Thiamine Degradation Off-Flavors in Energy Beverages and Their Reduction

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Dedication

This thesis is dedicated to my parents

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Chapter 1

Literature Review

1.1 Off-flavors in Foods

When it comes to flavor chemistry it is easy for one to focus on off-flavors as they are the main cause of complaints about foods and beverages (4). Three causes of objectionable flavors are 1.) degradation of a food component (e.g. lipid oxidation), 2.) environmental tainting (e.g. air, water, or packaging materials), and 3.) loss of characteristic flavor in a food (e.g. evaporation) (4). This latter situation is not considered to be tainted or to have developed an off-flavor.

Kilcast (2) states that off-flavors become a defect in a food when an undesirable flavor is perceived by a selected percent of the consumers and poses an economic risk to the producer of the food product. Undesirable flavors can occur through defects such as lipid oxidation, vitamin degradation, or other reactions in the food. Taints on the other hand occur through packaging leaching volatile and non-volatile flavor compounds into the product, microbial spoilage, and other external sources. The process of trying to identify the off-flavor and to determine how it occurred is complicated, mostly because of the different consumer sensitivities to various off-flavors.

1.1.1 Sensory Aspects of Off-flavors

Off-flavors can be studied using sensory and analytical techniques. When it comes to the detection of off-flavors using sensory techniques there is a wide range in human sensitivities. For instance, the sensitivity for 3-methyl-2-butene-1-thiol (prenyl mercaptan) can range anywhere from 10^{-6} g/mL to 0.01 g/mL which is a 10,000 fold difference (3). Kilcast (2) has illustrated this in a plot of the distribution of people who can detect an aroma with respect to the concentration of an odorant (Figure 1.1). Many compounds that impart off-flavors and taints have extremely low sensory thresholds such as 2,4,6-tricholoroanisole at 0.000001 ppb in water (1). Off-flavor components, such as 2,4,6-trichloroanisole, can be problematic as they lead to rejection of many foods and beverages because consumers deem them unacceptable.

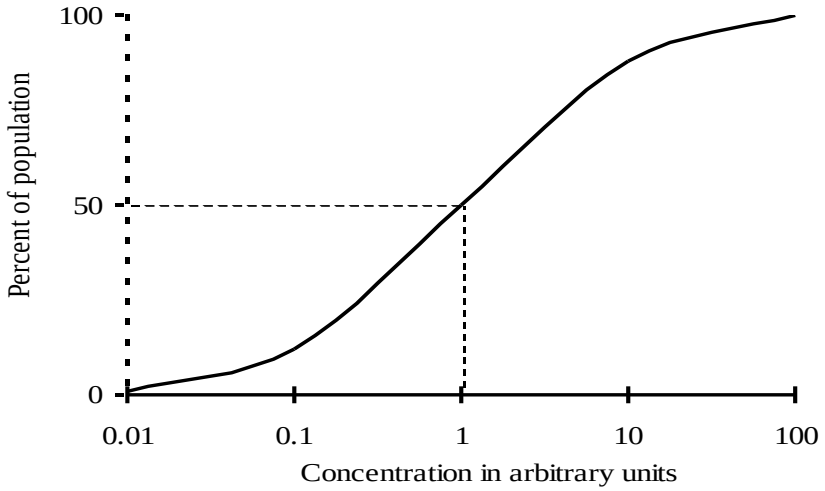

Figure 1.1. Relationship of percent of population that can detect an off-flavor with respect to concentration of an odorant (From ref. (2))

In Figure 1.1, Kilcast illustrates how some individuals may not detect an off-flavor while others may find the off-flavor offensive. At the sensory threshold concentration, 50% of the population can detect the chemical. This is considered a detection threshold because it is the minimum value that an off-flavor is perceived (2, 4). Further down the chart, at a concentration lower than the threshold, e.g. 5% of the population would still detect the aroma chemical whereas the other 95% cannot sense it (2, 4). This means that a consumer may come to the company with a complaint, yet other consumers may not detect the off-flavor.

Kilcast (2) has provided some guidelines for sensory testing of off-flavors and his points are briefly summarized below:

1. When the cause/identity of the off-flavor is unknown, assessors who are reliable and most sensitive to judging an off-flavor are used.
2. As many judges as possible must be used when no sensitive ones are available.
3. The most sensitive test possible is used.
4. The information from a single test is maximized by asking for additional information; e.g. description, degree of objection, etc.

5. Appropriate statistical methods are used and statistical significance is relaxed to 20%, not 1 or 5% as is traditional.
6. Minority opinion is looked at, especially if from reliable judges.
7. Finally, the cause of taints and off-flavors in foods is discussed.

1.2 Analysis of Off-flavors

In order to help analyze off-flavors that may be present in a food, compounds are first isolated by a selected extraction protocol and then detected by instrumental analysis. A good analytical protocol must be simple, sensitive, reproducible, and must take into consideration the composition, quantity, and physical state of the food.

1.2.1 Solid Phase Microextraction (SPME)

SPME is an aroma isolation technique that involves both the extraction of headspace volatiles and/or direct immersion into the sample. SPME fibers are fused silica fibers that can be coated with a variety of phases or polymers (29). Aroma compounds from the sample are adsorbed onto the fiber until equilibrium is reached (29). After a given time, the fiber is inserted in a gas chromatograph (GC) injection port at high temperature, thereby thermally desorbing the absorbed aroma compounds into the GC for analysis (Figure 1.2). SPME offers many advantages such as a solvent-free technique, and simple and rapid extraction. It requires little sample preparation, and is good for recovery of volatile analytes. However, this extraction method is not without disadvantages. Some of the most notable are a small amount of adsorbent material available on the fiber, competition between volatiles for binding sites which in turn may cause poor quantification (4).

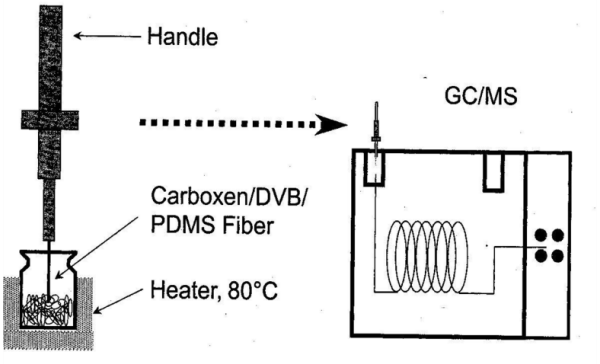

Figure 1.2. Schematic of SPME aroma isolation and analysis by GC (From ref. (30))

1.2.2 Stir-Bar Sorptive Extraction (SBSE, Twister®)

Stir-bar sorptive extraction is another flavor extraction technique based on the same technology as SPME. However, the stir bar has much more adsorbent coating than the SPME and the adsorbent phase is placed on an inert glass stir bar (Figure 1.3) (4). The coating, a polydimethylsiloxane or PDMS, acts as an extracting solvent as opposed to an adsorbent. PDMS stir bars can be coated with 25-250 µl of phase coating. The stir bar is immersed directly in the product, either a liquid/aqueous solution or headspace, and allowed to come into equilibrium with the sample being analyzed (4). After the stir bar extracts the sample for a pre-determined amount of time, 1 min to 24 hrs, it is taken out with tweezers, rinsed with water, blotted dry, and thermally desorbed into a GC for analysis. Figure 1.3 shows what the stir bar looks like and a schematic of the analytical process.

Figure 1.3. Schematic of stir-bar sorptive extraction (SBSE)

One of the main improvements that SBSE offers is having much more adsorbant material for the extraction of compounds than SPME fibers. In Figure 1.4 the graph clearly shows that there is a larger recovery of solutes and better extraction efficiencies with SBSE compared to SPME due to the larger mass of the phase on stir-bars. Stir-bar is generally used for direct sampling of foods that contain fat levels below 2-3%, beverages with alcohol levels below 10%, and other liquid samples (4). Some advantages of using this extraction method include simplicity, ease of use, no need for solvents, good quantifiable data, speed, and improved sensitivity; i.e. ability to detect compounds at lower limits. On the other hand, it still has the same disadvantages as the SPME fiber, in addition to it's cost.

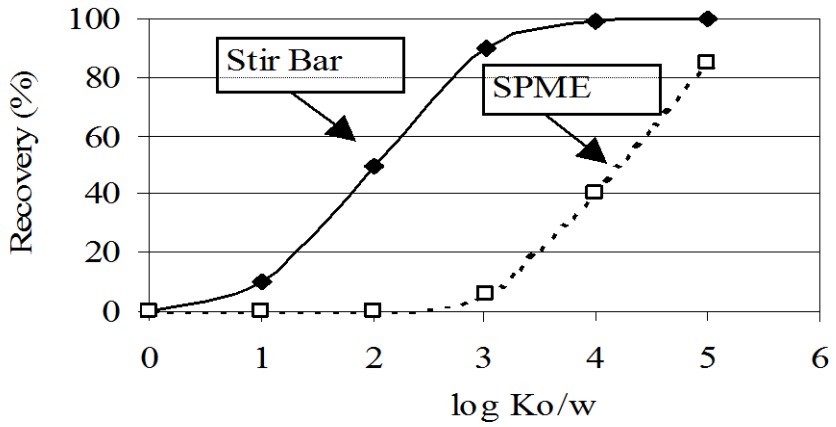

Figure 1.4. The ability of stir-bar verses SPME to extract compounds of different polarity from an aqueous solution (From ref. (41))

1.2.3 Gas Chromatography (GC), GC-MS, GC/O

Gas chromatography is the most widely used technique to analyze aroma isolates. The gas chromatograph does the separation and quantification of volatile compounds based on three basic processes: volatilization, separation, and detection (4, 32). The volatilization takes place in the injection port where the sample is introduced, the separation occurs in the column which includes different phases varying by polarity, and the detection occurs when a molecule is detected by an electric signal that sends data to a computer. Injection can be in split, splitless, and on column modes. The columns used depend on the polarity of analytes one is working with. The most commonly used columns are DB-Wax and DB-5. Finally, the detectors most commonly used are Flame Ionization Detector (FID) and/or Mass Spectrometer (MS) and these can be coupled with an olfactory unit which consists in the MS and olfactory unit allowing for better identification of components.

One of the most useful tools in the analysis of off-flavors is using the combination of gas chromatography-mass spectrometry (GC-MS) and olfactometry. After separation by GC, volatiles are split between the MS and the olfactometry port. In the MS, compounds are broken down by an electron beam and resulting fragment ions are sorted through the mass analyzer (a quadrupole or time of flight analyzer). The ion signal is thereafter amplified by an electron multiplier, which measures each ion signal amplitude

and generates a mass spectrum for each compound. MS possesses many ionization modes including Electron Ionization (EI) and Chemical Ionization (CI) mode. Both modes can be used for the analysis of off-flavors and to identify unknown compounds (29). In order to increase sensitivity, one can set the MS in a selected mode where it focuses only on a few ions and analyze co-eluting compounds from the GC (4).

Another way to analyze off-flavors is coupling the GC with an olfactory unit (GC/O) or using GC-MS/O. The use of GC-O allows for characterization of off-flavor compounds by assigning a given odor to a given GC peak. The premise of this method is to use odor characteristics to describe what one smells when compounds are being analyzed by the GC. One of the powerful tools with GC/O coupled with GC-MS is the ability to identify compounds by relating the aromagram to the GC peaks. An aromagram is a listing, usually in table form, of an odor character of each peak in a GC run. An aromagram can show that an odor was detected by a human subject and there appears to be a GC peak at around that same time. Or an aromagram can show that the most important area of the chromatogram is where there are no GC peaks. The latter can indicate that there may be something there that the nose could smell, but the GC couldn't detect because individuals have better detection thresholds than instruments. Overall, the addition of olfactometry to GC and GC-MS can prove to be a very useful tool for the identification of known and unknown off-flavor compounds.

1.3 Off-Flavors Due to Vitamin Degradation

Vitamin supplements are commonly added to beverages and foods for human consumption. However, there may be inherent problems with certain vitamins present in beverages and foods as vitamins can degrade and produce off-flavors. Vitamins that have been known to be problematic and degrade to produce off-flavors are Vitamin A, Vitamin C (ascorbic acid), and Vitamin B1 (Thiamine). Riboflavin (Vitamin B2) can't produce off-flavors, but it can catalyze reactions in the presence of light to break down specific food components into off-flavors.

1.3.1 Vitamin A

Vitamin A exhibits some potential to produce off-flavors. In a study done comparing whole milk to skim milk (6), it was found that Vitamin A degraded and produced a "hay-like" off-flavor in skim milk and whole milk had much less of the undesirable flavor. The lower concentrations of the "hay-like" flavor in whole milk formed because the presence of milk fat protected against Vitamin A degradation (6). Suyama et. al (8) found that Vitamin A in 2% milk undergoes photooxidation to produce the "haylike" flavor and is greatly increased if the milk is processed using high heat i.e. nonfat dry milk. Reineccius (4) points out that the oxidation of carotenoids yields highly unsaturated and cyclic compounds. The characteristic off-flavor of Vitamin A is due to the β-ionone and dihydroactinolide (7).

1.3.2 Vitamin C

Vitamin C, commonly called ascorbic acid, can create "stale" off-flavor problems because it will partake in nonenzymatic browning (4). This is common in fruit juices that naturally have ascorbic acid, citrus drinks, and any other beverage fortified with the vitamin. The "stale" term is not very descriptive when trying to figure out what exactly is causing those flavors and it is generally referring to a lack of fresh character in the product (4). Parks et al. (42) points out that the cause of the "stale" off-flavor in dry milk is due to benzothiazole and o-aminoacetophenone formation in dry products.

In fruit juices it is commonly debated which reaction causes the off-flavors- the Maillard reaction involving sugars or ascorbic acid degradation (4). Researchers found that ascorbic acid browning in fruit juices occurs around a pH of 2.0-3.5 (10). Rouseff et al. (9) points out that ascorbic acid browning creates chemicals such as furfural in citrus juices. Furfural, which has a threshold of 80 ppm, can help contribute to the product tasting "burnt," "sweetish," and "carmelized" (11). This compound produces deoxyfuroin and furil that gives citrus juices the characteristic off-flavor (11). Figure 1.5 shows the proposed mechanism by which ascorbic acid turns into dehydroascorbic acid to produce browning and furfural.

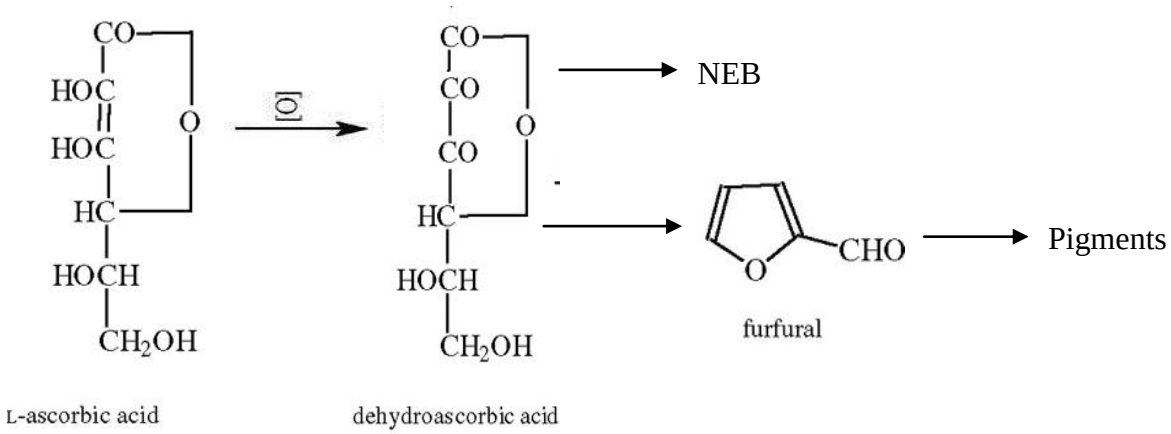

Figure 1.5. Proposed mechanism of ascorbic acid browning reaction creating characteristic off-flavors and pigments (Adapted from ref. (31))

1.3.3 Vitamin B2 (Riboflavin)

Riboflavin is a water soluble vitamin that is relatively stable during thermal and non-thermal food processing, but it is sensitive to light (12). Riboflavin in the presence of light catalyzes amino acid degradation in products such as milk or lipids in chips to produce an unpleasant "sunstruck" or "sunlight" off-flavor in products (4). Off-flavors due to this reaction have been significantly reduced by the advent of new packaging such as opaque plastics that prevent light from striking the product.

In milk, riboflavin content is about 1.7 ppm and it can form off-flavors either through a type I pathway involving free radical generation or a type II pathway involving a singlet oxygen oxidation (13, 5). In dairy products, amino acids are catalyzed by light and riboflavin to produce the off-flavors (4). It is pointed out by Reineccius (4) that the amino acid methionine may be more susceptible to undergo this reaction. This reaction is summarized in Figure 1.6 where the volatile compounds are most likely methional and dimethyldisulfide.

$$\text{Amino Acids} \xrightarrow[\text{Riboflavin}]{h\eta} \text{Volatile Compounds} + NH_3 + CO_2$$

Figure 1.6. Light and riboflavin catalyzing amino acids to produce volatile off-flavor compounds. (From ref (43))

Alcoholic beverages including beer and wine, mostly white wine and champagne, will have a “light struck” off-flavor due to riboflavin catalyzing the degradation of amino acids. Much like the mechanism that produces off-flavors in milk, riboflavin in beer and champagne catalyzes the degradation of methionine and photoxidation of lipids (4). Beer off-flavors are due to the photooxidation of lipids and the photo-catalyzed breakdown of isohumulones (14). Kamimura (14) points out that the isohumulones are oxidized to produce the off-flavor 3-methyl-2-butene-1-thiol which has a very low odor threshold of 7ng/L in beer. Today, packaging in amber bottles has significantly reduced this off-flavor in beer.

1.3.4 Vitamin B1 (Thiamine)

One vitamin that is more sensitive to degradation, producing objectionable off-flavors is thiamine, Vitamin B1. Thiamine and its ability to produce off-flavors is discussed in Section 1.4.2 and 1.6 in depth. Thiamine can degrade by multiple pathways to produce off-flavors. It is very unstable under alkaline and high heat (110°C-120°C) conditions (17). Though the degradation is clearly not as rapid, thiamine can also degrade under ambient room temperature storage as well as acidic conditions to produce off-flavors.

Thiamine degradation can produce potent meaty aromas and other sulfur compounds that make products fortified or naturally present with this vitamin undesirable. However, the meaty flavors associated with thiamine may make it desirable for meat flavor production and products such as beef, chicken, and pork. Overall, thiamine degradation to produce off-flavors is very complex.

1.4 Thiamine (Vitamin B1)

Thiamine is a water soluble form of the B complex that helps nervous system function, mental acuity, and converts carbohydrates in the body to produce energy (15). The Recommended Daily Intake (RDI) for thiamine is 1.4 mg per day. Its structure which is shown in Figure 1.7, is composed of a pyrmidine ring and a thiazole ring connected by a methylene bridge. Thiamine can come in many forms such as mononitrate, but the most common form is thiamine hydrochloride. Thiamine hydrochloride is a crystalline product that is used in fortifying food and various

beverages. It can be used in soups and gravies to impart a meaty, brothy character to the product.

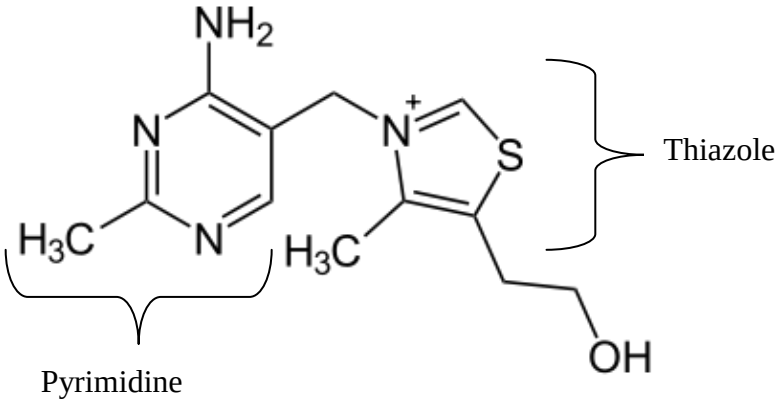

Figure 1.7. Basic structure of thiamine composed of a pyrimidine ring and a thiazole ring (From ref (16))

1.4.1 Foods that Contain Thiamine

Foods that are rich in thiamine include whole grain cereals, legumes (e.g., beans and lentils), nuts, lean pork, and yeast (18). Most foods such as rice, flour, bread, and pasta undergo processing that result in the loss of this vitamin. Therefore, these foods are fortified with thiamine to compensate for this loss. With a varied diet people should be able to obtain the RDI of thiamine. However, this is not always the case especially for people with limited food intake due to allergies, diet restrictions, and malnourishment. As a result, it may be desirable to incorporate the vitamin in various forms. Table 1.1 displays common foods that naturally contain thiamine. As can be seen in the table, an individual can fulfill the RDI of thiamine by eating a variety of foods: It is seldom a deficiency disease in the general population. However, consumers may prefer foods and beverages fortified with thiamine in order to obtain the whole RDI in one product or serving.

Table 1.1. Selected foods that naturally contain thiamine (From ref. (44))

Food	Serving	Thiamine (mg)
Lentils (cooked)	1/2 cup	0.17
Peas (cooked)	1/2 cup	0.21
Long grain brown rice (cooked)	1 cup	0.19
Long grain white rice, enriched (cooked)	1 cup	0.26
Long grain white rice, unenriched (cooked)	1 cup	0.04
Whole wheat bread	1 slice	0.10
White bread, enriched	1 slice	0.11
Fortified breakfast cereal	1 cup	0.5-2.0
Wheat germ breakfast cereal	1 cup	4.47
Pork, lean (cooked)	3 ounces*	0.72
Brazil nuts	1 ounce	0.18
Pecans	1 ounce	0.19
Spinach (cooked)	1/2 cup	0.09
Orange	1 fruit	0.10
Cantaloupe	1/2 fruit	0.11
Milk	1 cup	0.10
Egg (cooked)	1 large	0.03

1.4.2 Thiamine Degradation Off-flavors

Beverages that contain thiamine have the potential to produce undesirable off-flavors due to thiamine degradation in the product. In beverages with thiamine added such as fortified sport drinks, fruit-flavored beverages, meal replacement, and other drinks the thiamine degrades due to the acidic nature of the product. In a thiamine degradation study Pachapurkar (17) points out that under acidic conditions, thiamine becomes protonated and cleaved to form 2-methyl-4-amino-5-hydroxymethyl pyrimidine and 4-methyl-5β-hydroxyethyl thiazole. The same study also found that thiamine degrades rapidly at high temperatures around 110°C (17). Overall, in beverages containing a pH of 4, phosphate buffer was found to stabilize thiamine from degrading. Citrate buffer also helped stabilize thiamine from degrading in beverages with a high pH around 7 (17).

Thiamine in orange juice is unstable during processing (pasteurization) and storage at room temperature. During storage, some compounds that contribute characteristic fresh orange juice flavor decrease and off-flavors such as furaneol and α-terpineol are formed (19). According to Dreher et al. (18) the most significant thiamine thermal degradation products are 2-methyl-3-furanthiol (MFT) and its dimer bis(2-methyl-3-furyl) disulfide (MFT-MFT) which both impart a meaty, brothy flavor. These potent off-flavors are readily created during thermal processing, but also occur at ambient storage temperature, which would be problematic to foods and beverages stored in that sort of environment. MFT has an odor threshold of 6.14×10^{-8} mM in water (20) and MFT-MFT has an odor threshold of 8.9×10^{-11} mM in water (21). Since the dimer (MFT-MFT) is formed from the monomer (MFT), the formation of these compounds are linked. In a model orange juice study MFT and MFT-MFT, as well as other off-flavor compounds including 4,5-dimethylthiazole (fishy, earthy), 2-methyl-3-(methyldithio) furan (meaty), dimethyl sulfide (sulfury), and 2-acetylthiophene were identified (18). This led the researchers to believe that thiamine could be a precursor to those potent off-flavors (18).

Products other than orange juice in which MFT and MFT-MFT have been found include, but are not limited to cooked brown rice, meats, grapefruit juice, and coffee (18). Rouseff et. al (22) found that MFT and MFT-MFT were formed in original fresh squeezed grapefruit juice and not in concentrated juice. They postulated that the lack of MFT presence in concentrated juice could have been attributed to loss through evaporation (22). In coffee, MFT was formed due to the roasting of coffee beans and this compound had a significant reaction with the melanoidins present in the coffee solids (23).

1.5 Thiamine Degradation Kinetics

Degradation kinetics could be used to better predict the shelf life of thiamine and stability. Thiamine degradation could be a problem for nutritional labeling because if a producer declares a certain amount of thiamine in the product and it degrades, there could be less than what it claims and this would fall under "mislabeling." The shelf life of

thiamine under an accelerated storage temperature of 50°C is about 4 weeks which relates to about 9 months at ambient storage temperature (17).

According to Pachapurkar (17) thiamine degradation follows first-order kinetics. The thiamine degradation rate constant for an acidic solution was reported as 3.09×10^{-4} (17). In the study it was found that as pH increased, thiamine degradation increased as well. Therefore, for every 1 unit increase in pH, the rate constant increased by a factor of 10 (17). In an acidic solution, phosphate buffer helped significantly slow down thiamine degradation at a pH of 4 and extend the shelf life up to 1 year. In Figure 1.8, it is clear that under more alkaline conditions thiamine rapidly degrades. On the other hand, under acidic conditions, it degrades very slowly.

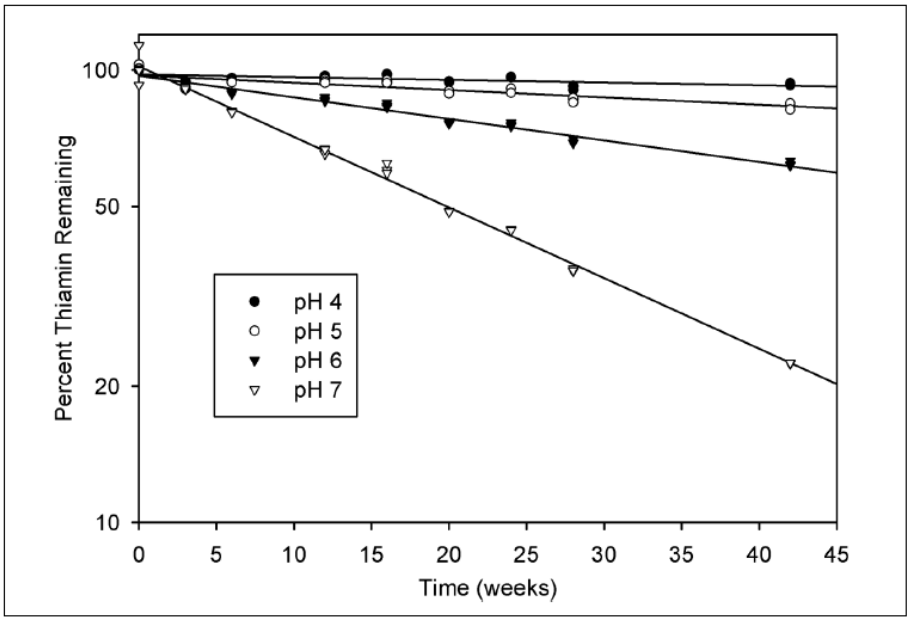

Figure 1.8. The effect of pH on the degradation of thiamine degradation in 0.1M phosphate buffer at 25°C (From ref. (17))

1.6 Chemistry of Thiamine Degradation

The chemistry of thiamine degradation is very complex as it can yield a wide variety of off-flavor compounds including MFT (26). MFT has been found to be produced as a result of the hydrolysis of thiamine under acidic conditions and also through the Maillard reaction involving cysteine and reducing sugars such as ribose (19, 24, 25).

During thermal processing such as baking, thiamine is cleaved to its pyrimidine and thiazole rings that create degradation compounds. Heating thiamine solutions at a pH of 6.0 or below cleaves thiamine at the methylene bridge between the rings and produces 4-methyl-5-(β-hydroxyethyl) thiazole as the main sulfur containing product (26). Dwivedi (26) confirmed that this compound is a product of thiamine in slightly acidic or alkaline conditions. In addition, hydrogen sulfide, H_2S, can be formed in a heated alkaline solution containing thiamine. This happens because the thiazole ring is broken releasing the thiol form of thiamine. Dwivedi (26) reported thiamine degradation off-flavors such as "heated onion" or "sulfury" odors were produced upon heating thiamine solutions. The main compounds that were identified to produce these off-flavors were hydrogen sulfide (H_2S), 2-methylthiophene and 4,5-dihydro-2-methylthiophene.

As stated before, both MFT and its dimer MFT-MFT are some of the most significant off-flavors related to the degradation of thiamine. In figure 1.9 it is illustrated that the thiol radicals from MFT can easily dimerize and produce bis(2-methyl-3-furyl) disulfide which is cleaved at higher temperatures (27). These compounds can also develop as a result of the Maillard reaction with cysteine and simple sugars, as well as a reaction of norfuraneol and cysteine (27). The cysteine and simple sugars can form MFT, but thiamine in the solution is required in order for it to react with S-cysteine to form MFT. Norfuraneol can also be a precursor in the development of MFT. According to Dreher et. al (18) norfuraneol is a degradation product of pentoses or hexoses. The addition of hydrogen sulfide to norfuraneol yields MFT after reduction by reductones from the Maillard reaction (27). These two potent "meaty" chemicals are important flavor compounds contributing to the characteristic flavor of meats such as beef, chicken, and pork. Thiamine which naturally is present in these products hydrolyzes to produce MFT and MFT-MFT.

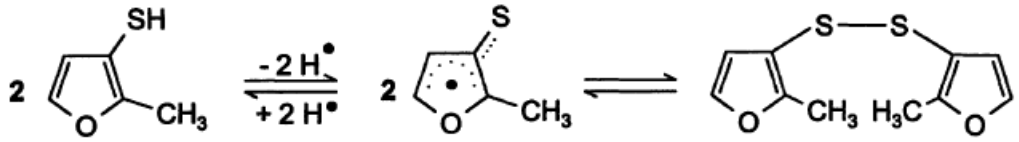

Figure 1.9. MFT readily dimerizing into the more potent chemical bis(2-methyl-3-furyl) disulfide or MFT-MFT under heated conditions (From ref. (27))

Without thiamine, MFT can still be formed under acidic conditions at a pH of 2.3. According to Zhang et al. (40) inosine-5'-monophosphate and hydrogen sulfide react. This reaction produces 5-hydroxy-3-mercaptopentan-2-one which is one of the main precursors for MFT (Figure 1.10). Other researchers suggest that MFT can be formed from the reaction of hydrogen sulfide and furanones at a pH of 5.7 (28). Researchers suggest that thiamine is one of the main contributors to MFT formation (24). They suggested that the precursor 5-hydroxy-3-mercaptopentan-2-one was stabilized in a H_2S rich environment (24).

Two pathways are involved in the thermal production of MFT (Figure 1.10). In one reaction the compound 5-hydroxy-3-mercaptopentan-2-one is cyclized into hemiacetal. Then the hemiacetal loses the water by dehydration to produce a potent aroma compound that is subsequently oxidized to produce the final product of MFT (45, 46). The other reaction involves thiamine producing the same compound 5-hydroxy-3-mercaptopentan-2-one in which the -SH group on that compound is replaced by a hydroxyl, -OH, group. When oxyfuran is produced, it reacts with hydrogen sulfide, H_2S, which is then oxidized to produce MFT (26, 45, 46). It is clear that the compound 2-methyl-4,5-hydro-3-furanthiol is a major precursor to the formation of MFT as an end product. Of the two pathways involved, it was found that the second pathway involving oxyfuran predominates more with respect to the thermal degradation of thiamine (28).

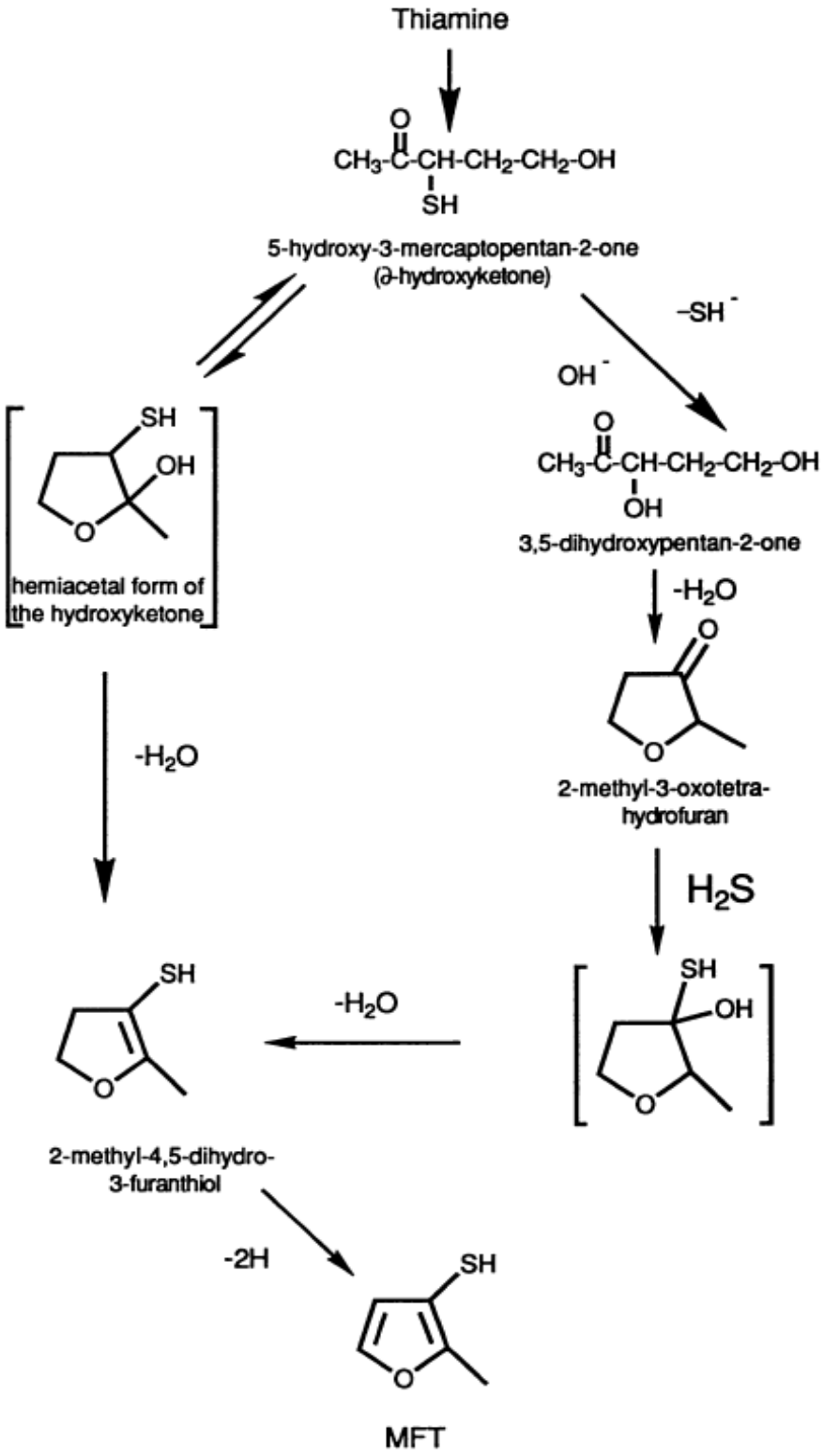

Figure 1.10. Formation of MFT off-flavor via two different chemical pathways (From ref. (28))

1.7 Cyclodextrins

Cyclodextrins (CyDs) are cyclic, non-reducing oligosaccharides that form crystalline complexes with a variety of chemicals. The molecular shape of cyclodextrins resemble that of a truncated cone (Figure 1.11). They have a hydrophilic exterior and a hydrophobic cavity, where small or medium-sized molecules can be included. Cyclodextrins are formed by the fermentation of starch using the enzyme CyD transglycosylase (4). There are three forms of CyDs: alpha (α-), beta (β-), and gamma (γ) cyclodextrin composed of 6, 7, and 8 D-glucose units respectively (Figure 1.11) (4). The cyclodextrins can trap a whole or part of a guest molecule inside the cavity by means of van der Waals forces, dipole-dipole interactions, and hydrogen bonding (4).

Inclusion complexes are formed when a flavoring material is mixed with cyclodextrins in a solution. The "guest" molecule may reside inside the cyclodextrin ring structure. The size of the ring can limit the complexation of a guest molecule. Polarity, hydrocarbon saturation, and the functional groups of the flavor components can also affect whether or not the molecule will be included in the cyclodextrin cavity or to what degree the inclusion will take place. β-CyD tends to be the best candidate for the inclusion of flavor chemicals as it can accommodate a wide variety of guest molecules, are readily available, reasonably priced, and provide the best stability once in the dry form (Table 1.2).

Table 1.2. Solubility and cost of cyclodextrins (From ref. (30))

Type	No. Glucose	M.W.	Solubility (g/100 mL H_2O)	Approx Cost $/kg (1000 kg purchase)
α	6	972	14.5	50
β	7	1136	1.85	8
γ	8	1298	23.2	90

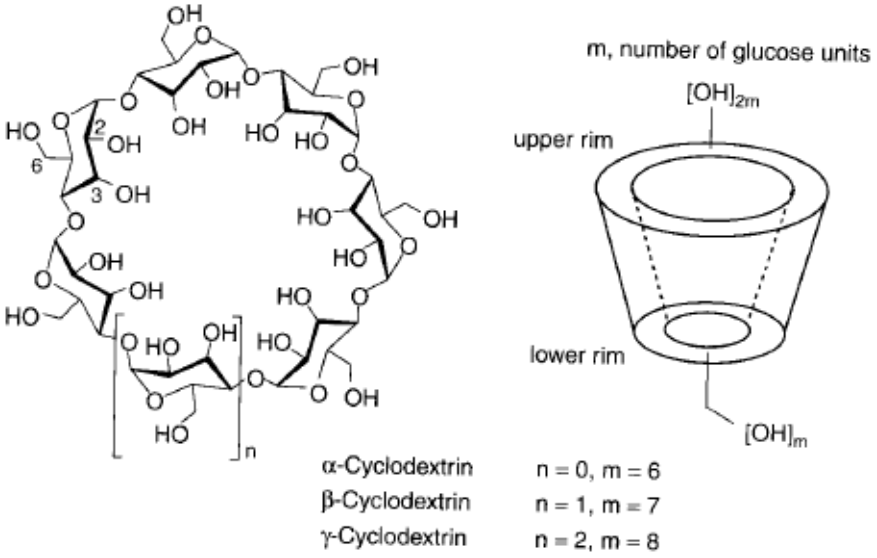

Figure 1.11. Structure of cyclodextrins (From ref. (30))

1.7.1 Cyclodextrins and Flavor Interactions

There is a lot of published research on using flavors with cyclodextrins. Cyclodextrin and flavor inclusion complexes can be prepared in a variety of ways including stirring the CyD and guest molecule in a beaker on a stir plate, as well as blending the CyD with guest molecules in a powerful mixer. A dry complex can be prepared by collecting the complex either by filtration or centrifugation and drying in an oven (36). Hedges et. al (47) claim that a slurry or paste method, which involves reducing the water content to 20-40% by filtering or centrifuging the complex, is good for commercial operations since it eliminates the drying process.

Flavor interactions with CyDs depend on the size of the flavor molecule, relative molar ratio of compound to CyD, the type of cyclodextrin, and the amount of CyD used. In a study using high temperatures (85°C), there was an increased interaction and binding of flavor compounds with CyDs (34). Reineccius et al. (33) found variable interactions between cyclodextrins and test volatiles. Only some of the cyclodextrins had interactions with the test volatiles, but β-CyD bound the volatiles to the greatest extent. Interactions between CyDs and flavor compounds can also be achieved by using more than one type of CyD rather than using one CyD individually (33). However, the use of CyDs may

have a negative impact on the sensory characteristics of foods and beverages. Sensory aspects such as decreasing flavor intensity and change in the characteristic flavor of a food has been reported (4, 34).

1.7.2 Reducing/Controlling Off-flavors by Cyclodextrins

Another application of cyclodextrins is to control or reduce off-flavors in foods, beverages, and drugs. Bitter, astringent components in foods such as soybeans, naringin in citrus fruit juice, and chlorogenic acid and polyphenols in coffee can be reduced or stabilized by the use of cyclodextrins (35). Soybeans have an astringent taste and a grassy smell that comes from aliphatic carbonyl compounds and volatile aliphatic alcohols. Adding CyDs to soy paste has been proven to form a complex with these carbonyl compounds and aliphatic alcohols to reduce the astringent taste and grassy smell in soybean food products (35). Cyclodextrins can also be used in citrus products that have undesirable off-flavors and bitterness. In orange juice, naringin, which is bitter, can be complexed with cyclodextrins to reduce the potential for these effects to occur. However, it has been found that the use of cyclodextrins in citrus products can be problematic because they can affect the desirable components of the juice (35).

1.7.3 Cyclodextrins and Use with Vitamins/Thiamine

Cyclodextrins can be used to stabilize vitamins and essential oils from deteriorating effects such as light, heat, oxygen, and other compounds that might react with them (36). For example, Vitamin A rapidly degrades when exposed to light and air. When this vitamin was complexed with β-CyD and after 24 hours of sitting in an oxygen environment, oxidation was reduced as compared to fresh Vitamin A (35, 36). The same concept can be utilized in the stabilization of thiamine from heat degradation. One of the foods that contains thiamine is brown rice (Table 1.1). Since rice is stored for more than one year, it acquires an unpleasant off-flavor due to the degradation of thiamine. It was found in a study that the characteristic odor of thiamine can be reduced by cooking the rice in water with 0.01-0.4% β-CyD (37).

1.7.4 Thiamine and Beta-Cyclodextrin Complex Formation

There are two ways CyDs may reduce off-flavors—first, by forming a CyD complex to stabilize the thiamine, the other to react with thiamine degradation products to

make them less intense (36, 38). β-CyD is the most economical cyclodextrin to potentially use in stabilizing thiamine as it is the cheapest form of cyclodextrin available, approved for food use, and it forms a stable complex with thiamine. It is therefore useful to review how well thiamine complexes with β-CyD to gain some insight into the potential of the complex to minimize off-flavor formation. Nuclear Magnetic Resonance (NMR) was used to determine how well different cyclodextrins complex with thiamine hydrochloride (38). When increasing levels of thiamine were complexed with α-CyD, the inclusion and stability of thiamine decreased. However, when it was incorporated with β-CyD a 1:1 complex was formed based on molar ratios. Results indicated that the inclusion of the hydrophobic functional group of thiamine occurred in the cavity of the β-CyD (38).

It was also concluded that by measuring the H-NMR and C-NMR chemical shifts, the thiamine hydrochloride and its derivatives were included in the complex. This was evidenced by H-3 protons of β-CyD showing a downfield shift and the H-5 protons exhibiting an upshield shift (38). Specifically, the pyrimidine ring of the thiamine hydrochloride molecule was included in the hydrophobic cavity of the β-CyD (38). This was determined because the results indicated that the ring proton signals of thiamine hydrochloride shifted downfield with increasing molar ratio of thiamine:β-CyD. Additionally, it was determined by High Performance Liquid Chromatography (HPLC) method that the thiamine hydrochloride and β-CyD complex showed a 1:1 stoichiometry because it had a linear relationship. Overall, it was found that the cavity of the β-CyD was better suited for including thiamine and achieved better stability compared to α-CyD (38).

1.8 Research Objectives

The main objectives of the present research were to create model energy beverages that contained thiamine, investigate the off-flavor compounds generated by thiamine degradation, and investigate ways to reduce these off-flavor compounds.

Chapter 2

Formation of Off-Flavors Due to Thiamine Degradation in Simple Model Beverage Solutions

Summary

Model energy beverage solutions prepared at a low pH (2.6) and containing 0.3g of thiamine hydrochloride were stored for 12 days at 50°C in the absence of light. Samples studied contained $1.83x10^{-3}$ mol/L (1g) β-CyD, $3.7x10^{-3}$ mol/L (2g) β-CyD, $1.6x10^{-3}$ mol/L (1g) γ-CyD, and $3.2x10^{-3}$ mol/L (2g) γ-CyD were compared to the control sample. The ability of the CyDs to reduce thiamine degradation off-flavor compounds was measured by monitoring the major off-flavor compounds. These compounds were quantified using gas chromatography (GC) with a flame ionization detector (FID). Compound identifications were confirmed by gas chromatography-mass spectrometry (GC-MS).

Several off-flavor compounds were identified in the samples including dimethyl sulfide, 4,5-dimethylthiazole, methional, and 2-acetylthiophene. However, the more sensorially significant compounds detected were 2-methyl-3-furanthiol (MFT) and its dimer bis(2-methyl-3-furyl) disulfide (MFT-MFT). Overall, the 1g ($1.83x10^{-3}$ mol/L) β-CyD sample and the 2g ($3.2x10^{-3}$ mol/L) γ-CyD sample were effective at reducing the off-flavor compounds in the sample by up to 80%. The 2g γ-CyD was the best at reducing all the identified off-flavor compounds. The results could prove to be useful for the incorporation of thiamine into energy beverage formulations.

2.1 Introduction

Thiamine will thermally decompose to produce sulfur compounds that render food and beverage products unacceptable to the consumer. Temperature, pH, and heating time all influence the decomposition pathways and the final products (17).

Thiamine is a water soluble B vitamin complex that helps nervous system function, mental acuity, and helps to convert carbohydrates in the body to produce energy (15). Some foods and beverages such as whole wheat bread, long grain brown rice, orange juice, and milk are good sources of thiamine (18). Thiamine can be added in the form of a crystalline food additive, thiamine hydrochloride, to fortify food products lacking the vitamin. It is sometimes added to soups and gravies to impart a meaty, brothy character to the product.

Some of the most significant thermal degradation products of thiamine include dimethyl sulfide, 4,5-dimethylthiazole, 2-acetylthiophene, 2-methyl-3-furanthiol (MFT), and its dimer bis(2-methyl-3-furyl) disulfide also known as MFT-MFT (18). Both MFT and MFT-MFT have very low odor thresholds that impart an objectionable meaty, savory aroma to beverage products.

Since thiamine has the potential to degrade into objectionable off-flavor compounds, it is seldom used in energy beverages. Most energy beverages are stored at room temperature and have relatively low pH's around 2.6, which is similar to the pH of many citrus juices such as orange juice. Recent research shows that the low pH of orange juice and storage at room temperature can degrade thiamine to produce off-flavor compounds (18). Since most producers of energy drinks may want to include thiamine into their energy beverages for the obvious benefits, the goal of this research was to reduce thiamine degradation off-flavor compounds. The main purpose of the study was to identify off-flavor compounds in thiamine fortified model energy beverages and to reduce these compounds by using different cyclodextrins (CyD's). It was hypothesized that the addition of β- and γ-cyclodextrin will stabilize thiamine thereby reducing the off-flavor compounds formed from it. The main objective of the study was to monitor off-flavor formation in the model beverage solution stored at 50°C over a 12 day shelf-life period.

2.2 Materials and Methods

Materials

The following compounds were obtained from Aldrich (Milwaukee, WI): 2-methyl-3-furanthiol, 4,5-dimethylthiazole, 2-acetylthiophene, bis(2-methyl-3-furyl) disulfide, and 3-(methylthio) propionaldehyde (methional). Thiamine hydrochloride, dimethyl sulfide, 1-pentanol and 4-methyl-5β-hydroxyethyl thiazole were obtained from Sigma (St. Louis, MO). Beta and gamma cyclodextrin was a gift from Robertet Flavors Inc. (Piscataway, New Jersey). Citric acid was purchased from Fisher Scientific (Pittsburgh, PA). Twister®/stir-bars were used for the extraction of solutions and purchased from Gerstel Inc. (Linthicum, Maryland).

Sample Preparation

Preparation of Thiamine Model Solutions

Model thiamine solutions contained the following ingredients: 480mL water adjusted to a pH of 2.6 using citric acid, 0.3g of thiamine hydrochloride, and ca. 50 ppb (5μL) of 4-heptanone as the internal standard. This solution served as the control. Four other solutions were prepared the same way, but cyclodextrins were added. One solution had 1g β-cyclodextrin ($8.8x10^{-4}$ mol), the second solution had 2g of β-cyclodextrin ($1.76 x10^{-3}$ mol), the third solution had 1g γ-cyclodextrin ($7.7x10^{-4}$ mol), and the fourth solution had 2g γ-cyclodextrin ($1.54x10^{-3}$ mol). These cyclodextrins were solubilized in 480mL water for 30 min at 450 rpm and stored for 24 hrs. Thiamine hydrochloride, citric acid, and the internal standard were added in the same amounts as stated above. Samples were made twice for two separate analyses.

Storage and Sampling Protocol

Samples were stored at 50°C in the absence of light and samples were analyzed on days 0, 3, 6, 9, and 12. Fifteen mL aliquots were taken and placed into a 20mL closed glass vials. Samples were extracted using stir-bar sorptive extraction (SBSE, Twister®).

Sample Analysis

Quantitative Analysis

Compounds were quantified by means of a multiple point standard calibration curve containing 50-100ppb of each of the compounds listed in materials. Each

compound was weighed out to 0.24g on an analytical balance and 100% ethanol was used to fill to a 50mL volumetric flask. Then 5-10µL of this was taken and put in 480mL water to obtain a final concentration of 50-100ppb. The samples were extracted using stir-bar sorptive extraction (SBSE) and analyzed in triplicate using a GC-FID. Then the relative response to an internal standard (4-heptanone) versus concentration was plotted to display the linear correlation.

Stir-bar Sorptive Extraction

A Twister® was directly immersed in 15mL of sample and was stirred on a stir plate at 250 rpm for 20 min. After the extraction, the Twister® was removed from the sample using a tweezer. The Twister® was rinsed with double distilled water and blotted dry with a Kimwipe. Then the Twister® was put in the injection port of the GC or GC-MS at 225°C and compounds were cryofocused by immersing a part of the GC column in a Dewar flask containing liquid nitrogen under splitless mode for 5 min. After 5 min., the liquid nitrogen was removed and the GC acquired data for analysis.

Gas Chromatography

An HP 5890 Series II GC (Agilent Technologies, Palo Alto, CA) with a flame ionization detector (FID) was used to analyze the model solutions. The GC was equipped with a DB-Wax column (30m x 0.25 mm i.d. x 0.25 µm film thickness, J&W Scientific (Folsom, CA)). The initial oven temperature was 50°C and it was increased at 10°C/min to a final temperature of 230°C. The GC was operated on splitless mode for 1 min. then thereafter it was in split mode at 50mL/min. Injection port temperature was set to 225°C and detection temperature set to 250°C. Data were collected and recorded using ChemStation Software. Samples were analyzed in triplicate.

Gas Chromatography-Mass Spectrometry

The samples were analyzed using a GC-MS system for further verification of the compound identity. Volatile separation was performed using a HP 5890 Series II GC with a HP 5970 Series Mass Selective Detector (Agilent Technologies, Palo Alto, CA). The GC was equipped with a DB-Wax column (30m x 0.25 mm i.d. x 0.25 µm film thickness, J&W Scientific (Folsom, CA)). The MSD was operated under EM Voltage: solvent delay of 0.50 min., EM Voltage 1400, low mass 29, high mass 350, and threshold

150. The transfer line temperature was set at 250°C. The GC oven parameters were under the same conditions as stated above. The MSD was operated in the selective ion monitoring (SIM) mode to continuously measure selected ions of the compounds. Data were collected using ChemStation Software.

Identification of Volatiles

Samples were identified using a GC-MS and compound chromatography relative to reference standards. The MS was used in the Selected Ion Chromatogram (SIC). Dimethyl sulfide had an m/z 62 (M+), 46. 4,5-dimethylthiazole had an m/z 113, 71, 85. Methional had an m/z 48, 104, 76, 61. 2-acetylthiophene had an m/z 111, 126. The potent compound MFT had an m/z 114 (M+), 85, and 45. The dimer MFT-MFT had an m/z 226 (M+), 113, and 43.

Artifact Formation during Analysis

To test the potential for the high injector temperatures to create thermal artifacts due to thiamine's sensitivity to heat, an artifact formation study was done. Headspace samples from the control and cyclodextrin samples were exposed to SPME: 50/30μm DVB/Carboxen/PDMS StableFlex (Supelco, Bellfonte, PA) for 20 min. Then the SPME fiber was desorbed as described above for the stir bar and the same GC parameters as stated above for compound separation.

Data Analysis

Samples were analyzed and underwent two-way ANOVA statistical analysis using R software version 2.10.1 (2009 The R Foundation for Statistical Computing ISBN 3-900051-07-0). Significance level was set at $\alpha=0.05$.

2.3 Results and Discussion

This study focused on examining the potential of CyDs to reduce the formation of thiamine degradation off-flavor compounds in simple model energy drinks containing water and thiamine hydrochloride. Two types of cyclodextrins were used in the formulation of the complex model beverages: β-CyD and γ-CyD. The recommended daily intake (RDI) of thiamine is 1.4 mg per day, but the concentrations in the beverages

were increased to 100x (140mg) to generate off-flavors at higher levels for better quantification.

Standard Calibration and Method Validation

Off-flavors in model energy beverages were quantified by using the internal standard method. In the standard calibration curve there was a good linear relationship between increasing concentration and relative amount of each compound to an internal standard (Figure 2.1). Thus, one can use the linear equation to calculate the amount of each off-flavor compound in the sample. All of the compounds had an average R^2 of 0.951, which indicated a strong linear relationship. The good correlation and high R^2 validated the stir-bar method (extraction time of 20 min).

Table 2.1. Statistics on the standard curves used in the quantification of selected thiamine degradation products

Compound	Retention Time (min)	Linear Equation	R^2
Dimethyl sulfide	3.85	y = 0.1533x - 3.7011	0.9521
2-methyl-3-furanthiol (MFT)	8.6	y = 0.7156x - 36.292	0.9789
4,5-dimethylthiazole	9.79	y = 0.2341x - 11.827	0.861
Methional	10.78	y = 0.3378x - 7.8145	0.9547
2-acetylthiophene	14.6	y = 0.0918x + 0.2781	0.9837
bis(2-methyl-3-furyl) disulfide (MFT-MFT)	18.38	y = 0.0112x + 0.0294	0.9729

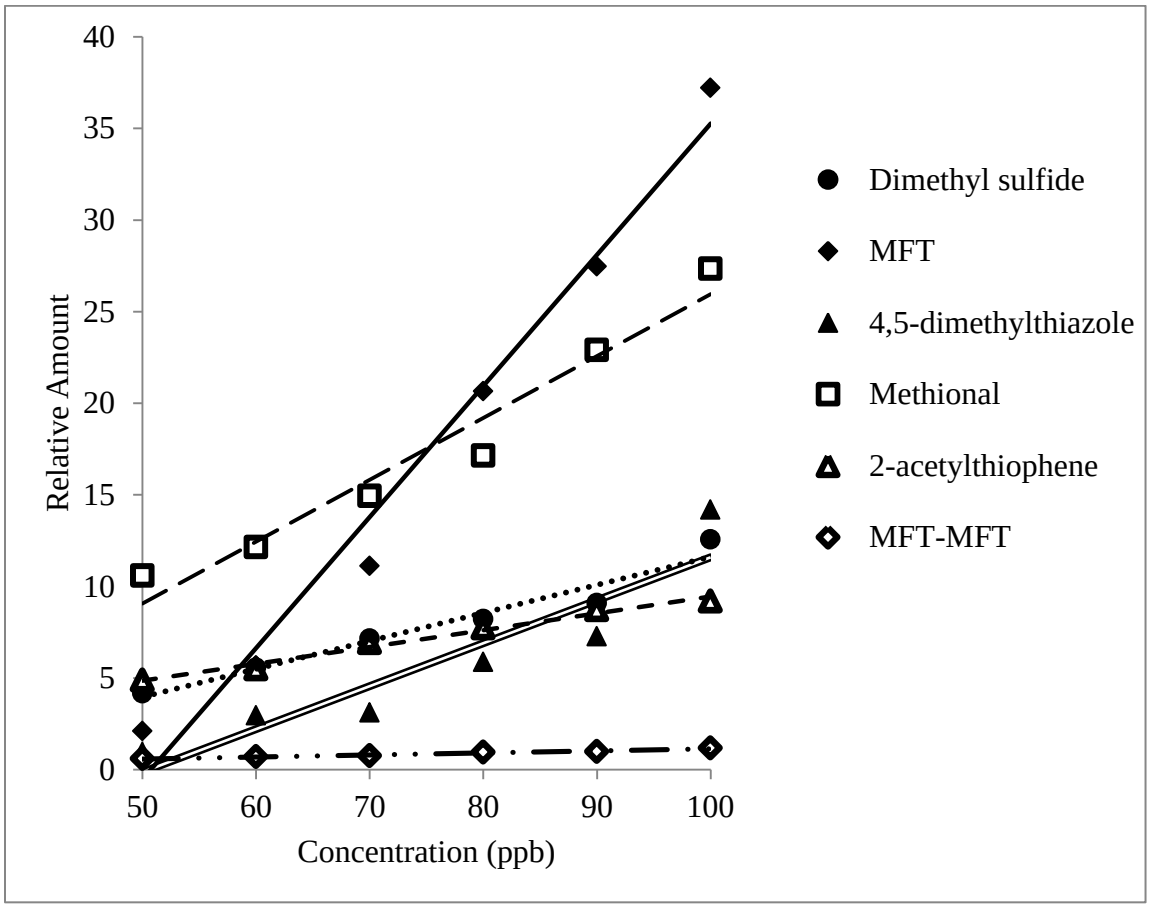

Figure 2.1. Multiple Point Calibration curve containing 50-100ppb of off-flavor compounds relative to the amount of a known concentration of an internal standard

In Table 2.1, most of the compounds showed a good linear regression. The compound with the lowest R^2 value, 4,5-dimethylthiazole, was a good fit except for the last point. This could have been due to some user error.

Use of β-cyclodextrin to reduce off-flavors

Overall, the β-cyclodextrin appeared to reduce concentrations of compounds that have been previously reported as thiamine off-flavors in literature (18). The $1.83x10^{-3}$ mol/L of β-CyD was used to form a complex with $1.83x10^{-3}$ mol/L thiamine in the sample. Cumulatively, 1g ($1.83x10^{-3}$ mol/L) of β-CyD was adequate in reducing $2.0x10^{-7}$ mol/L of off-flavor compounds in the sample. Specifically, the 1g of β-CyD reduced the concentrations of thiamine degradation off-flavor compounds better than the 2g ($3.7x10^{-3}$ mol/L) of β-CyD (Figures 2.2-2.5). One example of this is the significantly

higher amounts of 4,5-dimethylthiazole in the 2g of β-CyD sample (Figure 2.3). In the control sample, all the detected off-flavor compounds showed an increase in concentration over the 12 day shelf-life period (Figures 2.2-2.5). Compared to the control, 1g of β-CyD reduced concentrations of dimethyl sulfide after day 3 ($p<0.05$) (Figure 2.2), 4,5-dimethylthiazole after day 3 ($p<0.05$) (Figure 2.3), 2-acetylthiophene after day 6 ($p<0.05$) (Figure 2.4), and methional after day 6 ($p<0.05$) (Figure 2.5). One significant off-flavor, 4,5-dimethylthiazole was reduced by nearly 48% on day 12 in the 1g β-CyD sample.

On the other hand, 2g of β-CyD of beverage reduced concentrations of dimethyl sulfide after day 9 ($p<0.05$) (Figure 2.2), methional after day 9 (Figure 2.5), and 2-acetylthiophene after day 6 (Figure 2.5). The 2g of β-CyD actually had a negative effect on higher concentrations of 4,5-dimethylsulfide than the control sample. There is little rationale for the 1g of β-CyD sample working better than 2g β-CyD, which is problematic.

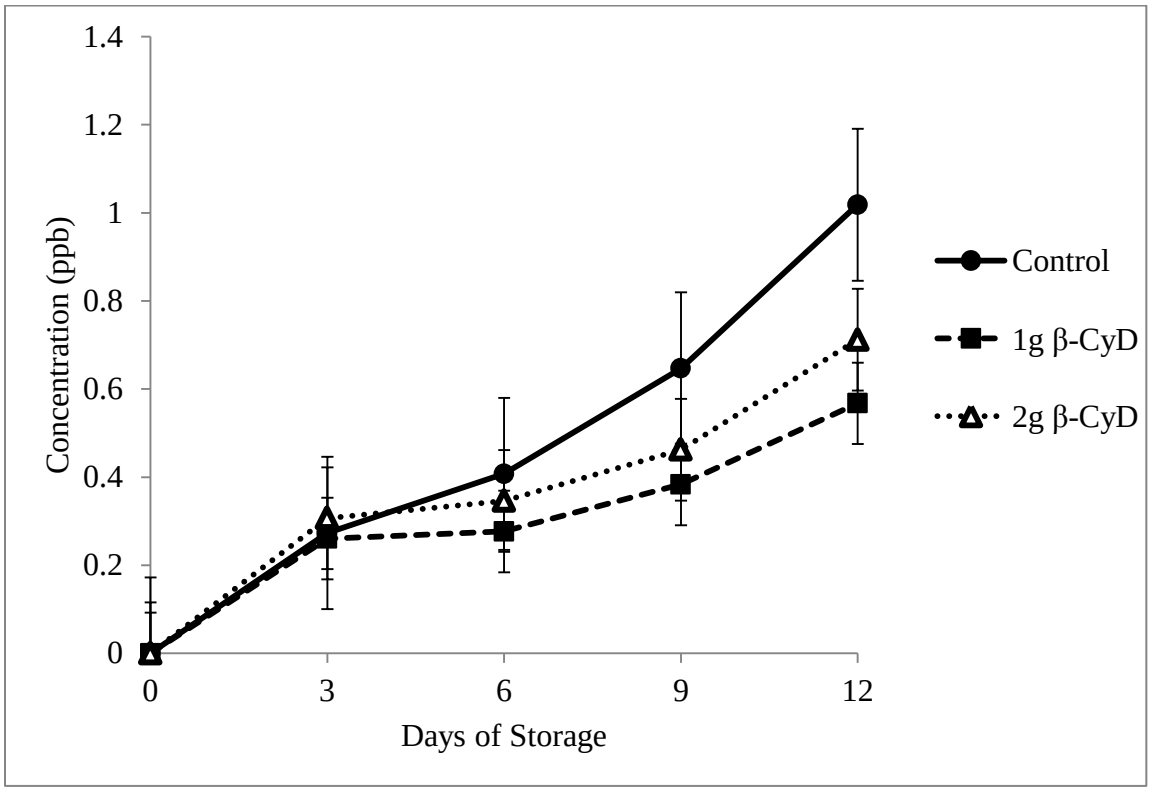

Figure 2.2. Influence of adding β-CyD on reducing concentrations of dimethyl sulfide in stored thiamine-containing model systems

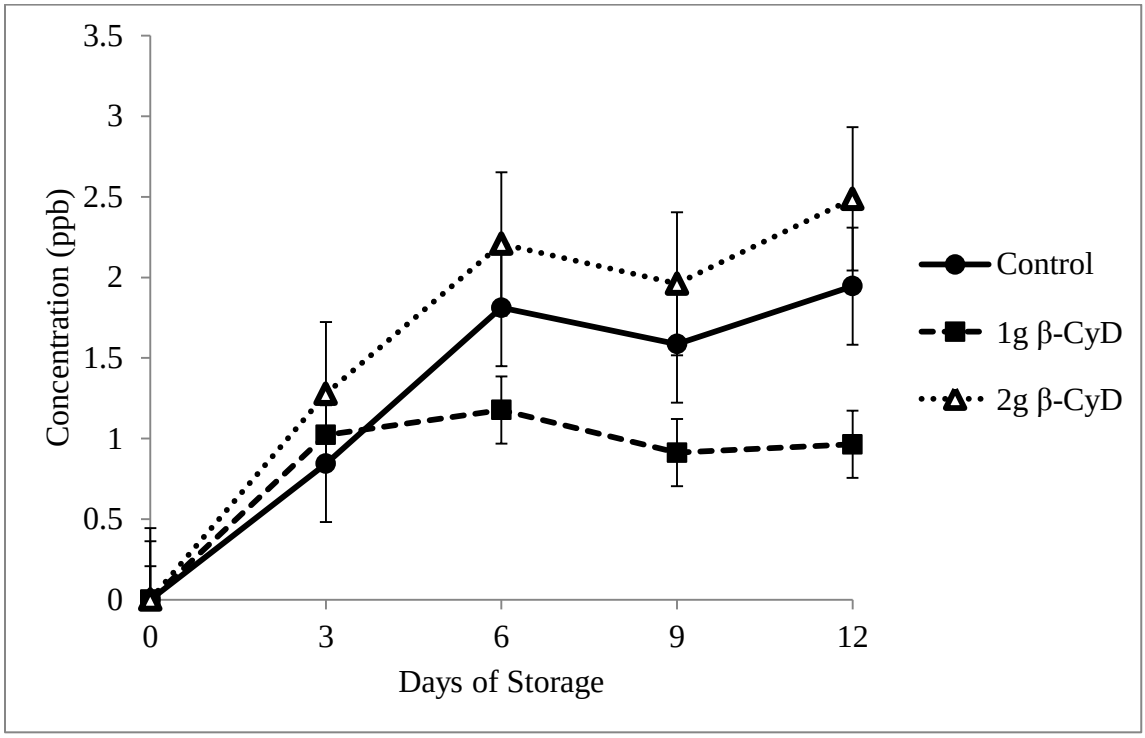

Figure 2.3. Influence of adding β-CyD on reducing concentrations of 4,5-dimethylthiazole in stored thiamine-containing model systems

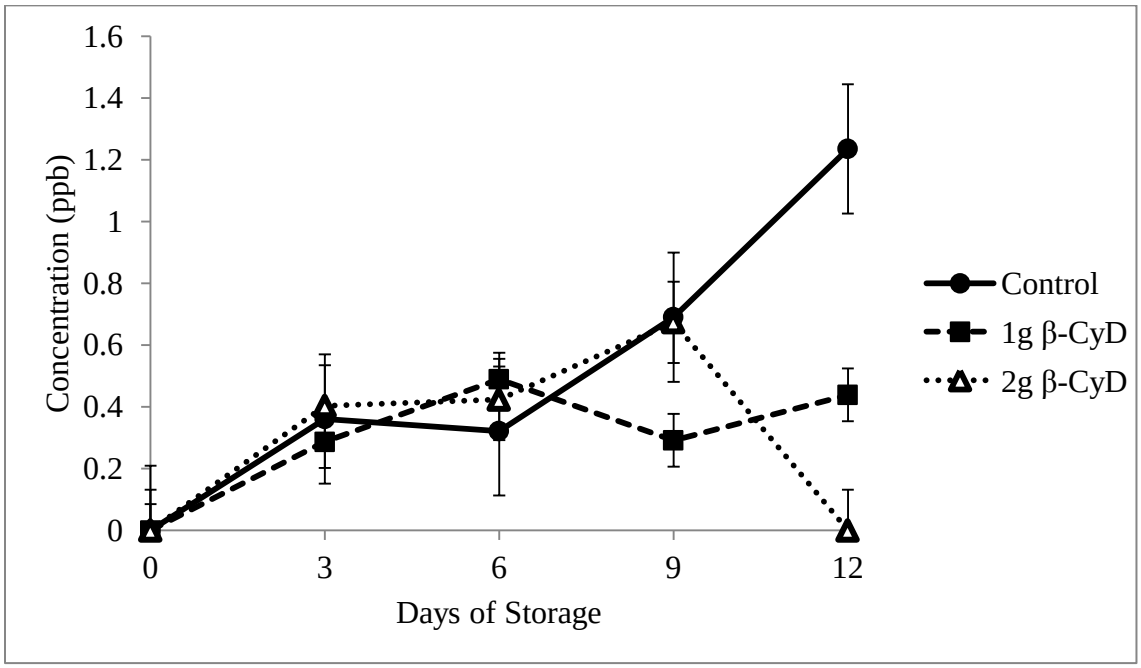

Figure 2.4. Influence of adding β-CyD on reducing concentrations of methional in stored thiamine-containing model systems

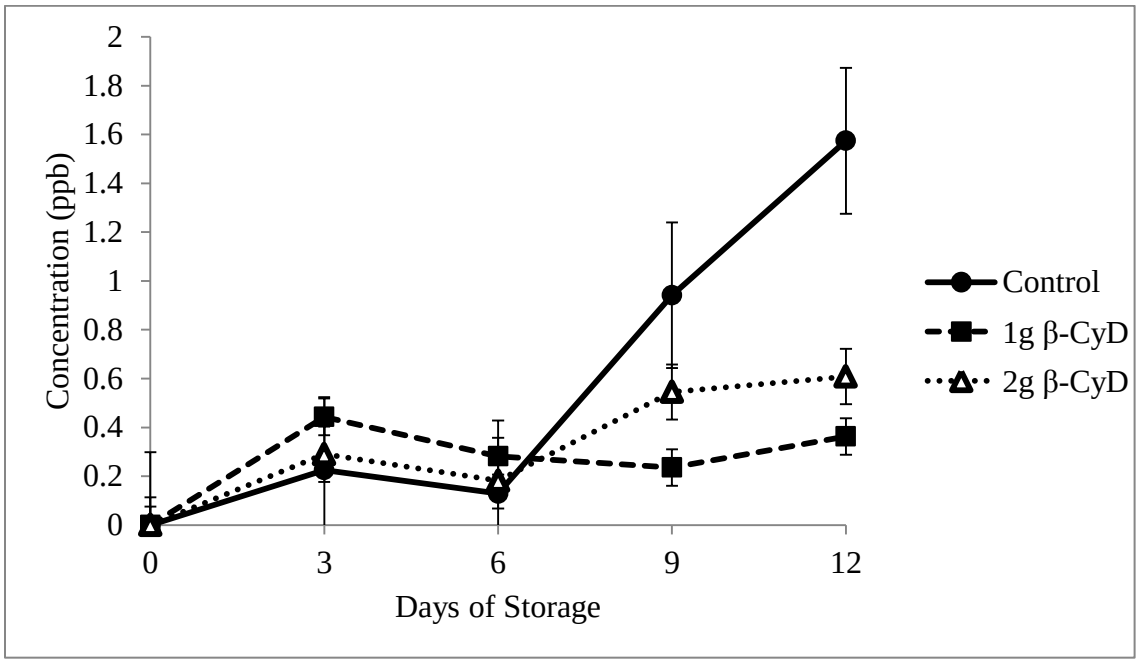

Figure 2.5. Influence of adding β-CyD on reducing concentrations of 2-acetylthiophene in stored thiamine-containing model systems

Use of γ-cyclodextrin to reduce off-flavors

When using γ-CyD, much different results were found. It was determined that 2g ($3.2x10^{-3}$ mol/L) of γ-CyD was more effective in reducing $2.0x10^{-7}$ mol/L of thiamine degradation off-flavor compounds compared to 1g ($1.6x10^{-3}$ mol/L) of γ-CyD (Figures 2.6-2.9). Compared to the control, 1g of γ-CyD reduced the concentrations of methional immediately ($p<0.05$) (Figure 2.8), dimethyl sulfide after day 9 ($p<0.05$) (Figure 2.6), and 2-acetylthiophene after day 9 ($p<0.05$) (Figure 2.9). The amount of 4,5-dimethylthiazole present in the 1g γ-CyD sample was lower than the control only on day 6.

In comparison, the samples containing a higher level 2g ($3.2x10^{-3}$ mol/L) of γ-CyD reduced concentrations of dimethyl sulfide after day 6 ($p=0.012$) (Figure 2.6), 4,5-dimethylthiazole immediately ($p<0.05$) (Figure 2.7), methional immediately ($p<0.05$) (Figure 2.8), and 2-acetylthiophene after day 12 ($p=0.042$) (Figure 2.9). It was very effective in reducing the formation of methional with there being no detectable amount in any samples. The major sulfur compounds dimethyl sulfide, 4,5-dimethylthiazole, and 2-

acetylthiophene were reduced 53%, 47%, and 45% respectively by day 12 using $3.2x10^{-3}$ mol/L of γ-CyD in the model solution.

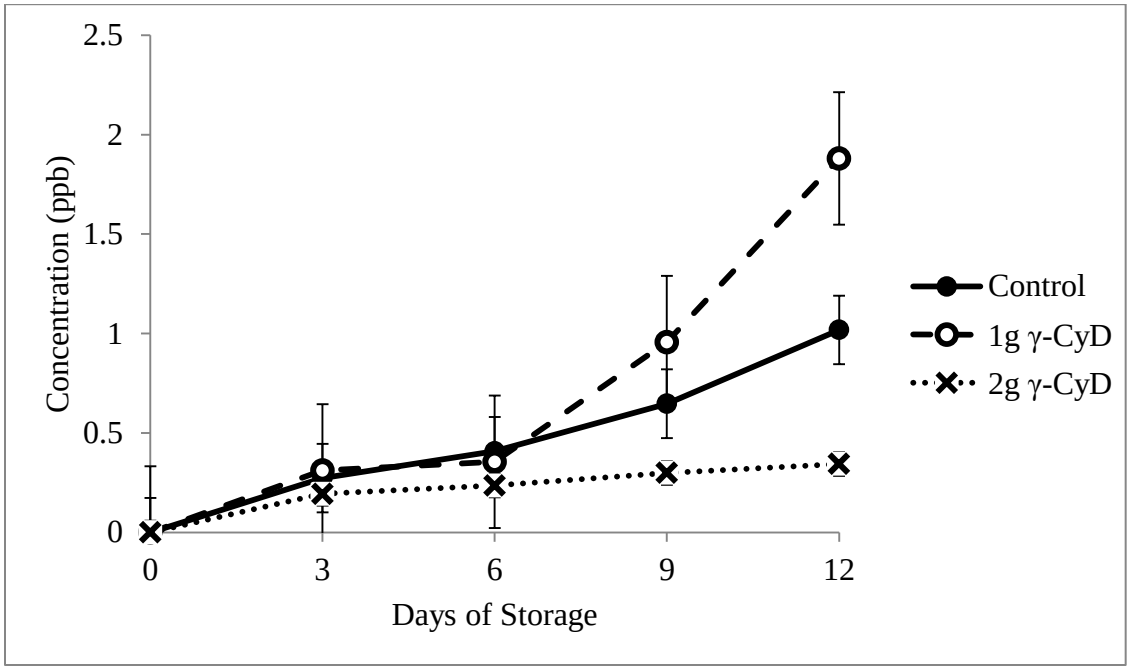

Figure 2.6. Influence of adding γ-CyD on reducing concentrations of dimethyl sulfide in stored thiamine-containing model systems

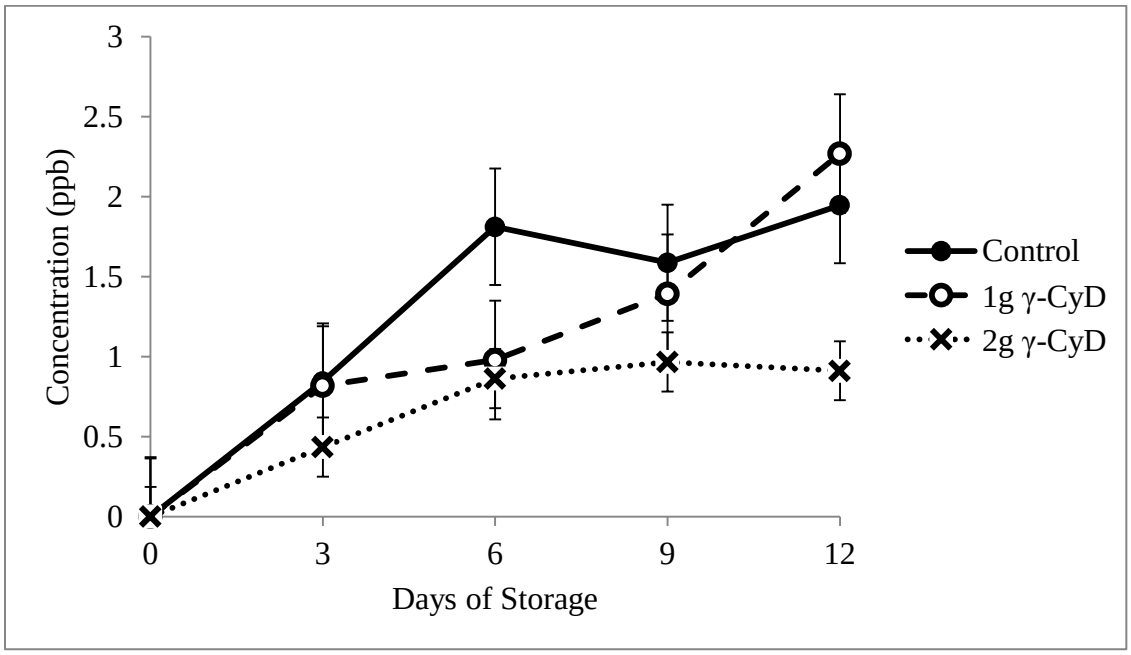

Figure 2.7. Influence of adding γ-CyD on reducing concentrations of 4,5-dimethylthiazole in stored thiamine-containing model systems

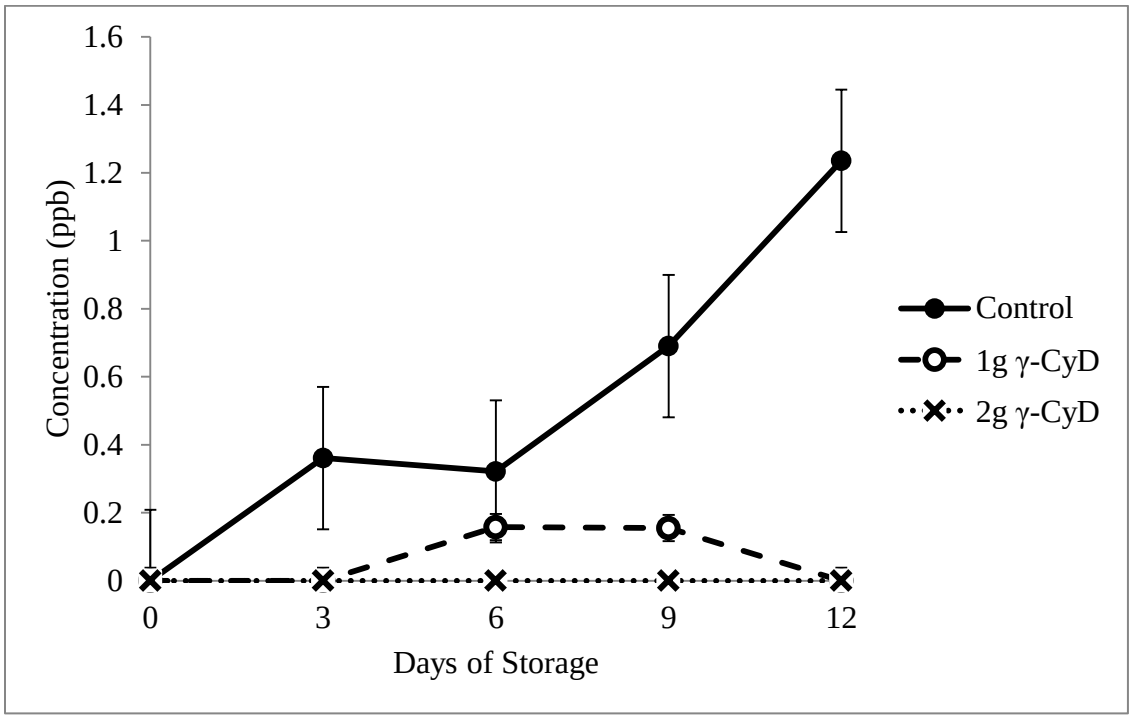

Figure 2.8. Influence of adding γ-CyD on reducing concentrations of methional in stored thiamine-containing model systems

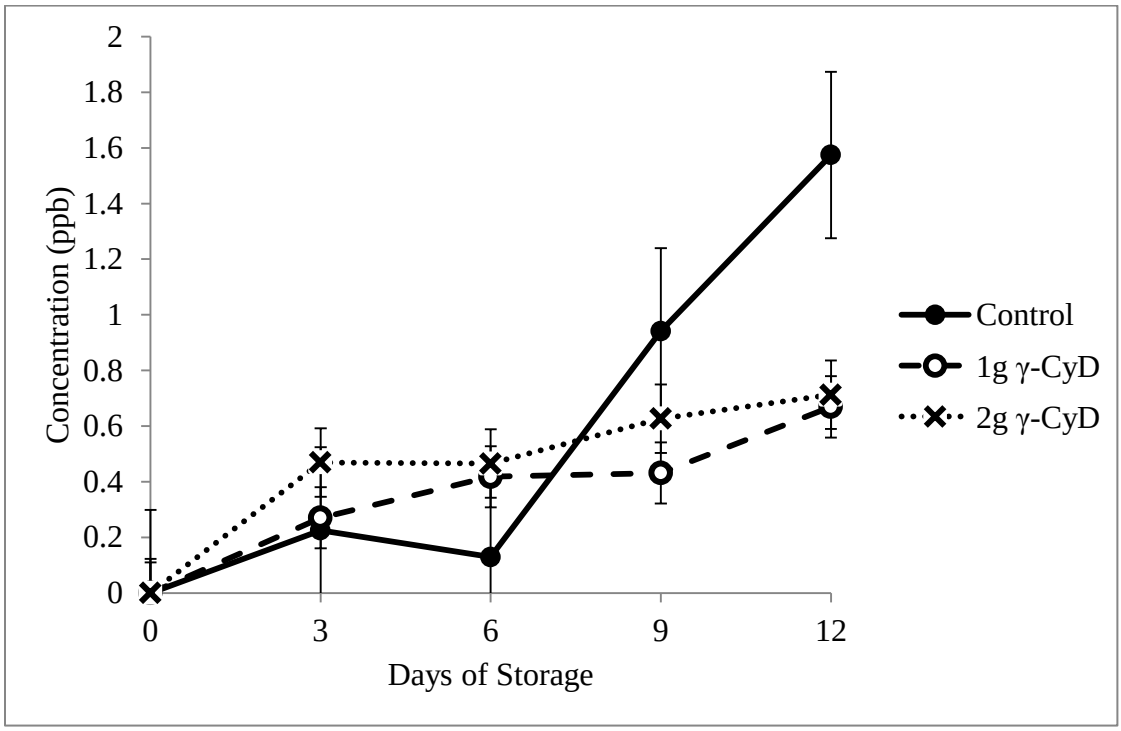

Figure 2.9. Influence of adding γ-CyD on reducing concentrations of 2-acetylthiophene in stored thiamine-containing model systems

In Figures 2.3-2.5 and 2.10 the concentrations of 4,5-dimethylthiazole, methional, and 2-acetylthiophene in the β- and γ-CyD containing samples were higher than that of the control in earlier days of testing. This does not appear to be logical but I will present a couple of explanations for this. One explanation could be a problem (error) in analysis. The data were normalized relative to an internal standard (4-heptanone) and thus, any factor that influenced the measured amount of this compound would introduce error into the quantification of all compounds being measured. For example, there could have been a co-chromatography of some unknown with this compound thereby changing its peak area. An alternative reason is more complicated and involves an appreciation for the interactions that may occur in the system being studied. For instance, the CyD is going to include thiamine which may protect it from degradation. However, the CyD will also potentially include some or all of the degradation products of the thiamine. Thus, there will be some equilibrium of thiamine and its degradation products with the CyD. We complicate this by adding a stir bar which competes with the CyD for degradation products. The final equilibrium that one has between CyD, stir bar and thaimine for the CyD is very complex and unknown. The data may in fact be correct or reflect the complexity of attempting to determine how much degradation product is formed when using a partitioning method for off flavor extraction when in the presence of CyD.

MFT and MFT-MFT Generation in Model Solutions

Two of the most significant thiamine thermal degradation products are 2-methyl-3-furanthiol and its dimer bis(2-methyl-3-furyl) disulfide. They have very low sensory thresholds and contribute a brothy, meaty aroma that is not desirable in a beverage product. Both are well documented contributors to meat flavors (23). MFT has an odor threshold of 0.007 ppb (6.14×10^{-8} mM) in water (20) and MFT-MFT has an odor threshold of 2.0×10^{-5} ppb (8.9×10^{-11} mM) in water (21). Since the dimer (MFT-MFT) is formed from the monomer (MFT), the formation of these compounds are linked. Due to the importance of these two compounds, this study focused more specifically on the effectiveness of β-and γ-cyclodextrin in reducing the concentrations of MFT and MFT-MFT in the model energy beverages.

In Figures 2.10 and 2.11, there is a pretty steady increase in the concentration of both MFT and MFT-MFT with storage in the control sample. Both 1g and 2g of β-CyD were effective in reducing the concentrations of MFT (after 6 days of storage) and MFT-MFT to about 0.04 ppb (Figure 2.10, Figure 2.11). With respect to MFT-MFT, both β-CyD concentrations decreased levels of MFT-MFT in the sample to roughly 7 ppb (Note there was no statistical significant difference at day 12, 2g CyD) (Figure 2.11). It is clear that the concentrations of MFT and MFT-MFT in the CyD samples are above their sensory thresholds. This may be due to my desire to use elevated levels of thiamine in the samples to make analysis easier. If the typical RDI of thiamine was used, the concentrations of MFT and MFT-MFT would be much lower but I expect still above their sensory thresholds.

In Figure 2.10, MFT increased faster initially in the CyD containing samples and then started to decrease. One explanation for this could be user error in analyzing the data. Unfortunately, there is little rationale for this occurrence especially since the β-CyD was able to reduce the concentrations of MFT after day 6.

In the experiments with γ-CyD, the control solution exhibited the same trends in MFT and MFT-MFT concentrations as for the β-CyD containing samples (Figures 2.12 and 2.13). This occurred because the control was the same as for the β-CyD experiments. When γ-CyD was added, the trends were more consistent with expectations: The 2g ($3.2x10^{-3}$ mol/L) of γ-CyD sample was more effective at reducing concentrations of both aroma compounds during storage than the 1g ($1.6x10^{-3}$ mol/L) of γ-CyD sample. Similar to β-CyD, the 1g γ-CyD sample reduced concentrations of MFT-MFT ($p=0.03$) up until day 12 where the concentration increased significantly (Figure 2.13). By day 12, 2g γ-CyD had about 0.02 ppb MFT and 2 ppb MFT-MFT.

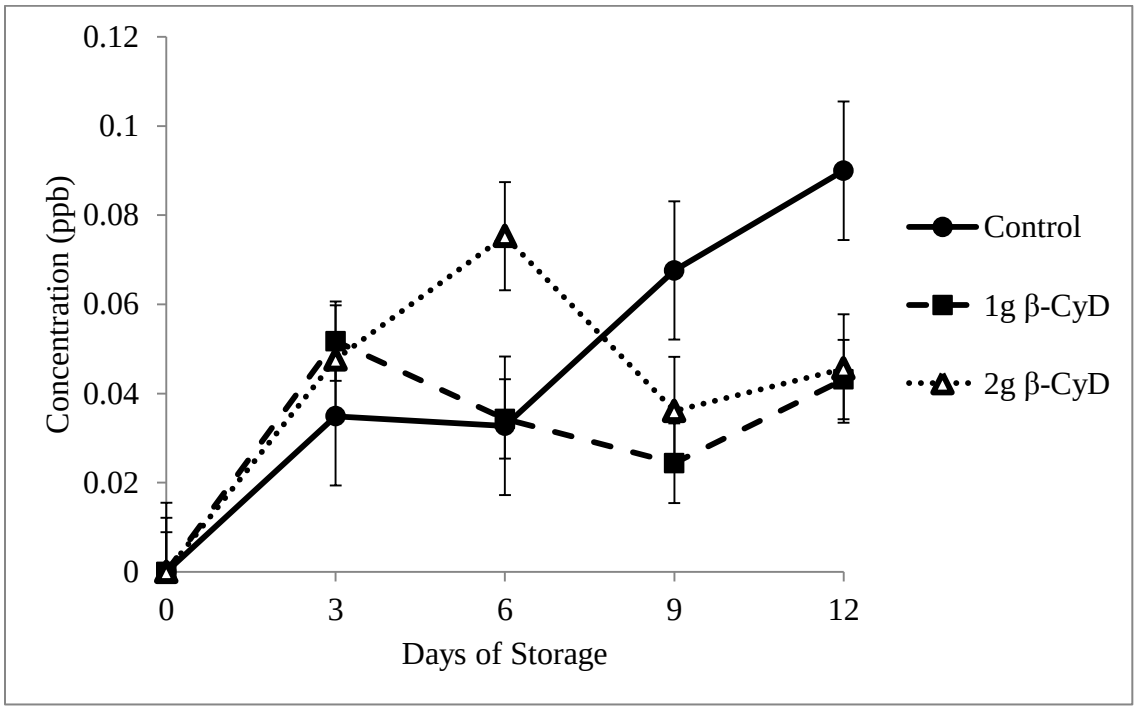

Figure 2.10. Influence of adding β-CyD on reducing concentrations of MFT in stored thiamine-containing model systems

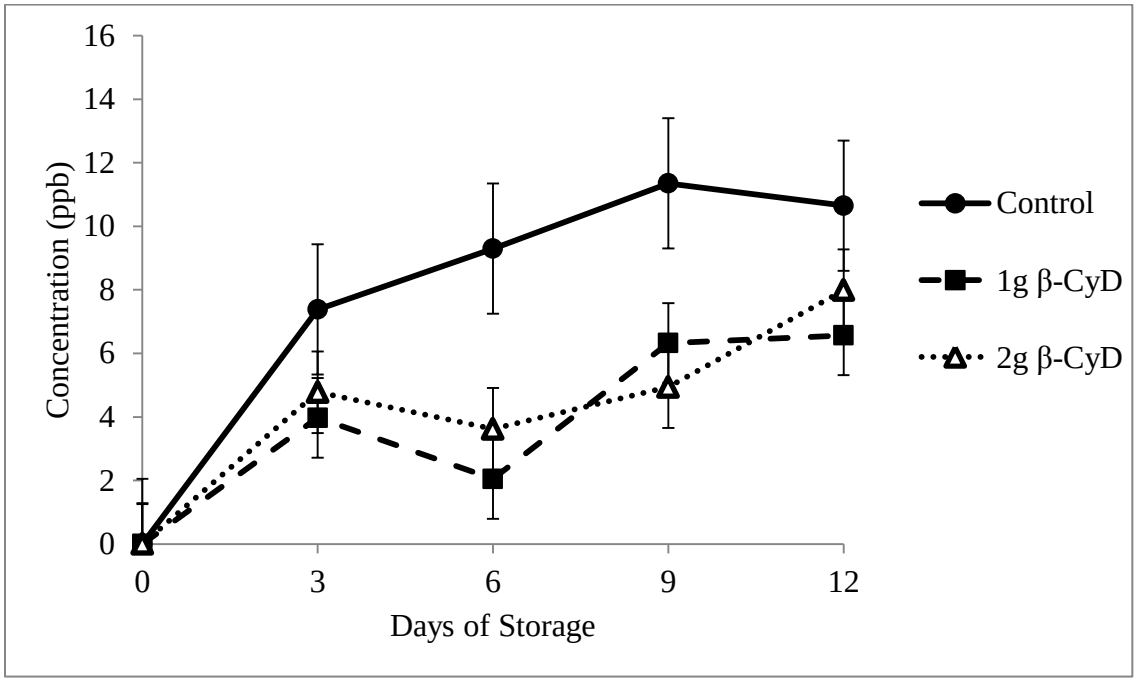

Figure 2.11. Influence of adding β-CyD on reducing concentrations of MFT-MFT in stored thiamine-containing model systems

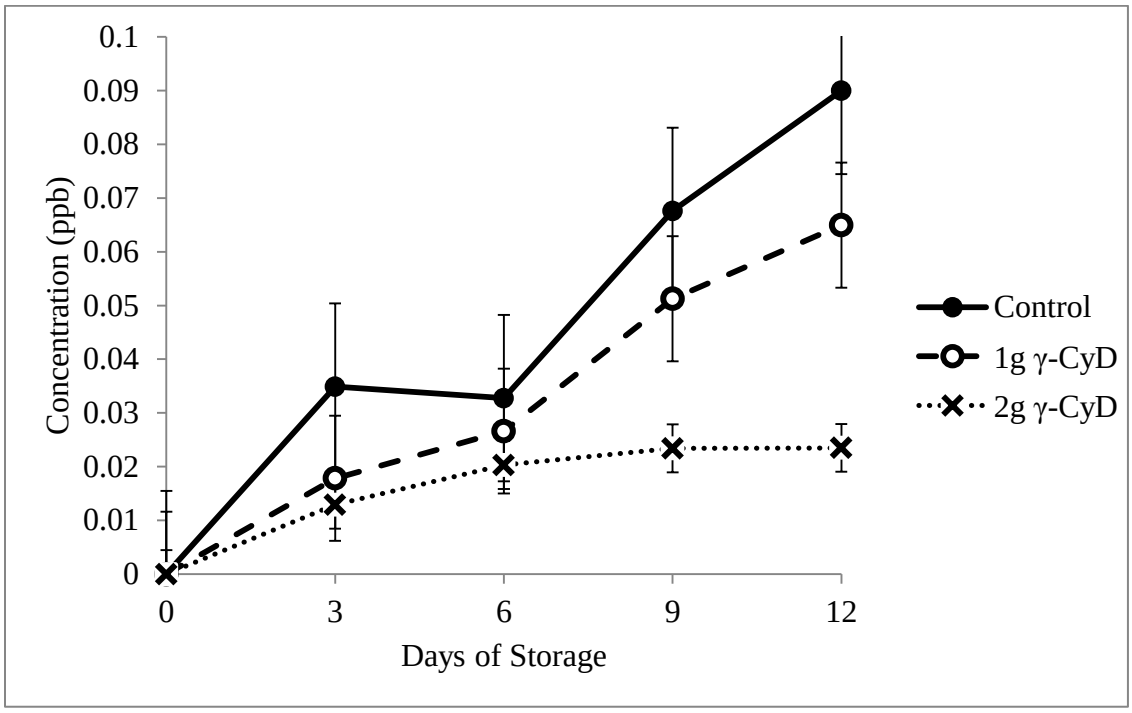

Figure 2.12. Influence of adding γ-CyD on reducing concentrations of MFT in stored thiamine-containing model systems

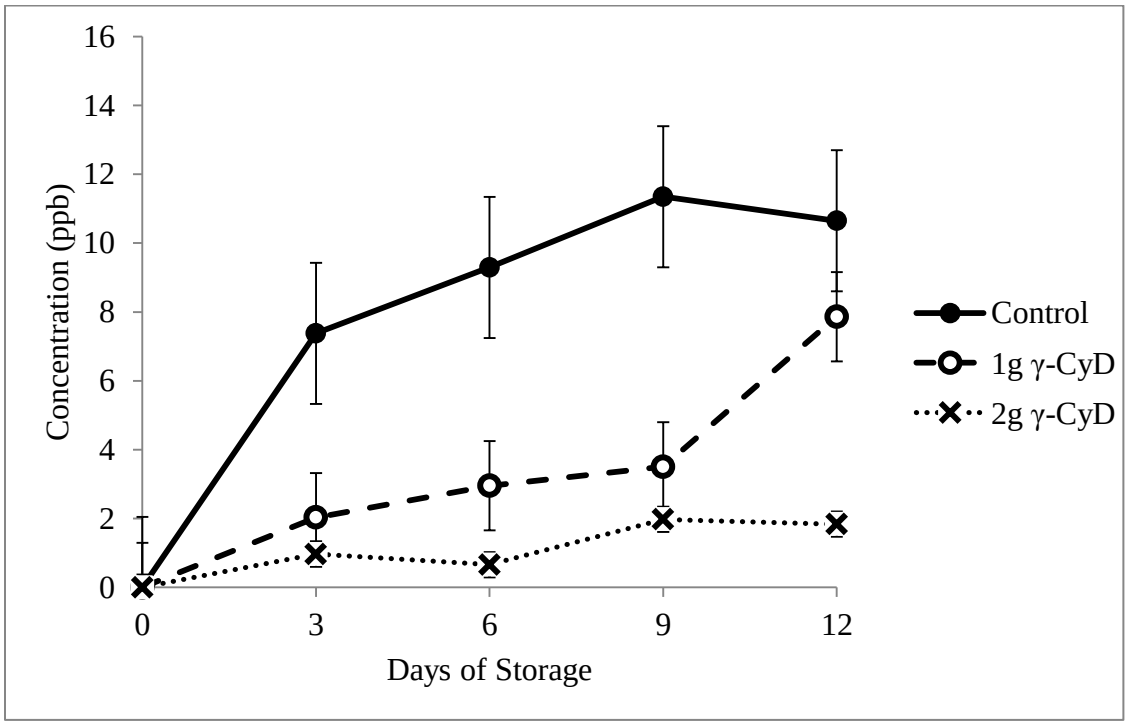

Figure 2.13. Influence of adding γ-CyD on reducing concentrations of MFT-MFT in stored thiamine-containing model systems

Artifact Formation during Analysis

Experiments were conducted to determine if any artifacts were created during the thermal desorption of the stir bars: If thiamine was absorbed to the stir bar and it was placed in the hot GC injection port, we would have expected to see degradation products being formed in analysis. Thus, the potential formation of artifacts was examined by measuring the headspace concentrations of typical thiamine degradation products (no stir bar immersion in the sample to form artifacts during thermal desorption) to see if the headspace concentrations of the volatiles paralleled their concentrations in the liquid. This was done using SPME. When using SPME, the off-flavor compounds were present in the same proportion as those determined using SBSE. Therefore, it appears that the thiamine was not degraded in the injector and the off-flavors analyzed were not injector port artifacts.

Comparison of Cyclodextrins

In all the samples, 2g of γ-cyclodextrin was the most effective in forming a complex with thiamine thereby reducing concentrations of off-flavor compounds associated with thiamine degradation. One of the expected outcomes with β-CyD was that the more cyclodextrin used, the better it would be able to reduce off-flavor compounds due to the thermal degradation of thiamine. However, this was not the case. Further studies need to be done investigating why 2g of β-CyD was less effective than 1g because there is little rationale for this observation. On the other hand, higher amounts of γ-CyD were more effective in reducing off-flavor compounds.

In comparison, the 1g β-CyD was better at reducing concentrations of off-flavor compounds than the 1g γ-CyD sample. This was especially true regarding the formation of MFT. The 1g of β-CyD solution reduced concentrations of MFT by 52% as opposed to 1g of γ-CyD reducing it only 28% by day 12 (Figure 2.12). However, this was opposite when implementing 2g of each of the cyclodextrins. Concentrations of MFT decreased 49% when using 2g β-CyD and in comparison, it decreased 75% when using 2g of γ-CyD. It is evident that the 2g γ-CyD is the best choice in stabilizing thiamine and thereby reducing concentrations of MFT.

The 2g of γ-CyD was also more effective at reducing concentrations of MFT-MFT. In the 1g solutions, the β-CyD reduced concentrations of MFT-MFT by 38% as opposed to the γ-CyD solution reducing it only 26%. With the 2g solutions, MFT-MFT concentrations were reduced 25% by using β-CyD and in comparison 82% by using γ-CyD. This confirms that again 2g of γ-CyD was best at associating with thiamine and reducing concentrations of MFT-MFT.

2.4 Conclusions

It was determined that cyclodextrins reduced concentrations of detected thiamine off-flavor compounds. One gram of β-CyD and 2g of γ-CyD solutions were best at reducing off-flavor compounds such as dimethyl sulfide, methional, 4,5-dimethylthiazole, and methional. The results suggest that 2g of γ-CyD sample was best at reducing concentrations of the potent aromas 2-methyl-3-furanthiol (MFT) and its dimer bis(2-methyl-3-furyl) disulfide (MFT-MFT). It has been suggested that there might be better performance if more than one cyclodextrin can be used in a system (33). Further research using NMR and/or Capillary Electrophoresis (CE) needs to be done to investigate the interactions that take place between CyD and thiamine and its degradation products.

Chapter 3

Formation of 2-Methyl-3-furanthiol and its dimer bis(2-methyl-3-furyl) disulfide in Complex Model Beverage Solutions containing Thiamine

Summary

Complex model energy beverages were prepared at pH of 2.6, with vitamins, sugar, caffeine, taurine, carbonated water, and contained 0.3g of thiamine hydrochloride. The beverages were stored at 50°C for 6 days in the absence of light. One sample lot was non-pasteurized and the other was pasteurized in can at 71°C for 10 min to see if pasteurization was a problem. Samples containing $1.83x10^{-3}$ mol/L (1g) β-CyD, $3.7x10^{-3}$ mol/L (2g) β-CyD, $1.6x10^{-3}$ mol/L (1g) γ-CyD, and $3.2x10^{-3}$ mol/L (2g) γ-CyD were compared to that of the control. Off-flavor compounds formed during storage were quantified using gas chromatography (GC) with a flame ionization detector (FID). Compound identifications were confirmed by gas chromatography-mass spectrometry (GC-MS).

Two major aroma compounds were identified, 2-methyl-3-furanthiol (MFT) and bis(2-methyl-3-furyl) disulfide (MFT-MFT) which both contribute sulfury, meat notes. Out of all the samples, the 1g ($1.6x10^{-3}$ mol/L) γ-CyD, and 2g ($3.2x10^{-3}$ mol/L) γ-CyD samples were effective at reducing concentrations of MFT and MFT-MFT in the sample. The results could prove to be useful in the formulation of energy beverages that intend on using thiamine to fortify the product.

3.1 Introduction

Since thiamine can thermally degrade and form objectionable sulfur compounds, it is omitted from energy beverages. Most energy beverages are stored at room temperature and have relatively pH's around 2.6, which is similar to the pH of many citrus juices such as orange juice. Since most producers of energy drinks may want to include thiamine into their energy beverages for the obvious benefits, the goal of this research was to reduce off-flavor compounds due to thiamine degradation in complex model energy beverages.

The hypothesis of this study was that the use of cyclodextrins will stabilize thiamine and therefore reduce concentrations of thiamine off-flavor compounds due to degradation. The main objective of the study was to monitor the off-flavor formation in a thiamine fortified complex model energy beverage and to reduce the off-flavor compounds by using different cyclodextrins (CyD's).

3.2 Materials and Methods

The methods used to analyze the samples are the same as used in Chapter 2, except for the following changes:

Materials

The following compounds were obtained from Aldrich (Milwaukee, WI): 2-methyl-3-furanthiol and bis(2-methyl-3-furyl) disulfide. Thiamine hydrochloride was obtained from Sigma (St. Louis, MO). Beta and gamma cyclodextrin was a gift from Robertet Flavors Inc. (Piscataway, New Jersey). Citric acid, Vitamin C, niacin, Vitamin B6, Vitamin B12, taurine, and caffeine were purchased from Fisher Scientific (Pittsburgh, PA). Carbonated water obtained from in-house carbonation system and sugar was purchased from a local store. Twister®/stir bars were used for the extraction of solutions and purchased from Gerstel Inc. (Linthicum, Maryland).

Sample Preparation

<u>Preparation of Complex Thiamine Model Solutions</u>

Complex model thiamine solutions were prepared in 12 oz. beverage cans. The 12oz. beverages were made by combining 1 part syrup (59.14mL) with 3 parts water

(177.4mL), and 2 parts carbonated seltzer water (118.28mL). The beverages were only lightly carbonated to avoid the possibility of explosion during pasteurization. The syrup and water were added by weight to ensure sufficient accuracy. The syrup and carbonated water were chilled prior to filling in an effort to keep the carbonation level consistent. The syrup contained 288g of sucrose, 1.2g of vitamin blend (Niacin, Vitamin B6, Vitamin B12, and Vitamin C), 2.15g of thiamine hydrochloride, 10g of energy blend (taurine and caffeine), 302.8g water, ca. 50ppb (31.25 μL) of 4-heptanone (internal standard), and 2.54g of citric acid. This solution served as the control. Four other solutions were prepared the same way, but cyclodextrins added. One solution had 1g β-cyclodextrin (8.8×10^{-4} mol), the second solution had 2g of β-cyclodextrin (1.76×10^{-3} mol), the third solution had 1g γ-cyclodextrin (7.7×10^{-4} mol), and the fourth solution had 2g γ-cyclodextrin (1.54×10^{-3} mol). These cyclodextrins were solubilized in 302.8g of water for 30 min. at 450 rpm and stored for 24 hrs. Thiamine hydrochloride, citric acid, and the internal standard were added in the same amounts as stated above. Samples underwent two treatments: one lot was non-pasteurized and the other lot was pasteurized in the can at 71°C for 10 min. Samples were made twice for two separate analyses.

Storage and Sampling Protocol

Samples were stored at room temperature in the absence of light and were analyzed in duplicate on days 0, 2, 4, and 6. Fifteen mL aliquots were then taken and placed into a 20mL closed glass vials. Samples were then exposed to stir-bar sorptive extraction (SBSE, Twister®).

3.3 Results and Discussion

This study focused on examining the potential of CyDs to reduce the formation of MFT and MFT-MFT in real energy drinks. Two types of cyclodextrins were used in the formulation of the complex model beverages: β-CyD and γ-CyD. The recommended daily intake (RDI) of thiamine is 1.4 mg per day, but they were increased by 100x to 140mg to generate off-flavor compounds at higher levels for better quantification.

Formation of MFT and MFT-MFT in Non-Pasteurized Samples

It appears that adding CyDs to a true, non-pasteurized energy beverage results in less MFT being formed (by day 4) and likely no effect on the formation of MFT-MFT (Fig 3.1 and 3.2). In fact, the data indicate that small amounts of CyD may have an adverse effect on the formation of MFT-MFT (Fig 3.2). The accelerated shelf-life study at 50°C for 6 days relates to about 2 months of storage at room temperature (17). All the samples except the 2g ($3.7x10^{-3}$ mol/L) β-CyD sample reduced concentrations of MFT ($p<0.05$) by Day 6 (Note there was no statistical significant difference at day 6, 2g β-CyD). With respect to MFT-MFT, all of the samples except the 2g β-CyD reduced concentrations of this compound by Day 6 ($p<0.05$) (Note there was no statistical significant difference at day 6, 2g β-CyD).

Overall, the 1g ($1.6x10^{-3}$ mol/L) of γ-CyD and 2g ($3.2x10^{-3}$ mol/L) of γ-CyD samples were most effective at reducing concentrations of MFT. The 1g γ-CyD was very effective in keeping the concentrations of MFT lower than the control during the study (Figure 3.1). The 1g of γ-CyD sample reduced concentrations of MFT by 33% and the 2g of γ-CyD sample reduced it by 47.5% by day 6. With respect to MFT-MFT both γ-CyD concentrations decreased levels of this MFT-MFT in the sample, with 2g γ-CyD being the most effective. The 1g γ-CyD reduced concentrations of MFT-MFT by 43% and the 2g γ-CyD reduced it by 37% by day 6. Clearly, the γ-CyD containing samples were more effective at reducing concentrations of MFT and MFT-MFT in the sample. It makes sense since γ-CyD is a larger molecule and better able to associate with thiamine and the other components of the beverage.

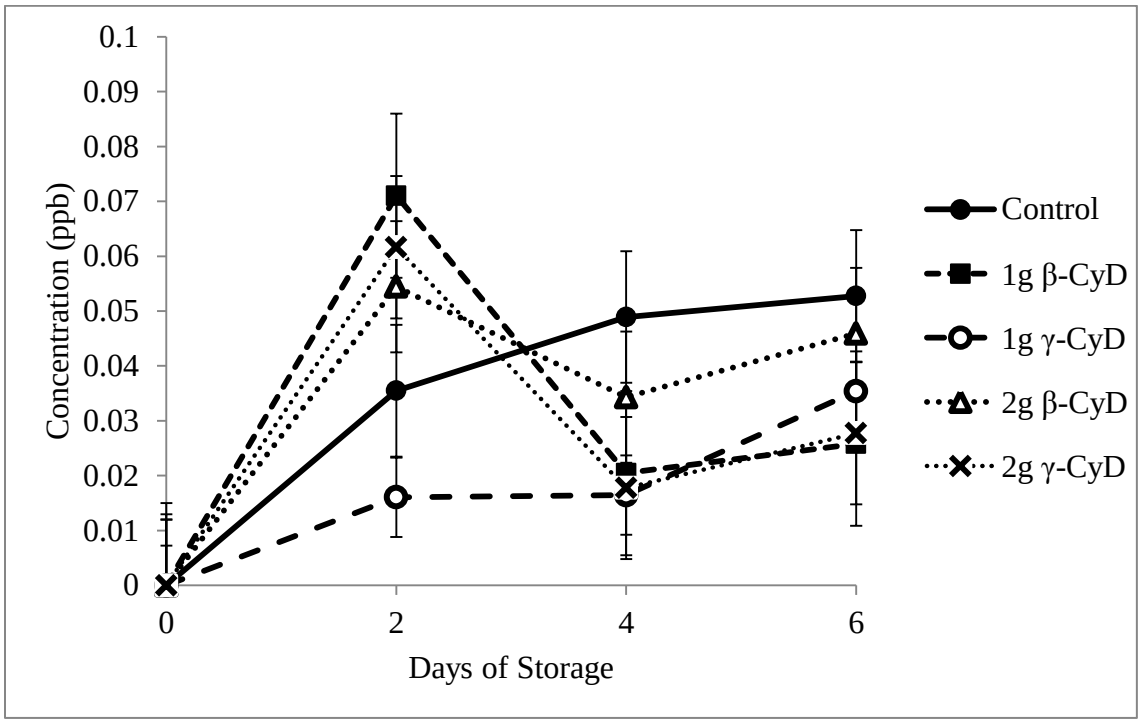

Figure 3.1. Influence of adding CyDs on reducing concentrations of MFT in non-pasteurized complex model energy beverages

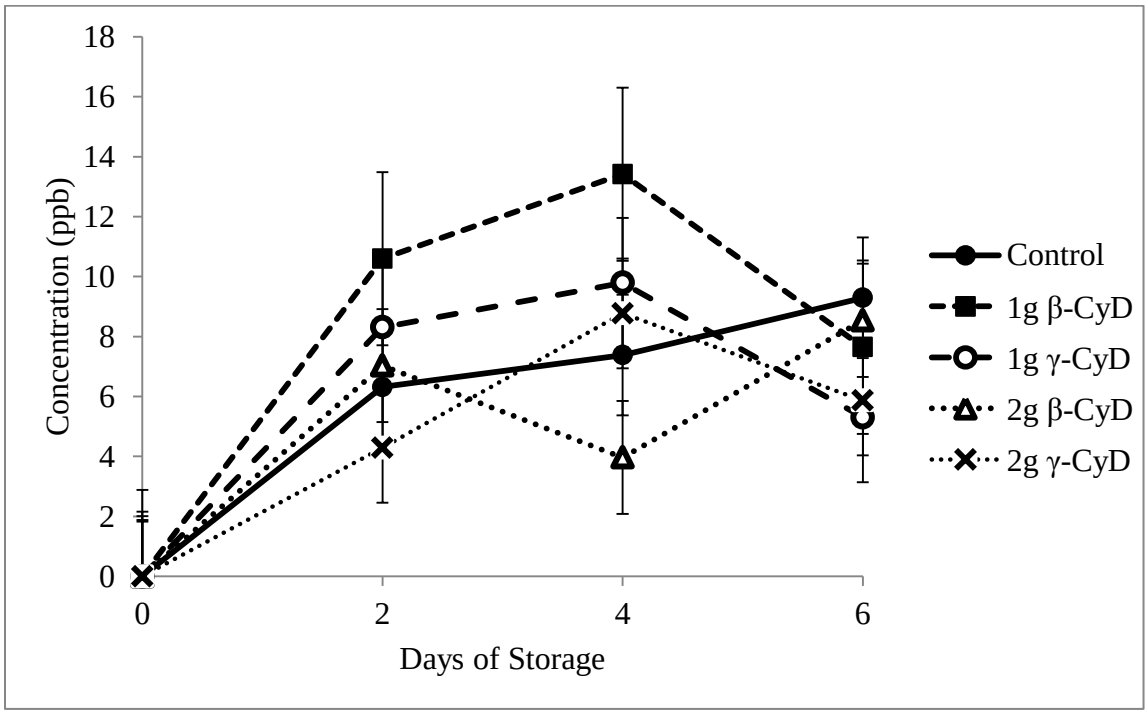

Figure 3.2. Influence of adding CyDs on reducing concentrations of MFT-MFT in non-pasteurized complex model energy beverages

Formation of MFT and MFT-MFT in Pasteurized Samples

The pasteurized samples were different compared to the other samples because pasteurization was used to get large initial concentrations of MFT and MFT-MFT and to investigate if pasteurization was a problem. As expected, there is a trend of an increase of MFT due to the high temperatures associated with pasteurization. With respect to MFT, the 1g β-CyD, 1g γ-CyD, and the 2g γ-CyD samples were able to effectively reduce concentrations of this compound by day 6 ($p<0.05$). This result was very similar to that seen with the non-pasteurized sample. In comparison, the 1g and 2g γ-CyD samples reduced concentrations of MFT-MFT on day 6 ($p<0.05$).

In the samples, the 1g of γ-CyD was the most effective treatment in reducing off-flavor compounds. The 1g of γ-CyD appeared to reduce concentrations of MFT by 33% and MFT-MFT by 21.5% by day 6. The more effective 2g γ-CyD sample reduced concentrations of MFT by 66% and MFT-MFT by 26% by day 6. Therefore, adding 2g of γ-CyD may be an acceptable amount to help stabilize thiamine and thereby reducing off-flavor compounds associated with it.

Early Days of Testing

In Figures 3.1-3.4, some of the samples appeared to have higher concentrations of MFT and MFT-MFT than the control in earlier days of testing. The formation of MFT and MFT-MFT appears to follow first-order kinetics. In the last chapter, some discussion was offered to explain such results: this will not be repeated here.

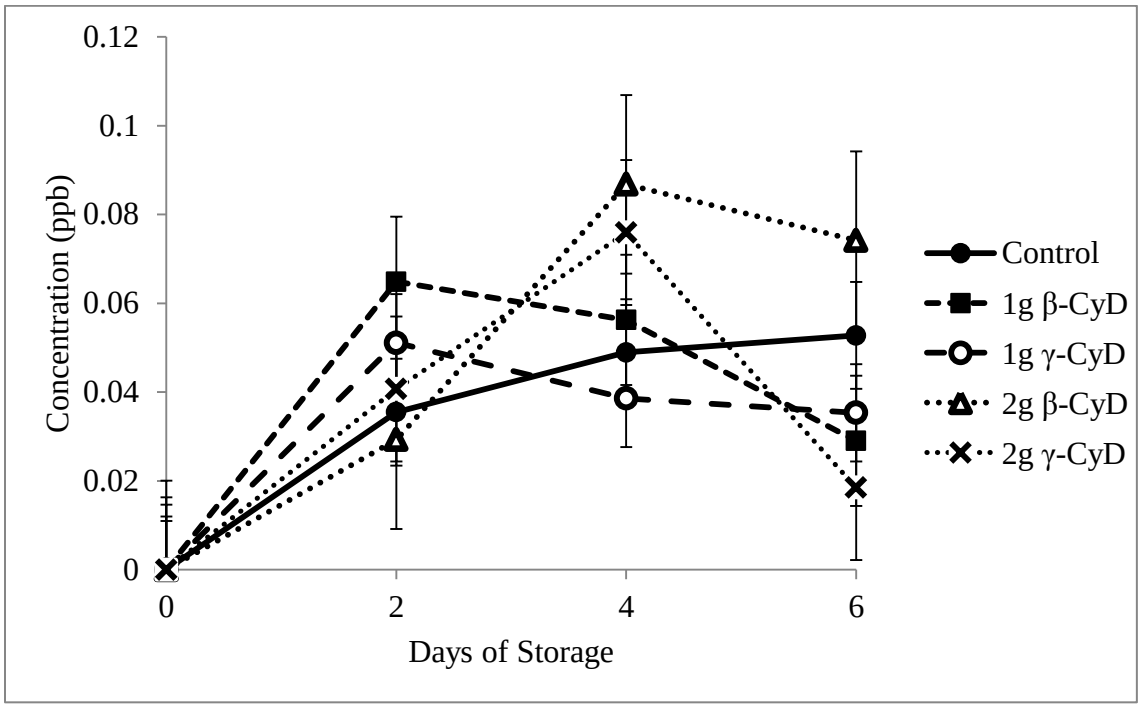

Figure 3.3. Influence of adding CyDs on reducing concentrations of MFT in pasteurized complex model energy beverages

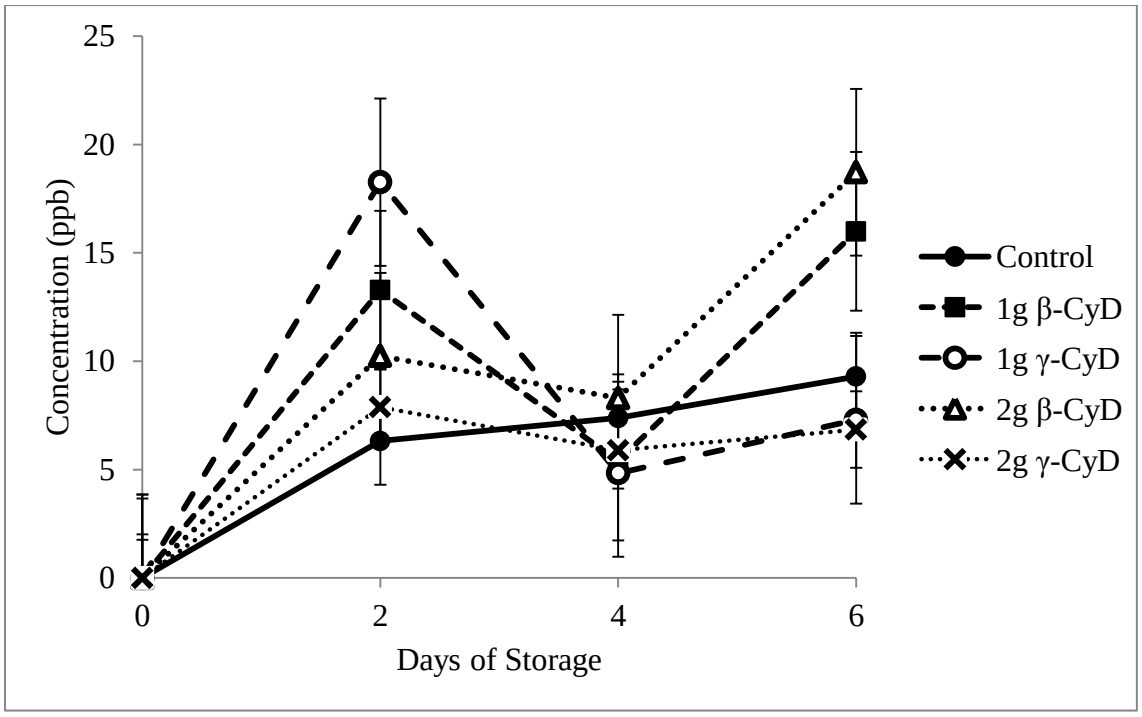

Figure 3.4. Influence of adding CyDs on reducing concentrations of MFT-MFT in pasteurized complex model energy beverages

3.4 Conclusions

It was determined that cyclodextrins were effective in complexing with thiamine and stabilizing it, therefore reducing the thiamine off-flavor compounds. The main thiamine off-flavor compounds identified in this study were 2-methyl-3-furanthiol (MFT) and its dimer bis(2-methyl-3-furyl) disulfide (MFT-MFT). The most effective cyclodextrins were the 1g and 2g γ-CyD samples at reducing thiamine off-flavor compounds.

References

1. J.M. Amon, J.M. Vandepeer, R.F. Simpson, Aust. N. Z. Wine Ind. J. 4 (1989) 62

2. Kilcast, D., *Food Taints and Off-Flavours,* M.J. Saxby, Ed., Blackie Acad. Prof., London, 1996

3. Margaria, Carlos., Goodner, K.L., Baldwin, Elizabeth A., *Detection and Identification Threshold Values for Key Flavor Components in an Orange Juice Matrix,* Proc. Fla. State Hort. Soc. 115:53-54, 2002

4. Reineccius, Gary. *Flavor Chemistry and Technology*: Second Edition. Boca Raton, Florida: CRC Press. 2006, 487 pages

5. Azzara, Daniel C., Campbell, Larry B., Off-Flavors of Dairy Products in *Off-Flavors in Foods and Beverages,* G. Charalambous, Ed., Elsevier Pub. B.V., 1992, p. 329

6. Whited L.J., Hammond, B.H., Chapman, K.W., and Boor, K.J. Vitamin A Degradation and Light-Oxidized Flavor Defects in Milk. *J. Dairy Sci*. 85:351–354, 2002

7. Ayers, J.E.. M.J. Fishwick, D.G. Land, T. Swain, Off-flavor in dehydrated carrot stored in oxygen, *Nature,* 203, p.81, 1964

8. Suyama, K., T. Yeow, and S. Nakai, Vitamin A Oxidation Products Responsible for Haylike Flavor Production in Nonfat Dry Milk. *J. Agric. Food Chem.* 31, 22-26, 1981

9. Roussef, R.L., S. Nagy, M. Naim, U. Zahavi, Off-Flavor Development in Citrus Fruit Juice Products in *Off-Flavors in Foods and Beverages,* G. Charalambous, Ed., Elsevier Pub. B.V., 1992, p. 211

10. Braverman, J.B.S. Introduction to the Biochemistry of Foods. Elsevier, Amsterdam, 1963

11. Shaw, P.E., J.H. Tatum, T.J. Kew, C.J. Wagner, R.E. Berry, *J. Agric. Food Chem.,* 18 (1970) 343-345

12. Choe, E., R. Huang, D. Min, Chemical Reactions and Stability of Riboflavin in Foods. *J. Food Sci*. 70(1): R28-R36, 2006

13. Lee, J.H., D.B. Min, Changes of Headspace Volatiles in Milk with Riboflavin Photosensitation, J. Food Chem. 84(7):C563-C568, 2009

14. Kamimura, M., H. Kaneda, Off-flavors in Beer in *Off-Flavors in Foods and Beverages*, G. Charalambous, Ed., Elsevier Pub. B.V., 1992, p. 433

15. Benton D. Thiamine supplementation improves mood and cognitive functioning. *Psychopharmacology* 1997;129:66-71

16. Kraut, J., H.J. Reed, The crystal structure of thiamine hydrochloride (vitamin B1), *Acta Cryst.* (1962). 15, 747-757

17. Pachapurkar, D., L. Bell, Kinetics of Thiamin Degradation in Solutions under Ambient Storage Conditions. *J. Food Sci.* 70(7):C423-C426, 2005

18. Dreher, J.G., R. Rouseff, M. Naim, GC-Olfactometric Characterization of Aroma Volatiles from the Thermal Degradation of Thiamin in Model Orange Juice. *J. Agric. Food Chem,* 51 (2003) 3097-3102

19. Bezman, Y., R. Rouseff, M. Naim, 2-Methyl-3-Furanthiol and Methional Are Possible Off-Flavors in Stored Orange Juice: Aroma-Similarity, NIF/SNIF GC-O, and GC-Analyses, *J. Agric. Food Chem*, 49 (2001) 5425-5432

20. Munch, P.; Schieberle, P. Quantitative Studies on the Formation of Key Odorants in Thermally Treated Yeast Extracts Using Stable Isotope Dilution Assays. *J. Agric. Food Chem.* 1998, 46**,** 4695-4701

21. Buttery, R. G.; Haddon, W. F.; Seifert, R. M.; Turnbaugh, J. G. Thiamin odor and bis(2-methyl-3-furyl) disulfide. *J. Agric. Food Chem.* 1984, 32, 674-6

22. Lin, J.; Rouseff, R. L.; Barros, S.; Naim, M. Aroma Compostion changes in Early Season Grapefruit Juice Produced from Thermal Concentration. *J. Agric. Food Chem.* 2002, *50*, 813-819

23. Hofmann, T.; Schieberle, P. Chemical Interactions between Odor-Active Thiols and Melanoidins Involved in the Aroma Staling of Coffee Beverages. *J. Agric. Food Chem.* 2002, *50*, 319-326

24. Grosch, W., Zeiler-Hilgart, G. Formation of Meatlike Flavor Compounds. In *Flavour precursors. Thermal and enzymatic conversions*; R. Teranishi; G. R. Takeoka and M. Güntert, Eds.; American Chemical Society: Washington DC, 1992; pp 183-192

25. Farmer, L. J.; Mottram, D. S. Recent Studies on the Formation of Meat-Like Aroma Compounds. In *Flavour Science and Technology;* Bessiere, Y., Thomas, A. F., Eds.; Wiley & Sons: Chichester, U.K., 1990; pp 113-116

26. Dwivedi, B. K.; Arnold, R. G. Chemistry of Thiamine Degradation in Food Products and Model Systems: A Review. *J. Agric. Food Chem.* 1973, *21*, 54-60

27. Belitz, H.D., Grosch, W., Schieberle, P. *Food Chemistry*: 4th Edition. Berlin, Heidelberg: Springer Berlin Heidelberg, 2009

28. Bolton, T.A., Reineccius, G., Liardon, R., Ba, T.H. Role of Cysteine in the Formation of 2-Methyl-3-furanthiol in a Thiamine-Cysteine Model System. In *Thermally Generated Flavors*; Parliment, T., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 1993

29. Reid, W.J., Instrumental methods in detecting taints and off-flavours. *In Taints and off-flavours in food,* Brian Baigrie, Ed., Woodhead Pub. Ltd., 2003, p. 31

30. Grimm, C., E.T. Champagne, K. Phtsubo, *Flavor Fragrance and Odor Analysis*, R. Marsili, Ed., Marcel Dekker, New York, 2002

31. Nursten, H. E, The Maillard reaction chemistry, biochemistry, and implications, Royal Society of Chemistry (Great Britain) Inc., Norwood Mass, 2005

32. Wampler, T., Analysis of Food Volatiles Using Headspace-GC Techniques. In *Flavor, Fragrance, and Odor Analysis,* Ray Marsili, Ed., Marcel Dekker, New York, 2002

33. Reineccius et al. Encapsulation of flavors using cyclodextrins: comparison of flavor retention in alpha, beta, and gamma types, *Journal of Food Science,* 2002, 67(9)

34. Reineccius et al. Flavor Release from Cyclodextrin Complexes: Comparison of Alpha, Beta, and Gamma Types. *J. Food Sci.,* 2002: 68(4) p. 1234-1239

35. Szejtli, J., Szente, L., Elimination of bitter, disgusting tastes of drugs and foods by cyclodextrins, *Eur J Pharm Biopharm* 61(2005) 115-125

36. Hedges, A., Shieh, W.J., Sikorski, C.T., Use of Cyclodextrins for Encapsulation in the Use and Treatment of Food Products. In *Encapsulation and Controlled Release of Food Ingredients,* Risch, S., et al., ACS Symposium Series, ACS: Washington, D.C., 1995

37. Takeda Chem. Int. Ltd.: Removal of off-flavor from old rice, Jpn Kokai JP 81127058, 1980 (CA:96:33613)

38. Im, I.S., Lee, W.K., Park, M.K., Studies on the Interaction of Thiamines and Cyclodextrins, Arch. Pharm. Res. 6(1), 35-44, 1983

39. Snyder, L.R., Kirkland, J.J., Dolan, J.W., *Introduction to Modern Liquid Chromatography*, John Wiley & Sons, Inc., New Jersey, 2010

40. Zhang, Y.; Ho, C.-T. J. *Agric. Food Chem.* 1991, *39*, 1145-1148

41. David, F.T., B. Tienpont, P. Sandra, Stir-bar sorptive extraction of trace organic compounds from aqueous matrices, LC/GC, 21(2), 2003

42. Parks, O.W., D.P. Schwartz, Identification of O-aminoacetophenone as a flavor compound in stale dry milk, *Nature*, 202, 1964

43. Patton, S., The mechanism of sunlight flavor formation in milk with special reference to methionine and riboflavin, J. Dairy Sci., 61, 1978

44. USDA National Nutrient Database for Standard Reference, Release 23

45. Van der Linde, L.M.; van Dort, J.M.; de Valois, P.; Boelens, H.; de Rijke, D. In *Progress in Flavor Research;* Land, D.G., Nursten, H.E.,Eds.; Applied Science Publishers: London, U.K., 1979; pp 219-224

46. MacLeod, G,; Seyyedain-Ardebili, M. *CRC Crit. Rev. Food Sci. Nutr.* 1981, *14*, 309-437