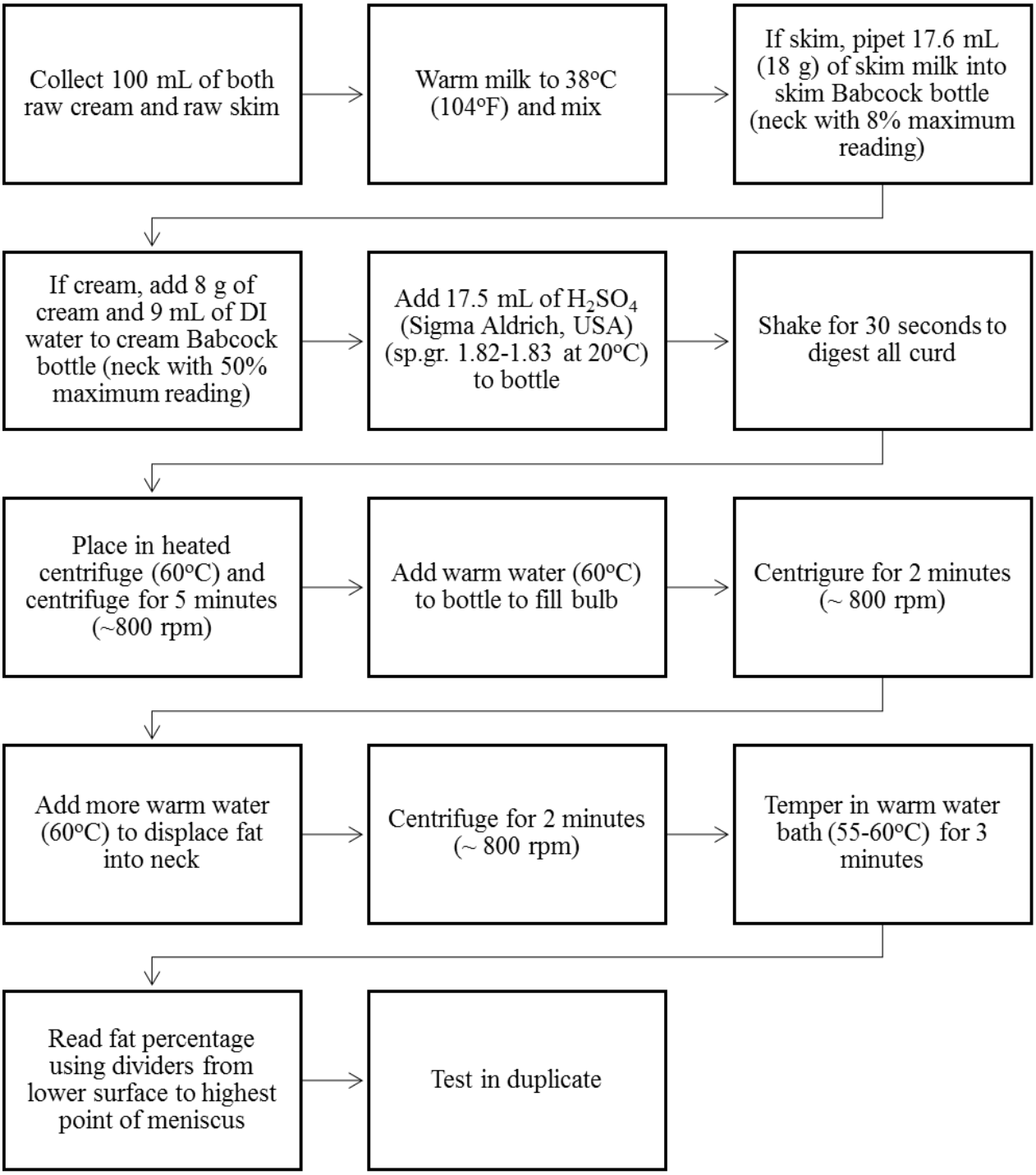

Figure 3.2.1. Babcock procedure outline for cream and skim products.

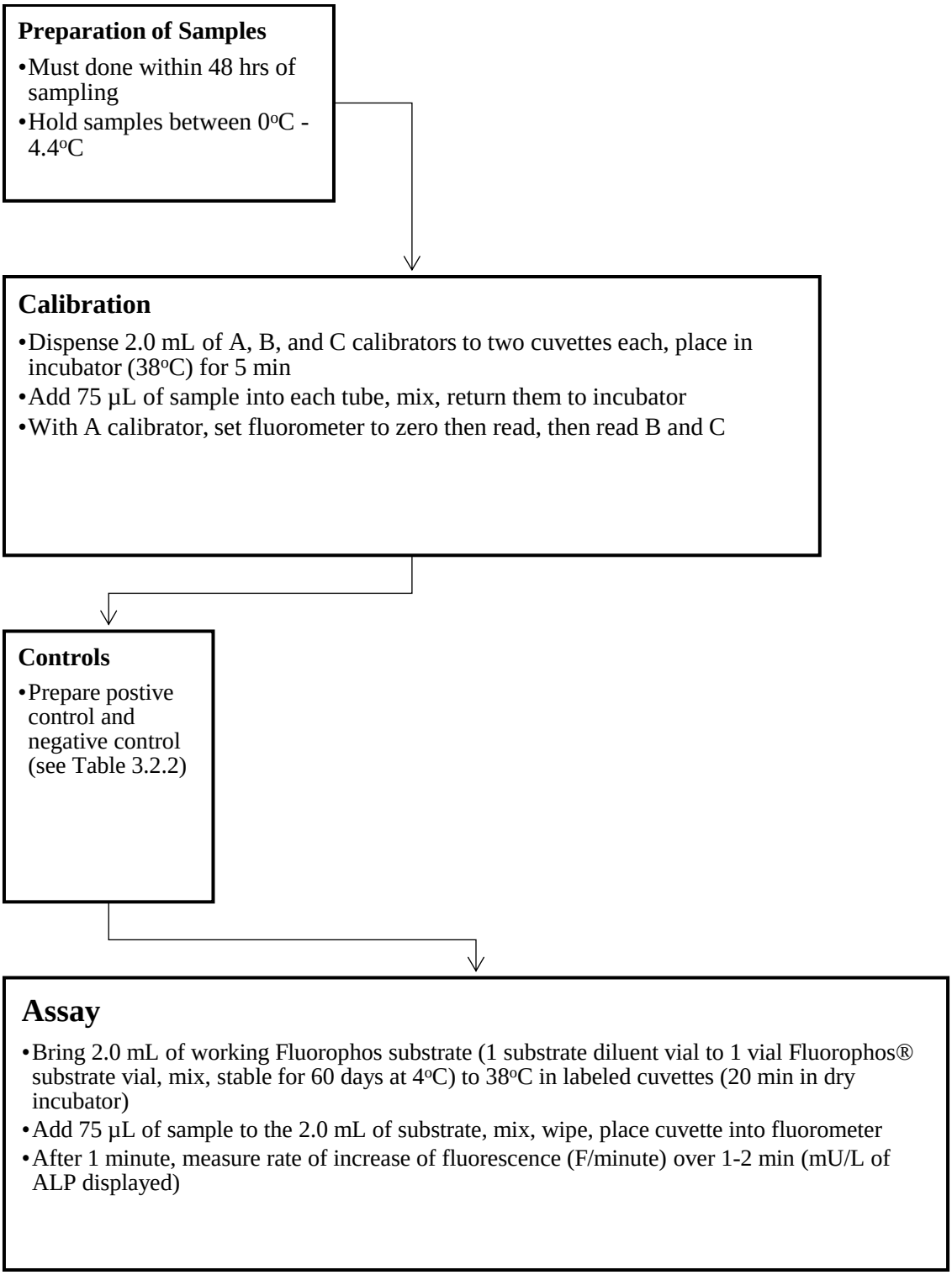

Figure 3.2.2. Outline of the procedure for conducting the Fluorophos® ALP test.

Table 3.2.2. Preparation of positive and negative control for the fluorometric ALP analysis (Rocco 2004).

Positive Control	Negative Control
• Add 0.1 mL of a fresh raw milk to a 100 mL volumetric flask containing 50 mL of negative control. Mix the flask gently and then add more negative control to reach the 100 mL mark • This positive control contains 0.1% raw milk (equivalent to roughly1 µg/phenol per mL/15 minutes) and can be stored in aliquots in the freezer for at least 1 month • Should be 350 to 500 mU/L	• Heat milk sample to 95°C, once it reaches targeted temperature, keep at temperature for 1 minute • Cool rapidly to room temperature and monitor • Should be less than 10 mU/L

3.2.3. Methods for Flavoring Interferences Objective

The main objective of this study was to determine the effects of milk-flavoring constituents (cocoa, sugar, and vanilla extract) on the fluorometric assay for bovine ALP. The experimental procedure is graphically outlined in **Figure 3.2.3.1** and images of some of the processing and testing equipment are shown in **Appendix: Figure A1, Figure A2, and Figure A3**. The raw milk was received, separated, and standardized to 3.25% fat according the same procedure adhered to in the preliminary objectives (**Figure 3.2**). After standardization, the milk was divided into four portions. Vanilla extract, cocoa powder, and sucrose were added individually to a 15 mL portion of raw milk (0.1, 1.5, and 8%, respectively, average amounts based on common levels of inclusion in flavored milk products), and one portion did not contain any ingredients, which served as the control. Next, the four portions were HTST pasteurized in the water bath (72°C for 15 seconds). Each treatment (vanilla extract, cocoa, sucrose, and control) were analyzed using the Fluorophos® ALP test in duplicate following the same procedures in **Figure 3.2.2**. The cocoa powder treatment was tested against a chocolate milk calibration, whereas all other treatments were tested against a whole (3.25% fat) milk calibration. This was due to the turbidity correctional factor for chocolate milk. The entire experimental procedure was independently repeated three times.

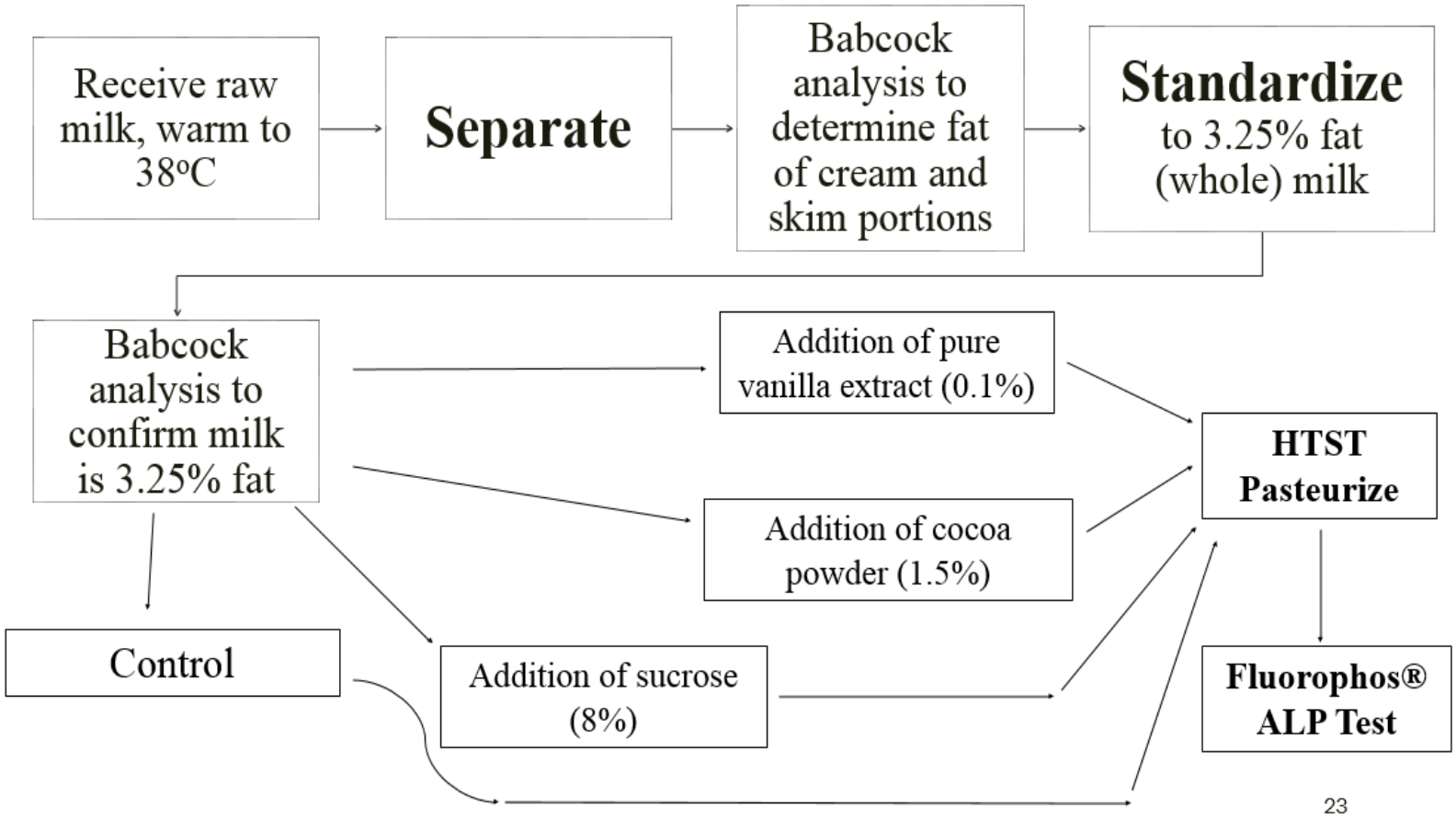

Figure 3.2.3. Graphical depiction of the proposed experimental design, independently replicated three times, to determine the effects of milk-flavoring constituents on the fluorometric assay of bovine ALP.

3.3 Statistical Analysis

All objectives were analyzed using XLSTAT-Base for Excel version 2017.2 (New York, NY, USA). Analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) was conducted for all objectives, and objectives one and two were analyzed together using two-way ANOVA with multiple comparisons. Fixed factors for objectives one were homogenized or unhomogenized and objective two's were LTLT or HTST pasteurization. Fixed factors for objective three were pure vanilla extract, cocoa powder, sucrose, or no ingredients (control). A confidence interval of 95% was used and differences were considered significant when $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Processing Conditions and ALP Activity

To further understand the effects of processing conditions on the enzyme, ALP, 3.25% fat raw milk was subjected to dual-stage homogenization (1,500 and 500 psi) or left unhomogenized followed by either LTLT or HTST pasteurization time and temperature conditions. The mean differences are seen in **Figure 4.1.1, Figure 4.1.2, and Table 4.1.1**. There were no significant differences in determined ALP activity as a function of varying physical and thermal processing conditions.

Table 4.1.1. Mean residual ALP activities, standard deviations, and *P*-values for 3.25% fat milk subjected to varying processing conditions. A *P*-value < 0.05 was considered significant.

		Mean ALP (mU/L)
Treatment	LTLT Homogenized	68.42±19.16
	HTST Homogenized	13.78±6.55
	LTLT Unhomogenized	104.15±125.68
	HTST Unhomogenized	98.07±152.54
		***P*-value**
Contrast	LTLT Unh vs HTST Hom	0.692
	LTLT Unh vs LTLT Hom	0.970
	LTLT Unh vs HTST Unh	1.000
	HTST Unh vs HTST Hom	0.733
	HTST Unh vs LTLT Hom	0.982
	LTLT Hom vs HTST Hom	0.904

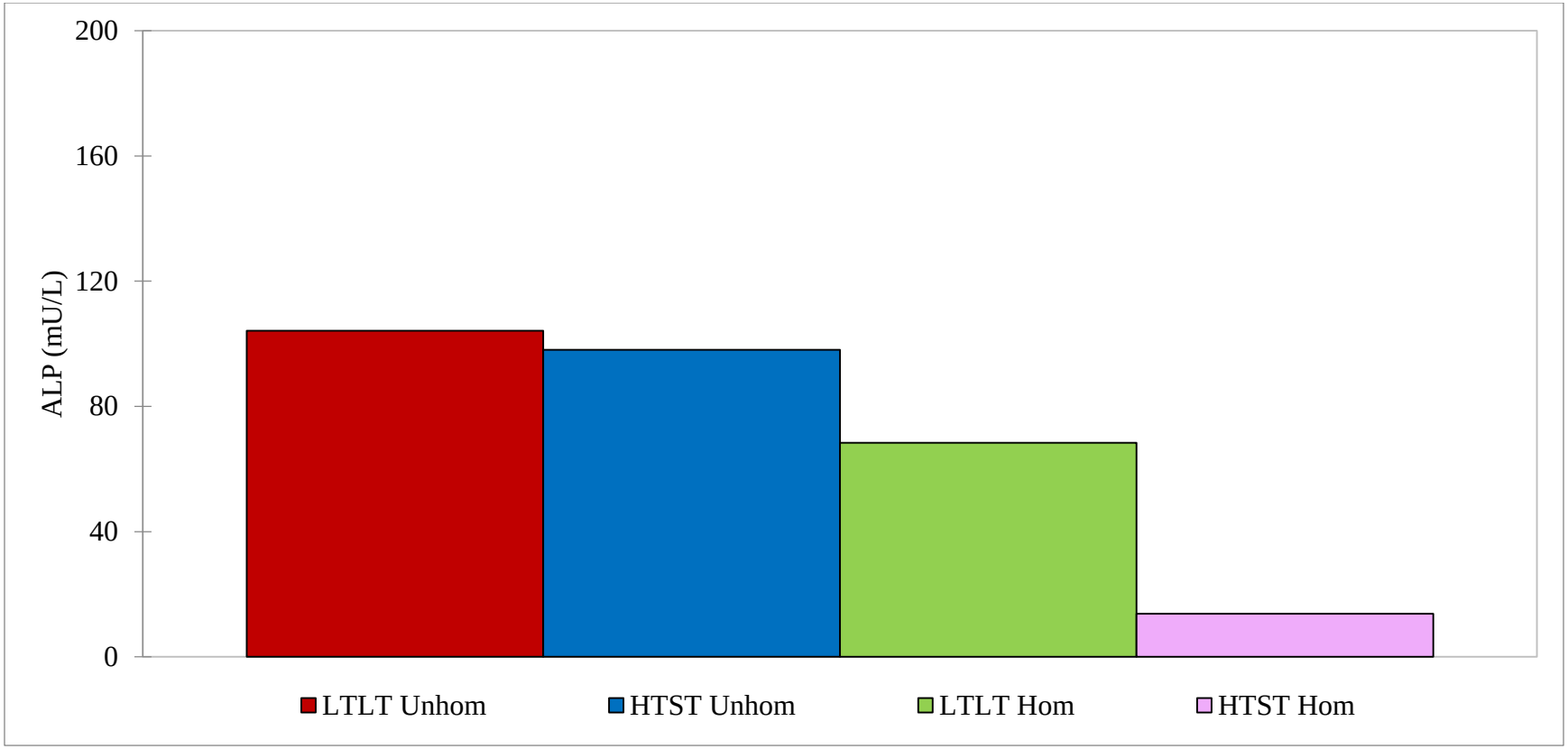

Figure 4.1.1. Mean differences in ALP activity among 3.25% fat milk subjected to varying processing conditions and methods: **unhomogenized LTLT** (63°C for 30 min) pasteurized milk, **unhomogenized HTST** (72°C for 15 s) pasteurized milk, **homogenized** (dual-stage; 1,500 and 500 psi) **LTLT** pasteurized milk, and **HTST** pasteurized milk. There were no significant differences in ALP (mU/L) among the treatment groups ($P > 0.05$); n = 12.

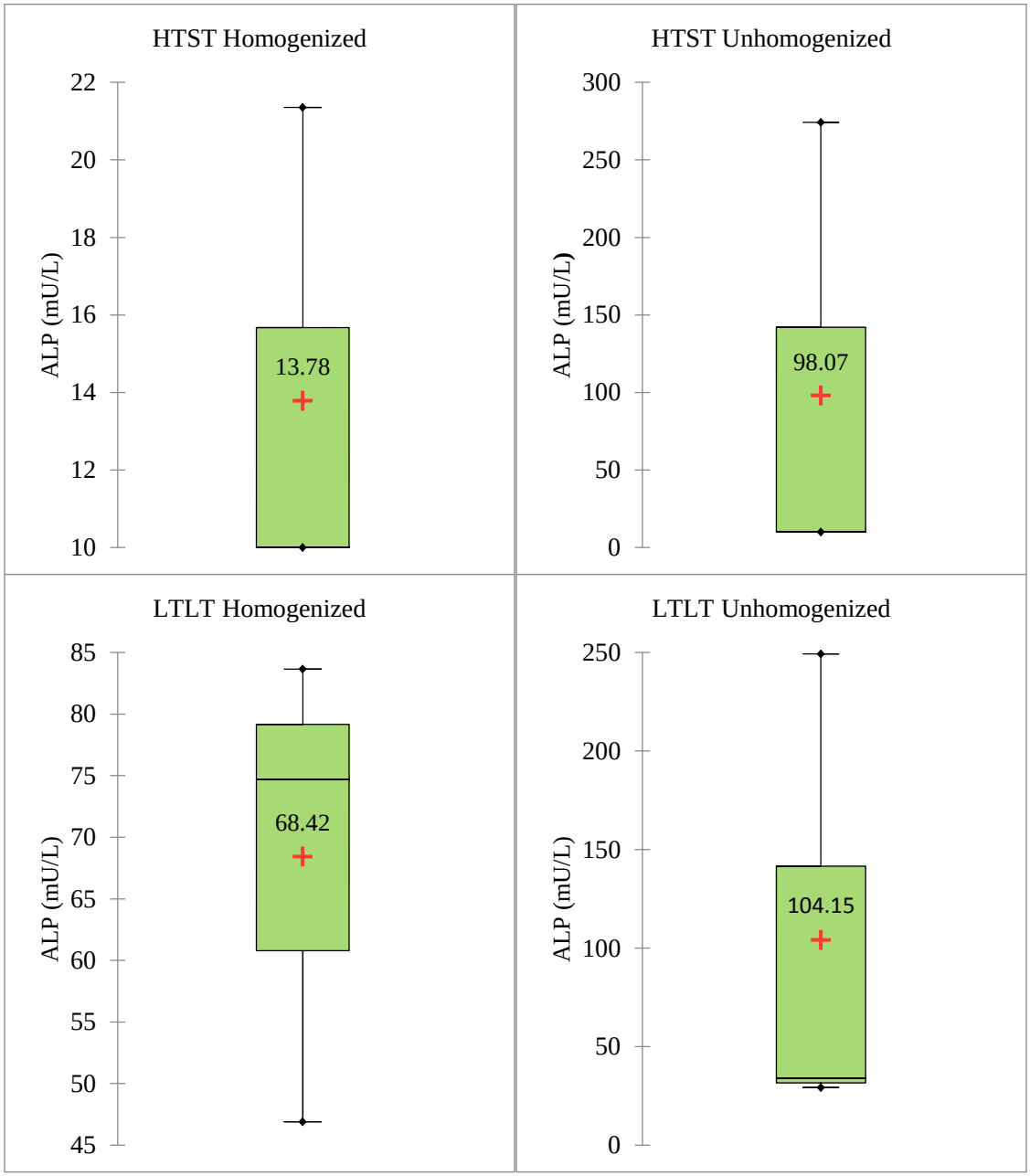

Figure 4.1.2. Box plots visually depicting ALP activity in 3.25% fat milk samples subjected to varying processing conditions and methods (unhomogenized or homogenized [dual-stage; 1,500 and 500 psi] samples either HTST [72°C for 15 s] or LTLT pasteurized [63°C for 30 min]) determined using the fluorometric assay.

Of the four treatments, HTST homogenized and LTLT homogenized had the lowest mean ALP activity at 13.78±6.55 and 68.42 mU/L±19.16, respectively. HTST unhomogenized milk had a mean ALP activity of 98.07±152.54 mU/L, and LTLT unhomogenized milk had a mean ALP activity of 104.15 mU/L±125.68. Notably, LTLT unhomogenized and HTST unhomogenized had outliers (non-significant) of high ALP activity (249.25 and 274.20 mU/L, respectively). Although under the ALP limit of 350 mU/L, the non-significant outliers warrant further discussion. According to the Pasteurized Milk Ordinance, ALP, end-product testing is recommended to be completed within 24-48 hours from the start of processing (FDA 2015b). Raw milk, in general, cannot remain in storage for more than 72 hours before processing (FDA 2015b). With respect to ALP testing, time sensitivity is of concern due to the potential for ALP to reactivate primarily during storage and hold times (Rankin and others 2010). ALP is a protein, that when subjected to elevated temperatures or mechanical shear, can denature (Li-Chan 2012). When a protein denatures, it unfolds from its native structure, essentially losing its tertiary, quaternary and/or secondary structure, into a different shape, thus affecting its activity and functionality (Culbertson 2012). Often times, this denaturation is irreversible; however, there is evidence that ALP can reactivate after denaturation during hold times or storage, which can yield a false positive.

In terms of the heightened levels of residual ALP activity in LTLT and HTST unhomogenized treatments (249.25 and 274.20 mU/L, respectively), experimental error is a probable cause for the heightened levels of ALP activity. Within this experimental study, the milk was processed and tested for ALP activity within a 12-hour window. However, it was noticed that when the milk was held in refrigerated (1.6^{o}C, 35°F) conditions post-pasteurization and before fluorometric testing for six hours, there was a general increase in ALP activity.

Immediately after pasteurization, the milk would cool to 38°C and be tested for ALP activity using the fluorometer. On the particular day where the LTLT unhomogenized milk reported an ALP activity of 249.25 mU/L, the milk was pasteurized and stored in refrigerated conditions (1.6^{o}C, 35°F) for six hours prior to fluorometric testing. Although the entire processing and testing was completed within 24 hours, the additional storage time may have contributed to possible reactivation of the ALP. This explanation is plausible based on the recognized potential for ALP to "renature" or reactivate during storage (Wright and Tramer 1953; Painter and Bradley 1997; Rankin and others 2010).

4.1.1 ALP Reactivation

When exposed to the time and temperature conditions of HTST and LTLT pasteurization, ALP denatures, thus losing its native form and activity, but the amino acid (primary) sequence remains intact. This denaturation, or unfolding, has been shown to be irreversible in many studies regarding ALP milk. Wright and Tramer (1953) were the first to observe a reactivation of ALP in pasteurized milk. ALP was reactivated to a higher degree of activity when subjected to very high pasteurization temperatures (85°C to 170°C) and when post-pasteurization storage conditions ranged from 18°C to 30°C (Wright and Tramer 1953). The potential for ALP reactivation poses a risk to the quality assurance and food safety practices for bovine milk processing. Even if the milk sample was tested for ALP activity one to two days after storage, there is potential for a false positive, which can be financially costing and lead to the unnecessary waste and disposal of said product. The 249.25 mU/L ALP activity of LTLT unhomogenized milk may have been due to some of the enzyme folding back into its native and active state due to various mechanisms.

One mechanism by which reactivation of ALP has been explained is through the abundance metal ions in the biochemical composition of milk, particularly magnesium, calcium, and zinc. Upon denaturation, if the active sites are still exposed on the surface of ALP to the abundance of metal ions in the milk, there is potential for the metal ions to bind back to the active sites and promote re-dimerization of ALP into its active form (Fransson and Lonnerdal 1983; Bortolato and others 1999). Magnesium and zinc have both been shown to contribute to the structural changes and reactivation of ALP (Bortolato and others 1999; Fox and Kelly 2006; Rankin and others 2010). At each active site, there are two zinc ions and one magnesium ion present; therefore, when ALP denatures and forms a monomer, metal ions can still bind back to the active site, thus inducing a conformational change of the enzyme and possibly reactivating it (Kuzuya and others 1982; Bortolato and others 1999).

Lyster and Aschaffenburg (1962) determined that the presence of magnesium, even as low as one mM, can promote reactivation of ALP in milk. This could be attributed to the important role of magnesium in ALP stability. Similar results were seen with the presence of zinc (Bortolato and others 1999). When ALP is exposed an environment containing a metal chelator, structural changes occur, particularly in the loss of α-helices accompanied with the loss of tertiary and quaternary structure (Bortolato and others 1999). Naturally, milk is abundant in zinc and magnesium, which increases the susceptibility for ALP reactivation, given the environmental conditions are optimal. However, it is important to consider the effect of other constituents in the milk that can promote reactivation along with the presence of divalent cations. Kresheck and Harper (1966) proposed a model for ALP reactivation involving magnesium, β-lactoglobulin, and casein, of which reactivation does not occur without all of three above

components. Morton (1954) also demonstrated that alanine, as a free amino acid, can have a protective effect on ALP activity and integrity.

Heat denaturation of ALP in an environment with sufficient zinc ions (i.e. milk composition, which contains 2,000-6,000 μg/L), can reactivate ALP produced by *Escherichia coli* (Bortolato and others 1999). This finding suggests that the ALP produced by psychotropic bacteria is more thermostabile and has higher potential to reactivate. In this present study, since microbial produced ALP was not differentiated from bovine ALP, it is possible that the microbial ALP was reactivated and the probable cause of the LTLT unhomogenized (249.25 mU/L) heightened residual ALP activity during the six hour storage time. Kresheck and Harper (1966) also suggested the role of sulfhydryl group formation in the reactivation of ALP. Less formation of sulfhydryl groups upon heating allows for a higher susceptibility for ALP reactivation (Kresheck and others 1966). Due to the differences in primary sequence between bovine ALP and microbial ALP, it can be concluded that there will differences in sulfhydryl groups formed once denatured. When an enzyme denatures, various amino acid side chains can become exposed and interact, thus rendering the enzyme's functionality (Culbertson 2012). Furthermore, without differentiation between microbial and bovine ALP, there is potential for a false positive to occur. Further research on storage reactivation of ALP is warranted due to the complex biochemical matrix of bovine milk.

From **Figure 4.1.1**, the HTST and LTLT unhomogenized treatments had higher mean activities of ALP (98.07±152.54 and 104.15±125.68 mU/L, respectively) when compared to the HTST and LTLT homogenized treatments. Although the mean differences are not significant, nor are they above the limit of 350 mU/L, it is important to discuss the relative variation in activity and the outliers, although non-statistically significant, among the unhomogenized and

homogenized treatments. For the HTST unhomogenized treatment, there was an outlier (non-significant) at 274.20 mU/L. This finding could be attributed to the fact that the samples were not homogenized. Milk that is not subjected to homogenization has shown a higher susceptibility for reactivation (Rankin and others 2010). In a general sense, ALP is localized on the outer membrane of the milkfat globule, but not a structural component (Fox and Kelly 2006; Picart and others 2006). Homogenization is a mechanical process involving a shear force that disrupts the integrity of the milkfat globule, which in some cases can inhibit ALP activity (Picart and others 2006).

High-pressure homogenization (14,000 psi and above) cannot only reduce microbial load, but can interfere with ALP activity in conjunction with short-time thermal treatments (Picart and others 2006). The ALP in the unhomogenized samples may have undergone different structural unfolding during thermal processing in comparison to the homogenized samples due to the absence of applied shear force, thus contributing to higher ALP activities and increased potential for reactivation in the unhomogenized samples. Additionally, it is possible that the unhomogenized samples had higher ALP activities due to sampling error in terms of the cream and skim phases not being well-mixed prior to sampling. Within this study, it is possible, but highly unlikely that dual-stage homogenization (1,500 and 500 psi) induced conformational change of ALP. According to Cano-Ruiz and Richter (1997), homogenization does not significantly affect the biochemical composition of the milk fat globule membrane, but rather the size of the globule. The structural integrity of the milkfat globule membrane is often well-maintained. However, ALP is not a structural component of the milkfat globule, so there is some potential for the shear force to impart structural change of the enzyme, but only to small degree.

Confirmatory tests for ALP reactivation were not completed in this study, but the findings suggest that there may be potential for ALP to reactivate in storage. From the results, a recommendation can be made that the fluorometric testing of ALP should be completed immediately after the milk samples have cooled to 38°C post-pasteurization. However, the intrinsic and extrinsic conditions of the milk sample must be optimal for significant reactivation to occur, thus the risk for a false positive is minimal in this case. For example, the milk sample must have sufficient levels of magnesium and zinc, sufficient levels of alanine as a free amino acid, limited sulfhydryl groups formed upon deactivation, an alkaline pH, and optimal interactions between casein and β-lactoglobulin with ALP still associated with the milkfat globule membrane (Lyster and Aschaffenburg 1962; Kresheck and Harper 1966; Kuzuya and others 1982; Bortolato and others 1999; Fox and Kelly 2006). As for extrinsic factors, reactivation is favored in milk samples that were subjected high-pressure homogenization and storage temperatures above 22 – 37°C (Wright and Tramer 1953; Picart and others 2006). To minimize the risk for potential ALP reactivation, whether it is the reactivation of microbial or bovine ALP in milk, it is imperative to complete the enzyme assay immediately after the milk has cooled to 38°C.

In summary, when comparing mean ALP activities between LTLT and HTST pasteurization, there were no significant differences ($P > 0.05$; **Table 4.1.1**). Therefore, it can be concluded that inactivation and denaturation of ALP was effective in both LTLT and HTST, with this finding, pasteurization conditions were excluded as an experimental treatment for the third objective. The mean ALP activities for HTST unhomogenized and HTST homogenized were 98.07$\pm$152.54 and 13.78 mU/L$\pm$6.55, respectively. The mean ALP activities for LTLT unhomogenized and LTLT homogenized were 104.15$\pm$125.68 and 68.42$\pm$19.16 mU/L,

respectively. From these findings, it is important to consider the general higher levels of ALP activity in the unhomogenized samples, which could be attributed to the effect of shear force on the milkfat globule where ALP is localized. Additionally, it was seen that when a milk sample was stored for six hours, post-pasteurization, prior to the fluorometric analysis, levels of ALP activity were generally higher, thus indicating that this deviation in the typical process flow might lead to the reactivation of ALP.

From this, it is recommended to test ALP activity immediately after the milk has cooled to 38°C post-pasteurization to obtain a more accurate depiction of the microbiological safety of the product. Moreover, the variations in the ALP activity among treatment groups, although below the limit of 350 mU/L, may be attributed to biological variability of the milk. Initial, or baseline, ALP activities in milk can vary among the breed of cow the milk was collected from, environmental conditions (i.e. seasonal), diet of the cow, storage and cooling times of the milk at the farm before shipment, and the age of the cow (Rankin and others 2010; Walsh and others 2012). Further research on the unfolding and subsequent reactivation of ALP is warranted.

4.2 Milk-Flavoring Constituents and ALP Activity

To determine the effects of common flavored milk ingredients on the fluorometric assay of bovine ALP, 3.25% fat milk was divided into four treatment groups. Each treatment group contained either pure vanilla extract, cocoa powder, or sucrose added at 0.1, 1.5, and 8% (w/v), respectively. The fourth group contained no additional ingredients and acted as the control. The mean differences of ALP activity between the four treatment groups are expressed in **Figure 4.2.1.** There were no significant differences in ALP activity found between the samples with ingredients and the control group. However, there are specific findings that are important to

consider. This section will discuss the findings (**Figure 4.2.1, Figure 4.2.2, and Table 4.2.1**) and provide a theoretically interpretation of possible interactions and interferences of flavored milk ingredients with ALP and the fluorometric assay.

Table 4.2.1. Mean residual ALP activities, standard deviations, and *P*-values for 3.25% fat milk with varying additional ingredients. A *P*-value < 0.05 was considered significant.

		Mean ALP (mU/L)
Treatment	Cocoa	106.70±158.53
	Sucrose	33.82±33.36
	Vanilla	31.55±16.37
	Control	19.32±16.14
		***P*-value**
Contrast	Cocoa vs Control	0.583
	Cocoa vs Vanilla	0.686
	Cocoa vs Sucrose	0.704
	Sucrose vs Control	0.996
	Sucrose vs Vanilla	1.000
	Vanilla vs Control	0.998

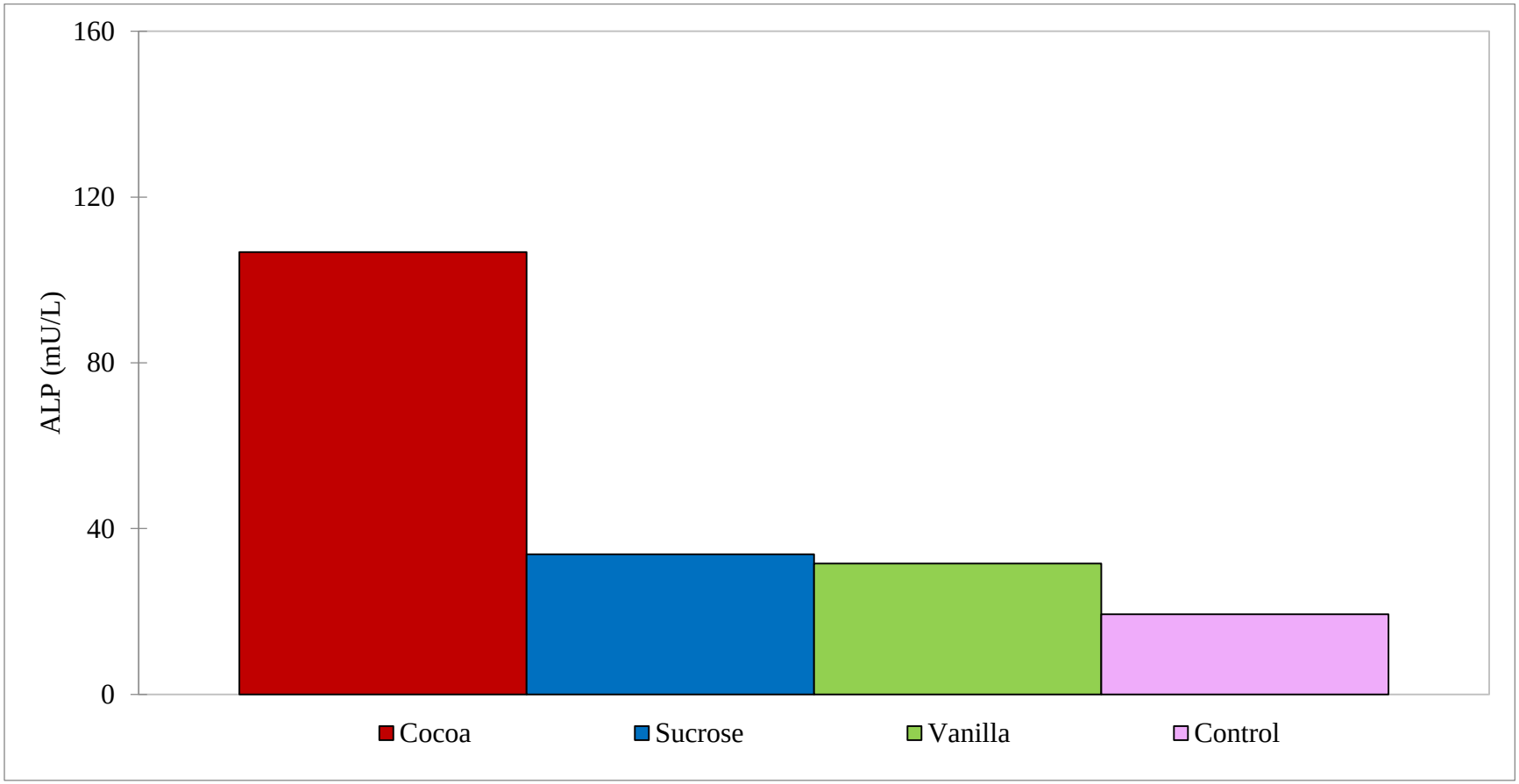

Figure 4.2.1. Mean differences in ALP activity among the four treatment groups: control (no added ingredients), cocoa powder (1.5% w/v), sucrose (8% w/v), or vanilla extract (0.1% w/v) added to standardized 3.25% fat milk. There were no significant differences in ALP (mU/L) among the treatment groups ($P > 0.05$); n = 12.

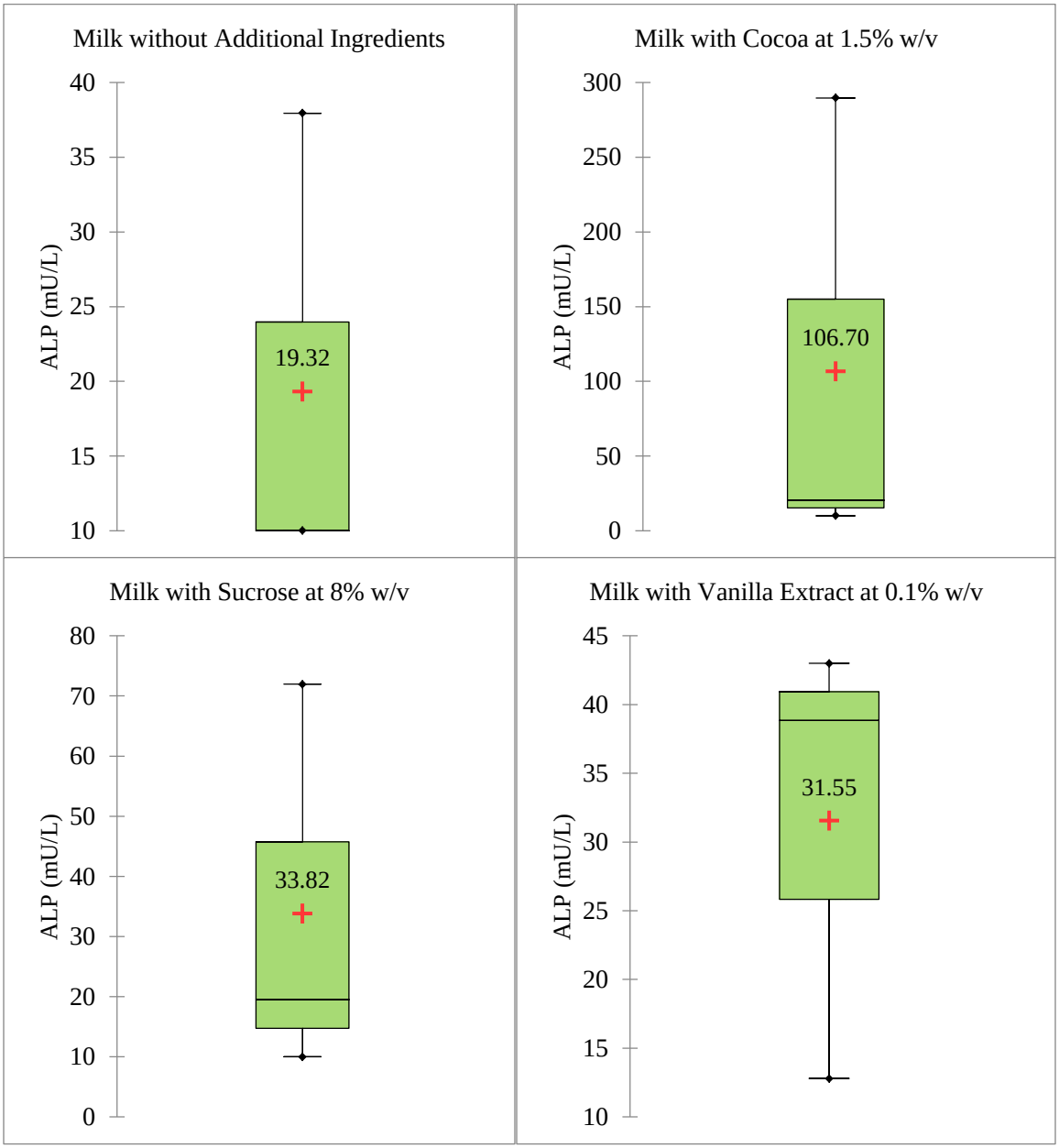

Figure 4.2.2. Box plot of ALP activity in the four separate treatment groups (control, cocoa powder, sucrose, or vanilla extract added to standardized 3.25% fat milk) determined using the fluorometric assay.

Based on the findings from the first two objectives, the milk was not homogenized and only pasteurized under HTST conditions. Since there were no significant differences in ALP activity as a function of varying processing conditions determined in the first two objectives, homogenization was eliminated as an experimental variable in this present objective. In **Figure 4.2.2**, the control had the lowest mean ALP activity (19.32±16.14 mU/L), followed by vanilla extract and sucrose (31.55±16.37 and 33.82 mU/L±33.36, respectively). Cocoa powder had the highest ALP activity (106.70±158.53 mU/L) compared with the control and other two treatments. Although there were no significant differences in ALP activity among the four treatment groups, it is important to discuss the potential interactions and the differentiating levels of activity seen in the results.

4.2.1 Cocoa Powder

Cocoa powder is a common ingredient included in chocolate milk formulations to attain the beverage's characteristic flavor and color. With a mean ALP activity of 106.70±158.53 mU/L, the 3.25% fat milk containing 1.5% (w/v) cocoa powder had the highest residual activity compared with the other two treatments and control. The addition of cocoa powder to the milk increased the turbidity and deepened the color of the sample to dark brown; therefore, a chocolate milk calibration setting was used to correct for any interferences based on turbidity. The calibration was done prior to testing using the Fluorophos® calibration standards with 75 µL of 3.25% fat milk with 1.5% (w/v) of cocoa powder added. Although not significantly different from the pure vanilla extract, sucrose, and control treatments, the milk samples with cocoa powder exhibited differing residual ALP activities that will be discussed further in this section.

Rocco (1990a) studied the effectiveness and reliability of the fluorometric assay in fluid dairy products. Chocolate milk samples were spiked with raw milk in varying percentages. In

comparison to the control spiked samples, the chocolate milk samples produced a residual ALP activity lower than that of the control spiked samples, at nearly 40% less, thus exhibiting potential for false negatives to arise (Rocco 1990a). Rocco (1990a) attributed these findings to interactions among the flavoring constituents in chocolate milk with the produced fluorescence, perhaps a quenching effect. It was also explained that ALP is possibly interacting with one or more of the ingredients in chocolate milk, thus leading to an inhibitory effect on the enzyme (Rocco 1990a). The flavoring components in the milk samples tested by Rocco (1990a) were not disclosed in the article; therefore, a theoretical explanation regarding the quenching or inhibitory mechanism of flavoring agents was not explained. A similar study, also completed by Rocco in 1990b, did not observe a lowered detection of residual ALP activity for 0.4% fat chocolate milk. Again, chocolate milk ingredients were not disclosed in the article. Additionally, Kuzuya and others (1982) found that flavonoids, namely morin, quercetin, and naringin, inhibited the activity of highly purified bovine ALP. Murthy and Peeler (1979) also concluded that polyphenolic compounds could inhibit ALP activity.

Cocoa powder is comprised of polyphenolic compounds, which appear in **Table 2.5**. This present study was the first to observe possible interferences of individual milk-flavoring ingredients on ALP activity and quantified fluorescence. Contrary to many findings in the literature suggesting cocoa powder may quench the fluorescence or inhibit ALP activity, this present study showed an increased detection of residual ALP activity in 3.25% fat milk containing 1.5% (w/v) of cocoa powder. This finding could be attributed to the interactions between the ALP enzyme and the polyphenolic compounds in the cocoa powder. Skrt and others (2012) studied interactions between milk proteins and polyphenols. They found that complexes could form between the protein and polyphenol. In the case of ALP, it is possible that when the

enzyme denatured during pasteurization, interactions between the newly exposed amino acid side chains and the cocoa powder polyphenolic compounds occurred. An exposed cysteine residue has been shown to act as the covalent binding site for both epicatechin and catechin (Gallo and others 2013). However, evidence has also shown that protein-polyphenolic complexes can also involve hydrogen and/or hydrophobic bonds, which could be largely attributed to the exposure of hydrophobic amino acid residues of a protein during heating (Siebert and others 1996; Skrt and others 2012; Wang and others 2012). Therefore, it is highly possible that upon heating, the main polyphenols in cocoa powder, epicatechin and catechin, were able to form hydrophobic bonds and/or bind covalently with an exposed cysteine residue and form a complex.

When the complexes are formed in the milk matrix and the polyphenols are the limiting factor, the formed complexes have potential to push the un-complexed ALP back into its native state regardless of heating due to molecular crowding around the milkfat globule membrane where the un-complexed ALP is bound. Theoretically, the protein-polyphenolic complexes could have a tendency to aggregate near the milkfat globule membrane, due to higher affinity for the area, and act as a protectant of the intact ALP on the membrane. This phenomenon could explain the higher levels of ALP activity in the cocoa powder treatments.

4.2.2 Sucrose and Pure Vanilla Extract

Both the sucrose and pure vanilla extract treatments exhibited mean residual ALP activities similar to that of the control. Sucrose and pure vanilla extract had mean ALP activities of 33.82$\pm$33.36 and 31.55 mU/L$\pm$16.37, respectively. It was expected that sucrose would have a protectant effect on the native ALP structure, therefore increasing enzyme stability. In pharmaceuticals and beverages, sucrose is used primarily for its stabilizing properties (Wang 1999). In a model system, sucrose tends to interact with proteins and enzymes by decreasing

solvent accessibility (Wang 1999). This effect was not observed in the present study, suggesting that sucrose has limited interaction or interference with ALP. Pure vanilla extract, which can contain oxidative products such as vanillic acid, can form complexes with either ALP, or the assay substrate, and produce false positive results, particularly in colorimetric testing (Anklam and others 1997; Rocco 2004). The addition of pure vanilla extract did not affect residual ALP activity in 3.25% fat milk.

In summary, the findings of the present study suggest that milk-flavoring constituents do not significantly affect residual ALP activity determined using the fluorometric assay. The heightened levels of ALP activity in the cocoa powder treatment, although not significantly different from the other two treatments and control, could be attributed to not only biochemical interactions, but also possible sampling error or experimental error. In terms of experimental error, it is possible that the introduction of cocoa powder into the 3.25% fat milk sample also introduced additional microbes with the ability to produce more thermostable ALP, which would lead to a higher residual ALP activity. However, cocoa powder has a generally low water activity; therefore, it is quite difficult for many microbes to survive in that environment. Furthermore, this study also concluded that pure vanilla extract and sucrose do not act as interferences to the fluorometric assay of bovine ALP.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

Milk processing conditions and flavoring constituents did not significantly affect residual ALP activity in 3.25% fat milk and all levels of activity were below the maximum limit of 350 mU/L. HTST pasteurization (72°C for 15 s) or LTLT (63°C for 30 min) were both effective in reducing ALP activity and there were no significant differences in ALP activity between the two methods of thermal processing. Homogenization (dual-stage; 1,500 and 500 psi) did not significantly affect ALP activity. However, unhomogenized samples generally had higher levels of ALP activity. The shear force of homogenization applied to the milkfat globule, where ALP is localized, could contribute to the lower levels of ALP activity in homogenized samples due the physical force possibly causing denaturation. Additionally, storage time of the milk samples that exceeded an hour contributed to higher levels of ALP activity, which leads to further scientific inquiries regarding the reactivation potential of ALP.

Milk-flavoring constituents (cocoa powder, pure vanilla extract, and sucrose) did not significantly affect residual ALP activity in 3.25% fat milk. All levels of activity were below the upper limit of 350 mU/L. However, the cocoa powder treatments had a marked increase in residual ALP activity in comparison to the control. The heightened level of activity could be attributed to a myriad of reasons, but most likely caused by the polyphenolic compounds present in chocolate milk. Polyphenols have potential to form complexes with proteins. It is possible that some of the ALP denaturing during pasteurization was able to form complexes with the polyphenols. If the polyphenols were the limiting factor in the formation of the complexes, then

not all ALP would complex with the polyphenols. Therefore, the complexes, with a high affinity for the milkfat globule membrane, could act as a protectant of the intact ALP and increase its stability during thermal processing, which would produce a higher level of residual ALP activity. It was expected that sucrose would have a protectant effect on ALP, but this phenomenon was not observed in this present study. Additionally, pure vanilla extract did not affect residual ALP activity or quantified fluorescence.

5.2 Future Directions and Study Limitations

This study was the first to observe the effects of three common milk-flavoring ingredients on bovine ALP activity using the fluorometric assay. This study also observed the effects of various thermal and physical processing methods on ALP using the fluorometric assay. From the findings, theoretical conclusions were made based on the biochemical properties of milk, ALP, and the added ingredients. There are many future directions for research based on this topic. Spiking the milk samples with a known concentration of active ALP would help eliminate the potential for biological variability and provide a more clear depiction of possible interferences with the fluorometric assay. Many of the findings in this study could be attributed to the compositional variability in milk. In this study, baseline ALP activities were unable to be determined due to the levels being above the detectable limit for the fluorometer. Knowing baseline ALP activities prior to processing, possibly quantifying the levels using another common assay would also reduce the confounding biological variability. However, one should be mindful when working solely with purified bovine intestinal or kidney ALP, as there are differences in functionality between the ALP extracted from cow organ tissues and the ALP present in milk (Morton 1954). It is recommended to purify ALP enzyme directly from raw milk

samples. Additionally, a limitation of this present study was the lack of differentiation between native bovine ALP and microbial ALP, which is also present in milk.

Further research regarding the potential for ALP reactivation is warranted. The recommended ALP testing window of 24-48 hours may be too large if ALP has potential to reactivate. In dairy processing, the possibility of a false positive derived from ALP reactivation can be extremely detrimental to a business. To study reactivation, it is recommended to first observe the unfolding characteristics of bovine ALP and sequence the enzyme as well. Techniques, such as circular dichroism and nuclear-magnetic resonance, can be used to observe the unfolding and exposure of amino acid side chains and charges. From those findings, one can build theoretical models regarding possible reactivation and interactions based on the other constituents comprising the milk matrix. Additionally, a combination of ingredients and other extrinsic and intrinsic parameters that have potential to promote a false positive or false negative ALP activity should be studied. From those findings, more specificied limits of ALP activity can be defined for dairy products, thus reducing the need to conduct time-consuming ALP interference tests or confirmatory microbial plating.

APPENDIX

Figure A1. APV SPX dual-stage homogenizer (SPX Flow Technology, Delavan, WI, USA).

Figure A2. Main components of the CENTRIWESTA LWA-205 multi-purpose centrifugal liquid-liquid separator (Centrico Inc, Englewood, NJ, USA).

Figure A3. Fluorophos® advanced filter fluorometer (Advanced Instruments Inc, Norwood, MA, USA).

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