STORAGE STABILITY OF TAGATOSE IN BUFFER SOLUTIONS

OF VARIOUS COMPOSITION

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STORAGE STABILITY OF TAGATOSE IN BUFFER SOLUTIONS OF VARIOUS COMPOSITION

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THESIS ABSTRACT

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Tagatose, an epimer of fructose, is a minimally absorbed monosaccharide that has been shown to function as a prebiotic in the human body. For this prebiotic effect to be achieved, tagatose in food and beverage products must not be lost during their processing and storage. However, data on the storage stability of tagatose is lacking. The objective of this study was to evaluate the storage stability of tagatose in solutions as affected by buffer type, buffer concentration, pH and temperature.

Tagatose solutions (0.05 M or about 1%) were prepared in 0.02 and 0.1 M phosphate and citrate buffers at pH 3 and 7. Triplicate vials were stored at 20, 30 and 40°C. These eight solutions were prepared again with the addition of 0.05 M

glycine. Aliquots were analyzed at regular time intervals for nine months. Tagatose analysis occurred via reverse-phase HPLC while browning was measured using a spectrophotometer at 420 nm.

In the solutions with no added glycine, no tagatose loss or browning was observed in 0.02 M phosphate and citrate buffers at pH 3. In 0.1 M buffers at pH 3 and 40°C, 5-10% tagatose was lost over nine months and slight browning occurred. Tagatose loss was enhanced at pH 7. Tagatose degraded in a biphasic manner, with a rapid initial decrease followed by a plateau. The pseudo-first order rate constants (k_{obs}) for the initial tagatose degradation at pH 7 were greater in phosphate buffer than citrate buffer. Higher buffer concentrations also increased k_{obs}. In phosphate buffers at pH 7, browning accompanied the tagatose loss, increasing to a maximum and then decreasing as tagatose loss plateaued.

In solutions with added glycine, tagatose also degraded faster at pH 7 than pH 3. However, glycine did not enhance tagatose loss. Glycine did enhance browning as compared to solutions without glycine. Tagatose degradation and browning occurred faster at higher phosphate buffer concentrations and at higher temperatures.

To deliver the prebiotic effect from tagatose, shelf-stable beverages should be formulated to the lowest buffer concentration and pH possible to optimize tagatose's stability.

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CHAPTER 1: INTRODUCTION

Nutraceuticals are substances found in foods that have been shown to positively impact human health by preventing diseases or improving physiological function. The nutraceutical classification includes vitamins, minerals, genestein, lycopene, flavonoids, inulin, and fructooligosaccharides, among others (Wildman and Kelley 2007). For nutraceuticals to be beneficial in the human body, they must not be degraded during food processing and storage. Significant data have been collected regarding the beneficial health effects of nutraceuticals; however, less data about their stability in foods exists.

The nutraceuticals inulin and fructooligosaccharide are also classified as prebiotics. Prebiotics are food components that stimulate the growth and/or activity of beneficial bacteria in the colon, and thus improve host health. Most prebiotics are not digested or absorbed in the small intestine, but pass into the large intestine where they are then fermented into short chain fatty acids by intestinal microflora. The short chain fatty acids help promote the growth of beneficial intestinal bacteria while inhibiting pathogenic bacteria.

Tagatose is a monosaccharide that also falls into both the nutraceutical and prebiotic categories. An epimer of fructose, tagatose displays a sweetness and bulk comparable to sucrose (Levin 2002). Tagatose obtained GRAS (generally

recognized as safe) status in 2000 in the United States (Levin 2002), and has also been approved for use in Australia, New Zealand, South Korea, Brazil, South Africa and the United Kingdom (FSANZ 2004; Skytte 2006; FSA 2005). Tagatose is currently found in several products in Europe; these include a cocoa drink, a chocolate-hazelnut spread, diet jams, and chocolate bars (Damhert 2008).

One reason tagatose may be utilized is for its positive health effects. It contributes less than 1.5 kcal/g, which is far less than other carbohydrates at 4 kcal/g (Levin 2002). Tagatose also does not elicit a glycemic response in diabetes mellitus patients (Donner and others 1999). It does not promote tooth decay (Levin and others 1995). Tagatose has also been shown to contribute positively to human health by increasing the production of short chain fatty acids in the intestines (Bertelsen and others 1999). It is this prebiotic effect that is most significant. For the prebiotic effect of tagatose to be achieved, it must not be degraded during food processing and storage.

The storage stability of tagatose has not been widely studied. Therefore, the objective of this study was to evaluate the stability of tagatose in solution under various conditions, such as buffer type and concentration, pH, and temperature.

CHAPTER 2: REVIEW OF LITERATURE

The literature review addresses nutraceuticals and then more specifically prebiotics. After discussing examples and effects of prebiotics, the properties and utilization of tagatose will be presented. Monosaccharide stability will then be addressed. The objectives of the current study will conclude this chapter.

Nutraceuticals

Over the past several years, numerous biologically active substances have been shown to contribute positively to human health; these substances have been designated "nutraceuticals" or "functional foods". Nutraceuticals have been defined as "chemicals found as a natural component of foods or other ingestible forms that have been determined to be beneficial to the human body in preventing or treating one or more diseases or improving physiological performance" (Wildman 2001). Nutraceuticals include an assortment of substances, from vitamins and minerals to antioxidants. Some examples of nutraceuticals are lycopene, flavonoids and tocotrienols. Lycopene, produced by some fruits and vegetables, has been shown to prevent free radical damage in the body (Bruno and Wildman 2001). Flavonoids, found in plants, also protect the body against oxidative damage (DiSilvestro 2001). Tocotrienols, naturally occurring analogues of tocopherol, function as antioxidants and have been shown to have anticancer and cholesterol lowering properties (Guthrie and Kurowska 2001). For these and any other nutraceutical to have a

healthful benefit, the substances must not be degraded during food processing or product storage. Significant data have been collected on the health effects of nutraceuticals; however, less information exists about their behavior and stability in foods.

Definition of Prebiotics and Probiotics

Another group of substances classified as nutraceuticals are the prebiotics and probiotics. Gibson and Roberfroid (1995) defined a prebiotic as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health". Prebiotics are metabolized by intestinal microorganisms and help those organisms already present in the gut to grow.

Many foods are marketed as containing probiotics, which are not the same as prebiotics. Fuller (1989) defined the term probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". The main difference between a prebiotic and probiotic is that the latter includes living microorganisms that are found in certain foods. Probiotics can be one or two different microorganisms in foods that help the body. Species of lactobacilli and bifidobacteria are commonly used probiotics that are components of fermented milk products, such as yogurt and sour cream (Gibson and Roberfroid 1995). It has been shown that probiotics have a role in alleviating lactose intolerance, enhancing the immune system, and in lowering cholesterol (Roberfroid 2000).

Prebiotic Examples and Effects

Prebiotics encompass a wide range of substances, including β-glucans, pectin, gums and resistant starch 3. Two of the more commonly studied prebiotics are inulin and fructooligosaccharides (Figure 2.1). Inulin is a carbohydrate composed of β-(2-1)-fructans where the degree of polymerization ranges from 2 to 60, but most commonly from 27-29 with a terminal glucose (Franck and De Leenheer 2002). The most common sources of inulin are wheat, onions and bananas. Inulin-type fructans can be used as sugar and fat replacers, and as a way of providing texture, improving mouth feel, and stabilizing foams. It has been suggested that inulin-type fructans can help alleviate constipation and diarrhea as well as reduce the risk of osteoporosis, atherosclerotic cardiovascular disease and obesity (Roberfroid 2000). Fructooligosaccharides consist of a terminal glucose molecule with 2 to 10 fructose units attached by a β-(2-1)-glycosidic linkage (Niness 1999). They occur naturally in plants such as onion, wheat, rye, asparagus and triticale and can be produced industrially through an enzymatic process (Bornet 1994). Fructooligosaccharides function mainly as sugar replacers. The primary difference between inulin and fructooligosaccharides is the degree of polymerization (Figure 2.1).

Most prebiotics that are ingested are not digested or absorbed in the small intestine. Molis and others (1996) determined that 89 ± 8.3% of the ingested fructooligosaccharides in healthy humans was never absorbed. Ellegård and others

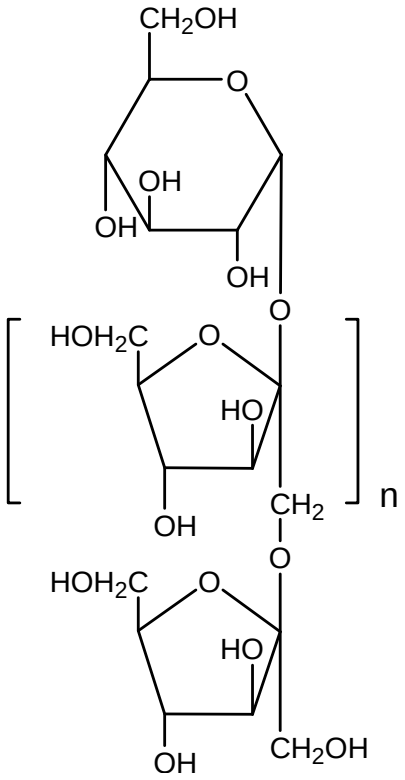

Figure 2.1. Structural Representation of Inulin (n=27-29) and Fructooligosaccharides (n=2-10).

(1997) demonstrated that 88% of inulin and 89% of oligofructose that was ingested by ileostomy patients was recovered intact. These prebiotics then pass to the large intestine where microflora ferment them.

Kleessen and others (1997) examined the effect of inulin ingestion on fecal microflora in humans. Inulin was given in doses of 20 g/d for the first eight days, gradually increased to 40 g/d for two days, and then set at 40 g/d for the last seven days. Bifidobacteria in the feces increased from 7.9 ± 0.4 $\log_{10}$/g before inulin administration to 9.2 ± 0.5 $\log_{10}$/g at the end of the study (Kleessen and others 1997).

Gibson and others (1995) demonstrated the laxative effect of prebiotics. When humans were supplemented with 15 g of oligofructose, stool output increased from 135.8 ± 22.8 g/d to 154.1 ± 22.9 g/d. By increasing stool output, oligofructose is acting like other non-digestible carbohydrates (i.e., fibers). The increase in stool output can be attributed to an increase in biomass, which was supported by an increase in nitrogen excretion.

Prebiotics can influence many different aspects of human health. The effect of prebiotic consumption on mineral metabolism, the immune system, and on humans in general will be discussed.

Effect on Mineral Absorption

Ohta and others (1994) examined phosphorus and magnesium balance in rats fed a diet containing different levels of fructooligosaccharides (FO). No difference in phosphorus balance in rats was found when they were fed 1% or 5% FO. The rats that were fed a control diet containing no FO had a phosphorus absorption of 80.1%. The rats that were fed 1% FO had a phosphorus absorption of 81.8%. Similarly, the rats that were fed 5% FO had a phosphorus absorption of 78.1%. Contrary to the phosphorus balance, Ohta and others found that magnesium absorption increased in rats that were fed a diet containing fructooligosaccharides. The rats fed a control diet (no FO) had a magnesium absorption ratio of 54.4% whereas the rats supplemented with 1% FO had an absorption ratio of 62.3%. A more significant increase was shown with the rats fed 5% FO. Their magnesium absorption ratio increased to 71.8%. Rats supplemented with FO showed an

increase in magnesium absorption while phosphorus absorption stayed relatively constant.

Delzenne and others (1995) showed that absorption of calcium, magnesium, iron and zinc all significantly improved when rats were fed a diet containing 10% fructooligosaccharides versus ones who were not fed fructooligosaccharides. Ohta and others (1995) demonstrated in rats that fructooligosaccharides improved recovery from anemia. This recovery was attributed to increased iron absorption, as shown by Delzenne and others (1995).

Scholz-Ahrens and Schrezenmeir (2002) also evaluated the effects of prebiotics on mineral metabolism in rats. To evaluate calcium balance, ovariectomized rats were fed a diet containing 0.5% calcium plus 0, 25, 50 or 100 grams (g) oligofructose/kg diet, or diets containing 1.0% Ca plus 0 or 50 g oligofructose/kg diet for 16 weeks. After 4, 8 and 16 weeks, there was a positive trend for increased calcium retention with oligofructose consumption. Consistent with other studies, they also found that phosphorus absorption and retention were unaffected by 0.5% or 1.0% dietary oligofructose (Scholz-Ahrens and Schrezenmeir 2002).

While several studies have determined the effect of prebiotics on mineral metabolism in rats, one study used humans as their subjects. Coudray and others (1997) evaluated the effect of inulin supplementation on the absorption of several minerals in humans. Inulin significantly increased calcium absorption (control, 21.3

± 12.5%; inulin, 33.7 ± 12.1%), but did not affect magnesium, iron, and zinc absorption (Coudray and others 1997).

Effect on Immune System

Gibson and Roberfroid (1995) demonstrated that inulin and oligofructose fermentation increased the production of short chain fatty acids, mainly acetate, butyrate and propionate in the gut. As mentioned previously, inulin and oligofructose increase the number of beneficial bacteria in the gut, which can help the body ward off infection (Kleessen and others 1997; Gibson and others 1995). Short chain fatty acid production may reduce the need for glutamine by the epithelial cells, which allows the cells in the immune system to use the glutamine (Jenkins and others 1999). Pratt and others (1996) showed that the natural killer cells in rats fed total parenteral nutrition supplemented with short chain fatty acids had significantly higher cytotoxic activity than the rats fed only the total parenteral nutrition. Therefore, it appears the fermentation of prebiotics into short chain fatty acids may improve the immune system.

Miscellaneous Human Studies

Cummings and Macfarlane (2002) reviewed the effect of oligofructose in humans. No change in blood glucose or insulin levels were observed when 25 g of oligofructose were fed to healthy subjects. With regards to fermentability, in pH controlled co-cultures of *B. infantis*, *E. coli* and *C. perfringens* with oligofructose being the only carbohydrate substrate, the bifidobacteria grew well and displayed an inhibitory effect on the growth of *E. coli* and *C. perfringens*. Human feeding

studies confirmed the effect of bifidobacteria on the inhibition of harmful bacteria (Cummings and Macfarlane 2002).

Saavedra and others (1999) evaluated the effects of supplementing infants with oligofructose. Infants (n=123) aged 4 to 24 months were divided into control and experimental groups. The control group received a commercially available infant cereal, while the experimental group received the same cereal supplemented with oligofructose at a concentration of 0.55 g per 15 g of dry cereal. The experimental group received a daily average of 1.1 g oligofructose. No significant differences were observed between the two groups in stool frequency and consistency or the occurrence of flatulence. The experimental group exhibited a significantly lower frequency of emesis, discomfort with bowel movements, and regurgitation. During a diarrheal episode, the supplemented group did not run a fever as often as the control group [8.25 and 21.4 (frequency per subject-year), respectively]. Saavedra and others (1999) concluded that supplementation with oligofructose resulted in a decrease in severity of symptoms with diarrheal disease.

Tagatose

In addition to the afore-mentioned prebiotic substances, there are other novel ingredients with prebiotic properties. One such ingredient is the carbohydrate, tagatose.

Chemical Properties

Tagatose is a monosaccharide that differs from fructose only in the positioning of the hydroxyl group on the fourth carbon (Levin and others 1995).

The structure of tagatose is shown in Figure 2.2. Tagatose is 92% as sweet as sucrose and has a similar bulk. Tagatose is a white anhydrous crystalline solid that has no odor. Its solubility in water is approximately 58% at 21°C, while its pH stability range is 2-7. It is found naturally in trace amounts in dairy products and some fruits (Levin 2002; Skytte 2006).

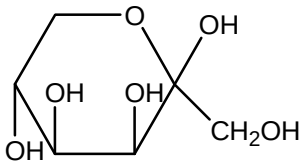

Figure 2.2. Structure of β-D-Tagatose.

Manufacturing

Tagatose must be able to be produced economically for it to be used as an ingredient in food. Although tagatose does occur in trace amounts in dairy products, its extraction would not be a practical option. A possible starting material for the production of tagatose is whey powder. Whey powder contains lactose, which could be hydrolyzed to release galactose. Galactose can then be converted into tagatose. The conversion of galactose into tagatose can occur one of two ways: using a chemical process (Beadle and others 1992) or an enzymatic process (Kim and others 2003). The chemical process involves two main steps: isomerization and neutralization. Isomerization is where galactose reacts with a basic metal hydroxide (preferably calcium hydroxide) in the presence of a catalyst at a low temperature to form an intermediate reaction product. The intermediate metal hydroxide-tagatose

complex is then neutralized with an acid to yield tagatose and a salt during the second step of the chemical process (Beadle and others 1992). The enzymatic process involves immobilizing a thermostable L-arabinose isomerase, Gali152, in alginate. The galactose isomerization reaction conditions were optimized for the conversion of galactose to tagatose (Kim and others 2003). Production of tagatose could be practical since it utilizes whey, a major byproduct of cheese manufacturing.

Health Benefits

The main health benefit of tagatose is that it is considered a prebiotic. Tagatose is not digested and is absorbed only minimally in the small intestine; the unabsorbed tagatose is fermented in the intestines to short chain fatty acids. Bertelsen and others (1999) determined that colonic butyrate increased in pigs fed a diet containing tagatose. The effect of tagatose consumption on colonic microflora has also been evaluated in humans. Colonic butyrate increased significantly in subjects after they consumed 10 g doses of tagatose three times a day. Also, the number of lactic acid bacteria increased while the number of coliform bacteria decreased (Bertelsen and others 1999). Prebiotics' role in improving immune function (Schley and Field 2002) may have aided in the reduction of coliform bacteria.

Tagatose provides several other health benefits. Tagatose has been proven to contribute less than 1.5 kcal/g, which is far less than sucrose at 4 kcal/g (Levin 2002). Livesey and Brown (1996) determined that the caloric value of tagatose in

rats was -2.2 kJ/g, which meant that tagatose basically had a zero energy value. Arla Foods had the caloric value of tagatose estimated using a pig model and factorial method; results ranged from 1.1-1.4 kcal/g. Therefore, Arla Foods requested that the U.S. Food and Drug Administration approve a conservative caloric value of 1.5 kcal/g (Levin 2002). The FDA responded with a "no objection" letter (Hoadley 1999). This officially set the legal caloric value of tagatose at 1.5 kcal/g, although the actual caloric value may be lower.

Donner and others (1999) demonstrated that tagatose does not elicit a glycemic response. After an oral loading of 75 g, tagatose was shown not to increase glucose or insulin concentrations in normal and diabetes mellitus patients. In diabetes mellitus patients, increases in blood glucose were lessened significantly by pretreatment with 75 g of tagatose before an oral loading of 75 g of glucose at 60 min, 120 min and 180 min. Because of its chemical properties discussed earlier, tagatose could possibly function as a sugar replacer. This function would be especially beneficial in foods for diabetic patients since tagatose does not cause a rise in blood sugar after consumption.

Tagatose obtained GRAS (generally recognized as safe) status in the United States for use as a sweetener in drugs and cosmetic products in 2000, and later obtained that status for use in foods and beverages (Levin 2002). Tagatose has been approved at maximum concentrations of 1% in diet carbonated beverages, 3% in light ice cream, 15% in regular and diabetic hard candies and 30% in icings or glazes used on baked goods in the U.S. (Rulis 2001). Tagatose use has also been

approved in Australia and New Zealand, as well as South Korea, Brazil, and South Africa (FSANZ 2004; Skytte 2006). In June 2004, the Joint Expert Committee of Food Additives approved an ADI of "not specified" for tagatose (JECFA 2004). The United Kingdom, and consequently the European Union, approved tagatose for use in foods in December 2005 (FSA 2005). Tagatose is currently found in several food products in Europe; these include a cocoa drink, chocolate-hazelnut spread, diet jam and chocolate bars (Damhert 2008). Tagatose appears to have potential as a sugar replacer and prebiotic in foods. However, tagatose must not break down during processing and storage to achieve these health benefits.

Adverse Side Effects

Donner and others (1999) evaluated the gastrointestinal effects of tagatose. None of 10 diabetes mellitus subjects had any side effects after consuming 10 g of tagatose. When they were given 20 g of tagatose, only one person experienced nausea. They found that 100% of subjects experienced adverse side effects after an oral load of 75 g of tagatose. Symptoms included diarrhea (81%), nausea (44%), flatulence (19%), bloating (31%), abdominal pain (25%) and headache (12%). The diarrhea occurred most commonly 2-3 hours after tagatose consumption and sudsided after approximately 4 hours. Bloating and nausea were reported to begin as early as 30 minutes after consumption.

Buemann and others (1999) also determined human gastrointestinal tolerance to tagatose. Seventy-three healthy males aged 21-40 years were given a piece of cake with 30 g of tagatose in the afternoon. Symptoms rated included

heartburn, stomach rumble, nausea, vomiting, stomach ache, flatulence and diarrhea. Most symptoms were reported to be light or moderate. No subjects reported vomiting. Nausea was reported to begin 1 to 2 hours after ingestion, lasting for 1 to 2 ½ hours. Flatulence was observed during a 4 to 5 hour period after ingestion.

Lee and Storey (1999) compared the side effects of tagatose and sucrose consumption in humans. Subjects received either a chocolate bar containing 20 g of tagatose or sucrose, and were instructed to eat two a day. The mean frequency of bathroom visits to pass watery feces was higher after consumption of the tagatose-containing chocolate bars than the sucrose chocolate. However, the increase was not statistically significant. Also, consumption of the tagatose chocolate was linked with significantly more subjects experiencing thirst, appetite loss, nausea, bloating, and flatulence.

Monosaccharide Reactions and Stability

Monosaccharide Degradation

Alkaline degradation

Monosaccharides participate in several rearrangements when placed in an aqueous alkaline solution. After going through ionization, mutarotation, enolization and isomerization, one monosaccharide transforms into another. In addition, monosaccharide degradation can occur.

The first transformation a monosaccharide goes through is ionization. Ionization is the removal or addition of an H^+ to change the molecule from an anion

to a cation or vice versa. The second rearrangement a monosaccharide participates in is mutarotation. This is simply a transition between the different hemiacetals of the monosaccharide. To achieve mutarotation, a sugar undergoes fast ionization, which is then followed by the opening of the acetal ring (de Bruijn and others 1986).

Enolization and isomerization follow ionization and mutarotation. These last two transformations involve three features. There must be a fast equilibrium between the cyclic sugar anions and their pseudo-cyclic carbonyl structures. Next, the formation of the enediol anion must occur. The formation of this anion occurs via an intramolecular proton shift from C_2 to pseudo-cyclic (Z)-enediol anions. The last feature to be observed is the reversal of the formation of the enediol anion, leading to isomerization (de Bruijn and others 1986). The reaction product is a different monosaccharide.

The starting intermediate in monosaccharide alkaline degradation reactions is the enediol anion. This intermediate will go through several reaction pathways before obtaining the final degradation products, carboxylic acids. The 1,2-enediol anion may participate in β-elimination as the first of five pathways leading to carboxylic acids. By undergoing β-elimination, the dicarbonyl compound 3-deoxy-erythro-hexosulose is formed. The dicarbonyl compound is unstable in basic conditions and may undergo either a benzilic acid rearrangement or a cleavage reaction. The benzilic acid rearrangement yields metasaccharinic, isosaccharinic and saccharinic acid. The cleavage reaction moves toward producing a carboxylic

acid and an aldehyde. The 1,2-enediol anion may also go through a retro-aldol reaction, producing two triose moieties. The trioses may participate in β-elimination, ultimately producing lactic acid, acetic acid and formic acid. Lastly, the dicarbonyl compound may undergo aldolization reactions (de Bruijn and others 1986).

As mentioned previously, monosaccharide degradation produces carboxylic acids. The majority of the monosaccharides are converted into low molecular weight carboxylic acids. These acids are composed of the same or smaller number of carbons as the original sugar. The low molecular weight acids are classified as ≤ C_6-carboxylic acids. Although monosaccharides primarily degrade into ≤ C_6-carboxylic acids, they may degrade into larger molecular weight acids referred to as > C_6-carboxylic acids. Monosaccharides may also degrade into miscellaneous products, but only a trace amount of them are formed (< 1%). These miscellaneous products include volatile non-acidic compounds and cyclic unsaturated aldehydes or ketones (de Bruijn and others 1986).

There are several kinetic features that are characteristic of monosaccharide alkaline degradation. The alkaline degradation reaction is considered a first order reaction. The hexose decomposition rate constant depends on the temperature and is proportional to the hydroxyl ion activity. Another feature notes the fact that divalent cations speed up the decomposition of monosaccharides and can possibly influence the composition of the final products (de Bruijn and others 1986).

There is a variety of reaction variables that may influence product formation. These include hydroxyl ion concentration, nature of the base and the concentration of the monosaccharide. When hydroxyl ion concentration is increased, there is an increase in lactic acid selectivity, while the total amount of formic, acetic, glycolic, glyceric and saccharinic acids decrease. It has also been noted that the cation of the base may influence the degradation pattern. The last variable that may influence product formation is the concentration of the monosaccharide. When a monosaccharide is diluted in solution, there is an almost complete conversion into $\leq C_6$-carboxylic acids. Contrary to this, there is significant formation of $> C_6$-carboxylic acids when the monosaccharide is concentrated in solution. One reaction variable, temperature, has been found to have no influence on product formation. It was determined that glucose degradation at 5°C and 80°C lead to the same product composition (de Bruijn and others 1986).

Acidic degradation

While monosaccharides may degrade in alkaline solution, they also undergo a set of very different reactions in acidic solution. Dehydration occurs when a monosaccharide is heated in a strong acidic solution. This dehydration reaction results in the formation of furfural compounds. Anhydro products may be formed in dilute acid solution (Wong 1989).

Furfural compounds form through a series of reactions. First, the carbonyl oxygen is protonated, followed by enolization to form the 1,2-enediol anion. Elimination at carbon three then leads to the formation of the enol form of 3-

deoxyglycosulose. This step is assisted by the protonation of the hydroxyl group at carbon three and nucleophilic addition at carbon one. Elimination at carbon four occurs next. This forms 3,4-unsaturated glycosulose. The conjugated system is extended by the protonation of the second carbon's carbonyl group, followed by enolization. Lastly, cyclodehydration of the oxygen at carbon two and five produces the furfural compound (Wong 1989).

A much simpler reaction occurs in dilute acid solution. When an aldohexose loses a water molecule, anhydro products are formed. The most common of these products are 1,6-anhydro sugars (Wong 1989).

Maillard Reaction

The Maillard reaction is a very common chemical reaction that occurs in food to produce brown discoloration, known as nonenzymatic browning. Foods that undergo this type of browning can also exhibit off-flavors and aromas. Understanding this reaction is critical to prevent it from occurring in foods. Tagatose is a reducing sugar, meaning it will react with amino acids to participate in the Maillard reaction and cause nonenzymatic browning. Tagatose is also lost during this reaction.

Basic overview

There are several steps that comprise the Maillard reaction. The first step to occur is the formation of glycosylamine. This proceeds via a Schiff-base formation between the amino group of the amine and the carbonyl group of the reducing sugar. The next step to occur is the Amadori rearrangement of the glycosylamine.

This compound is converted into a ketoseamine. The process by which this happens involves the nitrogen of the glycosylamine accepting a proton to form an amine salt. Rearrangement of the amine salt produces the enol form, which then may tautomerize to yield the keto form of the Amadori compound. The enol form undergoes elimination of the hydroxyl group at carbon three to form the 2,3-enol. The hydroxyl group at carbon four undergoes elimination to yield an unsaturated glycosulos-3-ene. This compound, glycosulos-3-ene, undergoes cyclodehydration to yield furaldehyde (Wong 1989). The production of furaldehyde leads to the formation of off-flavors associated with nonenzymatic browning. The brown coloration is developed from polymerization of the furaldehyde and copolymerization with amino compounds (Labuza and Baisier 1992). Many factors affect the Maillard reaction. The two factors relevant to the current study are pH and buffer properties.

pH effect

Ashoor and Zent (1984) determined the effect of pH on the intensity of nonenzymatic browning. Phosphate buffer solutions (0.05 M) at pH 6.0 and 7.5 and carbonate buffer solutions (0.05 M) at pH 8.0, 9.0, 9.5, 10.0, 11.0 and 12.0 were used in the study. The amino acids chosen to show the effect of pH were L-lysine, placed in the high browning group, L-alanine, from the intermediate browning group, and L-arginine, representing the low browning group. The sugars chosen were D-glucose, D-fructose and α-lactose. It was found that as the pH of the amino acid-sugar solution increased, the intensity of the Maillard browning increased.

This occurred to a maximum pH of 10.0, and then decreased at higher pH values. These results were similar for both D-glucose and D-fructose. Amino acids need to be unprotonated to react with the reducing sugars; a lesser extent of protonation occurs at the higher pH.

Buffer effect

Buffer type and concentration may also affect the Maillard reaction. As shown by Bell (1997), various buffers have differing effects on brown pigment formation. Solutions containing 0.1 M glucose and 0.1 M glycine in citrate and phosphate buffers at pH 7 and 25°C were analyzed. The buffer concentrations were 0.02, 0.05, 0.2 and 0.5 M. Results indicated that the rates of glycine loss and brown pigment formation increased with increasing phosphate buffer concentrations. Contrary to this, reaction rates did not differ significantly ($P > 0.05$) from zero in citrate buffer. Buffer concentration did not affect either the rate of glycine loss or the rate of brown pigment formation in the citrate buffer. Bell concluded that both rates were enhanced in phosphate buffer, while neither were affected in citrate buffer. These results indicate that buffer type and concentration can affect the rate of the Maillard reaction.

Justification and Objective

Nutraceuticals and prebiotics have made headlines in recent years for their health benefits. Tagatose is a monosaccharide that falls into both of these categories. For its prebiotic effect to be achieved, tagatose in food products must not be lost during their distribution and storage. However, data on the storage

stability of tagatose are lacking. Therefore, the objective of this study was to evaluate the storage stability of tagatose in various solutions as affected by pH, buffer type, buffer concentration and temperature.

CHAPTER 3: MATERIALS AND METHODS

Sample Preparation

Phosphate and citrate buffers were used in the experiment. Four different sodium phosphate buffer solutions were prepared: 0.02 M sodium phosphate monobasic, 0.02 M sodium phosphate dibasic, 0.1 M sodium phosphate monobasic and 0.1 M sodium phosphate dibasic. Prepared in the same manner, a set of four bulk citrate buffer solutions were made: 0.02 M sodium citrate, 0.02 M citric acid, 0.1 M sodium citrate and 0.1 M citric acid. Tagatose was added to each of these eight solutions at a concentration of 0.05 M (or about 1%). To evaluate the effect of the Maillard reaction, these eight buffer solutions were prepared again, containing 0.05 M tagatose and 0.05 M glycine.

Aliquots of the 0.02 M sodium phosphate monobasic and dibasic solutions containing 0.05 M tagatose were mixed to give a 0.02 M buffer solution at pH 7. This protocol was repeated for the 0.1 M phosphate buffer, yielding a 0.1 M buffer solution at pH 7. To obtain a 0.02 M phosphate buffer solution at pH 3, 0.02 M phosphoric acid was required. However, it was not desirable to add tagatose to this phosphoric acid solution. Therefore, the phosphoric acid solution was mixed with a 0.02 M phosphate monobasic solution to raise the pH to approximately 2.5. Tagatose was added to the pH 2.5 buffer solution. The phosphate monobasic solution and the pH 2.5 buffer solution, both containing 0.05 M tagatose, were then

mixed to obtain a final pH of 3. This procedure was repeated for the 0.1 M phosphate buffer at pH 3, with 0.1 M phosphoric acid replacing the 0.02 M phosphoric acid. These phosphate buffer solutions were prepared again containing the tagatose and glycine. The 0.02 M and 0.1 M citrate buffer solutions were obtained similarly by mixing the appropriate citric acid and sodium citrate solutions to yield 0.02 M and 0.1 M citrate buffers at pH 3 and 7. Eight buffer solutions containing tagatose resulted consisting of two buffer types, two buffer concentrations and two pH values. Also, eight buffer solutions containing tagatose and glycine resulted, consisting of two buffer types, two buffer concentrations and two pH values. Each of these 16 solution types was to be stored at three temperatures to give a total of 48 experiments.

A great amount of care was taken to prevent contamination of the solutions. Certified contaminant-free clear vials with septum-containing caps (I-Chem, Chase Scientific Glass, Rockwood, TN) were used in this study. A small amount (i.e., around 5 mL) of the sample solution was placed in a sterile syringe, with a sterile 0.20 μm nylon filter and sterile needle. The solution was injected into the vial via the septum-containing cap. The vial was then shaken to rinse away any potential microorganisms. The liquid was removed via the syringe and needle and then discarded. After rinsing, approximately 40 mL of each sample solution was transferred into triplicate vials using a sterile syringe, filter and needle. The resulting 144 vials (48 experimental solutions in triplicate) were placed in 20°, 30°

and 40°C incubators to determine the storage stabilities of tagatose as affected by buffer type, buffer concentration, pH and temperature.

Sampling Procedure

Aliquots of the experimental solutions were removed from storage for tagatose analysis 11-13 times over a nine month period. For example, the tagatose solutions in citrate buffer were removed for analysis at time intervals of 0, 20, 44, 55, 73, 95, 118, 153, 189, 224 and 265 days. The tagatose solutions in phosphate buffer at pH 7 were removed for analysis at time intervals of 0, 12, 20, 34, 44, 55, 73, 95, 118, 153, 189, 224 and 265 days. All three vials were sampled at time zero. Then, at each time, a 2-3 mL aliquot was removed from two of the three bottles using a sterile needle and syringe. Sampling rotated between the three vials. The sample was then passed through a 0.45 μm nylon filter and transferred into cryogenic vials. The vials were labeled with a code that consisted of buffer type, buffer concentration, pH, temperature and data point number. For example, "02P-3-T20-A1" meant that the sample was 0.02 M phosphate buffer at pH 3 and 20°C. The "T" stood for "tagatose", the "A" signified which vial in the triplicate series (A, B, or C) it was, and the "1" represented the particular data point. "TG" was used for tagatose/glycine solutions.

Tagatose Analysis

The tagatose concentrations of the solutions were analyzed using reverse-phase high performance liquid chromatography (HPLC). The column used was a 250 x 4.6 mm LUNA 5μ Amino column (Phenomenex, Torrance, CA). The mobile

phase consisted of a 85/15 (v/v) acetonitrile/deionized water solution flowing at 2.5 mL/min. Tagatose was detected using refractive index measurements. Data were integrated by a Hewlett-Packard integrator. Using tagatose standard curves, the concentrations of tagatose in the experimental solutions were determined. A sample chromatogram is shown in Figure 3.1 while Figure 3.2 shows a typical calibration curve.

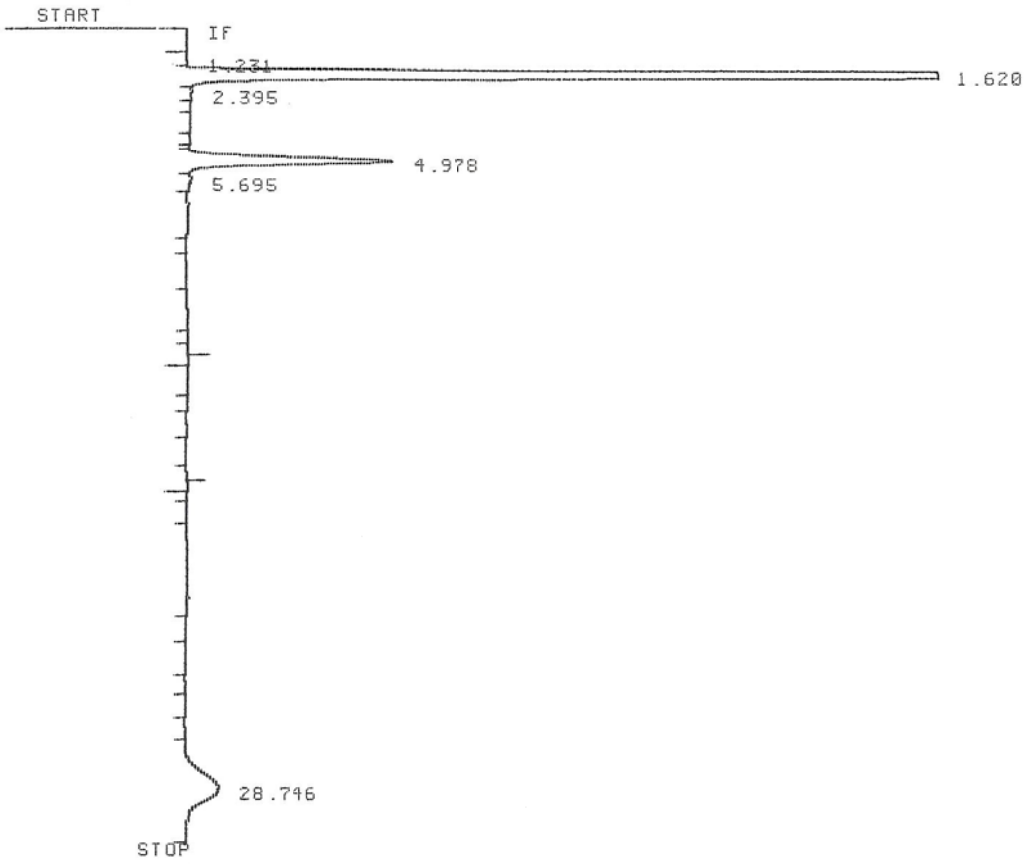

Figure 3.1. Sample HPLC Chromatogram for the Analysis of Tagatose in Buffer with 0.05 M Glycine using a Luna Amino Column (Phenomenex, Torrance, CA) and Mobile Phase Consisting of 85/15 (v/v) Acetonitrile/Water Running at 2.5 mL/min. Tagatose Elutes at Approximately 4.9 min and Glycine at 28.7 min.

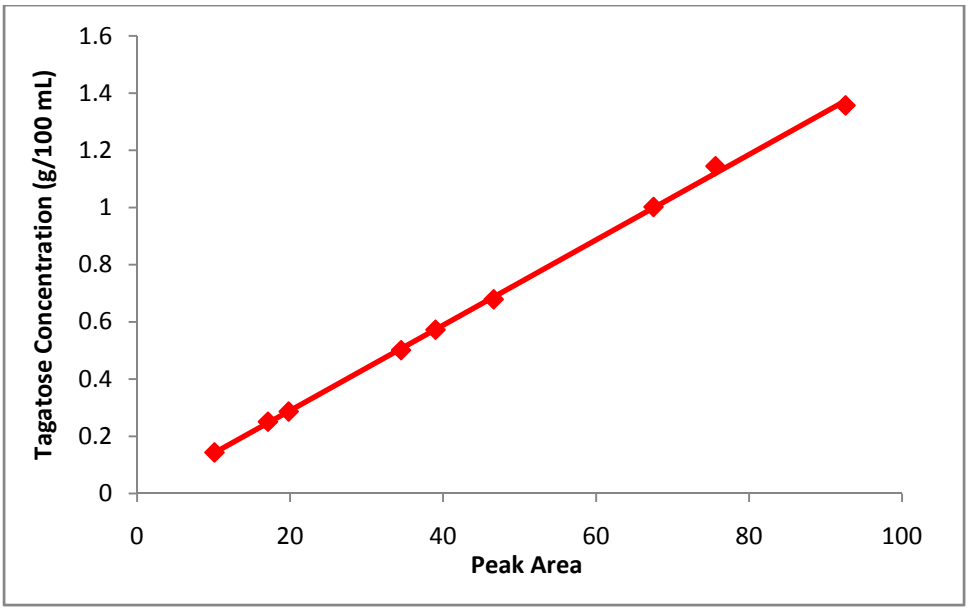

Figure 3.2. Sample Calibration Curve for Tagatose Analysis by HPLC.

Browning Analysis

Brown pigmentation was measured via spectrophotometry. Aliquots (1 mL) of pre-filtered samples were placed in semi-micro methacrylate cuvettes. Absorption was determined at 420 nm.

Data Analysis

Because the kinetic data did not follow a definite kinetic model, it was necessary to evaluate initial reaction rates. Therefore, the loss of tagatose for the initial 100 days was modeled using pseudo-first order kinetics. Rate constants with 95% confidence limits were calcuated using computerized least-squares analysis (Labuza and Kamman 1983). The same model was used whether or not glycine was present.

CHAPTER 4: RESULTS AND DISCUSSION

Tagatose Degradation

The degradation of tagatose in solution is affected by several variables, including pH, buffer type and concentration, and temperature. Degradation profiles were created to demonstrate these effects. Figures 4.1 and 4.2 illustrate the effect of pH on tagatose degradation in various phosphate and citrate buffers. Figures 4.3 and 4.4 show the effect of buffer type and concentration on the loss of tagatose in solutions at pH 3 and 7, respectively. The effect of temperature on tagatose degradation in solutions is shown in Figures 4.5 and 4.6.

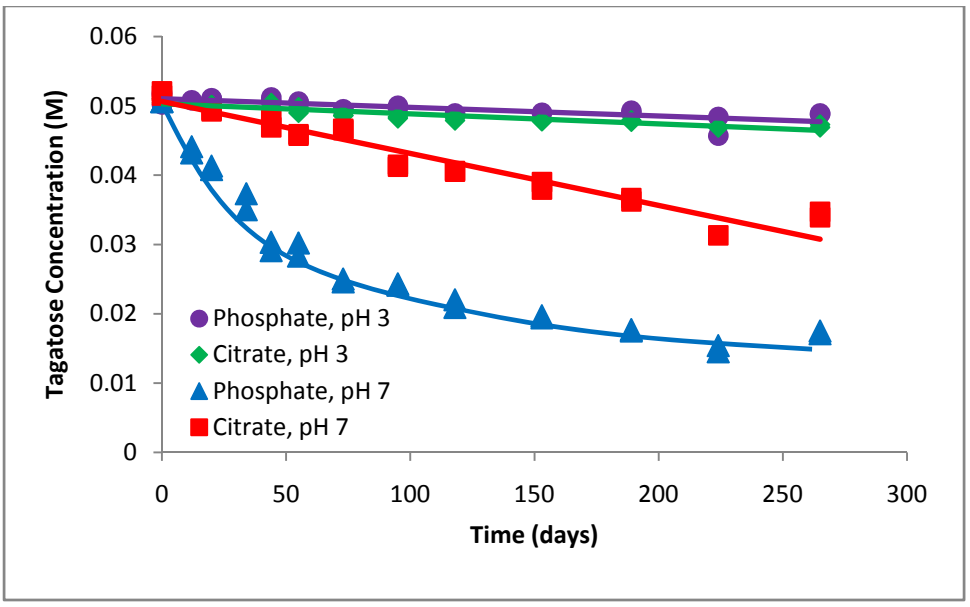

Figure 4.1. Tagatose Loss in 0.1 M Buffer Solutions at 40°C as Affected by pH.

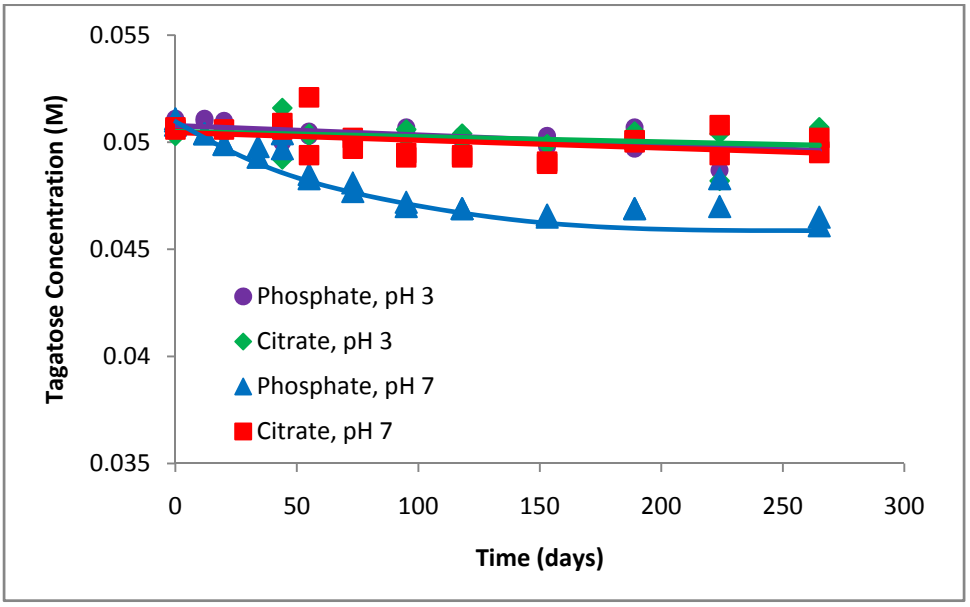

Figure 4.2. Tagatose Loss in 0.02 M Buffer Solutions at 20°C as Affected by pH.

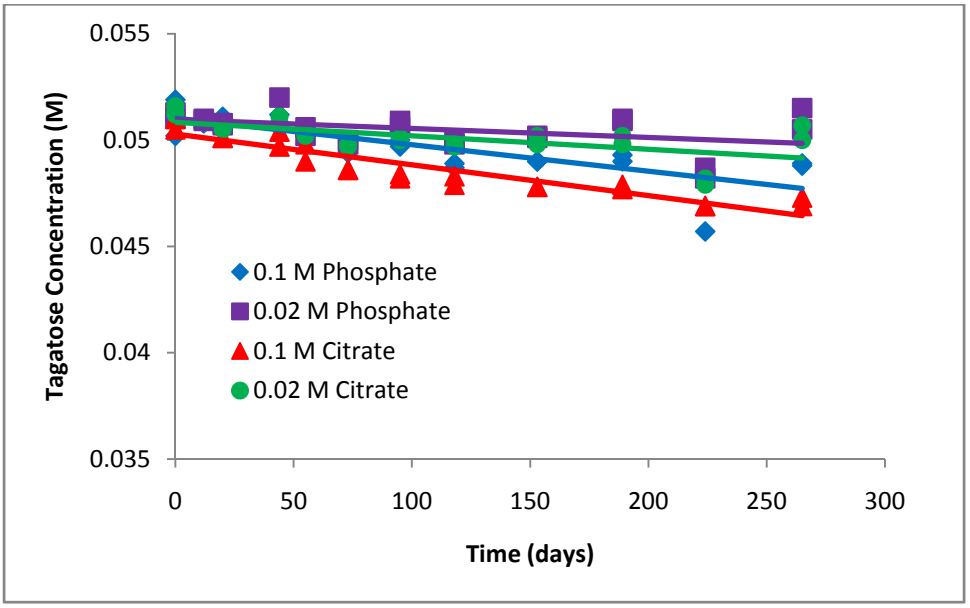

Figure 4.3. Tagatose Loss in Solution at pH 3 and 40°C as Affected by Buffer Type and Concentration.

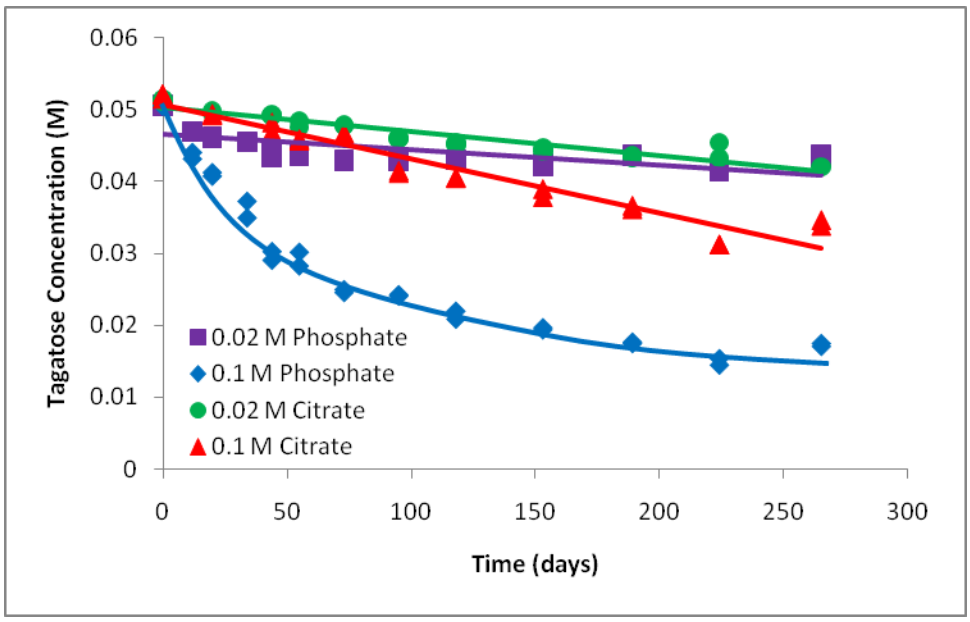

Figure 4.4. Tagatose Loss in Solution at pH 7 and 40°C as Affected by Buffer Type and Concentration.

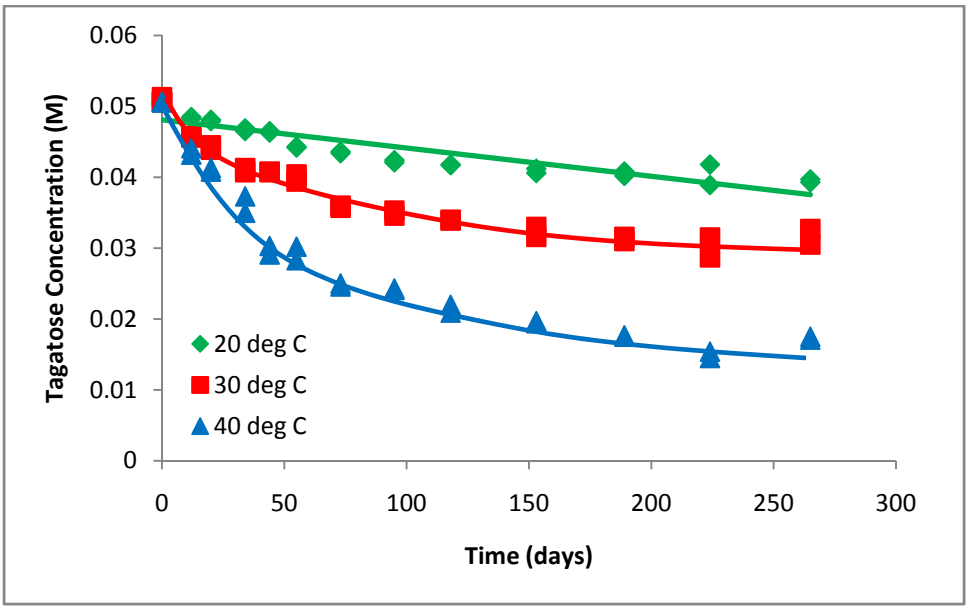

Figure 4.5. Tagatose Loss in 0.1 M Phosphate Buffer at pH 7 as Affected by Temperature.

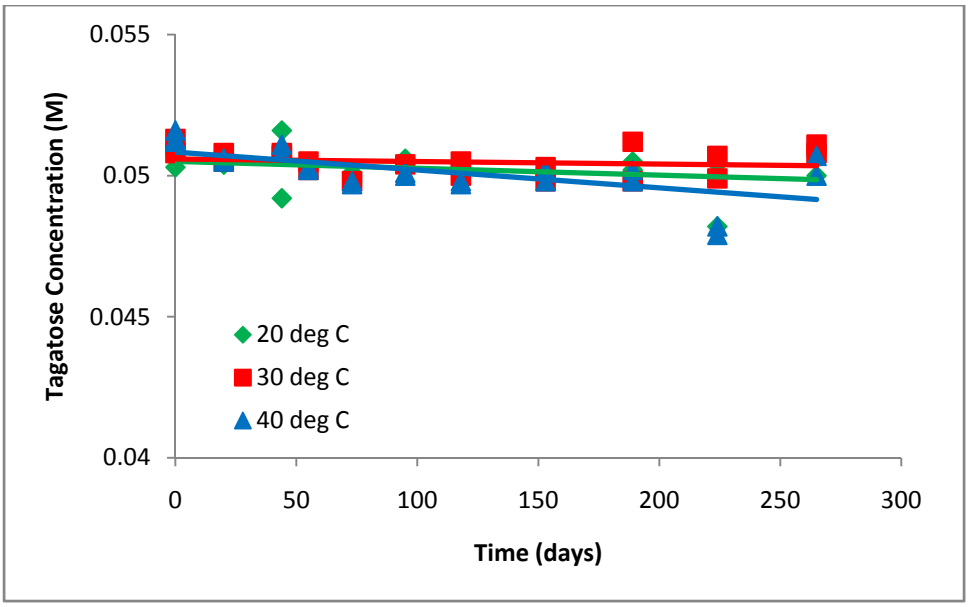

Figure 4.6. Tagatose Loss in 0.02 M Citrate Buffer at pH 3 as Affected by Temperature.

As shown in these figures, especially in phosphate buffer at pH 7, the degradation of tagatose appeared to plateau at approximately one hundred days of storage. Measuring the pH of select solutions indicated a drop in pH had also occurred. This drop in pH could explain the plateau, as will be discussed later. However, the existence of the plateau made traditional kinetic modeling difficult. Therefore, the initial loss of tagatose (i.e., the first 100 d) was modeled using pseudo-first order kinetics to determine degradation rate constants. Figure 4.7 illustrates the initial loss of tagatose over 100 days modeled using pseudo-first order kinetics. The slopes of these plots are equivalent to the pseudo-first order rate constants (k_{obs}). Pseudo-first order rate constants for the initial loss of tagatose in

solution were calculated using least-squares analysis and are shown in Table 4.1. Using the pseudo-first order kinetic model, ln (% tagatose remaining) = $-k_{obs}$(time), and the data in Table 4.1, the predicted percent losses of tagatose in solution after 100 d of storage were calculated. These percentages are listed in Table 4.2.

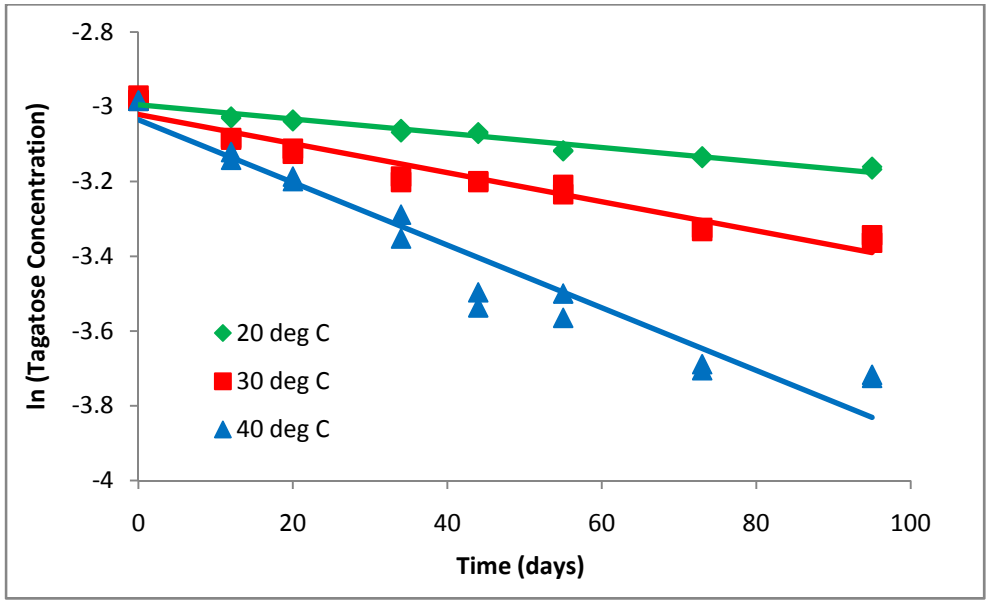

Figure 4.7. Initial Loss of Tagatose in 0.1 M Phosphate Buffer at pH 7 modeled using Pseudo-First Order Kinetics as Affected by Temperature.

Table 4.1. Pseudo-First Order Rate Constants (d^{-1}) with 95% Confidence Limits for the Initial Loss of Tagatose in Solution.

Sample	Temperature		
	20°C	30°C	40°C
pH 3			
0.02 M Phosphate	1.41 ± 1.01*	1.39 ± 1.76[#]	1.37 ± 1.99[#]
0.1 M Phosphate	0.35 ± 1.07[#]	2.13 ± 1.62	3.27 ± 1.68
0.02 M Citrate	0.76 ± 2.03[#]	1.96 ± 1.22	2.97 ± 1.23
0.1 M Citrate	1.65 ± 2.04[#]	2.61 ± 1.92	5.47 ± 1.28
pH 7			
0.02 M Phosphate	8.22 ± 1.38	10.5 ± 2.48	16.7 ± 4.63
0.1 M Phosphate	19.0 ± 1.92	38.9 ± 5.40	83.8 ± 11.6
0.02 M Citrate	2.29 ± 2.50[#]	4.89 ± 1.50	10.9 ± 1.56
0.1 M Citrate	4.10 ± 2.18	7.39 ± 2.81	21.0 ± 4.55

*Rate constants (± 95% CL) have been multiplied by 10^4.
[#] Not different from zero.

Table 4.2. Predicted Percent Loss of Tagatose in Solution at 100 days.

Sample	Temperature		
	20°C	30°C	40°C
pH 3			
0.02 M Phosphate	1.4%	1.4%	1.4%
0.1 M Phosphate	0.4%	2.1%	3.2%
0.02 M Citrate	0.8%	1.9%	2.9%
0.1 M Citrate	1.6%	2.6%	5.3%
pH 7			
0.02 M Phosphate	7.9%	10.0%	15.4%
0.1 M Phosphate	17.3%	32.2%	56.7%
0.02 M Citrate	2.3%	4.8%	10.3%
0.1 M Citrate	4.0%	7.1%	18.9%

Effect of pH

The effect of pH on tagatose degradation is exemplified in Figures 4.1 and 4.2 and quantified in Tables 4.1 and 4.2. Tagatose degradation was very slow in all solutions at pH 3. At pH 7, faster degradation was observed. For example, in 0.1 M phosphate buffer at pH 7 and 40°C, 56.7% of tagatose was lost at 100 d compared

to only 3.2% at pH 3. Similar results were seen for tagatose loss in citrate buffer as well. In 0.02 M citrate buffer at pH 7 and 20°C, 2.3% of tagatose was lost in comparison to 0.8% at pH 3. The pH effect was more dramatic in phosphate buffer at elevated temperatures.

At pH 7, tagatose is probably undergoing a mild alkaline degradation reaction that would not occur at pH 3. As described by de Bruijn and others (1986), monosaccharides placed in an aqueous alkaline solution undergo various rearrangements before degradation takes place. Through the processes of ionization, mutarotation, and enolization, an enediol anion is formed. The enediol intermediate goes through several reaction pathways before obtaining the final degradation product, carboxylic acids (de Bruijn and others 1986). These acidic products decrease the pH of the solution, and cause the tagatose degradation rate to slow down. As mentioned previously, the pH of some solutions initially at pH 7 decreased during storage. In Figures 4.1 and 4.2, the plateau after storing the pH 7 solutions for 100 d could be explained by the decreased pH associated with the formation of acidic products reducing subsequent tagatose loss.

Monosaccharides can also degrade in acidic solutions. When a monosaccharide is heated in a strong acid solution, dehydration occurs and furfural compounds are formed (Wong 1989). Under the milder conditions of this study, such a reaction was minimal. The greatest loss of tagatose at pH 3 was in 0.1 M citrate buffer at 40°C, where about 5% was lost after 100 days.

Effect of Buffer Type and Concentration

Figures 4.3 and 4.4 illustrate the effect of buffer type and concentration on tagatose degradation. At pH 3, both buffer types and concentrations showed similar results. The tagatose in the 0.1 M citrate buffer solution degraded slightly more than the other solutions, as shown in Figure 4.3. A greater effect of buffer type was seen at a higher pH. At pH 7 and 40°C, tagatose degraded more in the 0.1 M phosphate buffer solution with 56.7% lost at 100 d in comparison to 18.9% lost in the 0.1 M citrate buffer. More tagatose was lost at a higher buffer concentration as well. In the 0.1 M phosphate buffer at pH 7 and 30°C, 32.2% of tagatose was lost at 100 d as compared to 10.0% loss in 0.02 M phosphate buffer at the same conditions. These results are similar to those reported by Bell and Wetzel (1995) and Pachapurkar and Bell (2005) for other reactions.

Bell and Wetzel (1995) showed that the rate of aspartame degradation increased as buffer concentration increased. Aspartame in phosphate buffer was found to degrade faster than in citrate buffer at the same conditions. Also, degradation occurred faster at pH 7 rather than pH 3. The fastest degradation was in 0.1 M phosphate buffer at pH 7, which is similar to the tagatose data.

Pachapurkar and Bell (2005) found that thiamin also degraded fastest in 0.1 M phosphate buffer at pH 7. Similar to the findings of Bell and Wetzel (1995), Pachapurkar and Bell (2005) found that the rate of degradation increased as the buffer concentration increased. Thiamin degradation also occurred faster in phosphate buffer than citrate buffer at pH 7.

The mechanisms of both aspartame and thiamin degradation at pH 7 involve the transfer of protons. The primary component in phosphate buffer at pH 7 is the dibasic anion (HPO_4^{-2}), which appears to be much more efficient at the required proton transfers than citrate anions. Thus, the reactions proceed faster in phosphate buffer than citrate buffer at pH 7. Similarly, based on the discussion of glucose degradation (de Bruijn and others 1986; Wong 1989; Robyt 1998), the initial ionization and mutarotation of tagatose requires the removal of a proton from the hydroxyl at carbon 2 to form the anion. The subsequent opening of the ring (i.e., mutarotation) requires moving a proton from carbon 3 to the oxygen on carbon 6. The resulting product is the enediol anion, which then proceeds into additional degradation pathways. In the current study, the phosphate dibasic anion at pH 7 is a better facilitator of the intramolecular proton shift than the citrate anion, leading to the faster formation of the enediol anion and the degradation that follows.

Effect of Temperature

The effect of temperature on tagatose degradation is shown in Figures 4.5 and 4.6. Both figures show that the rate of tagatose degradation increases as temperature is increased. In 0.02 M phosphate buffer at pH 7, 7.9% of tagatose is lost at 20°C, 10.0% at 30°C and 15.4% at 40°C at 100 d of storage. The effect of temperature on degradation is less noticeable at pH 3 due to very little tagatose loss and the accompanied scatter of the data. Although not as noticeable, the higher temperature does result in more tagatose degradation.

The activation energy (E_A) gives an indication of the sensitivity of the reaction rates to temperature. A higher E_A value means reaction rates change more with temperature whereas a lower E_A value means rates will change less with temperature. Because tagatose loss was minimal at pH 3 (Tables 4.1 and 4.2), these activation energies may be quite error prone and were therefore not calculated. The greater tagatose loss at pH 7 makes these activation energies more reliable. Table 4.3 lists the activation energies for the initial loss of tagatose in pH 7 buffer solutions. Most activation energies for the initial loss of tagatose at pH 7 ranged from 13.5-14.8 kcal/mol. It is interesting that the E_A value for tagatose loss in 0.02 M phosphate buffer was much lower at 6.4 kcal/mol. The reason for this lower E_A value is unclear and warrants further investigation.

Table 4.3. Activation Energies (E_A) for the Initial 100 d of Tagatose Degradation in Solution at pH 7

Sample	E_A (kcal/mol)
0.02 M Phosphate	6.4
0.1 M Phosphate	13.5
0.02 M Citrate	14.2
0.1 M Citrate	14.8

Participation of Tagatose in Maillard Reaction

Degradation profiles were created to demonstrate the effects of pH, buffer type and concentration, and temperature on tagatose degradation in the presence of added glycine. Figures 4.8 and 4.9 illustrate the effect of pH on tagatose degradation. Figures 4.10 and 4.11 show the effect of buffer type and concentration

on the loss of tagatose. The effect of temperature on tagatose degradation is seen in Figures 4.12 and 4.13. Again, due to the plateau that was observed after 100 d, only the data for the initial 100 d was used to model the loss via pseudo-first order kinetics. An example of the pseudo-first order plot is shown in Figure 4.14, the rate constants are listed in Table 4.4, and values for percent loss are listed in Table 4.5.

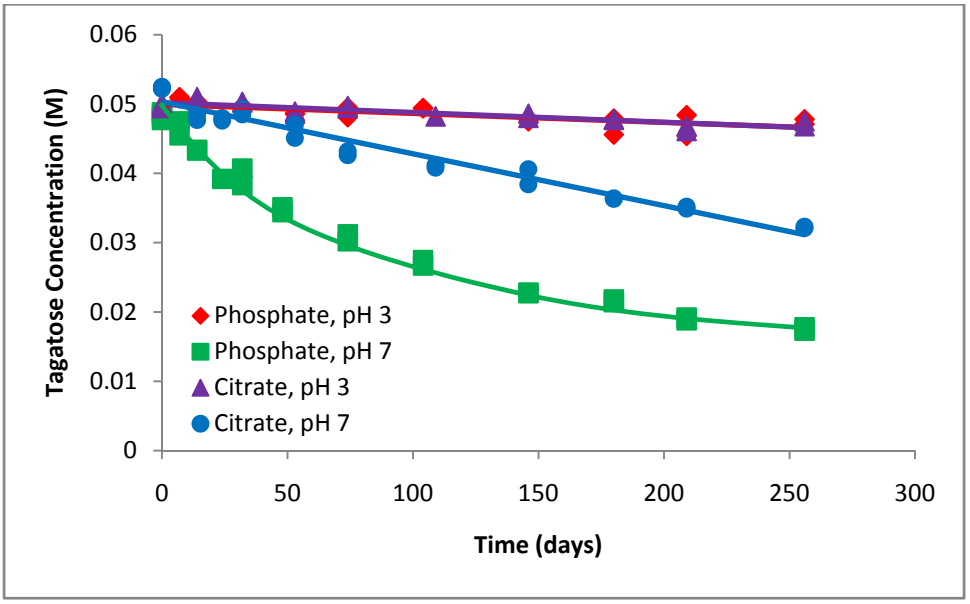

Figure 4.8. Tagatose Loss in 0.1 M Buffer Solutions also containing 0.05 M Glycine at 40°C as Affected by pH.

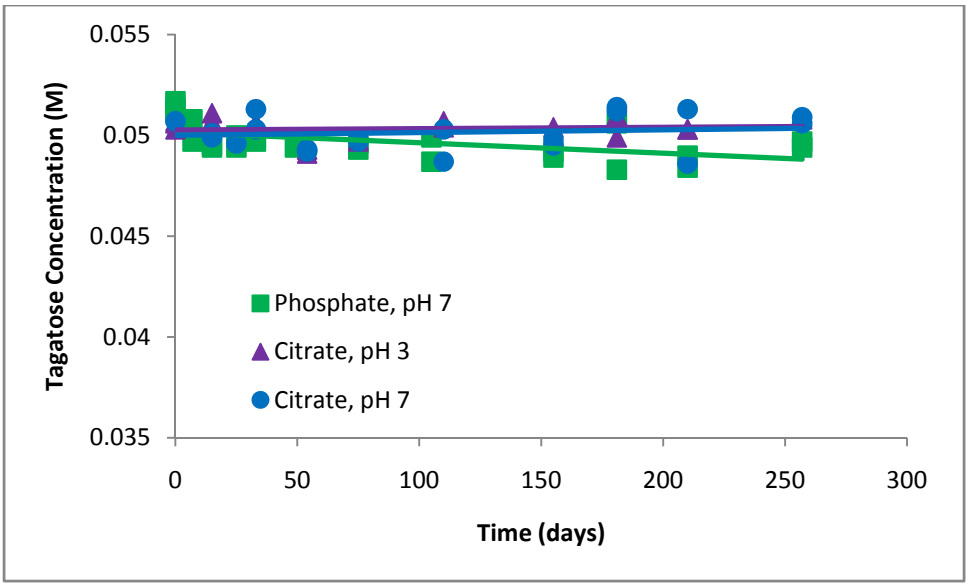

Figure 4.9. Tagatose Loss in 0.02 M Buffer Solutions also containing 0.05 M Glycine at 20°C as Affected by pH (No phosphate pH 3 due to mold).

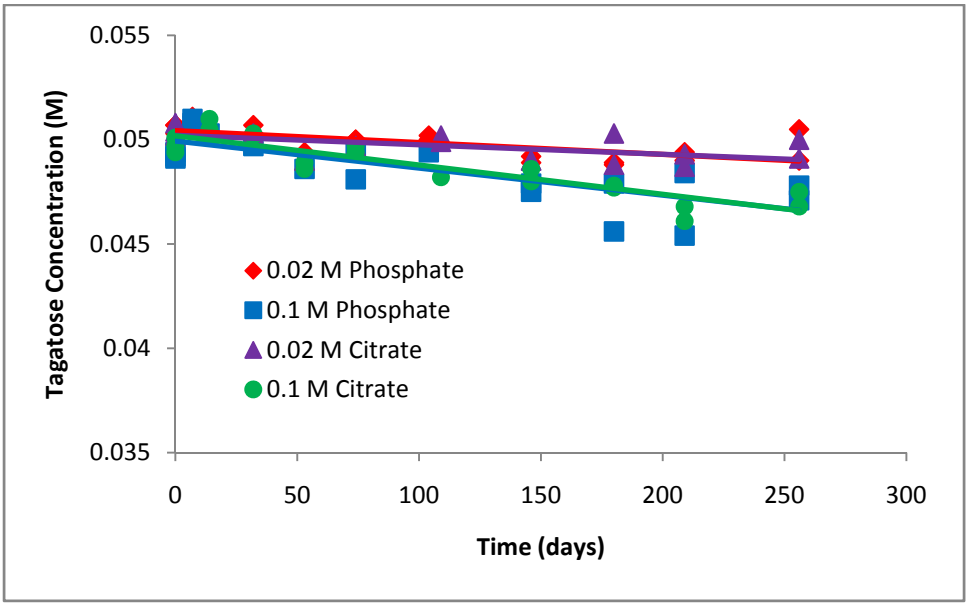

Figure 4.10. Tagatose Loss in Solution at pH 3 and 40°C also containing 0.05 M Glycine as Affected by Buffer Type and Concentration.

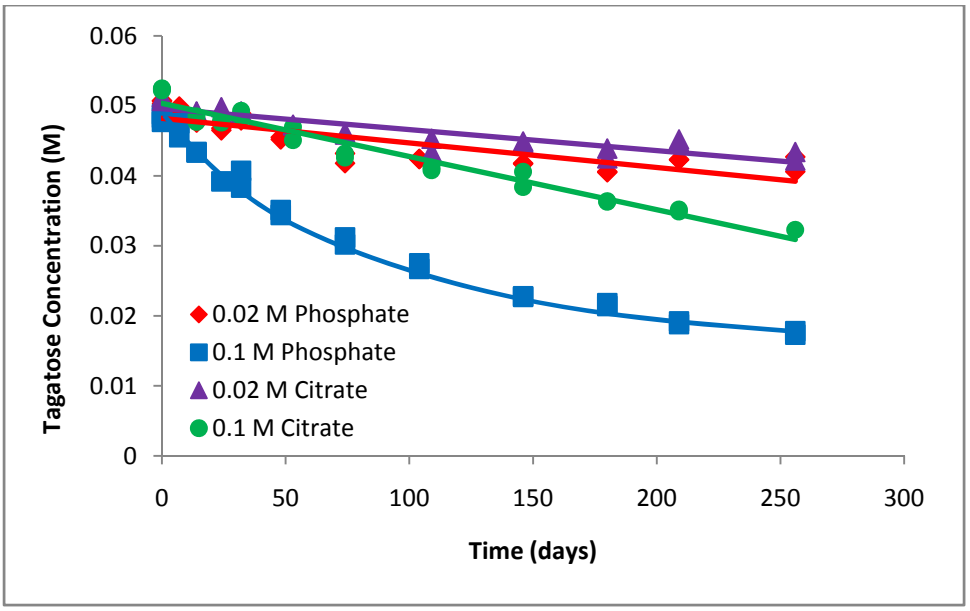

Figure 4.11. Tagatose Loss in Solution at pH 7 and 40°C also containing 0.05 M Glycine as Affected by Buffer Type and Concentration.

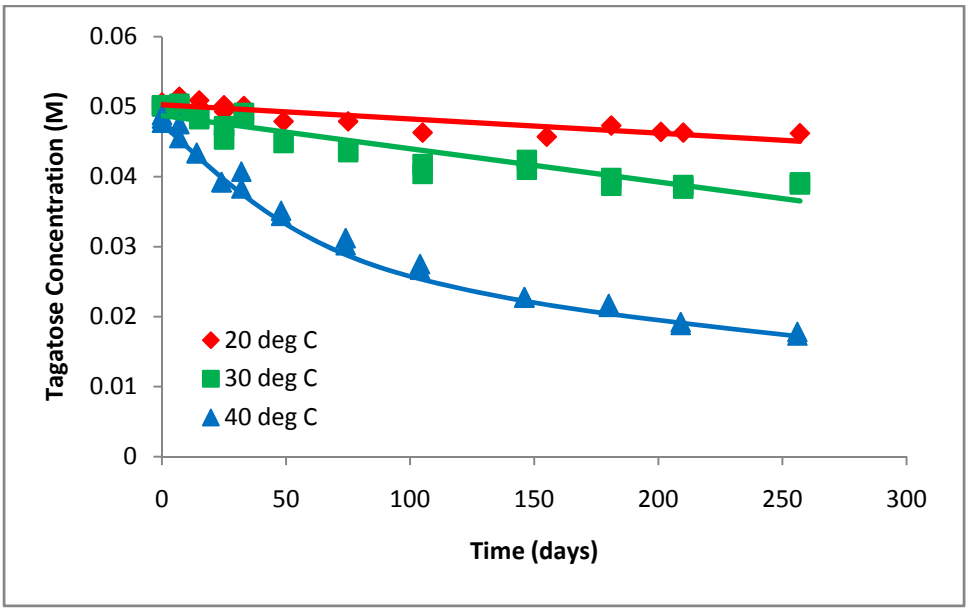

Figure 4.12. Tagatose Loss in 0.1 M Phosphate Buffer also containing 0.05 M Glycine at pH 7 as Affected by Temperature.

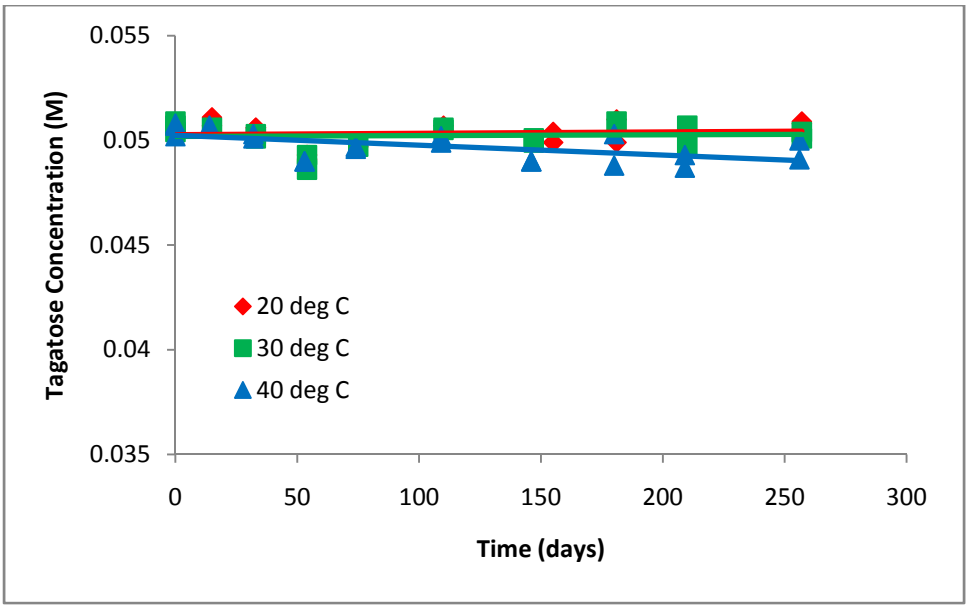

Figure 4.13. Tagatose Loss in 0.02 M Citrate Buffer also containing 0.05 M Glycine at pH 3 as Affected by Temperature.

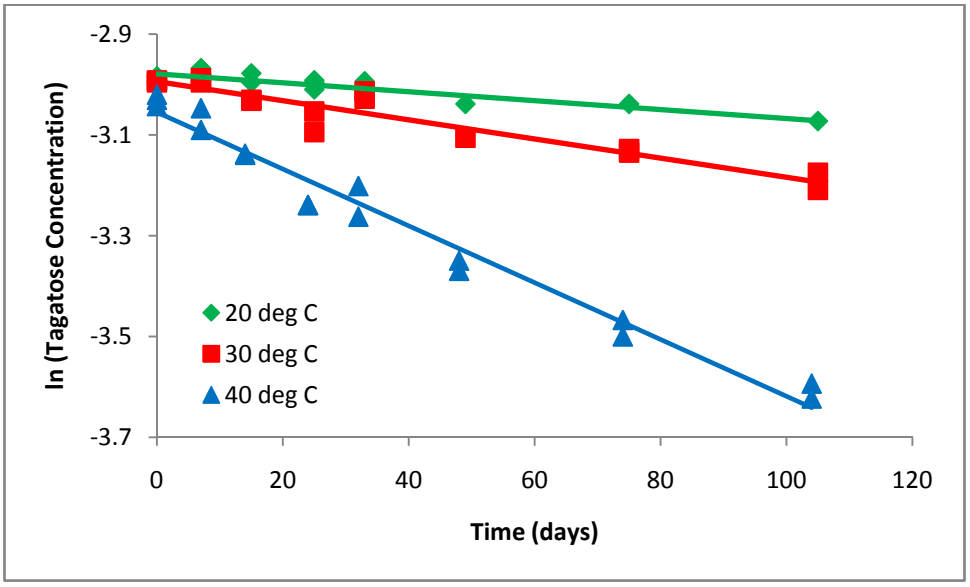

Figure 4.14. Initial Loss of Tagatose in 0.1 M Phosphate Buffer also containing 0.05 M Glycine at pH 7 modeled using Pseudo-First Order Kinetics as Affected by Temperature.

Table 4.4. Pseudo-First Order Rate Constants (d^{-1}) with 95% Confidence Limits for the Initial Loss of Tagatose in Solutions also containing 0.05 M Glycine.

Sample	Temperature		
	20°C	30°C	40°C
pH 3			
0.02 M Phosphate	N/A*	N/A	1.55 ± 1.31**[#]
0.1 M Phosphate	1.59 ± 2.30[#]	1.29 ± 9.23[#]	1.97 ± 2.36[#]
0.02 M Citrate	1.02 ± 1.94[#]	1.02 ± 2.13[#]	1.50 ± 1.72[#]
0.1 M Citrate	0.30 ± 1.56[#]	0.16 ± 1.93[#]	3.07 ± 2.50
pH 7			
0.02 M Phosphate	2.97 ± 2.03	8.70 ± 1.64	17.5 ± 3.55
0.1 M Phosphate	8.87 ± 2.65	19.0 ± 3.43	56.4 ± 5.14
0.02 M Citrate	2.00 ± 1.88	4.28 ± 3.64	12.7 ± 1.93
0.1 M Citrate	3.26 ± 1.48	3.89 ± 2.20	21.6 ± 3.79

*N/A: Not available due to mold contamination.
**Rate constants (± 95% CL) have been multiplied by 10^4.
[#] Not different from zero.

Table 4.5. Predicted Percent Loss of Tagatose in Solutions containing 0.05 M Glycine at 100 days.

Sample	Temperature		
	20°C	30°C	40°C
pH 3			
0.02 M Phosphate	N/A*	N/A	1.5%
0.1 M Phosphate	1.6%	1.3%	2.0%
0.02 M Citrate	1.0%	1.0%	1.5%
0.1 M Citrate	0.3%	0.2%	3.0%
pH 7			
0.02 M Phosphate	2.9%	8.3%	16.1%
0.1 M Phosphate	8.5%	17.3%	43.1%
0.02 M Citrate	2.0%	4.2%	11.9%
0.1 M Citrate	3.2%	3.8%	19.4%

*N/A due to mold contamination.

Effect of pH

The effect of pH is shown in Figures 4.8 and 4.9 as well as in Tables 4.4 and 4.5. Tagatose degradation in the presence of glycine occurred more rapidly at

pH 7 rather than pH 3. In fact, the 95% confidence limits of k_{obs} at pH 3 were larger than the rate constants themselves meaning that these rate constants were not different from zero. This pH effect could be due to a combination of alkaline degradation (discussed previously) and the Maillard reaction. At pH 7, a larger amount of glycine contains the unprotonated amine, which is more reactive with tagatose than the protonated amine at lower pH values. This pH effect on the Maillard reaction is consistent with that reported previously (Labuza and Baisier 1992).

Effect of Buffer Type and Concentration

Figures 4.10 and 4.11 demonstrate the effect of buffer type and concentration on tagatose degradation. Figure 4.10, Table 4.4, and Table 4.5 show that the degradation rates of tagatose in 0.1 M phosphate and citrate buffers at pH 3 are very similar. Very little degradation is observed at pH 3. Figure 4.11 shows that degradation at pH 7 occurred faster in 0.1 M phosphate buffer. At 100 d of storage, the percent of tagatose loss in 0.1 M phosphate buffer at pH 7 and 40°C was 43.1%, compared to 19.4% in 0.1 M citrate buffer at pH 7 and 40°C. Overall, tagatose degradation occurred faster in 0.1 M phosphate buffer solution. These results are similar to those reported by Bell (1997). The initial step of the Maillard reaction requires proton transfers to form the glycosyl amine, which phosphate dibasic anions do better than citrate anions.

Effect of Temperature

The effect of temperature on tagatose degradation in the presence of glycine is shown in Figures 4.12 and 4.13. Both graphs show that degradation of tagatose increases as the temperature of the solution is increased. In 0.02 M citrate buffer at pH 7, tagatose loss is 2.0% at 20°C, 4.2% at 30°C and 11.9% at 40°C. This result is consistent with basic principles of reaction kinetics where rates are faster at higher temperatures.

To evaluate the temperature sensitivity, the activation energies (E_A) for tagatose loss in the tagatose/glycine solutions were calculated. Because the rate constants for tagatose loss were basically zero at pH 3 (Table 4.4), the activation energies were unable to be calculated with much certainty. At pH 7, all E_A values were 16-17 kcal/mol (Table 4.6). Thus, regardless of buffer type or concentration, the temperature sensitivity for tagatose loss is similar at pH 7 in the presence of glycine. These E_A values could be used to predict tagatose loss at any other temperature.

Table 4.6. Activation Energies (E_A) for Tagatose Degradation in 0.05 M Glycine Solutions at pH 7.

Sample	E_A (kcal/mol)
0.02 M Phosphate	16.2
0.1 M Phosphate	16.8
0.02 M Citrate	16.8
0.1 M Citrate	17.1

Browning

Both solutions of tagatose alone and with glycine displayed some extent of browning. This browning was most pronounced at 40°C. The effect of buffer type and pH on browning of tagatose solutions is shown in Figure 4.15.

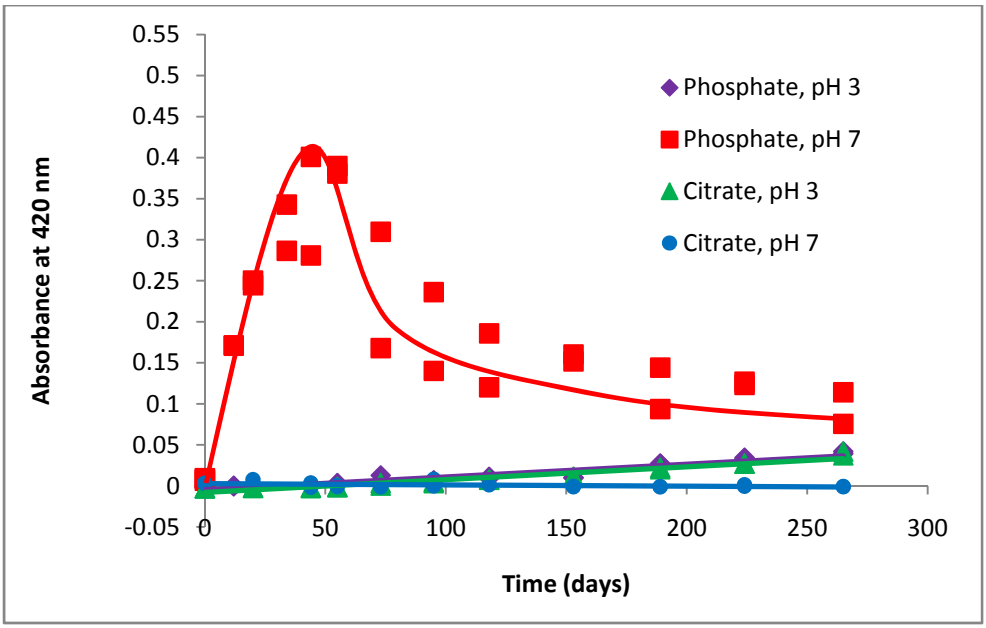

Figure 4.15. Browning of Tagatose in 0.1 M Buffer Solutions at 40°C as Affected by Buffer Type and pH.

More browning occurred in the 0.1 M phosphate buffer solution at pH 7 and 40°C. There was a steep increase before a decrease and leveling off. The other solutions had virtually no browning (data in Appendix C). The brown pigment formed from tagatose degradation in 0.1 M phosphate buffer at pH 7 is apparently unstable and deteriorates over time.

In comparison, the tagatose-glycine solutions showed a more continual increase in browning (Figure 4.16). Similar to the tagatose only solutions, more

browning was seen in the 0.1 M phosphate buffer solution containing 0.05 M glycine at pH 7 and 40°C. While the other solutions had a gradual increase in browning, the phosphate pH 7 buffer solution increased dramatically before somewhat leveling off. This brown pigment was more stable, remaining for the entire storage period. In the presence of glycine, tagatose participates in a Maillard type reaction leading to brown pigment formation, which apparently is a different type of brown pigment from that produced by tagatose degradation in the absence of glycine. Browning is more favored at pH 7, in phosphate buffer rather than citrate buffer, and at higher rather than lower buffer concentrations. This browning data can be found in Appendix D. These findings are consistent with those reported previously (Bell 1997).

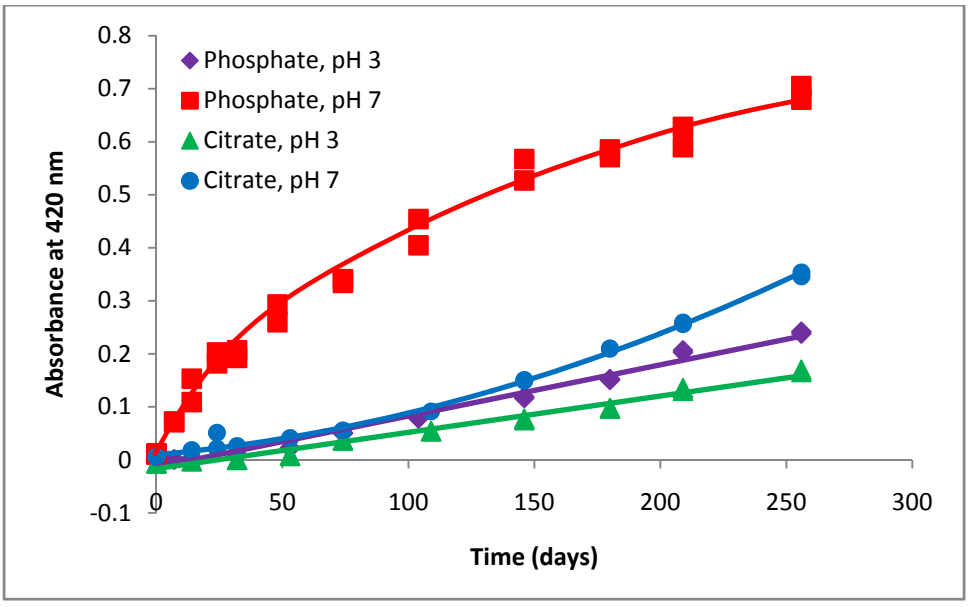

Figure 4.16. Browning of Tagatose and Glycine in 0.1 M Buffer Solutions at 40°C as Affected by Buffer Type and pH.

Comparing Tagatose Reactivity with and without Glycine

An interesting comparison to make is the effect of glycine on the loss of tagatose and the resultant browning. Because tagatose undergoes degradation in buffer solutions at pH 7 in the absence of glycine and because reducing sugars (e.g., tagatose) react with amino acids (e.g., glycine) through the Maillard reaction, it was thought that the addition of glycine to the tagatose solution would enhance its degradation beyond that observed in solutions without glycine. However, as shown in Figures 4.17 and 4.18, the loss of tagatose either remained unchanged in presence of glycine or even decreased. As shown in Figures 4.19 and 4.20, glycine is reacting with tagatose to increase the intensity of the brown pigment over a longer time, but apparently is not increasing the degradation of tagatose itself.

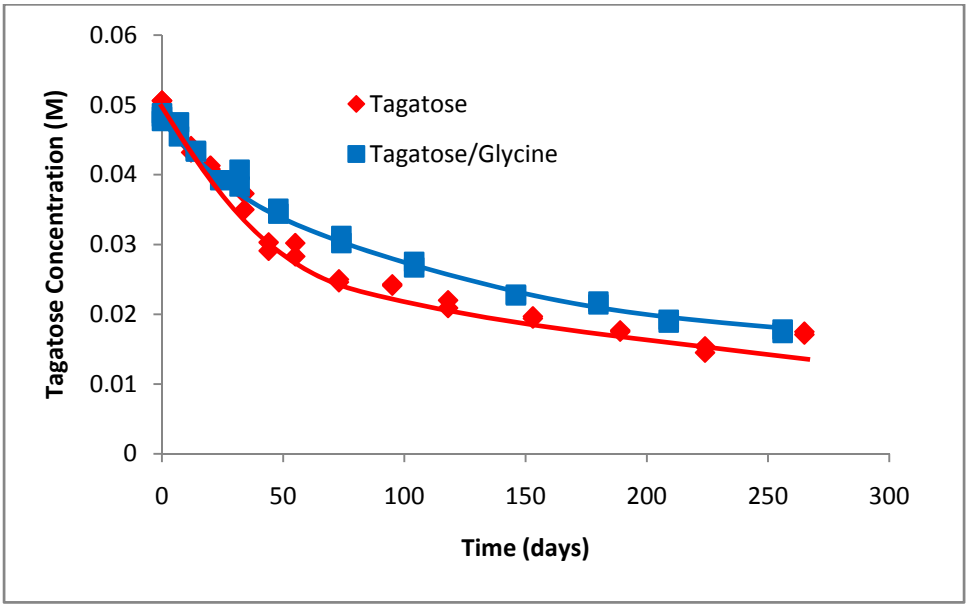

Figure 4.17. Effect of Glycine on Tagatose Loss in 0.1 M Phosphate Buffer at pH 7 and 40°C.

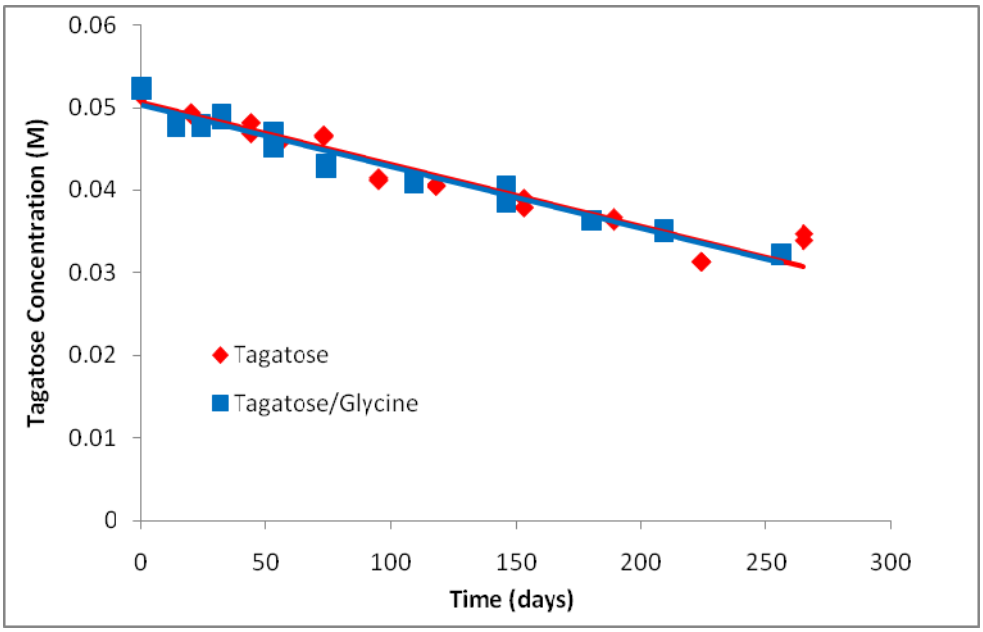

Figure 4.18. Effect of Glycine on Tagatose Loss in 0.1 M Citrate Buffer at pH 7 and 40°C.

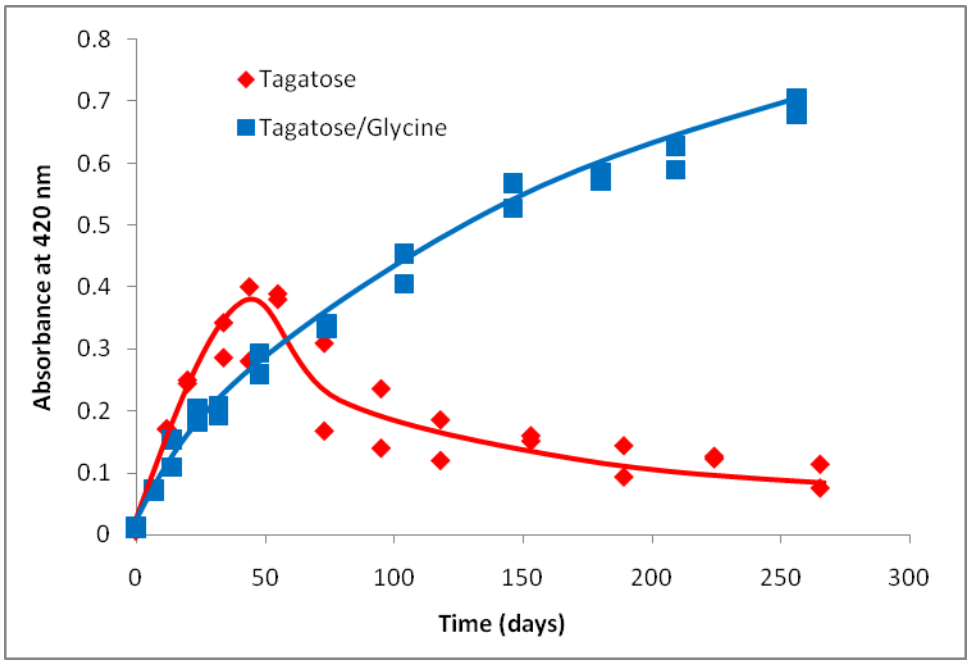

Figure 4.19. Effect of Glycine on Tagatose Browning in 0.1 M Phosphate Buffer at pH 7 and 40°C.

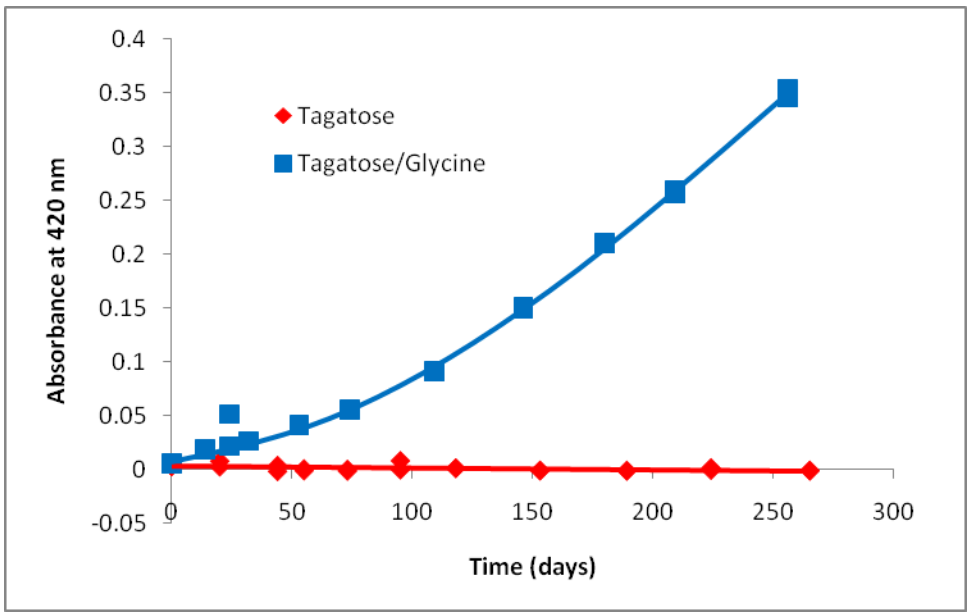

Figure 4.20. Effect of Glycine on Tagatose Browning in 0.1 M Citrate Buffer at pH 7 and 40°C.

The reason why the addition of a known reactant (glycine) would not cause enhanced tagatose degradation is unclear. At pH 7, both the degradation of the monosaccharide and the early steps of the Maillard reaction involve the acyclic sugar derived from mutarotation. The steps leading to the formation of the enediol (alkaline degradation) are reversible as are the steps leading to the glycosylamine (Maillard reaction). Eventually, both reaction pathways continue toward various degradation products and some extent of brown pigment formation.

As mentioned, both pathways involve the same intermediate and a series of reversible steps. One possible explanation may involve the manner in which glycine disturbs the equilibrium positioning of these reversible steps. Reaction steps which are reversible can shift in both direction and magnitude by the addition or removal of either reactants or products. Thus, adding glycine as a reactant can

alter the equilibrium and direct some of the tagatose toward the Maillard reaction (as seen by enhanced production of browning in Figures 4.19 and 4.20). The combination of re-established equilibrium occurring in competing reaction pathways may yield the net result of similar or slower tagatose loss in the presence of glycine.

It is also interesting to note that the most extreme "slowing down" of tagatose loss in the presence of glycine was in 0.1 M phosphate buffer; tagatose loss in citrate buffer was similar regardless of whether or not glycine was present (Figures 4.17 and 4.18). The importance of phosphate buffer anions as catalysts has been discussed. Perhaps an interaction occurred between the buffer and the glycine, reducing the buffer's catalytic ability. So while glycine may enhance the reactivity of tagatose via the Maillard reaction, its possible interaction with phosphate anions may decrease the tagatose loss more than the glycine enhances it.

This interesting result is worthy of further evaluation. However, regardless of the mechanism, tagatose is being lost both in the presence and absence of glycine at pH 7, but browning is more pronounced when glycine is available.

CHAPTER 5: SUMMARY AND CONCLUSIONS

Tagatose in solution degrades during storage both with and without glycine. The degradation of tagatose depends upon buffer type, buffer concentration, pH and temperature, with the greatest loss occurring in 0.1 M phosphate buffer at pH 7 and 40°C. The rate of tagatose loss is faster at higher temperatures, higher buffer concentrations, and higher pH values. It is also faster in phosphate buffer than in citrate buffer.

The loss of tagatose is accompanied by brown pigment formation. This discoloration occurs both in the presence and absence of glycine. In the solutions with added glycine, browning is more extensive and stable than that produced in the absence of glycine. Without glycine, the brown pigment formed deteriorates over time.

These stability issues need to be considered by manufacturers who desire to use tagatose as a prebiotic in their food and beverage products. To obtain the prebiotic effect from tagatose, it should not degrade during storage of the products. Shelf-stable beverages should be made with the lowest buffer concentration and pH possible to deliver the maximum amount of tagatose with the minimal discoloration from browning.

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APPENDIX A

TAGATOSE LOSS IN BUFFER SOLUTIONS WITHOUT GLYCINE

Table A1. Tagatose Degradation in 0.02 M Phosphate Buffer at pH 3 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	0.0507	0.0512	0.0513
0	0.0509	0.0510	0.0510
0	0.0511	0.0511	0.0510
12	0.0510	0.0510	0.0509
12	0.0511	0.0509	0.0510
20	0.0510	0.0508	0.0508
20	0.0507	0.0507	0.0507
44	0.0500	0.0508	0.0520
44	0.0504	0.0519	0.0520
55	0.0505	0.0502	0.0502
55	0.0503	0.0504	0.0506
73	0.0502	0.0497	0.0498
73	0.0500	0.0498	0.0500
95	0.0505	0.0508	0.0509
95	0.0507	0.0510	0.0506
118	0.0501	0.0503	0.0498
118	0.0503	0.0502	0.0500
153	0.0498	0.0506	0.0502
153	0.0503	0.0502	0.0501
189	0.0507	0.0507	0.0509
189	0.0497	0.0512	0.0510
224	0.0487	0.0510	0.0482
224	0.0495	0.0500	0.0487
265	0.0501	0.0501	0.0515
265	0.0502	0.0528	0.0505

Table A2. Tagatose Degradation in 0.02 M Phosphate Buffer at pH 7 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	0.0511	0.0508	0.0505
0	0.0510	0.0508	0.0507
0	0.0508	0.0510	0.0506
12	0.0504	0.0486	0.0468
12	0.0504	0.0487	0.0469
20	0.0499	0.0480	0.0462
20	0.0499	0.0482	0.0459
34	0.0493	0.0475	0.0455
34	0.0498	0.0472	0.0455
44	0.0497	0.0481	0.0433
44	0.0504	0.0471	0.0443
55	0.0485	0.0467	0.0442
55	0.0483	0.0470	0.0435
73	0.0477	0.0458	0.0428
73	0.0481	0.0470	0.0430
95	0.0472	0.0454	0.0428
95	0.0470	0.0455	0.0430
118	0.0469	0.0447	0.0430
118	0.0469	0.0455	0.0429
153	0.0465	0.0454	0.0421
153	0.0466	0.0449	0.0425
189	0.0469	0.0452	0.0434
189	0.0469	0.0453	0.0438
224	0.0483	0.0434	0.0413
224	0.0470	0.0437	0.0414
265	0.0465	0.0450	0.0433
265	0.0461	0.0463	0.0437

Table A3. Tagatose Degradation in 0.1 M Phosphate Buffer at pH 3 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	0.0511	0.0516	0.0516
0	0.0511	0.0516	0.0519
0	0.0509	0.0515	0.0502
12	0.0509	0.0512	0.0508
12	0.0509	0.0510	0.0508
20	0.0513	0.0511	0.0510
20	0.0511	0.0510	0.0511
44	0.0511	0.0510	0.0512
44	0.0509	0.0522	0.0506
55	0.0509	0.0503	0.0505
55	0.0513	0.0503	0.0506
73	0.0504	0.0502	0.0495
73	0.0502	0.0502	0.0494
95	0.0513	0.0509	0.0500
95	0.0511	0.0506	0.0497
118	0.0501	0.0504	0.0489
118	0.0500	0.0501	0.0487
153	0.0513	0.0506	0.0490
153	0.0508	0.0502	0.0490
189	0.0508	0.0510	0.0493
189	0.0508	0.0504	0.0490
224	0.0522	0.0491	0.0484
224	0.0506	0.0495	0.0457
265	0.0508	0.0509	0.0488
265	0.0530	0.0514	0.0489

Table A4. Tagatose Degradation in 0.1 M Phosphate Buffer at pH 7 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	0.0508	0.0507	0.0506
0	0.0506	0.0506	0.0506
0	0.0506	0.0513	0.0506
12	0.0485	0.0456	0.0432
12	0.0483	0.0458	0.0441
20	0.0481	0.0445	0.0413
20	0.0479	0.0439	0.0408
34	0.0465	0.0407	0.0350
34	0.0469	0.0413	0.0373
44	0.0465	0.0407	0.0291
44	0.0463	0.0408	0.0303
55	0.0442	0.0404	0.0302
55	0.0443	0.0394	0.0283
73	0.0434	0.0360	0.0246
73	0.0436	0.0357	0.0250
95	0.0424	0.0353	0.0241
95	0.0421	0.0346	0.0243
118	0.0418	0.0340	0.0220
118	0.0417	0.0339	0.0209
153	0.0412	0.0330	0.0197
153	0.0406	0.0316	0.0194
189	0.0402	0.0316	0.0175
189	0.0408	0.0310	0.0177
224	0.0389	0.0315	0.0145
224	0.0418	0.0287	0.0154
265	0.0393	0.0327	0.0175
265	0.0397	0.0305	0.0171

Table A5. Tagatose Degradation in 0.02 M Citrate Buffer at pH 3 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	0.0509	0.0513	0.0512
0	0.0508	0.0513	0.0516
0	0.0503	0.0508	0.0513
20	0.0504	0.0508	0.0506
20	0.0504	0.0505	0.0505
44	0.0492	0.0507	0.0508
44	0.0516	0.0508	0.05011
55	0.0503	0.0505	0.0502
55	0.0504	0.0502	0.0503
73	0.0501	0.0498	0.0498
73	0.0501	0.0498	0.0497
95	0.0506	0.0504	0.0500
95	0.0502	0.0505	0.0501
118	0.0504	0.0505	0.0497
118	0.0501	0.0500	0.0498
153	0.0499	0.0503	0.0498
153	0.0499	0.0498	0.0502
189	0.0502	0.0499	0.0498
189	0.0505	0.0512	0.0502
224	0.0504	0.0507	0.0482
224	0.0482	0.0499	0.0479
265	0.0500	0.0508	0.0500
265	0.0507	0.0511	0.0507

Table A6. Tagatose Degradation in 0.02 M Citrate Buffer at pH 7 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	0.0506	0.0513	0.0515
0	0.0506	0.0513	0.0512
0	0.0507	0.0510	0.0514
20	0.0506	0.0504	0.0498
20	0.0506	0.0504	0.0496
44	0.0506	0.0501	0.0492
44	0.0509	0.0509	0.0493
55	0.0521	0.0498	0.0476
55	0.0494	0.0492	0.0485
73	0.0502	0.0493	0.0477
73	0.0497	0.0492	0.0478
95	0.0493	0.0484	0.0459
95	0.0495	0.0494	0.0460
118	0.0494	0.0491	0.0451
118	0.0493	0.0495	0.0453
153	0.0490	0.0483	0.0441
153	0.0491	0.0485	0.0446
189	0.0501	0.0483	0.0436
189	0.0500	0.0482	0.0433
224	0.0508	0.0480	0.0454
224	0.0494	0.0492	0.0432
265	0.0502	0.0488	0.0420
265	0.0495	0.0500	0.0420

Table A7. Tagatose Degradation in 0.1 M Citrate Buffer at pH 3 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	0.0502	0.0506	0.0510
0	0.0503	0.0506	0.0505
0	0.0504	0.0508	0.0510
20	0.0503	0.0503	0.0501
20	0.0502	0.0503	0.0501
44	0.0488	0.0513	0.0497
44	0.0500	0.0509	0.0504
55	0.0490	0.0498	0.0489
55	0.0488	0.0501	0.0490
73	0.0503	0.0491	0.0486
73	0.0497	0.0492	0.0486
95	0.0497	0.0495	0.0484
95	0.0495	0.0499	0.0482
118	0.0493	0.0496	0.0483
118	0.0496	0.0494	0.0479
153	0.0502	0.0492	0.0478
153	0.0496	0.0483	0.0478
189	0.0498	0.0493	0.0477
189	0.0500	0.0491	0.0479
224	0.0496	0.0486	0.0469
224	0.0492	0.0472	0.0469
265	0.0490	0.0500	0.0469
265	0.0501	0.0506	0.0473

Table A8. Tagatose Degradation in 0.1 M Citrate Buffer at pH 7 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	0.0517	0.0516	0.0521
0	0.0517	0.0510	0.0521
0	0.0519	0.0520	0.0515
20	0.0511	0.0504	0.0494
20	0.0508	0.0506	0.0492
44	0.0503	0.0505	0.0482
44	0.0503	0.0497	0.0469
55	0.0522	0.0493	0.0457
55	0.0502	0.0487	0.0459
73	0.0506	0.0500	0.0465
73	0.0500	0.0499	0.0467
95	0.0494	0.0475	0.0412
95	0.0495	0.0475	0.0415
118	0.0498	0.0477	0.0405
118	0.0500	0.0475	0.0406
153	0.0485	0.0465	0.0390
153	0.0493	0.0468	0.0379
189	0.0491	0.0477	0.0362
189	0.0496	0.0460	0.0367
224	0.0495	0.0435	0.0313
224	0.0504	0.0441	0.0313
265	0.0496	0.0476	0.0339
265	0.0492	0.0463	0.0347

APPENDIX B

TAGATOSE LOSS IN BUFFER SOLUTIONS WITH GLYCINE

Table B1. Tagatose Degradation in 0.02 M Phosphate Buffer (with 0.05 M Glycine) at pH 3 as Affected by Temperature

Time	Tagatose Concentration (M)		
(days)	20°C	30°C	40°C
0	N/A*	N/A	0.0507
0			0.0507
0			0.0503
7			0.0508
7			0.0511
14			0.0504
14			0.0502
32			0.0507
32			0.0503
53			0.0493
53			0.0494
74			0.0503
74			0.0519
104			0.0528
104			0.0531
146			0.0492
146			0.0489
180			0.0489
180			0.0488
209			0.0494
209			0.0490
256			0.0490
256			0.0505

*N/A: Not available due to mold contamination.

Table B2. Tagatose Degradation in 0.02 M Phosphate Buffer (with 0.05 M Glycine) at pH 7 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Concentration (M)	Time (days)	Concentration (M)	Time (days)	Concentration (M)
0	0.0512	0	0.0512	0	0.0507
0	0.0514	0	0.0517	0	0.0503
0	0.0517	0	0.0514	0	0.0507
7	0.0497	7	0.0510	7	0.0499
7	0.0508	7	0.0511	7	0.0486
15	0.0494	15	0.0497	14	0.0476
15	0.0495	15	0.0497	14	0.0477
25	0.0500	25	0.0491	24	0.0465
25	0.0494	25	0.0488	24	0.0468
33	0.0497	33	0.0493	32	0.0479
33	0.0497	33	0.0492	32	0.0489
49	0.0494	49	0.0486	48	0.0455
49	0.0494	49	0.0482	48	0.0452
75	0.0496	75	0.0478	74	0.0432
75	0.0493	75	0.0479	74	0.0418
105	0.0487	105	0.0470	104	0.0424
105	0.0499	105	0.0464	104	0.0424
155	0.0498	147	0.0474	146	0.0417
155	0.0492	147	0.0482	146	0.0418
181	0.0506	181	0.0457	180	0.0406
181	0.0483	181	0.0473	180	0.0405
210	0.0484	210	0.0470	209	0.0423
210	0.0490	210	0.0467	209	0.0423
257	0.0497	257	0.0481	256	0.0427
257	0.0494	257	0.0458	256	0.0406

Table B3. Tagatose Degradation in 0.1 M Phosphate Buffer (with 0.05 M Glycine) at pH 3 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Concentration (M)	Time (days)	Concentration (M)	Time (days)	Concentration (M)
0	0.0507	0	0.0505	0	0.0491
0	0.0506	0	0.0505	0	0.0494
0	0.0505	0	0.0506	0	0.0492
7	0.0519	7	0.0519	7	0.0510
7	0.0518	7	0.0512	7	0.0508
15	0.0503	15	0.0510	14	0.0503
15	0.0503	15	0.0504	14	0.0503
33	0.0504	33	0.0498	32	0.0497
49	0.0503	33	0.0498	32	0.0497
75	0.0501	54	0.0498	53	0.0486
105	0.0503	54	0.0495	53	0.0486
155	0.0503	75	0.0501	74	0.0481
181	0.0515	75	0.0505	74	0.0495
210	0.0503	105	0.0503	104	0.0494
257	0.0508	105	0.0504	104	0.0494
		147	0.0503	146	0.0479
		147	0.0492	146	0.0475
		181	0.0516	180	0.0479
		181	0.0507	180	0.0456
		210	0.0498	209	0.0454
		210	0.0510	209	0.0484
		257	0.0497	256	0.0471
		257	0.0504	256	0.0478

Table B4. Tagatose Degradation in 0.1 M Phosphate Buffer (with 0.05 M Glycine) at pH 7 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Concentration (M)	Time (days)	Concentration (M)	Time (days)	Concentration (M)
0	0.0500	0	0.0500	0	0.0477
0	0.0502	0	0.0502	0	0.0483
0	0.0506	0	0.0502	0	0.0488
7	0.0514	7	0.0504	7	0.0475
7	0.0514	7	0.0500	7	0.0455
15	0.0500	15	0.0483	14	0.0433
15	0.0509	15	0.0482	14	0.0434
25	0.0500	25	0.0472	24	0.0392
25	0.0493	25	0.0453	24	0.0392
25	0.0502	33	0.0484	32	0.0383
33	0.0501	33	0.0491	32	0.0407
33	0.0484	49	0.0448	48	0.0351
49	0.0479	49	0.0448	48	0.0344
75	0.0479	75	0.0435	74	0.0302
105	0.0463	75	0.0438	74	0.0312
155	0.0457	105	0.0404	104	0.0267
181	0.0473	105	0.0418	104	0.0275
201	0.0464	147	0.0424	146	0.0227
210	0.0463	147	0.0410	146	0.0228
257	0.0462	181	0.0398	180	0.0214
		181	0.0387	180	0.0218
		210	0.0388	209	0.0188
		210	0.0383	209	0.0192
		257	0.0392	256	0.0178
		257	0.0389	256	0.0173

Table B5. Tagatose Degradation in 0.02 M Citrate Buffer (with 0.05 M Glycine) at pH 3 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Concentration (M)	Time (days)	Concentration (M)	Time (days)	Concentration (M)
0	0.0503	0	0.0504	0	0.0502
0	0.0506	0	0.0509	0	0.0504
0	0.0507	0	0.0507	0	0.0508
15	0.0511	15	0.0506	14	0.0506
15	0.0507	15	0.0506	14	0.0507
33	0.0506	33	0.0503	32	0.0501
33	0.0506	33	0.0501	32	0.0503
54	0.0493	54	0.0493	53	0.0490
54	0.0491	54	0.0486	53	0.0490
75	0.0497	75	0.0497	74	0.0497
75	0.0498	75	0.0497	74	0.0496
110	0.0504	110	0.0506	109	0.0499
110	0.0507	110	0.0505	109	0.0502
155	0.0499	147	0.0499	146	0.0490
155	0.0504	147	0.0501	146	0.0490
181	0.0510	181	0.0509	180	0.0488
181	0.0499	181	0.0509	180	0.0503
210	0.0503	210	0.0498	209	0.0493
210	0.0503	210	0.0507	209	0.0487
257	0.0509	257	0.0504	256	0.0491
257	0.0508	257	0.0501	256	0.0500

Table B6. Tagatose Degradation in 0.02 M Citrate Buffer (with 0.05 M Glycine) at pH 7 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Concentration (M)	Time (days)	Concentration (M)	Time (days)	Concentration (M)
0	0.0507	0	0.0510	0	0.0513
0	0.0507	0	0.0506	0	0.0512
0	0.0507	0	0.0508	0	0.0507
15	0.0501	15	0.0499	14	0.0492
15	0.0499	15	0.0499	14	0.0490
25	0.0496	25	0.0494	24	0.0498
25	0.0496	25	0.0539	24	0.0489
33	0.0513	33	0.0501	32	0.0489
33	0.0503	33	0.0499	32	0.0492
54	0.0493	54	0.0486	53	0.0472
54	0.0492	54	0.0486	53	0.0473
75	0.0497	75	0.0501	74	0.0459
75	0.0498	75	0.0499	74	0.0462
110	0.0503	110	0.0479	109	0.0453
110	0.0487	110	0.0488	109	0.0434
155	0.0495	147	0.0488	146	0.0447
155	0.0498	147	0.0486	146	0.0449
181	0.0514	181	0.0507	180	0.0439
181	0.0512	181	0.0480	180	0.0425
210	0.0513	210	0.0497	209	0.0452
210	0.0486	210	0.0484	209	0.0448
257	0.0509	257	0.0495	256	0.0422
257	0.0506	257	0.0494	256	0.0434

Table B7. Tagatose Degradation in 0.1 M Citrate Buffer (with 0.05 M Glycine) at pH 3 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Concentration (M)	Time (days)	Concentration (M)	Time (days)	Concentration (M)
0	0.0504	0	0.0505	0	0.0501
0	0.0509	0	0.0501	0	0.0497
0	0.0502	0	0.0502	0	0.0494
15	0.0505	15	0.0503	14	0.0505
15	0.0510	15	0.0504	14	0.0510
33	0.0501	33	0.0501	32	0.0502
36	0.0501	33	0.0501	32	0.0503
54	0.0499	54	0.0491	53	0.0489
54	0.0494	54	0.0490	53	0.0486
75	0.0505	75	0.0497	74	0.0494
75	0.0497	75	0.0501	74	0.0497
110	0.0506	110	0.0512	109	0.0482
110	0.0507	110	0.0503	146	0.0480
155	0.0496	147	0.0497	146	0.0486
155	0.0498	147	0.0497	180	0.0477
181	0.0499	181	0.0486	180	0.0478
181	0.0492	181	0.0494	209	0.0461
210	0.0499	210	0.0488	209	0.0468
210	0.0505	210	0.0497	256	0.0468
257	0.0507	257	0.0500	256	0.0475
257	0.0499	257	0.0512		

Table B8. Tagatose Degradation in 0.1 M Citrate Buffer (with 0.05 M Glycine) at pH 7 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Concentration (M)	Time (days)	Concentration (M)	Time (days)	Concentration (M)
0	0.0507	0	0.0503	0	0.0525
0	0.0504	0	0.0506	0	0.0524
0	0.0505	0	0.0505	0	0.0522
15	0.0508	15	0.0499	14	0.0485
15	0.0509	15	0.0506	14	0.0477
25	0.0505	25	0.0506	24	0.0480
25	0.0501	25	0.0500	24	0.0476
33	0.0499	33	0.0499	32	0.0493
33	0.0498	33	0.0507	32	0.0485
54	0.0508	54	0.0495	53	0.0451
54	0.0505	54	0.0496	53	0.0470
75	0.0502	75	0.0473	74	0.0426
75	0.0491	75	0.0489	74	0.0432
110	0.0488	110	0.0497	109	0.0408
110	0.0485	110	0.0482	109	0.0412
155	0.0490	147	0.0478	146	0.0406
155	0.0498	147	0.0482	146	0.0384
181	0.0492	181	0.0484	180	0.0363
181	0.0489	181	0.0449	180	0.0364
210	0.0487	210	0.0478	209	0.0349
210	0.0493	210	0.0471	209	0.0352
257	0.0502	257	0.0468	256	0.0323
257	0.0497	257	0.0470	256	0.0321

APPENDIX C

BROWNING OF TAGATOSE IN BUFFER SOLUTIONS WITHOUT GLYCINE

Table C1. Browning of 0.05 M Tagatose in 0.02 M Phosphate Buffer at pH 3 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	-0.0027	-0.0020	-0.0037
0	-0.0021	-0.0021	-0.0033
0	-0.0014	-0.0020	-0.0034
12	-0.0011	-0.0018	-0.0029
12	0.0002	-0.0018	-0.0028
20	-0.0023	-0.0015	-0.0039
20	-0.0006	-0.0017	-0.0035
44	-0.0018	-0.0020	-0.0034
44	-0.0015	-0.0019	-0.0034
55	-0.0003	-0.0018	-0.0025
55	-0.0015	-0.0015	-0.0025
73	-0.0039	-0.0024	-0.0029
73	-0.0038	-0.0026	-0.0029
95	-0.0038	-0.0021	-0.0020
95	-0.0038	-0.0021	-0.0018
118	-0.0035	-0.0018	-0.0011
118	-0.0035	-0.0018	-0.0013
153	-0.0037	-0.0023	-0.0012
153	-0.0038	-0.0024	-0.0004
189	-0.0034	-0.0018	0.0023
189	-0.0035	-0.0021	0.0030
224	-0.0033	-0.0016	0.0029
224	-0.0034	-0.0019	0.0040
265	-0.0037	-0.0018	0.0032
265	-0.0037	-0.0019	0.0037

Table C2. Browning of 0.05 M Tagatose in 0.02 M Phosphate Buffer at pH 7 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	-0.0008	-0.0013	-0.0035
0	-0.0002	-0.0018	-0.0028
0	0.0002	-0.0008	-0.0021
12	0.0006	0.0013	0.0112
12	0.0017	0.0022	0.0162
20	-0.0008	-0.0018	0.0108
20	-0.0002	-0.0011	0.0066
34	-0.0014	-0.0001	0.0035
34	0.0029	-0.0003	0.0031
44	0.0018	0.0026	0.0027
44	0.0011	0.0007	0.0058
55	-0.0006	-0.0023	0.0078
55	-0.0022	-0.0016	0.0036
73	0.0002	0.0002	0.0009
73	0.0006	-0.0015	0.0011
95	0.0034	0.0012	0.0042
95	0.0021	0.0006	0.0045
118	0.0022	-0.0001	0.0020
118	0.0013	0.0001	0.0015
153	0.0009	-0.0012	0.0011
153	0.0002	-0.0011	0.0002
189	0.0021	0.0017	0.0056
189	0.0006	-0.0001	0.0048
224	0.0001	-0.0008	0.0048
224	0.0007	-0.0008	0.0039
265	0.0015	-0.0010	0.0025
265	0.0004	-0.0010	0.0042

Table C3. Browning of 0.05 M Tagatose in 0.1 M Phosphate Buffer at pH 3 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	0.0020	-0.0020	-0.0018
0	0.0068	-0.0010	-0.0012
0	0.0024	-0.0017	0.0020
12	0.0024	-0.0017	-0.0006
12	0.0025	-0.0018	0.0002
20	0.0009	-0.0024	0.0008
20	0.0013	-0.0017	-0.0016
44	0.0006	-0.0019	-0.0001
44	0.0025	-0.0019	0.0001
55	0.0032	-0.0014	0.0034
55	0.0065	-0.0011	0.0016
73	-0.0004	-0.0021	0.0018
73	0.0003	0.0028	0.0127
95	-0.0003	-0.0020	0.0063
95	0.0015	-0.0017	0.0066
118	0.0016	-0.0004	0.0110
118	0.0012	-0.0013	0.0093
153	-0.0009	-0.0022	0.0097
153	0.0001	-0.0019	0.0101
189	-0.0001	-0.0014	0.0269
189	0.0006	-0.0011	0.0271
224	0.0015	-0.0006	0.0332
224	0.0006	-0.0007	0.0343
265	0.0003	-0.0003	0.0391
265	0.0006	-0.0005	0.0419

Table C4. Browning of 0.05 M Tagatose in 0.1 M Phosphate Buffer at pH 7 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	-0.0041	0.0008	0.0093
0	-0.0039	0.0003	0.0062
0	-0.0037	0.0008	0.0087
12	0.0012	0.0416	0.1713
12	0.0025	0.0516	0.1708
20	0.0030	0.0509	0.2503
20	0.0024	0.0322	0.2446
34	0.0024	0.0250	0.2865
34	0.0019	0.0253	0.3429
44	0.0082	0.0229	0.2808
44	0.0065	0.0406	0.4010
55	0.0048	0.0328	0.3898
55	0.0026	0.0184	0.3807
73	0.0034	0.0149	0.1679
73	0.0027	0.0150	0.3097
95	0.0059	0.0158	0.1401
95	0.0200	0.0234	0.2362
118	0.0020	0.0093	0.1201
118	0.0024	0.0100	0.1858
153	0.0038	0.0137	0.1602
153	0.0026	0.0080	0.1516
189	0.0047	0.0070	0.0936
189	0.0031	0.0113	0.1441
224	0.0011	0.0113	0.1269
224	-0.0004	0.0055	0.1230
265	0.0017	0.0051	0.0756
265	0.0023	0.0086	0.1141

Table C5. Browning of 0.05 M Tagatose in 0.02 M Citrate Buffer at pH 3 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	-0.0032	-0.0012	-0.0006
0	-0.0030	0.0677	-0.0006
0	-0.0020	0.0001	-0.0002
20	-0.0017	0.0003	0.0003
20	-0.0005	0.0019	0.0060
44	-0.0033	-0.0011	-0.0007
44	-0.0030	-0.0006	-0.0008
55	-0.0022	0.0003	0.0025
55	-0.0022	0.0001	0.0007
73	0.0005	0.0016	0.0014
73	-0.0020	0.0004	0.0007
95	-0.0038	-0.0011	-0.0002
95	-0.0031	-0.0008	0.0003
118	-0.0025	0.0001	0.0013
118	-0.0025	0.0002	0.0007
153	-0.0015	0.0042	0.0031
153	-0.0023	0.0018	0.0013
189	-0.0033	-0.0008	0.0023
189	-0.0027	-0.0003	0.0026
224	-0.0019	0.0007	0.0041
224	-0.0020	0.0007	0.0038
265	0.0012	0.0068	0.0071
265	-0.0020	0.0004	0.0062

Table C6. Browning of 0.05 M Tagatose in 0.02 M Citrate Buffer at pH 7 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	-0.0021	-0.0010	-0.0002
0	-0.0026	-0.0006	0.0006
0	-0.0011	0.0007	0.0011
20	-0.0013	0.0000	0.0004
20	-0.0003	0.0029	0.0016
44	-0.0028	-0.0005	-0.0008
44	-0.0018	-0.0009	0.0000
55	-0.0018	-0.0007	0.0019
55	-0.0006	-0.0003	0.0033
73	-0.0004	0.0002	0.0047
73	0.0008	-0.0009	0.0005
95	-0.0029	-0.0019	0.0008
95	-0.0022	-0.0013	0.0043
118	-0.0023	-0.0011	0.0030
118	-0.0013	-0.0006	0.0022
153	0.0007	0.0007	0.0030
153	-0.0004	0.0005	0.0013
189	-0.0026	-0.0018	0.0009
189	-0.0023	-0.0011	0.0014
224	-0.0013	-0.0010	0.0028
224	-0.0015	0.0046	0.0023
265	-0.0007	0.0024	0.0054
265	0.0035	-0.0007	0.0066

Table C7. Browning of 0.05 M Tagatose in 0.1 M Citrate Buffer at pH 3 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	-0.0034	-0.0013	-0.0030
0	-0.0029	-0.0010	-0.0031
0	-0.0029	-0.0005	-0.0026
20	-0.0025	-0.0002	-0.0023
20	-0.0026	0.0009	-0.0025
44	-0.0032	-0.0018	-0.0025
44	-0.0029	-0.0011	-0.0025
55	-0.0034	-0.0005	-0.0015
55	-0.0035	0.0003	-0.0015
73	0.0001	0.0011	0.0004
73	-0.0029	-0.0004	0.0036
95	-0.0035	-0.0026	0.0050
95	-0.0035	-0.0022	0.0033
118	-0.0034	-0.0021	0.0074
118	-0.0033	-0.0020	0.0076
153	-0.0027	-0.0018	0.0112
153	-0.0032	-0.0020	0.0098
189	-0.0038	-0.0006	0.0223
189	-0.0037	-0.0007	0.0202
224	-0.0036	-0.0002	0.0272
224	-0.0031	-0.0007	0.0271
265	-0.0023	-0.0003	0.0418
265	-0.0033	-0.0001	0.0373

Table C8. Browning of 0.05 M Tagatose in 0.1 M Citrate Buffer at pH 7 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	-0.0028	-0.0030	0.0031
0	0.0003	-0.0022	0.0032
0	-0.0014	-0.0023	0.0032
20	-0.0025	-0.0017	0.0026
20	-0.0027	-0.0023	0.0077
44	-0.0004	-0.0009	0.0037
44	0.0014	-0.0025	-0.0017
55	0.0021	0.0003	-0.0010
55	-0.0002	-0.0029	0.0006
73	0.0001	0.0000	-0.0013
73	0.0008	0.0009	0.0001
95	-0.0024	-0.0019	0.0081
95	-0.0024	-0.0011	-0.0001
118	-0.0016	-0.0020	0.0013
118	0.0001	-0.0013	0.0012
153	-0.0021	-0.0007	-0.0011
153	-0.0022	-0.0006	-0.0007
189	-0.0016	-0.0031	-0.0008
189	-0.0037	-0.0036	-0.0013
224	-0.0012	-0.0024	0.0015
224	-0.0008	-0.0020	-0.0005
265	-0.0021	-0.0033	-0.0007
265	-0.0025	-0.0031	-0.0012

APPENDIX D

BROWNING OF TAGATOSE AND GLYCINE IN BUFFER SOLUTIONS

Table D1. Browning of 0.05 M Tagatose and 0.05 M Glycine in 0.02 M Phosphate Buffer at pH 3 as Affected by Temperature

Time	Absorbance at 420 nm		
(days)	20°C	30°C	40°C
0	N/A*	N/A	-0.0002
0			-0.0003
0			-0.0002
7			0.0003
7			0.0004
14			0.0015
14			0.0014
32			0.0041
32			0.0042
53			0.0094
53			0.0094
74			0.0303
74			0.0318
104			0.0479
104			0.0479
146			0.0558
146			0.0560
180			0.0653
180			0.0655
209			0.0981
209			0.0968
256			0.0981
256			0.0981

*N/A: Not available due to mold contamination.

Table D2. Browning of 0.05 M Tagatose and 0.05 M Glycine in 0.02 Phosphate Buffer at pH 7 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm
0	0.0026	0	0.0019	0	0.0028
0	0.0026	0	0.0020	0	0.0030
0	0.0049	0	0.0024	0	0.0030
7	0.0028	7	0.0041	7	0.0237
7	0.0038	7	0.0043	7	0.0223
15	0.0021	15	0.0077	14	0.0326
15	0.0026	15	0.0073	14	0.0352
25	0.0038	25	0.0106	24	0.0507
25	0.0040	25	0.0104	24	0.0461
33	0.0029	33	0.0123	32	0.0593
33	0.0052	33	0.0122	32	0.0545
49	0.0051	49	0.0150	48	0.0757
49	0.0056	49	0.0151	48	0.0718
75	0.0113	75	0.0196	74	0.0997
75	0.0089	75	0.0185	74	0.0966
105	0.0103	105	0.0230	104	0.1352
105	0.0100	105	0.0239	104	0.1312
155	0.0092	147	0.0274	146	0.1828
155	0.0094	147	0.0268	146	0.1787
181	0.0112	181	0.0314	180	0.2081
181	0.0077	181	0.0299	180	0.2094
210	0.0098	210	0.0346	209	0.2263
210	0.0097	210	0.0356	209	0.2231
257	0.0097	257	0.0404	256	0.2577
257	0.0091	257	0.0384	256	0.2563

Table D3. Browning of 0.05 M Tagatose and 0.05 M Glycine in 0.1 M Phosphate Buffer at pH 3 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm
0	0.0001	0	0.0001	0	0.0007
0	0.0008	0	-0.0006	0	-0.0002
0	0.0009	0	-0.0010	0	0.0001
7	0.0005	7	-0.0003	7	0.0009
7	0.0014	7	-0.0005	7	0.0009
15	-0.0003	15	-0.0008	14	0.0033
15	0.0006	15	-0.0010	14	0.0033
33	0.0006	33	-0.0001	32	0.0098
49	0.0007	33	0.0001	32	0.0102
75	0.0002	54	0.0036	53	0.0225
105	0.0016	54	0.0006	53	0.0223
155	0.0028	75	0.0028	74	0.0509
181	0.0048	75	0.0026	74	0.0504
210	0.0021	105	0.0044	104	0.0785
257	0.0018	105	0.0055	104	0.0785
		147	0.0081	146	0.1190
		147	0.0074	146	0.1166
		181	0.0092	180	0.1525
		181	0.0087	180	0.1507
		210	0.0137	209	0.2031
		210	0.0152	209	0.2065
		257	0.0152	256	0.2419
		257	0.0148	256	0.2384

Table D4. Browning of 0.05 M Tagatose and 0.05 M Glycine in 0.1 M Phosphate Buffer at pH 7 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm
0	0.0006	0	-0.0035	0	0.0102
0	0.0005	0	-0.0039	0	0.0100
0	0.0010	0	-0.0036	0	0.0124
7	0.0034	7	0.0116	7	0.0725
7	0.0042	7	0.0109	7	0.0711
15	0.0078	15	0.0157	14	0.1091
15	0.0082	15	0.0155	14	0.1532
25	0.0107	25	0.0194	24	0.1821
25	0.0111	25	0.0214	24	0.2028
25	0.0121	33	0.0224	32	0.2068
33	0.0116	33	0.0228	32	0.1923
33	0.0114	49	0.0269	48	0.2931
49	0.0130	49	0.0279	48	0.2596
75	0.0139	75	0.0338	74	0.3340
105	0.0155	75	0.0364	74	0.3409
155	0.0169	105	0.0459	104	0.4544
181	0.0177	105	0.0492	104	0.4049
201	0.0182	147	0.0639	146	0.5675
210	0.0192	147	0.0671	146	0.5271
257	0.0213	181	0.0776	180	0.5711
		181	0.0811	180	0.5856
		210	0.0862	209	0.6281
		210	0.0914	209	0.5899
		257	0.1059	256	0.7052
		257	0.1115	256	0.6795

Table D5. Browning of 0.05 M Tagatose and 0.05 M Glycine in 0.02 M Citrate Buffer at pH 3 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm
0	-0.0064	0	-0.0003	0	-0.0071
0	-0.0060	0	-0.0005	0	-0.0065
0	-0.0062	0	-0.0007	0	-0.0055
15	-0.0039	15	-0.0001	14	-0.0046
15	-0.0052	15	-0.0001	14	-0.0009
33	-0.0063	33	-0.0005	32	-0.0042
33	-0.0057	33	0.0001	32	-0.0038
54	-0.0057	54	0.0001	53	-0.0003
54	-0.0056	54	0.0005	53	0.0002
75	-0.0049	75	0.0052	74	0.0214
75	-0.0050	75	0.0024	74	0.0193
110	-0.0053	110	0.0026	109	0.0312
110	-0.0053	110	0.0030	109	0.0316
155	-0.0047	147	0.0032	146	0.0430
155	-0.0048	147	0.0042	146	0.0430
181	-0.0048	181	0.0038	180	0.0519
181	-0.0045	181	0.0037	180	0.0517
210	-0.0046	210	0.0070	209	0.0734
210	-0.0050	210	0.0067	209	0.0746
257	-0.0052	257	0.0056	256	0.0788
257	-0.0050	257	0.0058	256	0.0794

Table D6. Browning of 0.05 M Tagatose and 0.05 M Glycine in 0.02 M Citrate Buffer at pH 7 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm
0	0.0012	0	-0.0044	0	0.0034
0	0.0011	0	-0.0044	0	0.0105
0	0.0009	0	-0.0038	0	0.0046
15	0.0015	15	-0.0026	14	0.0090
15	0.0017	15	-0.0015	14	0.0087
25	0.0014	25	-0.0033	24	0.0112
25	0.0016	25	-0.0032	24	0.0114
33	0.0014	33	-0.0025	32	0.0148
33	0.0015	33	-0.0017	32	0.0147
54	0.0023	54	0.0004	53	0.0227
54	0.0038	54	-0.0011	53	0.0223
75	0.0027	75	-0.0010	74	0.0369
75	0.0026	75	0.0013	74	0.0302
110	0.0024	110	-0.0008	109	0.0494
110	0.0029	110	0.0008	109	0.0497
155	0.0028	147	0.0043	146	0.0805
155	0.0051	147	0.0035	146	0.0816
181	0.0028	181	0.0026	180	0.1098
181	0.0030	181	0.0020	180	0.1087
210	0.0070	210	0.0047	209	0.1339
210	0.0038	210	0.0051	209	0.1318
257	0.0050	257	0.0080	256	0.1785
257	0.0090	257	0.0071	256	0.1798

Table D7. Browning of 0.05 M Tagatose and 0.05 M Glycine in 0.1 M Citrate Buffer at pH 3 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm
0	0.0000	0	-0.0154	0	-0.0065
0	0.0001	0	-0.0156	0	-0.0063
0	-0.0001	0	-0.0154	0	-0.0060
15	0.0001	15	-0.0149	14	-0.0027
15	0.0003	15	-0.0151	14	-0.0021
33	0.0004	33	-0.0153	32	0.0001
36	0.0002	33	-0.0151	32	0.0001
54	0.0003	54	-0.0137	53	0.0085
54	0.0005	54	-0.0130	53	0.0075
75	0.0010	75	-0.0092	74	0.0375
75	0.0011	75	-0.0104	74	0.0365
110	0.0006	110	-0.0080	109	0.0544
110	0.0011	110	-0.0067	109	0.0539
155	0.0010	147	-0.0061	146	0.0778
155	0.0014	147	-0.0065	146	0.0750
181	0.0014	181	-0.0044	180	0.0968
181	0.0017	181	-0.0052	180	0.0979
210	0.0012	210	-0.0017	209	0.1356
210	0.0012	210	-0.0013	209	0.1304
257	0.0012	257	-0.0004	256	0.1658
257	0.0017	257	-0.0005	256	0.1702

Table D8. Browning of 0.05 M Tagatose and 0.05 M Glycine in 0.1 M Citrate Buffer at pH 7 as Affected by Temperature

20°C		30°C		40°C	
Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm	Time (days)	Absorbance at 420 nm
0	0.0018	0	0.0027	0	0.0052
0	0.0017	0	0.0025	0	0.0057
0	0.0015	0	0.0025	0	0.0054
15	0.0024	15	0.0071	14	0.0182
15	0.0030	15	0.0067	14	0.0184
25	0.0021	25	0.0088	24	0.0215
25	0.0021	25	0.0082	24	0.0213
33	0.0028	33	0.0093	32	0.0262
33	0.0032	33	0.0094	32	0.0264
54	0.0093	54	0.0139	53	0.0413
54	0.0040	54	0.0106	53	0.0415
75	0.0050	75	0.0100	74	0.0552
75	0.0046	75	0.0097	74	0.0555
110	0.0051	110	0.0106	109	0.0915
110	0.0055	110	0.0107	109	0.0914
155	0.0062	147	0.0126	146	0.1493
155	0.0061	147	0.0124	146	0.1510
181	0.0056	181	0.0136	180	0.2097
181	0.0057	181	0.0137	180	0.2104
210	0.0056	210	0.0149	209	0.2591
210	0.0059	210	0.0158	209	0.2560
257	0.0060	257	0.0189	256	0.3461
257	0.0059	257	0.0188	256	0.3536