THE EFFECT OF SPICES ON CARBOXY METHYLLYINSE LEVELS IN BISCUITS

by

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Abstract

Carboxymethyllysine (CML) and other advanced glycation end products (AGEs) have been shown to affect diseases such as diabetes, cancer, and Alzheimer's by inducing oxidative stress, inflammation, and tissue damage. CML is formed in foods through Maillard browning reactions and through various mechanisms that are affected by time, temperature, pH, water activity, trace metals, and antioxidants. Natural antioxidants can be found in plant and fruit extracts, as well as in spices. The research contained herein is in two parts. The first part includes preliminary work, which examines the percent recovery of CML from various solid phase extraction columns, the analysis of CML in maple syrup, barbeque sauce, honey, and infant formula. The data show that solid phase extraction with a C-18 cartridge produced the highest percent recovery when using a CML standard at 100 ppb, with a recovery of 69%. Furthermore, the analysis of the syrups and sauces is inconclusive, due to the very low levels of CML detected in the infant formula, and the complications due to the high levels of reducing sugars. The second part of the research examines the effect that cinnamon, onion, garlic, black pepper, and rosemary have on CML levels in biscuits. The data show that all of the spices inhibit the formation of CML, at each of the 0.5%, 1%, and 2% levels used in the experiment, by a minimum of 3% in 2% onion samples and a maximum of 79% in 0.5% cinnamon samples when looking at the cumulative data. When looking subsets of the data, the CML inhibition was a minimum of 59% in 2% onion samples and a maximum of 74% in 0.5% cinnamon samples. Other trends can be observed in the chroma values in the CML color data, which suggest that chroma values decrease as the spice level increases, but these are not statistically significant. They may be due to color from the spices themselves, or to the chemical changes in the Maillard reaction.

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CHAPTER 1- Literature Review

Introduction

Maillard browning has been studied since the early 1900's. Studies by Maillard, for whom the reaction is now named, were conducted in 1912, and the very foundations of the field continued into the 1950's (Seaver and Kertesz 1946). The complexity and mechanism of Maillard reactions is still not fully understood, but the body of evidence of suggests that Maillard Browning and AGEs form in food as well as in the body, and that these AGEs may play a role in disease pathologies. The literature review below will summarize the current research on AGEs and their effect on disease, their mechanism of formation and factors that affect formation, AGE levels in foods, and controlling AGEs through the use of spices.

Advanced Glycation End Products and Disease

Diabetes

Insulin resistance is one of the defining characteristics of Type 2 diabetes. Consumption of AGEs has been linked to the decrease of adiponectin proteins, which regulate glucose levels in the blood (Stirban and others 2007). The study found that a meal that was fried/broiled compared to an identical meal that was boiled/steamed contained higher levels of AGEs, and observed a decrease in adiponectin levels in the blood, and increased levels of glucose 2 hours (hrs) after the meal. The ingestion of high levels of AGEs could aggravate symptoms of the illness, which could lead to other complications.

One of these complications is damage in blood vessels. Damage to these blood vessels can also occur through more than one mechanism. Some studies have shown that AGEs, formed on red blood cells because of high levels of glucose in the body, can change cell signaling. AGEs on blood cells can bind to a Receptors for AGEs (RAGE), which creates free radicals and oxidative stress) (Wautier and others 1994; Wautier and others 2001). When AGEs form on the blood vessels in the eyes, these glycations can restrict blood flow and cause damage (Hughes and others 2004). Furthermore, glycation in eye tissues can increase the levels of some receptors in the eyes used for carrying signals, which may, again, lead to damage of the tissues (McKenna

and others 2001). The oxidative stress damage in tissue due to AGEs can also affect diabetes induced heart disease through similar mechanisms. Studies on heart cells show that AGEs increased NADPH oxidase activity, and produced similar oxidative damage markers as eye blood vessels (Zhang and others 2003). Still other studies have found that glycated proteins in the body can form on the innermost layers of the nervous system (endothelium), and that these modified proteins have localized high concentrations in the kidney and vascular endothelium. There is an accumulation of AGEs in tissues that suffer inadequate blood supplies, which is a not uncommon symptom in severely diabetic patients (Oya and others 1999). It is also interesting to note that similar types of damage can be induced by a Maillard-type reaction of glucose with unsaturated lipids, which results in an advanced lipid-oxidation endproduct (ALE). ALEs will not be discussed in detail here, but it is worthwhile to mention that ALEs can initiate oxidative damage, and that circulating levels of ALEs, as well as AGEs, can be found in people with diabetes (Bucala and others 1993).

Cancer

Preliminary data from the CDC suggests that cancer was the second leading cause of death in the United States in 2011 (Hoyert and Xu 2012). RAGE, as stated above, binds to AGEs to have an oxidative effect. Soluble RAGE (sRAGE) is produced by the body to remove AGEs and prevent cellular oxidation (Hanford and others 2004). Relative levels of RAGE and sRAGE may affect cancer. For example, prostate cancer cells express RAGE on the cell surface, and can be activated by AGEs (Allmen and others 2008). In another study on breast cancer patients, the researchers found that blood levels of AGEs were higher, and that levels of sRAGE were lower, which may help accelerate the progress of the cancer (Tesarova and others 2007). RAGE activation in colon cancer cells has also been shown to activate inflammation pathways and promote growth of the cancer cells (Fuentes and others 2007). AGEs were found to be tumor promoting in mouse fibroblasts (Wang and others 1998), possibly through RAGE. This effect was also observed in pancreatic cancers, where inhibition of RAGE slowed tumor growth (Arumugam and others 2006). The opposite effect was seen in lung cancers, where RAGE inhibition inhibited tumor formation in the lung (Bartling and others 2006). However, the trend in cancers seems to be that activation of RAGE with AGEs can promote growth of cancers. It is

therefore important to see if a reduction of AGEs in foods is possible, because it could have a preventative effect in cancer development.

Alzheimer's

Alzheimer's disease is a brain degenerative disorder characterized by neuronal degeneration, brain lesions, extracellular plaques, and neuron tangles in the brain. The plaques in the brain are characterized by amyloid-beta (Aβ) proteins, while the neuronal tangles are characterized by a τ protein (Sayre and others 2008). When AGEs are formed in the body, or endogenously, they can attach sugars, or glycate, to many tissues in the body. In Alzheimer's, when endogenous glycation occurs on τ-proteins, it can stabilize them as they aggregate together, enhancing neuronal tangle formation (Necula and Kuret 2004). AGEs in the body can bind at RAGE. In the case of Alzheimer's disease, there is a larger expression of RAGE in brain tissues that can activate the NF-κβ pathways and cause oxidative stress (Yan and others 2009), and RAGE activation is able to help transport Aβ proteins associated with Alzheimer's disease through the blood brain barrier(Candela and others 2010). RAGE not only interacts with food AGEs, but it can also interact with the Aβ proteins themselves, and can change the macrophage concentrations in the brain (Yan and others 2009), and increase the toxicity of Aβ proteins (Mruthinti and others 2007). Activated RAGE receptors in the brain can enhance IL-1β, TNF-α, NF-κβ, and nitric oxide production, all of which are inflammation inducing molecules, which may accelerate Alzheimer's progression (Fang and others 2010; Cuevas and others 2011). The link between Alzheimer's and AGEs is supported by studies that blocked the RAGE receptor, using inhibitors or antibodies, and found a decrease in Aβ protein toxicity, and protected neuronal tissue from damage. (Onyango and others 2005; Businaro and others 2006; Sturchler and others 2008). From a food science prospective, decreasing the AGEs in food stuffs could decrease the activation of the RAGE receptors, and thereby delay or prevent the onset of Alzheimer's disease.

Other Complications Influenced by AGEs

Diabetes, cancer, and Alzheimer's disease are very common pathologies that affect many people, but AGEs have been associated with other diseases as well. AGE accumulation can

occur in patients with arthritis as well as in patients with kidney failure who are on dialysis (Matsumoto and others 2007).

AGEs, in addition to causing damage to blood vessels and vessel linings, can also cause damage to other tissues in the body, usually through protein modification. Fu and others (1994) showed that AGEs formed in the body can crosslink collagen tissues, which would impair their function. Two studies conducted by Lederer, Buhler, and Lederer and Klaiber (1999, 1999), show that when glucose is present, as well as the early state AGEs glyoxal and methylglyoxal, lysine and arginine could crosslink in proteins causing damage to functionality.

A common thread among all of the complications associated with AGEs seems to be the presence of inflammation and oxidative stress. Oxidative stress can be due to activation of RAGE found in the body. As described above, RAGE was associated with diabetes, cancer and Alzheimer's, but may also affect patients with many polyneuropathies through the activation of RAGE with AGEs. RAGE activation increases levels of inflammation markers such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), which are responsible for regulating the immune system (Haslbeck and others 2007). Studies have found elevated levels of AGEs such as pentosidine and carboxymethyllysine (CML) and NF-κβ activation in muscle tissues and blood sera of patients with fibromyalgia (Hein and Franke 2002; Ruster and others 2005). In healthy males, one study did find that there was no statistical difference in oxidative stress markers, although baseline levels of radicals in the serum were higher than in patients who consumed less AGEs (Seiquer and others 2008).

One study by Carlo-Stella (2009) and others, does suggest that the immune system is responsible for the production of RAGE. The immune system tries to mitigate the effect of AGEs by producing soluble forms of RAGE (sRAGE), and Endocrine Secreted RAGE (esRAGE). The sRAGE and esRAGE proteins act to bind to AGEs and help clear them from circulation. Dysfunction of the immune system may contribute to elevated levels of RAGE and incidence of diseases such as chronic fatigue syndrome. Fasshauer and others (2008) showed that women with preeclampsia during pregnancy will have elevated levels of esRAGE during pregnancy, but low levels of esRAGE six-months after delivery, when compared to controls. This suggests that AGEs or an immune response may contribute to the development of the disease.

Through all of the evidence of the effect of disease, it is important to remember that AGEs and activation of RAGE does not occur only through endogenous Maillard Browning. The study by Uribarri showed that high levels of dietary AGEs also correlate with higher levels of oxidative stress and inflammation (Uribarri and others 2007a). Animal studies have also shown that oxidative stress can be reduced in the body by controlling the diet and dietary levels of AGEs (Cai and others 2008). It is therefore important to understand how AGEs are formed, factors that affect their formation, and how levels of AGEs can be controlled.

Formation of Advanced Glycation End Products

Formation Mechanism

The condensation of amino acids and reducing sugars has been studied since Maillard looked at the reaction in the early 1900's. In the late 1940's and early 50's a series of studies by Wolfrom and others (1947), looked at browning in terms of methylation conversion of glucose to hydroxymethylfuraldehyde (1948), the effect of furnas on color formation (1949), xylose and glucose formation of melanoidins and other pigments (Tan and others 1950), and polymerization of Maillard products to melanoidin pigments (Wolfrom and others 1953). The formation mechanism of AGEs, particularly CML that is associated with disease, is still not entirely known, but there are two main proposed mechanisms. One is a non-free radical mechanism; the other is a free radical formation mechanism.

The non free-radical mechanism was proposed by Kasper and Schieberle (2005), when they used radio labeled glucose in incubations with hippuryllysine. The proposed mechanism suggested that the nitrogen group from the lysine side chain forms a Schiff base through dehydration with the aldehyde group from glucose, which then undergoes an Amadori rearrangement. The Amadori rearrangement shifts from an enol form to a 2,4-diketone group. The Kasper and Schieberle mechanism then suggests that hydration of this diketone compound causes cleavage into CML. The proposed mechanism is summarized in Figure 1. The mechanism proposed below is supported in the study by Culbertson and others (2003), who found that CML is formed, to some extent, even in the presence of strong antioxidants and free radical trapping agents, which they attributed to a non-radical mechanism.

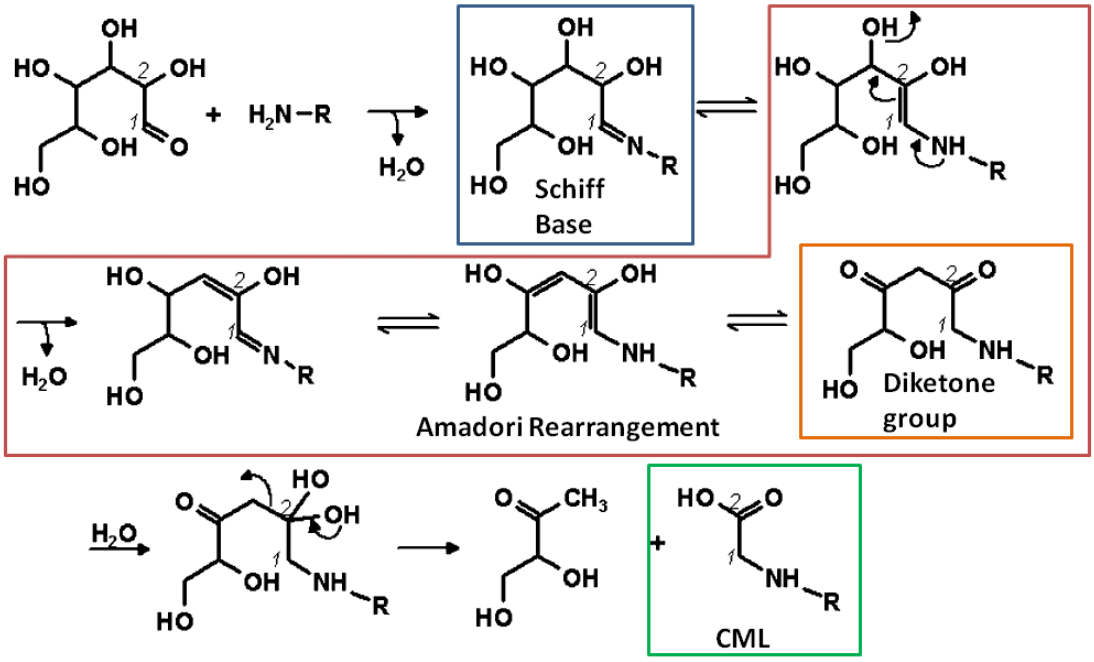

Figure 1: CML non free radical formation mechanism, as adapted from Kasper and Schieberle (2005).

However, some studies do suggest that the formation of AGEs occur through a free-radical mechanism, which was supported by the reduction of brown pigment formation when the study removed oxygen from the glucose-lysozyme model systems (Hayase and others 1996). They suggested that the free radicals either react with glucose directly, or with the Amadori compounds, in the presence of a transition metal, and that these reactive intermediates could further react with proteins to form AGEs. The proposed mechanism is summarized in Figure 2.

The free radical mechanism is supported by a study by Nagai and others (2002), who found that incubating serum albumin with peroxynitrite, a free radical generator, increased the levels of CML on the protein, and that the CML levels increased as the concentration of peroxynitrite also increased. In addition, they found that the intermediate of this reaction is glucosone, which was also proposed as an intermediate by Hayase.

In addition to glucose, one study by Hamada and others (1996) suggests that other modified sugar molecules such as fructose-3-phosphate, fructose-6-phosphate, glucose-6-phosphate, and 3-deoxyglucosone can form AGEs on proteins. These modified sugars are

derived from the glycolytic and polyol pathways, which convert excess glucose to sorbitol and D-glyceraldehyde-3-phosphate for glycolysis (Garrett and Grisham 2010). Furthermore, the study suggests that these metabolites can modify proteins faster than glucose alone.

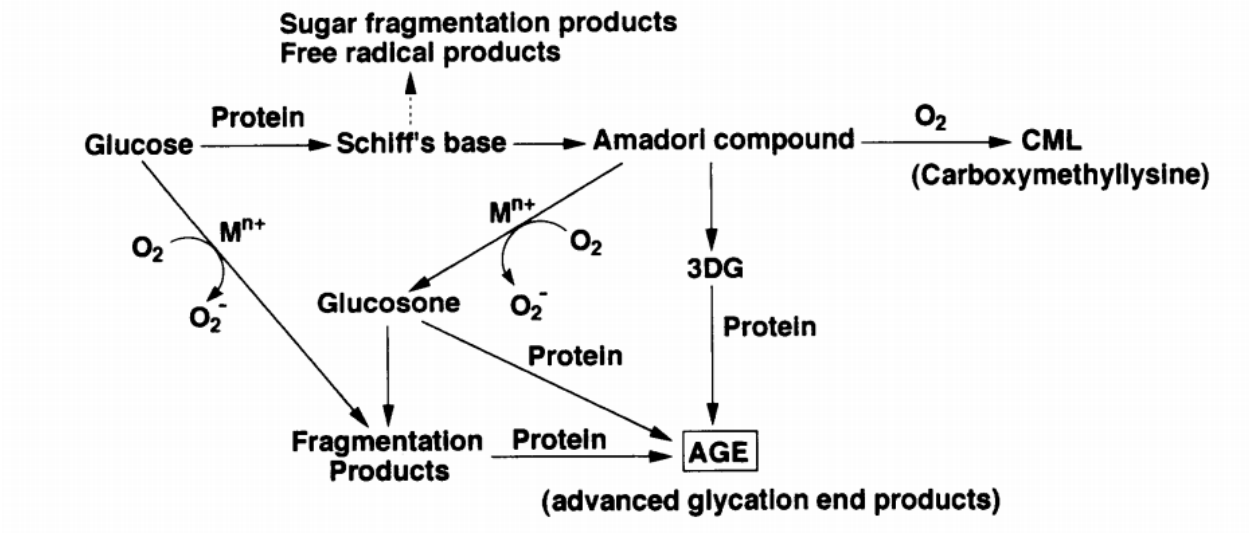

Figure 2: Free radical mechanism for AGE formation proposed by Hayase and others (1996).

Alabed and Bucala (1995) suggest that CML can be formed using glyoxal instead of glucose. Glyoxal is another Maillard product seen in Figure 3. They suggest that CML is formed through a direct reaction of glyoxal with lysine. Furthermore, the study suggests that the presence of cysteine in the protein can help the formation of CML by forming a temporary crosslink that may stabilize the system.

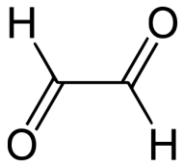

Figure 3: Glyoxal, a Maillard Browning intermediate that may be used to form other AGEs.

During formation of AGEs, it is possible to form CML from the Amadori mechanism described above, or through lipid peroxidation (Fu and others 1996). Lipid peroxidation also produces other aldehydes; however, model studies suggest that glucose does not react with these

aldehydes, but does work to catalyze lipid degradation (Adams and others 2011). On the other hand, some lipid oxidation products such as carboxymethylethanolamine can be used to monitor AGE formation *in vivo* (Requena and others 1997).

Cross-links in tissues are one of the complications that result from AGE formation. Studies have shown that formation of lysine-arginine cross-links, mainly pentosidine, is mediated by precursors and intermediates, which include pentosinane and glucosepane, (Biemel and others 2001a), and that the formation of these cross-links can be formed not just with glucose, but fructose, ribose, and ascorbate (Grandhee and Monnier 1991).

Some studies have even suggested that antioxidants can have some glycating ability. Hasenkopf and others (2002), incubated proteins with ascorbic acid or glucose, and found carboxymethyllysine as well as other AGEs in the ascorbic acid systems, as demonstrated in Figure 4.

Modeling the rate of Maillard browning has also been attempted in model studies of glucose and glycine. The data suggested that at 37 °C, the browning rate is first order in sugars, first order in amino acids, and second order overall, and brown pigment formation follows pseudo-zero order kinetics (Baisier and Labuza 1992).

Effect of Temperature on Advanced Glycation End Product Formation

Cooking temperature and time play one of the most important roles in Maillard browning and the formation of brown pigments. Franke and Strijowski (2011) demonstrated this very clearly in their study to standardize french fry processes. When they increased the frying temperature from 160 °F to 175 °F, the products were darker in overall color, and the effect was enhanced further when the frying time increased from 2 to 6 minutes. These data were supported by Park and others (2011), who found that as the roasting temperature of perilla seed oil increased, so did the primary oxidation markers. Franke and Strijowsi were also supported by Durmaz and Gokmen (2011), who found that as roasting time of *Pistacia terebinthus* oil increased, levels of oxidation markers increased. Augustin and others (2006) found similar effects in model systems with glucose and sodium caseinate. They found that as the temperature and time of heating increased, the level of dark colored products also increased. Delgado-

Andrade and others (2010) also found that as the severity of cooking method increases, the more Maillard browning markers are produced.

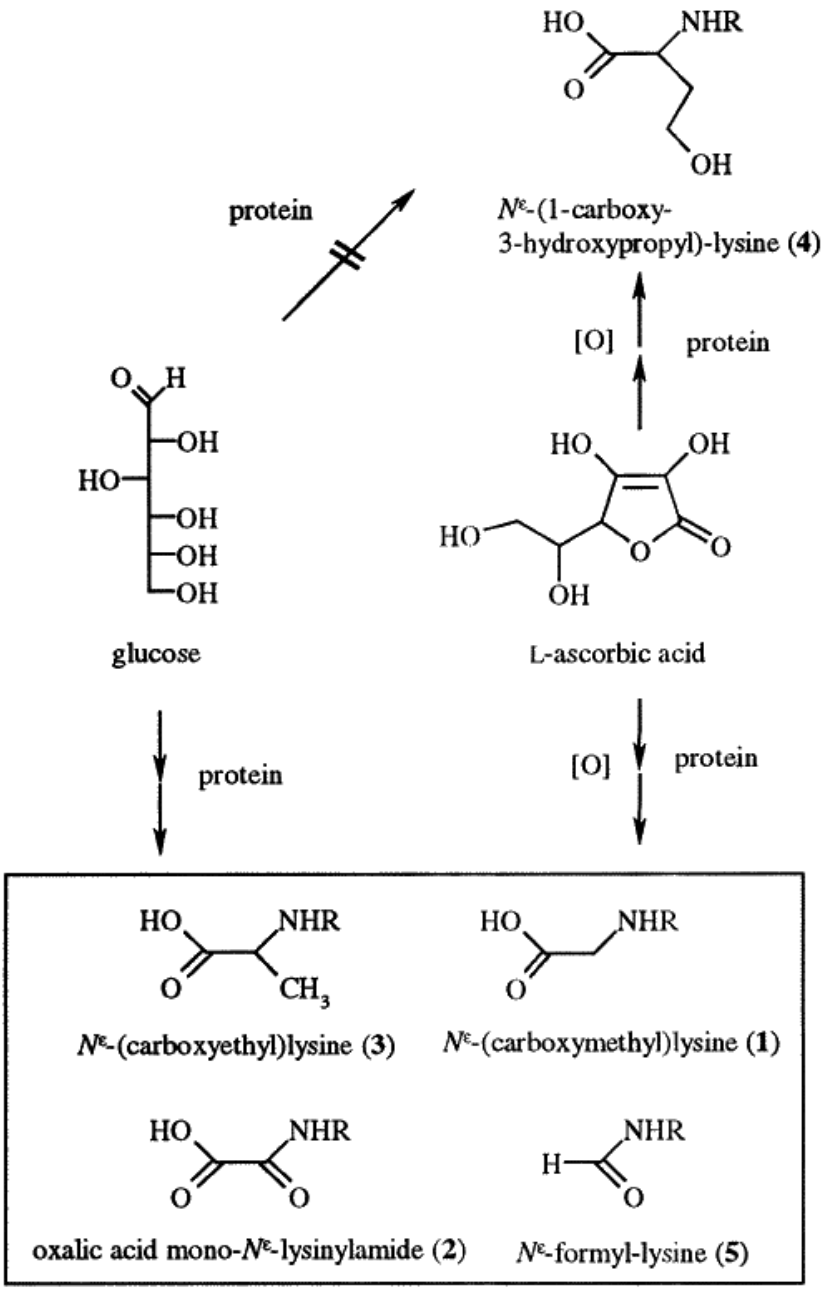

Figure 4: Formation of CML and other AGEs form ascorbic acid and glucose as adapted from Hasenkopf and others (2002).

Storage temperature is also important for the shelf life of products where Maillard browning could occur. In storage studies of royal jelly, a honey-like substance used in beauty

care products, changing the storage temperature from 4 °C to 20 °C increased the level of browning 3-4 fold after 1 month of storage (Chen and Chen, 1995).

Fermentation temperature was also shown to affect the Maillard browning in soy sauces, as Kim and Lee (2008) showed in their study. During their 360 day fermentation, the melanoidin fluorescence was consistently higher at 20 °C over 4 °C, and the concentration of 3-deoxyglucosone, another Maillard intermediate, 20 mg/L more concentrated after the fermentation period.

Effect of pH on Advanced Glycation End Product Formation

Aside from temperature, pH also plays a role in the browning rate of the Maillard reaction. Because dehydration or deprotination steps are necessary for the formation of some AGEs, a basic pH can enhance the rate of browning, and it can be become inhibitory to browning as the pH decreases (Wolfrom 1974). Model studies on glucose and fructose found that the maximum browning occurred between pH 9-10 (Ashoor and Zent 1984). Wolfram and others (1953) studied the optical density of a model system of equimolar 0.25 M solutions of glycine and xylose, and the data clearly show that as the pH increases, the optical density of the solution increases, as seen in Figure 5, adopted from the Wolfrom paper.

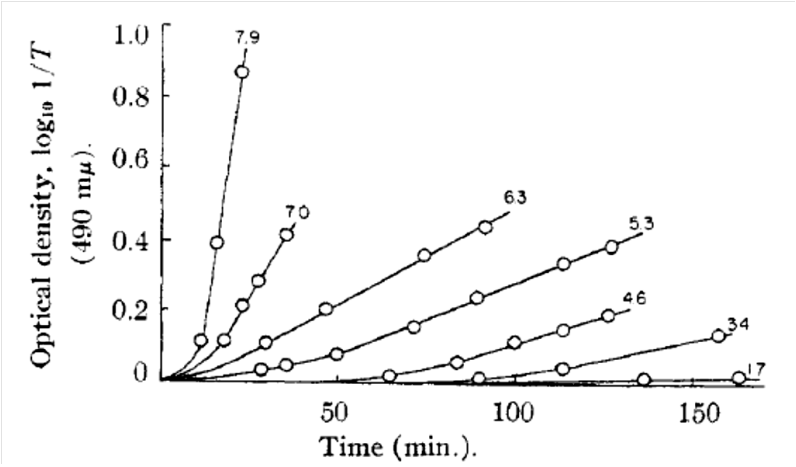

Figure 5: Rate of coloration of 0.25 M xylose and 0.25 M glycine at 100 °C at varying pH values, adapted from Wolfrom and others (1953).

Furthermore, they also calculated the first order rate constant for the linear sections of Figure 6 and found that there is an inflection point followed by a sharp increase in the rate

constant value, as seen in Figure 6, suggesting a faster reaction rate and higher Maillard product formation as the pH increases.

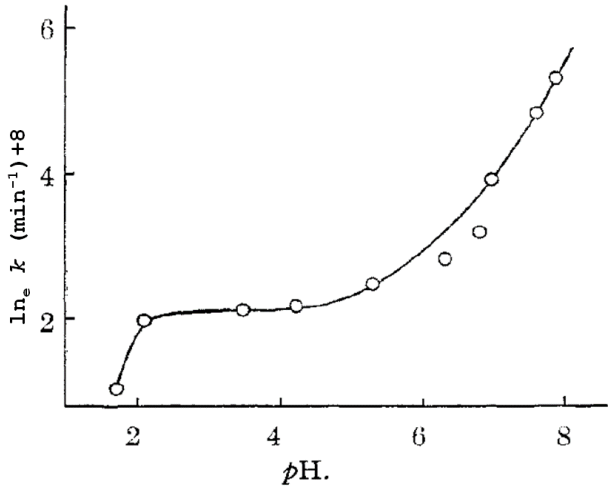

Figure 6: First order kinetic rate constant for Maillard reaction product formation as a function of pH, adapted from Wolfrom and others (1953).

The authors of this study suggest the reason for this dramatic increase in reaction rate and formation of Maillard products is because the isoelectric point of glycine is near pH 6 (Wolfrom and others 1953). These observations are supported by Yamaguchi and others (2009) who found that the amount of browning in a glucose/lysine model system increased as pH increased from 4.5-7. Other kinetic studies by Rozycki and others (2010), also suggest that browning kinetics are increased as pH is increased.

Because the formation mechanism includes deprotination and dehydration of the intermediates during the formation of AGEs, the pH of the system will fall as the reaction progresses. One study by Shih-Chuan Lu and others (2011) demonstrated this effect in a model fructose/glycine system with respect to temperatures that ranged from 45-90 °C and fructose concentrations that varied from 0.035-0.28 M. Their data suggested that the rates of pH change fell linearly between 45° and 60 °C, in two distinct linear stages (quick then slow) between 75°-90 °C, with the pH falling faster as the temperature increased and/or as the concentration of fructose increased. They suggested that the reaction rate slows during higher temperature reactions because of an "auto-inhibitory" effect. Their results suggest that to control browning, the pH of the food system should be below 6 (Liu and others 2011). Kwak and others (2005)

also suggested that maintaining a constant pH can increase the formation of melanoidins, because the auto-inhibitory effect is suppressed.

Ajandouz and Puigserver (1999) also studied the effects of pH (ranging from 4.0-12) on the Maillard reaction, using equimolar 0.05 M glucose and lysine, methionine, or threonine solutions. The data shows that a solution of glucose and lysine heated together to 100 °C for 120 minutes at pH 6 will show little browning, whereas large amounts of browning will occur at pH 12, as seen in Figure 7. The maximum browning was observed at pH 11 instead of pH 12, which they attributed to complete lack of protons necessary for the condensations to occur. The authors suggest that interactions between lysine and glucose are very strong at very high pH's, due to the complete deprotonation of the alpha-amino group. Methionine and threonine also showed similar trends, though not as exaggerated as those for lysine, which the authors attribute to the two available amino groups of lysine, as well as the partial negative charge on the threonine side chain. It is also interesting to note some studies have shown that the effect seems to be enhanced by increasing the temperature and pressure (Renn and Sathe 1997), while other studies seem to suggest that increasing the pressure will inhibit the formation of brown pigments (De Vleeschouwer and others 2010).

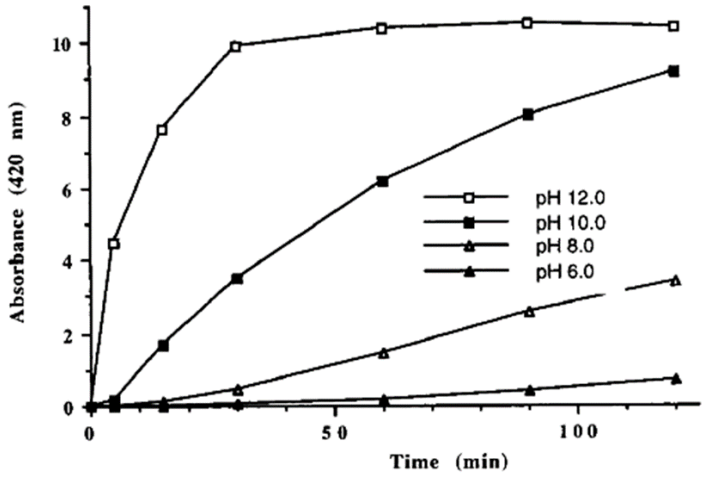

Figure 7: Brown color of lysine-glucose solutions at 100 °C from pH 6-12, as adapted from Ajandouz and Puigserver (1999).

The addition of pH buffering salts, such as phosphate, can further enhance browning because it can slow the drop on pH during browning (DeRoos 1992). Interestingly, the effect of pH on a solution is enhanced when pressure is also applied. A pressure of 600 MPa on a solution

of lysine and glucose dramatically increases the amount of browning at pHs above 8, but will decrease the amount of browning at pHs below 6 (Hill and others 1996).

Effect of Water Activity on Advanced Glycation End Product Formation

Water activity can pay a key role in determining the rate of Maillard browning. Model studies on glycine and xylose have shown that increasing the water content from 0%-30% rapidly increases the amount of browning, but this decreases again as the water content increases and is completely inhibited after 90% water content (Wolfrom and Rooney 1953). This is supported by studies in food systems, such as with Agudelo Laverde and others (2011), who found that dried apples and pears showed increased "degrees of browning" as the relative humidity (RH) increased from 11-75%, and a decrease in browning after 75% RH. They attributed these effects to water being unavailable in the reaction at lower RH and water acting as a diluent at high RH. Wolfrom and others (1974), observed the same effects in dehydrated orange juices, showing that increasing the water content past 65% water content decreased the amount of browning. These observations were further supported by Acevedo and others (2008), who found that in dried potato systems, the rate of browning increased until the water activity reached 0.75 in powdered potatoes, and 0.84 in potato discs. Studies on meat products have also found that non-enzymatic browning reaches its maximum rate when the water activity is between 0.6 and 0.8 (Tanaka and others 1994).

Addition of compounds that act as water binders, or creating conditions where water activity is reduced, can also reduce the amount of browning in a system. Lutz and others (2005), found that browning is decreased when it occurs in an emulsified state, and that the addition of surfactants and alcohols that act as water binders also inhibits water migration and lower the rate of browning. In liquid trehalose systems, addition of salts such as $MgCl_2$ will increase the rate of browning because it binds water and lowers the water activity; but in freeze dried systems, the addition of salts will inhibit browning because it decreases the reactivity of the reducing sugars through sugar-salt interactions (Matiacevich and others 2010). In model systems where glycerol was added as a plasticizer, increased rates of browning were observed at water activities of 0.4 compared to a control system without glycerol. However, this effect was still inhibited at water activities of 0.32, where the study suggests mobility of reactants is preventing interaction (Eichner and Karel 1972). This same study also observed that addition of gum arabic decreased

the rate of browning at low water activities, possibly through increasing the viscosity of the solution. The effect of glycerol was also observed by Warmbier and others (1976), who found that addition of glycerol to model systems decreased the maximum browning rate from water activities of 0.65-0.75 to water activities of 0.4-0.5.

Water activity can also affect the glass transition temperature of food systems, which would affect the mobility of reducing sugars in the system. One model study found that browning can occur in a glassy state, but as the water activity increases and the system transitions from glassy to rubbery there is an increase in browning rate. Furthermore, they suggested that a system with a higher glass transition temperature will exhibit lower rates of browning compared to a system with a lower glass transition temperature in the same transitional state (Bell 1995). These observations were supported by kinetics studies on model systems where large differences in the optical density were observed after the systems transitioned from rubbery to glassy (Karmas 1992).

Other Factors That Affect Advanced Glycation End Product Formation

Substrate type can also affect formation of AGEs. Specifically in protein substrates, lysine and arginine are preferentially used over other amino acids (Fu and others 1994), followed by glycine, alanine, serine, proline and aspartic acid in decreasing order (Wolfrom 1974). Glycine also seems to be more reactive than cysteine, and is more pH sensitive (DeRoos 1992), possibly due to cysteine's larger structure, and cross-link potential through sulfur-sulfur interactions. Protein size also seems to affect the rate of browning, with smaller proteins being more reactive to Maillard browning than larger proteins (Lertsiri and others 2001).

As with amino acid type in proteins, sugar type also changes the browning rate. CML can be formed during heating with hexoses and ketoses (Ruttkat and Erbersdobler 1995). Model studies by Laroque and others (2008) found that pentoses are more reactive than hexoses, in the following series: ribose>xylose>arabinose>fructose>glucose. This was supported by DeRoos (1992), with the exception that glucose was more reactive than fructose. However, other studies suggest that glucose and fructose are equally reactive, and that sucrose does not readily brown, most likely because it is not a reducing sugar (Wolfrom 1974). It is possible sugar reactivity depends on amino acids. For example, one model study used glucose and fructose with arginine

or glutamine, and observed that fructose produced more browning with glutamine over arginine, while the opposite was observed in the glucose systems (Gogus and others 1998).

The ratio of sugars to amino acids can also affect the amount of browning in a system. Reducing sugar can be readily used to start the formation of AGEs. Therefore, increasing the soluble solids and the reducing sugar content may increase the browning intensity (Davids and others 2003; Ibarz and others 2011). Model studies on glucose-glycine reactions have shown that glucose concentrations are not degraded as dramatically when the glycine concentration increases, but the amino acid degradation seems to exponentially increase as the glucose concentration is increased, and will still increase dramatically as the glycine concentration increases (Peterson and others 2010). Other model studies suggest that browning is most intense when the amino acid to sugar ratio is 5:1 (Wolfrom 1974).

Salt type can affect the Maillard browning in food systems. Salts can act to facilitate sugar degradation and water activity as seen above, but model studies on cysteine and glucose show sodium chloride will enhance browning more than calcium chloride, sodium sulfate, or ferrous chloride (Yeo and Shibamoto 1991). However, it has also been suggested that group II cationic species will enhance browning more than group I cationic species in model systems of ribose and glycine (Rizzi 2008). Additionally, it has been observed that extremely high concentrations of salt, 0.8-1.6 M, can actually decrease the amount of browning in food systems (Yamaguchi and others 2009), possibly due to interactions between the salt and the sugars.

Buffer type can affect the rate of browning as well. In model systems with glycine and glucose, citrate buffers inhibited the formation of browning products, while phosphate buffers, particularly at concentrations greater than 0.05 M, can actually act as a catalyst for browning (Bell 1997). Phosphate buffers, in addition to stabilizing pH, also act to promote formation of carbonyl compounds that are reactive in Maillard browning reactions (Rizzi 2004). Nitrite present in foods can also enhance browning (Russell 1983).

The presence or absence of trace metals and dissolved oxygen can also act to enhance browning. Models that have added strong chelating agents to remove trace metal ions found a reduction in the levels of browning. Furthermore, that same study found that the presence of dissolved oxygen in the sample may promote formation of free radicals and therefore enhance

browning. (Hayase and others 1996). It is possible that these trace metal ions may be able to induce oxidative stress through the formation of coordination complexes with CML and other AGE modified proteins (Seifert and others 2004).

Pressure in a food processing system, particularly extrusion systems, can also have an effect on browning. In model systems of asparagine and glucose, melanoidin content was reduced roughly by half after 1 hr of incubation at ambient pressure or at 600 MPa (De Vleeschouwer and others 2010).

Advanced Glycation End Products in Foods

AGEs, as seen above, can have an impact on health and disease. Furthermore, because AGEs are part of Maillard browning, they do not occur only in body tissues, but in food stuffs as well. The processing methods in food are important, also as previously discussed, where the greater the surface temperature, the more quickly the AGEs form. Consumption of AGEs in food is very difficult to avoid, but diet does play a role in the levels of AGEs in the body. One study looked at CML levels in two similar diets, with one diet being "processed softer," using boiling or steaming methods, and not including crusts on breads. They found that participants who ate the "softer" diet consumed 5.3 CML/kg protein compared to the "traditional diet," which contained an average 15.7 mg CML/kg protein (Delgado-Andrade and others 2007).

Dairy Products

AGE's in dairy products have been widely studied and observed. Hull and others (2012), looked at the CML content in 29 dairy samples, including milk, eggs, ice cream, coffee creamer, and cheese. They found that CML can range from 0.02-1.76 mg CML/100g food, and had an average of 0.44 mg CML/100g food in the samples analyzed. A select list is summarized in Table 1. It is of interest to note in the milk samples that the CML content increases as the milk fat increases, both in pasteurized and UHT pasteurized milks.

Goldberg and others (2004), looked at CML in a large number of dairy products. A summary of their findings are listed in Table 2 below. Similar to the Hull study, the more severe heat treatment produces more AGEs, which is exemplified in the fat free milk samples. There is also a similarity between both studies in parmesan cheese, in which both samples have a very high level of CML compared with other dairy products.

Drusch, Faist, and Ebersodobler (1999) found CML in milk, powdered milk, flavored milk, chocolate milk, coffee cream, ice cream mix, and whey cheese. The results are similar to the Hull (2012) study when comparing chocolate milk in that the values found in the Hull study are in the range of values found by Drusch; 172.36 mg CML/kg protein and 0-413 mg CML/kg protein, respectively. However some of the Drusch results differ from Hull; i.e. no CML could be detected from UHT pasteurized milk samples. However, other studies agree with Hull, showing ultra-high temperature milk will also brown during storage (Schamberger and Labuza 2007). In addition, no CML was detected in any of the 50 cheese samples analyzed in the Drusch study, while parmesan cheese had the highest amount of CML (1.76 mg CML/100 g food) of any of the dairy products in the Hull study. Differences between the Drusch and Hull studies could arise from method of analysis (RP-HPLC vs. ULPC- MS/MS) or through variation in processing from location to location (Germany vs. England/Ireland) (Drusch and others 1999).

Table 1: Select list of CML in dairy products as analyzed by Hull and others (2012)

Food item	Cooking Method	mg CML/100 g food
Cheddar cheese		1.18
Cheese, parmesan		1.76
Cream, fresh, single	Raw	0.12
Eggs, chicken, whole	Fried	0.63
Eggs, chicken, whole, raw	Raw	0.05
Ice cream, dairy, vanilla	Frozen	0.5
Ice cream, non dairy, vanilla	Frozen	0.54
Semi-skimmed milk	Pasteurized	0.03
Semi-skimmed milk	UHT Pasteurized	0.17
Skimmed milk	Pasteurized	0.02
Skimmed milk UHT	UHT Pasteurized	0.12
Whole milk	Pasteurized	0.05
Whole milk, UHT	UHT Pasteurized	0.22

A study by Patel and others (1996) looked at browning in sweetened condensed milk, although it did not quantify CML specifically. Briefly, sweetened condensed milk was stored for 135 days, at 7, 15, 35, 45, or 55 °C, and browning was determined by percent absorbance at 450 nm. Within 30 days, % absorbance had increased by 30% at 55 °C and by 15% at 45 °C. After the full 135 days, % absorbance had increased by 8% at 15 °C, and by 3% at 7 and 15 °C. Even though CML was not explicitly quantified, the increase in the percent absorbance does suggest

that browning can occur in this dairy product, especially if it is exposed to higher/abuse temperatures (Patel and others 1996).

Table 2: Summary of select findings of CML in kU/serving from Goldberg and others (2004)

Food Stuff	kU AGE/serving
Cheese, parmesan	169,020
Egg yolk, boiled, 10 min	12,134
Egg yolk, boiled, 12 min	18,616
Fat Free milk, microwave 1 min	5
Fat free milk, microwave 2 min	21
Fat free milk, microwave, 3 min	75
Ice cream, vanilla	352

Browning can also occur in cheeses and cheese powders. Kilic and others (1997), studied nonenzymatic browning in cheddar cheese powders at 20°, 30°, and 40 °C at water activities of 0.51, 0.62, 0.75, and 0.83, over a six month shelf life. All of the powders exhibited some browning. However, browning occurred more rapidly at the higher temperatures and lower water activities. The local maximum was observed at 0.63 water activity and 20 °C.

Infant formula is another dairy product in which Maillard browning can occur. Contreras-Calderón and others (2009), monitored Maillard browning products in four different infant formulas via fluorescence. The carbohydrate and protein sources for each formula are summarized in Table 3. Their study found that all of the infant formula had some fluorescent compound at any stage during production, in an industrial or pilot plant scale production line. The highest increase in fluorescence intensity was during spray drying, when processing temperatures were between 215-230 °C. However, they observed that Formula A and B exhibited less browning due to the use of whole versus partially hydrolyzed proteins.

Model studies have been used to study Maillard browning in milk. Morales and Boekel (1998) used two model systems to compare browning between glucose and lactose under browning temperatures ranging from 110-150 °C. They also looked at browning between the free amino acids and the caseinate bound browning fractions. Their study showed that browning in lactose and glucose were similar in behavior, but their systems showed a visual color change difference between lactose and glucose using an L*, a*, b* colorimeter to calculate chroma values for their samples. Increasing the thermal processing temperature of the system increased the observed amount of browning, and bound protein fractions of AGEs were much higher than

free browning products, indicating that browning will be most likely to occur in bound proteins. This is a typical result in milk, because there is not an abundance of free amino acids.

Table 3: Carbohydrate and protein source of infant formulae used by Contreras-Calderón (2009)

Infant Formula	Carbohydrate Source	Protein Source
Formula A	Sucrose, Maltose-Dextrin	Whey Protein with Low Lactose, Calcium Caseinate
Formula B	Lactose	Whey Protein Milk, Calcium Caseinate
Formula C	Sucrose, Maltose-Dextrin	Partially hydrolyzed whey protein, low lactose
Formula D	Sucrose, lactose	Partially hydrolyzed whey protein, low lactose

Fruits and Vegetables

While Millard browning is very low in fruits and vegetables when compared to other foods, particularly in fresh fruits and vegetables where the highest was seen as 0.08 mg CML/100g food in oranges, browning on the proteins is still possible (Hull and others 2012). One study on pea-proteins found that the digestibility (and potential absorption) is decreased when the proteins are glycated with glucose or lactose, while digestibility is actually increased when the proteins are glycated with fructose or glucosamine (Marciniak-Darmochwal and Kostyra 2009). A drop in lysine content is also observed in baby foods during storage (Alonso and Zapico 1995). Nonenzymatic degradation is also observed in fruit juices. Increasing the soluble solids in the juice, and increasing the temperature of the thermal processing will increase the level of brown pigment formation (Ibarz and others 2011). In cauliflower soup powder protein degradation is observed, and it is more important than lipid oxidation in consumer studies (Raitio and others 2011).

Another study by Lavelli (2009) looked at the effect of storage temperature and water activity on antioxidants, radical scavenging activity, and AGE inhibition in dried apples at varying water activities of 0.1, 0.2, 0.3, 0.5, and 0.7. Over the course of the storage period, either 120 days at 20 °C, 50 days at 30 °C, or 22 days at 40 °C, there was decrease in antioxidants in the powders, as well as a decrease in radical scavenging activity and AGE inhibition. As expected, these decreases were exaggerated as the temperature increased, and as the water activity increased. This result is supported by Agudelo-Laverde and others (2009), who found that dried apples and pears showed increased degrees of browning as the relative

humidity (RH) increased from 11-75%. However, they also observed that the browning rate decreased after 75% RH, which they attributed to water being unavailable at lower RH and water acting as a diluent at high RH. Fruit juice also exhibits browning during heating, which degrades not only the sensory characteristics, but the natural antioxidant capacity of juice (Anese and others 1999).

Meat and Poultry Products

Meat and meat products are consumed as a source of protein in the diet. However, cooking meat can induce Maillard browning in the food, though the cooking method does affect the levels of AGEs that form, as seen above. Studies that evaluated meat products have found that beef, poultry, and fish extracts had 30x higher levels of AGEs than did pastas or vegetables, and that these food extracts had cross linking activity in collagen tissues, and increased the levels of cellular oxidation in endothelial cells (Cai and others 2002). Chao and others (2009) found similar results, in that cooking beef, pork, chicken, salmon and cod at least doubles the level of AGEs in the food over the raw levels. However, the levels of CML found by Chao differ from those found by Hull and others (2012), who found higher levels of CML overall when the meats were cooked, as seen in Table 4.

In addition to CML, other Maillard browning products such as furosine, and hydroxymethylfurfural (HMF), have been detected in meat and meat dishes, such as pork loin, roasted chicken, and cooked anchovies, and at levels higher than reported by Chao (2009) or Hull (2012), i.e. roasted pork loin had 16 mg Amadori compounds/100g dry matter (Delgado-Andrade and others 2010). However, these levels may be higher because the food is "dry matter," instead of the whole foods, and because of the introduction of reducing sugars into the food matrices during cooking. It was observed that the introduction of sugars, even non-reducing sucrose, can increase the Maillard browning in meat products (Tanaka and others 1994; Chen and others 2002). Even meats from the same animal can have different Maillard browning depending on the diet fed to the animal, most likely due to the change in the fatty acid profile and the impact on the fatty acid profiles of the meats (Ventanas and others 2007).

Table 4: Summary of CML levels found in meats adapted from Chao (2009) and Hull (2012)

Food Sample	CML (mg/100 g food)	Reference
chicken, raw	0.018	Chao, 2009
chicken, baked	0.07	Chao, 2009
Chicken, light meat, roasted	0.46	Hull, 2012
pork, raw	0.02	Chao, 2009
pork, baked	0.076	Chao, 2009
pork, leg joint, baked, well done	0.27	Hull, 2012
beef, raw	0.013	Chao, 2009
beef, baked	0.062	Chao, 2009
beef joint, roasted, well done	0.42	Hull, 2012
salmon, raw	0.01	Chao, 2009
salmon, baked	0.068	Chao, 2009
salmon, grilled	3.35	Hull, 2012
cod, raw	0.018	Chao, 2009
cod, baked	0.055	Chao, 2009
cod, baked	0.06	Hull, 2012

Grain Products

Michalska and others (2008), were unable to detect any CML in their study of rye bread. They calculated that the GC- MS method used had a detection limit of 3.35 and 11.16 mg CML/100 g protein. However, they were able to detect other fluorescent and non-fluorescent AGEs, suggesting that CML levels in their bread samples were below the detection limit for their method. Charissou and others (2007), used a GC- MS method that detected CML levels that ranged from 5-35 ng/mg protein in cookies, 0-13 ng/mg protein in toasted bread, and 6-8 ng/mg protein in corn flakes. A large study conducted by Goldberg and others (2004), found CML in many starch and sugar based products using an ELISA method, a summary of which is provided in Table 5.

Table 5: CML in Starch and sugar based foods, as detected by Goldberg and others (2004)

Foodstuff	AGE U/g food
Lay's potato chips	28,818
Frozen, toasted waffle	28,711
Goldfish Cheddar crackers	21,760
Vanilla Wafers	5,096
Corn Flakes	2,320
Mini-Wheats	2,085
Popcorn, air-popped, with butter	1,340
Italian, white bread, crust	366
Italian, white bread, crumb	225

An even larger study conducted by Hull and others (2012), included 40 different "bread and savory biscuit" samples, 13 cereals, 28 potato, rice, and pea samples, and 36 sweets and snacks samples. The CML content of each sample was determined in triplicate using a UPLC-MS/MS analysis, and reported in mg CML/100 g food. The mean, and standard deviation of the categories mentioned above, are summarized in Table 6. The large standard deviation of the samples is due to the wide variety of analyzed samples.

Table 6: CML Levels (mg/100 g food) for selected food categories, as adapted from Hull and others (2012)

Food Category	mg CML/100 g food ± standard deviation
Bread and Savory Biscuits	1.29 ± 1.22
Cereals	2.55 ± 1.36
Potatoes, Rice and Pasta	0.13 ± 0.12
Sweets and Snacks	1.81 ± 0.173

Other studies that analyze bread samples find values similar to the Hull study. Assar and others (2009), used a UPLC- MS method on white and whole meal breads, and found similar values. These trends were corroborated by Peng and others (2010a), in his HPLC study of grapeseed extract fortification on the antioxidant activity and quality attributes or bread, and by Srey and others (2010), in the UPLC- MS/MS evaluation of CML in model sponge cakes. A comparison of CML values for bread from the Hull, Assar, Peng and Srey studies are summarized in Table 7.

Table 7: Comparison of CML value in bread samples from various studies

Food Stuff	Study	Reported mg CML/100 g food
Basic white bread crust	Peng, and others 2010	0.35
Model sponge cakes	Srey and others 2010	0.357 (avg. of all samples)
White bread	Hull and others 2012	0.66
White bread crumb	Assar and other 2009	0.225
White bread crust	Assar and other 2009	3.71
White bread, fried	Hull and others 2012	1.00
White bread, toasted	Hull and others 2012	1.37
White rolls, crusty	Hull and others 2012	1.02
White rolls, soft	Hull and others 2012	0.68
Wholemeal bread	Hull and others 2012	1.40
Wholemeal bread crumb	Assar and other 2009	0.445
Wholemeal bread crust	Assar and other 2009	4.61
Wholemeal bread, Toasted	Hull and others 2012	3.10

Among the studies where the bread crumb and crust were analyzed separately, the crust showed dramatically more mg CML/100 g food than did the crumb. This result is expected because browning in bread is most intense on the surface, which is why the outside of bread loaves are brown. Other studies have looked at melanoidins, AGE polymers, in bread, Spanish muffins, and dark specialty malts (Coghe and others 2004; Gonzalez-Mateo and others 2009; Fogliano and Morales 2011). All of these studies reported some level of melanoidins, which also suggests that CML was present, although none of the studies quantified CML specifically.

Non-enzymatic browning produces CML, which occurs when foods are processed. Several studies have looked at quantification of CML in starches and grains and found that mg CML/100 g of food can range from 0.13-4.61 in bread and starchy products. Further studies could be conducted to verify these results and expand the existing database of CML in grains/starch based foods.

According to Hull, and others (2012), the browning in grain products ranges from 0.08 mg CML/100g food in potato bread, to 6.18 mg/100g food in shortbread. Grain products with more oils and sugars have more CML than did products without, most likely due to the oxidation of oils during cooking, and the inclusion of additional reducing sugars in the case of sweetbreads Also, studies have found that more severe cooking methods will produce more browning products, such as frying in the case of potatoes (Delgado-Andrade and others 2010), and toasting bread (Goldberg and others 2004).

Alcoholic beverages are fermented grain products. Nonenzymatic browning also occurs in these solutions. Studies have found that in solutions where phenolic compounds are present, degradation of these phenols can be a major contributor in the browning (Chuang and others 2011).

Fats, Oils, Sauces, Syrups

Fats, oils, sauces and syrups are usually considered condiments or ingredients that are used sparingly in food recipes. However, because they still contain reducing sugars and amino acids, they are still subject to Maillard browning. Maillard browning can affect the shelf life of product due to darkening of the color and generation of off flavors and odors. Maillard browning affects the shelf life of white chocolate (Vercet 2003), and royal jelly (Chen and Chen 1995), by darkening the color and changing the protein solubility through glycation and pigment formation.

Model studies that have looked at oil/water emulsions have found that browning does occur when amino acids and reducing sugars are present, and even to a lesser extent if only sugar or only amino acids are present in the emulsion. However, it was also observed that the presence of the Maillard products decreased the incidence of lipid peroxidation in the emulsion (Severini and others 1998). This effect was also observed in model systems that looked at the addition of starch, glucose and lysine to soybean oil, which found that even though browning did occur in the glucose and lysine, the peroxide values of the soybean oil remained low (Mastrocola and others 2000). Spray dried microcapsules of fish oil also showed dramatic improvement of lipid stability when dextrins and sodium caseinate were incorporated into the structure found increased stability in fish oil microemulsions that included Maillard products from sodium caseinate (Kagami and others 2003; Augustin, and others 2006). A similar effect was seen in studies on crushed hazelnuts; defatted samples had the lowest amount of browning compared to non-defatted samples, and when defatted hazlenuts were enriched with sucrose and/or hexanal solutions, the hydroxymethylfurfural (HMF) contents, used as browning indices, were more than double the controls (Fallico and others 2003).

This lipid protective effect could be due to the formation of phenols during the later stages of the Maillard browning, as indicated by Shyu and others (2009), who found that extracts

from sesame meal increased in antioxidant capacity as the sesame seed roasting conditions increased from 180-220 °C, and the roasting time increased from 5-30 minutes. A very mild protective effect on lipids from volatiles of the Maillard reaction was observed in almonds that were packaged in air saturated with those volatiles. However, it was not as effective vacuum packaging after the almonds were already cool, suggesting that removal of the oxidant is a better option than the addition of trace antioxidants (Severini and others 2000). It also appears that some of the oxidative products could have some radical scaveng(ing activity, as seen in the study by Park and others (2011), who found that as the roasting temperature of parilla oil increased, so did the levels of radical scavenging activity. Increasing the roasting time (instead of temperature) also can increase the levels of phenolic compounds and radical scavenging activity in oils (Durmaz and Gokmen 2011). However, it is also worthwhile to note that as the radical scavenging activity increased in these oils, so did the oxidation markers, which have also been shown to create AGEs such as CML (Fu and others 1996).

Chao and others (2009) examined CML levels in commercially available condiments, such as soy sauce, sweet-sour sauce, tomato sauce, and barbeque sauce. They found that they do contain some level of CML (an indicator AGE) most likely due to their processing. They found that barbeque sauce contained the highest average levels of CML, with 352 µg/100 mL sauce, and the lowest was soy sauce, with an average CML level of 224 µg CML/100 mL sauce (Chao and others 2009). The CML levels of sweet and sour sauce were higher than the 100 µg/100g sauce found by Hull, and others (2012), but they were in the average range for soups and sauces. As the study by Kim and Lee (2008) showed, however, processing conditions such as the fermentation time and temperature of soy-sauce will affect the amount of Maillard browning that occurs.

A synergistic effect was observed on the CML levels with the addition of a sauce to meat, as observed by Chao and others (2009). They noted that in every instance where the meat was treated with sauce, the CML levels were greater than the meat or sauce alone, more than four times greater in the case of boiled chicken treated with soy sauce. Chao attributed this effect to the addition of reducing sugars from the sauce to the amino acids in the meat proteins. There have been some attempts to minimize this synergistic effect in soups by thermally processing the protein components and the carbohydrate components separately, then mixing

them together prior to packaging (Assinder and others 1987). However, with AGEs present in foods during cooking, processing, and storage, and with synergistic effects observed when cooking different foods together, the next topic of interest is the control of AGE; specifically what do common spices have that can inhibit AGE formation, and what do spices do to AGE levels in foods and in the body.

Coffee

Studying browning in coffee is difficult, as it is a complex beverage. Beans of the coffee plant are harvested, stored, roasted to varying degrees, ground, and brewed through a wide array of processes, each of which has an effect on the browning. During storage of green coffee beans Maillard browning reduces the number of free amino acids and reducing sugars, and develops some brown pigmentation without roasting. However, it is worthwhile to note that this storage also betters the sensory characteristics of the beans after they are roasted and brewed (Pokorny and others 1975). Roasting provides the greatest amount of browning, as expected. As coffee beans are roasted Maillard browning occurs quickly, but slows as the degree of roasting increases (Sacchetti and others 2009). During the browning process, some compounds that are produced do have antioxidant capacity (Verzelloni and others 2011). However, as expected, browning products such as methylglyoxal, which have been associated with disease, increase with degree of roasting as well (Sacchetti and others 2009).

Controlling AGEs

Antioxidant Capacity of Common Spices

Many spices have the ability quench free radical formation, or prevent oxidation. The antioxidant capacity of spices is commonly measured through the ferric reducing antioxidant potential (FRAP) assay (Dearlove and others 2008), the α,α-Diphenyl-β-picrylhydrazyl (DPPH) assay (Lo and others 2002), or the oxygen radical absorbance capacity (ORAC) assay (Jensen and others 2011). Each of these tests measures the relative ability of a compound to stop oxidation relative to a standard compound.

Some of the antioxidant potential for common spices has been attributed to the presence of phenolic, or polyphenolic, compounds present in these spices. The common assay to measure

total phenolic content is by using the Folin-Ciocalteu reagent, and a gallic acid standard (Singleton and others 1999; Dearlove and others 2008).

Dearlove and others (2008), conducted an extensive study of culinary spices and their total phenolic content, and their antioxidant capacity. A summary of their findings is in Table 8. Cloves and cinnamon have the highest phenolic content per gram, as well as the highest radical scavenging activity, according to the FRAP assay. Rosemary has much less phenolic content than cinnamon with only 48.2 mg GAE/g of phenols, and more than five times less radical scavenging activity. At the bottom of the list is black pepper, with only 5.1 mg GAE/g spice, and a FRAP value of 4.7. This study suggests that to maximize phenolic content and radical scavenging ability, spices such as cinnamon or rosemary, with high and intermediate phenolic contents, should be used preferentially over spices such as black pepper (Dearlove and others 2008).

The McCormick Science Institute (2012), also characterized the antioxidant capacity in spices. However, instead of a FRAP assay, they used an Oxygen Radical Absorbance Capacity (ORAC) assay. ORAC assays measure in µmol trolox equivalence/g sample. The McCormick Sicnece Institute results are similar, but not identical to the phenolic content and FRAP results of Dearlove (2008). Spices such as sage and rosemary have a higher relative ORAC value, compared to their relative FRAP values, suggesting that other compounds may be responsible for stabilizing the radicals. This is particularly true in the black pepper values, where there is a relatively low FRAP value and total phenolic content, yet still has an ORAC value close to that of ginger and nutmeg.

Table 8: Total phenolic content and FRAP values for common culinary spices. NT= not tested (Dearlove and others 2008; McCormick Science Institute 2012)

Herb or Spice	Phenolic Content (mg of GAE/g)	FRAP value (mmol/100 g)	Total ORAC µmol TE/g
Cloves	296 ± 3.7	271 ± 6.9	2996
Cinnamon, ground	183 ± 11.5	137 ± 3.1	1900
Oregano, ground	82.3 ± 0.9	84.0 ± 0.7	2657
Sage	59.8 ±3.0	62.8 ± 3.1	2401
Thyme	52.0 ± 25.0	59.1 ± 0.6	1637
Mint	50.9 ± 2.3	60.9 ± 0.6	NT
Rosemary	48.2 ± 1.0	47.9 ± 2.7	1704
Turmeric	25.9 ± 1.6	10.2 ± 0.06	1832
Basil	18.0 ± 0.5	18.9 ± 0.9	NT
Nutmeg	17.6 ± 0.9	6.8 ± 0.2	511
Ginger	17.7 ± 1.7	19.5 ± 0.3	264
Black pepper	5.1 ± 0.3	4.7 ± 0.2	424

Another study by Friedman and others (2000) tried to characterize antioxidant levels in herbs. They found that cinnamon cassia and cinnamon bark contained 81 and 62 mg cinnamaldehyde/100 µL, respectively, with cinnamaldehyde being an antioxidant responsible for cinnamon flavor, and contributing to the total phenolic content reported by Dearlove. They also found thyme and oregano had thymol levels of 37 and 8 mg/100 µL, respectively.

In addition to herbs and spices, fruits and plant extracts can also have compounds with antioxidant activities. Lavelli (2009) found that dehydrated apple powder contained ascorbic acid, epicatechin, procyanidin B2, phloridzin, chlorogenic acid and derivatives of p-coumaric acid, all of which have some antioxidant capacity. The antioxidants in the apple powders degraded during storage, losing some of their radical scavenging activity and ability to inhibit AGE formation. Phloridzin, and phloretin from apple polyphenols have been shown to trap some of the intermediates for AGE formation, such as glyoxal and methyl glyoxal, by reacting with the carbonyl groups and binding them to the polyphenol (Shao and others 2008). Green tea has several major compounds with antioxidant and free radical scavenging activity including catechin, epicatechin, epigallocatechin, and epigallocatechin gallate (Yamamoto and others

1997). However, some studies have also suggested that, at concentrations above 50 µM, epicatechin and gallic acid may actually generate hydrogen peroxide as they are oxidized, and work to enhance rather than inhibit CML formation, as do concentrations below 50µM (Fujiwara and others 2011).

Green coffee beans do possess vanillic acid, ferulic acid, and chlorogenic acids, which are polyphenols with antioxidant abilities. However as roasting occurs, these polyphenols degrade (Gomez-Ruiz and others 2008). Studies have shown that the advanced stage browning melanoidins that are formed during roasting will induce some CML formation, but they also show that coffee fractions with a low molecular weight (< 10 kDa), have antioxidant activity and can prevent glycation in serum albumin (Verzelloni and others 2011). These low molecular weight compounds most likely form in the "light" and "medium" roast coffees, as the radical scavenging activity of coffee increases from "green" beans to light and medium roasted coffee. However, high molecular weight compounds in coffee also have some antioxidant activity, and they may be simply complexes of the lower molecular weight melanoidins agglomerated with polysaccharides, possibly galactomannans in coffee (Nunes and others 2006; Gniechwitz and others 2008). As the coffee roasting degree increases, the coffees show decreased radical scavenging activity, probably through the degradation of the natural polyphenols in the coffee, after the melanoidins have formed (Sacchetti and others 2009).

The melanoidins in roasted coffee have been shown to have a protective effect against oxidation in low-density lipoproteins (LDL) (Gomez-Ruiz and others 2008), and model liver tissues (Goya and others 2007). However, this lipid protective effect is not limited to coffee alone. A lipid protective effect was seen turkey slices with the addition of dried honey prior to thermal treatment (Antony and others 2006). However, this addition of honey would also increase the levels of harmful Maillard products as well as the antioxidants found in the melanoidins.

Other model studies have looked the effect of cumin, and caraway oils on the formation of Maillard browning products between glucose and glycine, and found that caraway oil inhibited the formation during the 1 hr reflux incubation (Chawla and Sahu 2007).

Use of Antioxidants to Control AGEs

Antioxidants have been used to increase the shelf-life of products on the market by preventing lipid oxidation and protein degradation. Studies on Agave nectar found that addition of free amino acids, such as cysteine, or green tea polyphenols at levels of 400 ppm can reduce the levels of color change and preserve the taste and ascorbic acid content after six months as much as do other "unnatural" preservatives such as sodium metabisulfite (Abd El-Hady and others 2009). Jensen and others (2011), tried to improve the shelf-life of whole wheat bread by adding α-tocopherol, and rosemary extracts. However, addition of extracts in this context did not improve the shelf-life of the product when added to the formulation at 1.0 g/kg flour, and even enhanced rancid flavors and aromas associated with lipid peroxidation in the breads with the added α-tocopherol. In milk products, particularly ultra-high temperature pasteurized milk, green tea flavonoids epicatechin and epigallocatechin have been added to decrease the Maillard browning over the course of a 90 day storage period, without impacting consumer acceptability (Schamberger and Labuza 2007). It is interesting to note that addition of antioxidant phenols to ethanolic solutions may actually enhance browning as the phenols are degraded (Chuang and others 2011). Mustard seed oil and cinnamon oil have even been shown to inhibit fungal growth of bread molds, with mustard seed having a minimum inhibitory concentration of 1.8-3.5 μg/mL gas phase (Nielsen and Rios 2000).

Besides extending the shelf-life of products, the introduction of antioxidants into food products can also provide health benefits. Some studies have tried to increase the antioxidant content in foods, meaning more antioxidants would be ingested. For example, fruit extracts have been used to try to improve the total phenolic content in foods. Sivam and others (2011), looked at the addition of pectin fiber and phenolic extracts of apple, blackcurrant, and kiwifruit extracts and found that addition of these ingredients did increase the total phenolic content of the breads, but would also significantly affect bread loaf weight, color and volume. Decreased loaf volume was also seen in studies where caffeic and ferulic acid were added to bread formulations at a concentration of 4.44 μM/g flour, which was attributed to weakening of the gluten elasticity (Hye Min Han and Bong-Kyung Koh 2011). Ferulic acid and thiamine derivatives have also been added to model sponge cakes to test their ability to inhibit CML formation. Both showed

an ability to inhibit formation of CML by at least 25% in model sponge cakes (Srey and others 2010).

Hoye and Ross (2011) evaluated the consumer acceptance and phenolic content of bread fortified with grape seed flour, and found that consumers could accept up to 5g of grape seed flour for every 100g of wheat flour, which would provide 1.43 mg tannic acid/g dry weight. Balestra and others (2011), also looked as sensory acceptance and rheological properties of bread, though they used ginger powder in levels from 0-6%. On the surface the ginger powder did not affect the color, but the crumb decreased in lightness, hue angle and chroma as the level of ginger increased. As expected as the level of radical scavenging activity and total phenolic content increased with increasing levels of ginger, with the highest levels of total phenolics being 0.710 mg GAE/g dry weight at the 6% level in the bread crust, and 0.485 mg GAE/g dry weight in the crumb. However, the highest consumer acceptability was at the 3% ginger powder level, which would still provide two times the total phenolics and the radical scavenging activity of the control breads (Balestra and others 2011). Another study fortified bread with turmeric powder, and found that as the percent turmeric in the formulation increased the loaf volume decreased, and the cell volume of the loaf increased. The total phenolic content increased from 40 to 150 mg GAE/100 g dry weight from 2-8% turmeric, as expected, but consumer acceptance differed from the control only in 2% turmeric samples, which only provided 75 mg GAE/100 g dry weight (Lim and others 2011). Even commonly consumed cereals and breads such as Cinnamon Toast Crunch, Cinnamon Cheerios, and Cinnamon Swirl bread have detectable levels of antioxidants, in particular cinnamaldehyde (Friedman and others 2000).

Breads can also be enriched with antioxidants through the addition of other cereals or by processing ingredients of the existing flour. Mateo-Anson and others (2011), looked at fermentation of wheat bran in whole wheat bread as a way of increasing the antioxidant bioavailability in men, and found that the fermentation increased the blood concentrations of antioxidants by two-fold and decreased levels of inflammatory markers over the controls. Another study found that substituting buckwheat flour incorporated at 150 g buckwheat flout/ 500g total flour nearly tripled the antioxidant capacity and free-radical scavenging activity, but did not affect the consumer acceptance of the bread (Chlopicka and others 2012).

In the body, antioxidants from foods can help mitigate disease. For example, rosemary is a common spice used in Mediterranean cooking. One of the phenolic compounds in rosemary that has been researched for its health benefits is carnosol (Figure 8). Lo and others (2002), analyzed the antioxidant capacity of carnosol using a DPPH assay, and observed its effect in mouse white blood cells. They found that carnosol is capable of quenching 50% of DPPH radicals at concentrations near 0.59 μM. They also found that carnosol suppressed activation of NF-κβ in blood cells which, in turn, inhibited the formation of nitric oxide synthase, a protein that creates the free radical nitric oxide (NO) in the body. Their study suggested that carnosol in rosemary could prevent free radical damage and provide a protective effect in the body. These data were supported by that of Zeng, and others (2001) who showed that carnosol, as well as rosmanol and epirosmanol could inhibit oxidative damage to LDL cholesterol.

Two metabolites derived from rutin in fruits, 4-dihydroxyphenylacetic acid and 3,4-dihydroxytoluene, seen in Figure 9, inhibited CML formation in model systems of glyoxal and histone-1 by more than 90% at concentrations of 400 μmol/L (Pashikanti and others 2010). Catechin and epicatechin were also shown to inhibit CML formation (Peng and others 2010b). These data were supported by Wu and others (2009), who found that catechin, epigallocatechin gallate, rutin, luteolin and quercetin inhibited CML formation in cells, and reduced the levels of inflammatory markers that may promote disease. These data were supported by Urios and others (2007), who found rutin, catechin, and kaempferol inhibit the formation of AGE cross-link damage in collagen.

Studies have also looked at using herbs and spices for their anti-AGE properties. The tea polyphenol, epigallocatechin gallate (EGCG), was found to have a protective effect against AGEs by acting as an antioxidant, and through inhibiting the expression of RAGE in neuronal tissues (Lee and Lee 2007). Other studies suggest that herbs such as mint, rosemary, sage, verbena and lemongrass can be steeped in water like tea and ingested, and that these "herbal infusions" can help to provide antioxidants to prevent AGE formation (Ho and others 2010).

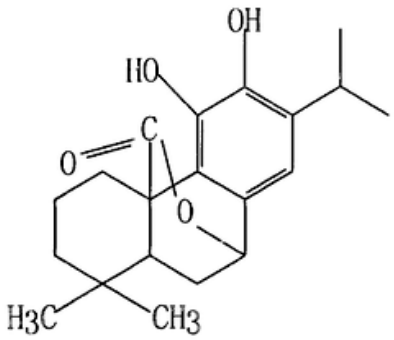

Figure 8: Carnosol, a phenolic found in rosemary adapted from Lo and others (2002)

Studies by Stirban and others (2006, 2007), used benfotiamine, a derivative of the B1 vitamin, as a dietary supplement and examined the effect produced on individuals with type 2 diabetes after eating meals containing higher levels of AGEs. Their studies found that use of this synthetic antioxidant helped to decrease the loss of adiponectin protein, used to regulate glucose levels, and reduce levels of endothelium damage from AGE induced oxidative stress. Aged garlic extract also could be used to inhibit AGE formation in the body, as it showed inhibition of CML as well as cross-linking activity during *in vitro* studies (Ahmad and others 2007). Alagebrium, an AGE cross-link breaker, has been shown to improve flow mediated dilation in men with high blood pressure when 210 mg were administered twice a day (Zieman and others 2007). Other studies found that aminoguanidine, seen in Figure 11, reduces the level of AGEs absorbed by the body by 50%, and increased clearance of AGEs from the kidneys liver (He and others 1999). Another study used lemon balm extracts and found that the antioxidant and metal chelating ability inhibited the formation of AGEs in albumin, and could possibly inhibit the activation of RAGE (Miroliaei and others 2011).

The animal studies on diabetic mice found that the Chinese prescription, Kangen-karyu, significantly reduced the levels of CML, CEL, and RAGE, and inflammatory markers in kidney and liver tissues when administered at 200 mg/kg body weight, per day. However, these markers were still higher than non-diabetic controls (Kim and others 2009; Okamoto and others 2011). Diabetic mice also showed decreased levels of AGEs, better clearance of AGEs, and decreased inflammatory markers when fed caffeic and ellagic acids, antioxidants found in fruits and vegetables (Chao and others 2010). Oleanic and ursolic acid extracts from hawthorn fruits have

also shown an ability to inhibit CML and free radical formation (Mei-Chin Yin and Kung-Chi Chan 2007). In model studies on rat collagen, 1m M of each diethylenetriaminepentaacetic and phytic acids were capable of inhibiting formation of AGE cross-links in the tissues through the inhibition of free radicals in the system (Fu and others 1994).

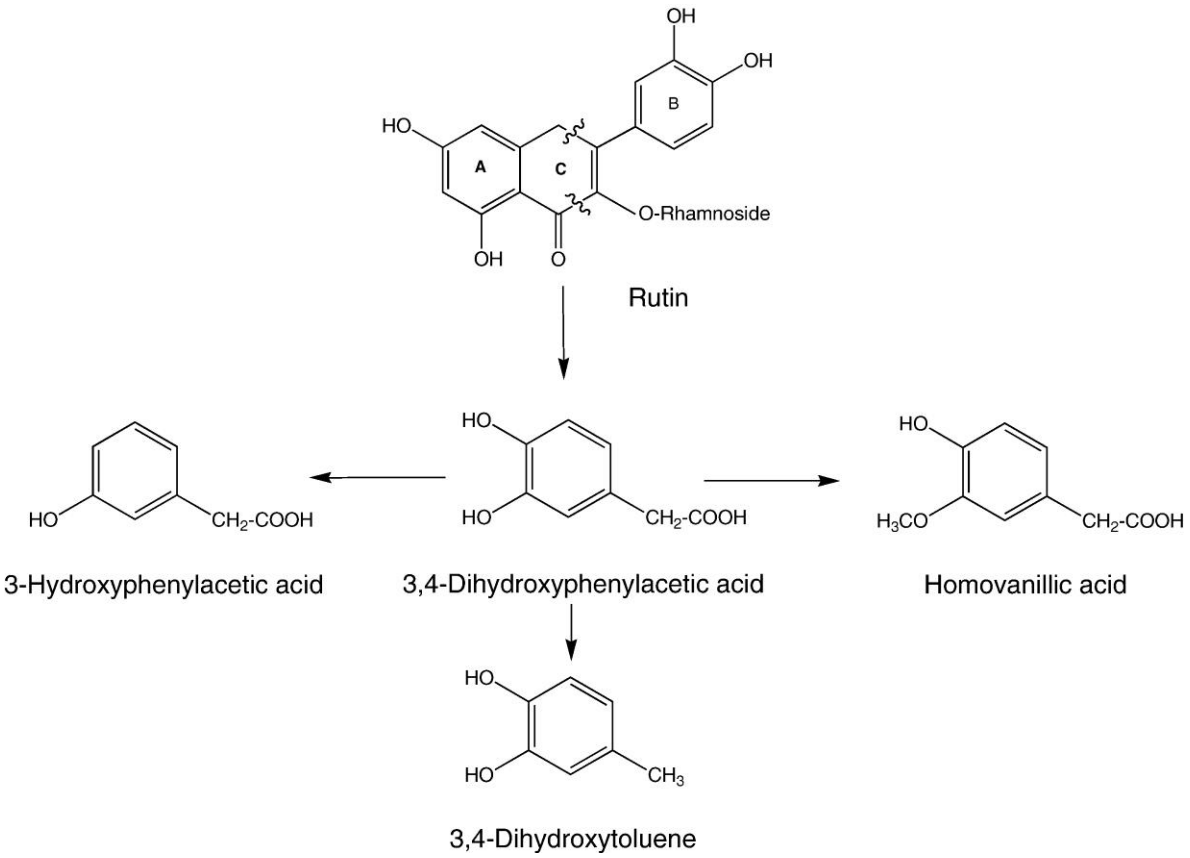

Figure 9: Rutin metabolites, 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxytoluene, used to inhibit AGE formation as adapted from Pashikanti and others (2010).

Ingestion of alcohol and fermentation byproducts may also help with controlling AGE levels in the body. Ingestion of ethanol may act as a late-stage AGE inhibitor by preventing glycated hemoglobin from cross-linking in people with diabetes, due to the breakdown of ethanol to acetaldehyde (Al-Abed and others 1999). A study by Ye and others (2010), on recycled deionized residues from alcohol distillation, and vinegars produced from those residues, showed the ability to reduce CML formation by 50%, though the active compounds were not characterized.

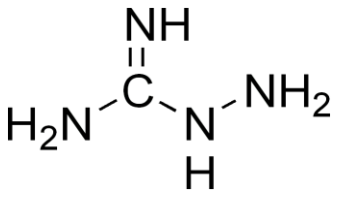

Figure 10 Structure of aminoguanidine as adapted from PubChem

CHAPTER 2- High Performance Liquid Chromatography of Syrups/Sauces and Solid Phase Extraction (SPE) of AGE's

Carboxymethyllysine (CML) is a product of Maillard browning, an Advanced Glycation Endproduct (AGE) (Xanthis and others 2007). AGEs, when ingested into the body react at Receptors for AGEs (RAGE), and exhibit pro-oxidant effect. These effects include exacerbation of diseases such as fibromyalgia, diabetes and Alzheimer's, as described above in Chapter 1.

Cooking, which does produce some of the desirable colors and flavors associated with certain foods, also produces CML in a food system (Goldberg and others 2004). CML must be liberated from the food matrix in order to be analyzed. Enzyme or acid digestion can hydrolyze peptide bonds in proteins to release free amino acids (Goldberg and others 2005; Hull and others 2012). Free amino acids can then be isolated through solid phase extraction (SPE). Solid phase extractions can be achieved through size exclusion, polarity, or through cationic/anionic exchange.

This chapter includes preliminary work and experiments prior to the main experiment in Chapter 3. The purpose of these efforts was to identify the most efficient method of CML analysis in samples. Initially, a high performance liquid chromatography method (HPLC) method was used for analysis, as established by previous work (Assar and others, 2009; Peng and others 2010). After the HPLC work, several solid phase extraction (SPE) experiments were conducted to establish, and improve, the recovery of CML from the food matrices. Different methods and SPE columns were used, including the prepacked Agilent PRS and C_{18} columns, and a hand packed lysozyme-linked cyanogen bromide activated Sepharose column.

High Performance Liquid Chromatography of Syrups, Sauces and Infant Formula

The purposes of this experiment was to establish an HPLC method for the determination of CML in foods, as well as to compare the method with samples that had established CML levels. In this case infant formula was used for comparison.

Materials and Methods

Sodium borate crystals, sodium borohydride powder, and sodium acetate powder were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide pellets, trace metal grade 37% hydrochloric acid, glacial acetic acid, HPLC grade acetonitrile, HPLC grade methanol, HPLC grade chloroform, orthofluoraldehyde (OPA), 0.45µm nylon syringe filters, 0.45µm filter discs were purchased from Fisher Scientific (Rockford, IL). Carboxymethyllysine powder was purchased from PolyPeptide Group (San Diego, CA). Kraft honey barbeque sauce, Koger brand honey, Maple Pure dark amber pure maple syrup, and Similac and Parent's Choice powdered infant formula were purchased from a local supermarket.

Prior to sample analysis, a standard curve was developed by derivitizing CML standards with OPA reagent, filtering them through a 0.45µm syringe filter, and injecting them directly into the HPLC using the HPLC method for samples as described below. Standards were prepared at 1.0, 0.75, 0.5, 0.195, 0.1, and 0.49 ppm concentrations, and the standard curve was evaluated by linear regression.

The method used to prepare the samples for HPLC analysis was adapted from Peng and others (2010a). First, 1g or 1 mL of sample was defatted by placing it in a test tube with 9 mL of 2:1 (v:v) chloroform methanol solution. The tube was capped and shaken, then centrifuging it for 20 minutes at 3,233x *g*. The liquid was then removed and the process repeated. After defatting, the sample was dried to complete dryness at 50 °C in an incubator. The reducing sugars in the dried samples were then converted to sugar alcohols by adding 4 mL of 0.2 M sodium borate buffer (pH= 9.4) and 2 mL of 1 M sodium borohydride ($NaBH_4$) dissolved in 0.1 M sodium hydroxide (NaOH) and reacting them for 4 hrs at room temperature. After the sugar reductions, the samples were digested by adding concentrated hydrochloric acid to each sample until the concentration was 6 M, then the sample headspace was flushed with nitrogen, and allowed to incubate at 110 °C for 20 hours. Digested, samples were dried by rotary evaporation, then re-dissolved in 10.0 mL of water and filtered through a 0.45µm nylon filter. Two mL of the filtered samples were again dried by rotary evaporation, and redissovled in 2 mL of 0.2 M borate buffer. Five minutes prior to HPLC analysis, 100 µL of sample were mixed with 200 µL of orthofluoraldehyde (OPA) derivitizing agent in a 1 mL vial and allowed to react.

For the HPLC analysis, 60 µL of the derivatized sample was injected into a 20 µL injection loop to ensure the proper volume was injected into the column. HPLC was performed on a HP Series II 1090 LC, using a reverse phase Biochi TOSOH ODS-80^{TM} column. The mobile phases were a 0.5 M sodium acetate buffer (pH=6.5) in 10% acetonitrile, and 100% acetonitrile, which were filtered with a 0.45µm filter to remove dust, and sparged with helium prior to use. The mobile phase gradient followed the following time program, as shown in Table 9, at 1 mL/min.

Table 9: Mobile phase gradient program used in HPLC analysis of syrups, sauces, and infant formula

Time (min)	% Acetonitrile	% Sodium Acetate Buffer
0.00	5.0	95.0
9.00	5.0	95.0
10.00	18.0	82.0
11.00	31.0	69.0
12.00	44.0	56.0
13.00	57.0	43.0
14.0	70.0	30.0
17.0	70.0	30.0
18.0	5.0	95.0

The column was allowed to equilibrate with the 5% acetonitrile: 95% acetate buffer for 10 minutes after each sample, with the maximum allowable pressure as 300 bar. Carboxymethyllysine-OPA derivatives were detected with a fluorescence detector using an excitation wavelength of 340nm and an emission wavelength of 455nm. Data analyses were performed on Chemstation software.

Results and Discussion

The standard curve for the CML can be seen in Figure 11. There is a high level of linear correlation between CML and the concentration.

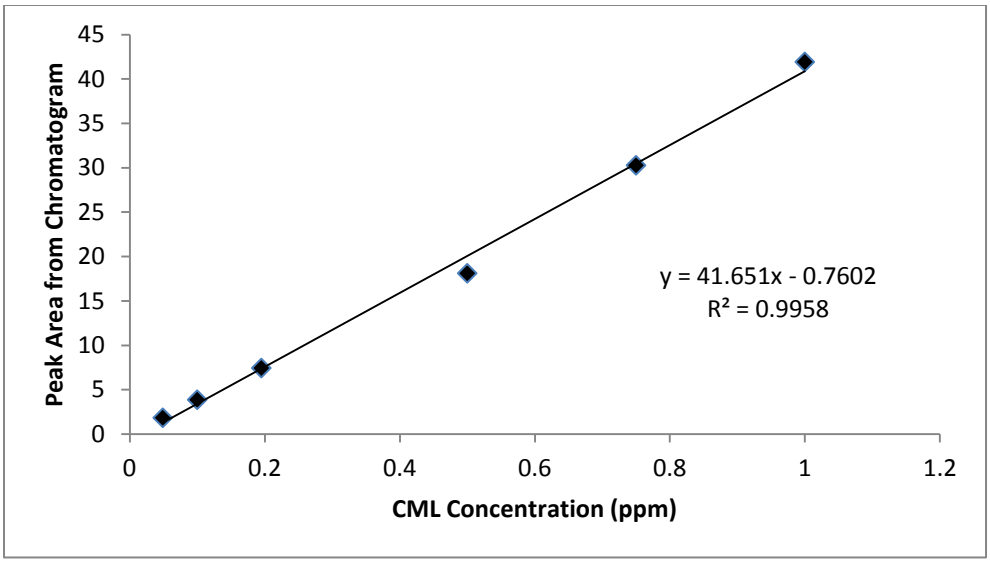

Figure 11: Peak Area vs. CML concentration standard curve. Equation on the graph is the formula for the regression trend line, and its corresponding R^2 correlation coefficient.

Due to the high concentrations of reducing sugars in the honey samples, 79.79 g/100 g (USDA NAL Nutrient Data Laboratory 2011), the $NaBH_4$ solutions reacted violently in most cases, resulting in massive sample loss due to foaming. Samples where barbeque sauce was analyzed congealed at the top of the analysis test tube during the addition of the defatting reagents, which resulted in significant mass loss. It is possible that this congealing is due to the guar gum thickeners present in the barbeque sauce.

Evaporation of water from a highly concentrated sugar solution results in a crust forming on the surface of the liquid, which prevents any further evaporation. Even with a rotary evaporation technique, which prevents this crust, a point is reached where no further water can be removed because of the water binding capacity of sugar. Sugar crusts and water binding from sugar concentrations were observed in both honey and syrup samples.

This analysis method was inadequate for samples with high levels of reduing sugars and liquid samples with thickeners. Foaming, mass loss, and crust formation will decrease the percent recovery of the sample and increase the variability of the data. Future work would need to develop a method that would improve the recovery without the use of extremely reactive borohydride, or extensive drying. These samples would ideally be analyzed directly by HPLC or through a simple solid phase extraction.

The Parent's Choice and Similac were the only two samples that allowed successful sample preparation. However, while these samples showed the presence of CML, the values were lower than the analytical software could detect to integrate. Because the fluorescence spectra could not be saved in the analysis software, samples could not be integrated manually. A sample chromatogram showing a Parent's Choice chromatogram compared to a 10 ppm CML standard is shown in Figure 12. Because these values were lower than the detection limit of the software, it is logical to assume that the average CML concentration was less that 0.049 ppm or 49 ng/mL. These values are significantly less than the 9-12 and 5-25 ng CML/mg protein reported by Charissou and others (2007), the 50 and 175 mg CML/kg protein reported by Hartkopf and others (1994), and the 514-11372 ng CML/mL infant formula reported by Dittrich and others (2006).

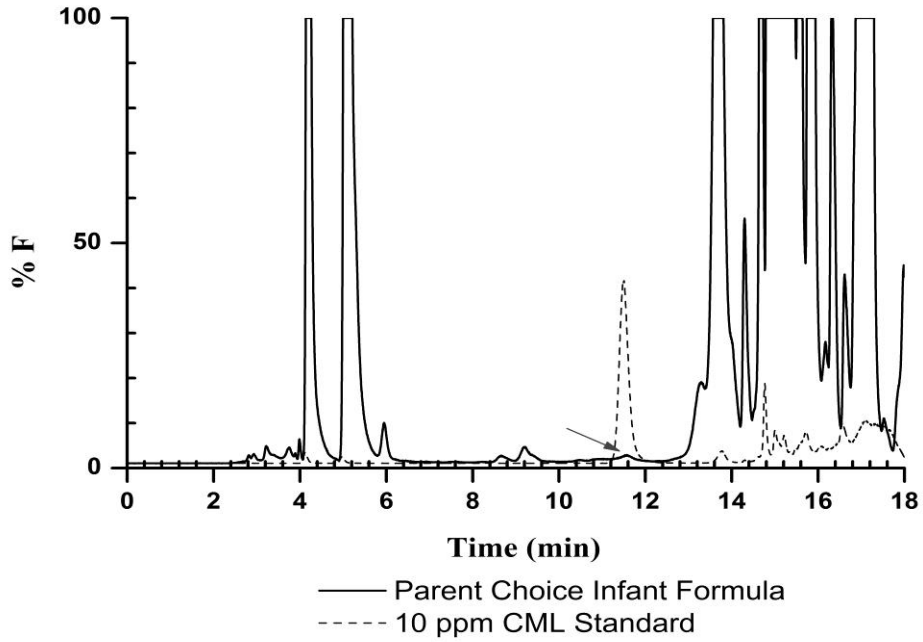

Figure 12: Sample chromatogram of a Parent Choice infant formula and a 10 ppm CML standard. The arrow indicates the CML peak.

Conclusions

From the data above, it was determined that samples which contained high levels of sugars, such as barbeque sauce, syrup, and honey, are not suitable for the method proposed by

Peng and others (2010), and should not be used with this method of analysis. The infant formula data also suggested that a method that was capable of reaching lower concentrations of CML may be needed determine the CML content in foods.

Solid Phase Extractions Using a Lysozyme-Linked Cyanogen-Bromide Activated Sepharose Column (Sepharose Column)

After analysis of infant formula, another method was adapted from Goldberg and others (2004), and Mitsuhashi and others (1997) to try to analyze the CML content in foods. In this method the solid phase extraction (SPE) column used a lysozyme-linked cyanogen bromide activated sepharose matrix. An experiment was conducted that both constructed the column, and tried to determine the percent recovery of CML during extraction.

Preparation of the Sepharose Column and Percent Recovery Procedure

The preparation procedure for the lysozyme-linked cyanogenbromide activated Sepharose 4B (Sepharose) solid phase extraction (SPE) column was adapted from several sources (Mitsuhashi and others 1997; GE Healthcare Bio-Sciences AB 2009; Sigma-Aldrich). First, 10 mg of lysozyme powder was dissolved in 0.1 M sodium hydrogen carbonate ($NaHCO_3$) and 0.5 M sodium chloride (NaCl) solution (coupling buffer). Next, 1 g of cyanogen bromide activated sepharose 4B beads were rehydrated in 30 mL of 1 mM hydrochloric acid (HCl) over a sintered glass filter, then rinsed with 200 mL of 1 mM HCl followed by 25 mL of deionized water and 25 mL of coupling buffer. The clean, reconstituted resin was then added to the flask with the dissolved lysozyme and allowed to react for 2 hours with mixing using a wrist-action shaker. After coupling the lysozyme to the Sepharose column, the mixture was transferred back to the sintered glass filter and rinsed with 25 mL of coupling buffer. To block un-reacted sites on the sepharose column, the resin was then transferred to a 0.2 M glycine solution at pH 8, and allowed to react for 2 hrs. The final resin was then transferred again to the sintered glass filter and rinsed with 25 mL of coupling buffer, then 25 mL of a 0.1 M sodium acetate (NaAc) buffer at pH 4.0 containing 0.5 M NaCl (acetate buffer). The coupling buffer/acetate buffer rinse cycle was repeated a total of five times.

A slurry was made with completed resin and coupling buffer, and degassed with nitrogen. The slurry was then poured into a 10 mL SPE column with a glass wool plug, previously rinsed

with coupling buffer, to prevent loss of the resin during extraction. A vacuum was applied and the column was rinsed with coupling buffer until the bed volume had reached a steady state value. Finally, the column was equilibrated with 1.0 M NaCl in 80:20 water: ethanol, which served as a storage solution. The column was stored at 4 °C in 2 inches of storage solution when not in use.

To test the binding capacity of the lysozyme SPE column, the lysozyme column was placed on a vacuum manifold box, and vacuum was applied such that the flow rate through the column was roughly 1 drop/sec, with the liquid level never dropping below the top of the sepharose matrix. Next, the column was equilibrated with 30 mL of coupling buffer. Then, 5 mL of 100 ppm or 2 ppm, 1 ppm, 100 ppb, or 10 ppb CML standard were loaded onto the column, and washed with 15 mL of coupling buffer. The bound CML was then eluted from the column with 15 mL of 0.1 M NaOH, immediately neutralized HCl, then collected and saved for analysis. The column was then regenerated by washing the column with 10 mL of coupling buffer, and 10 mL of acetate buffer three times.

The collected samples were diluted in 0.01 M phosphate buffer, pH=7.4 (1XPBS) in the following way: 1/10,000 for the 100 ppm samples, 1/1000 for the 2 ppm and 1 ppm samples, 1/100 for the 100 ppb samples, and 1/10 for the 10 ppb CML samples. Diluted samples were then analyzed using an Oxi-Select CML ELISA by Cell Biolabs using the manufacturer's instructions.

According to the instructions, 100 µL aliquots were transferred to their corresponding well on the ELISA microwell plate. These were then covered and allowed to incubate overnight at 4 °C. The next day, the microwell plate was inverted and shaken to remove the samples, tapped on an absorbent towel to remove excess liquid, and 200 µL of the provided assay diluent were added to each well and incubated for 2 hours on an orbital shaker, at 500 rpm. During this incubation, a 1X wash buffer (1XWash) was made by adding 20 mL of the provided 10X wash buffer to a 200 mL volumetric flask, diluting to the 200 mL mark with deionized water, and inverting several times to achieve homogeneity. Also during this incubation, the 1000X secondary anti-CML antibody provided by the kit was diluted to 1X by adding 7 µL of the 1000X antibody to 6993 µL of assay diluent in a silanized vial, as per the instructions. Two of

these 1X- secondary antibodies were made to ensure that enough liquid was available for each microwell.

After the 2hr assay diluent incubation, the microwell plate was inverted over a sink, shaken to remove the bulk of the liquid, and dried by tapping the plate on an absorbent towel. Next each well was washed wit 250 µL of 1XWash buffer, then inverted, shaken and dried by tapping on a towel. The wash process was repeated a total of three times, then 100 µL of the 1X-secondary antibodies were added to each well, and the plate incubated for one hour on an orbital shaker at 500rpm.

When the 1hr secondary antibody incubation was complete, the wash process was completed 3 times, then 100 µL of the conjugated antibodies were added to each well, and the plate was again incubated for 1 hr on the orbital shaker at 500rpm. During this incubation, the provided substrate stop solutions were removed from storage at 4 °C and allowed to warm to room temperature.

After the last 1 hr incubation was complete, the plate was washed a total of five times with the 1XWash buffer, and 100 µL of the substrate solution was added to each well, and allowed to incubate for 20 minutes at 500rpm on the orbital shaker, to develop the color. Then, without removing any of the substrate solution, 100 µL of the stop solution was added to each well to arrest color development. The absorbance of each well on the plate was then immediately read on a Biotek microplate reader and recorded for later analysis on data collection software. The absorbance was measured at 450nm, with a reference absorbance taken at 900nm. These two absorbance values were subtracted to determine the corrected absorbance, which was used in later analysis, and will herewith be referred to "absorbance."

Each plate was read in triplicate on the Biotek microplate reader, and the average absorbance was used to calculate the levels of CML in the vial. A standard curve was constructed by making CML-BSA standards with the materials and instructions included with the kit. Briefly, a 2.2 ppb CML solution was made by diluting a provided CML-bovine serum albumin (BSA) stock solution, then serially diluting the 2.2 ppb solution 1:1 with reduced BSA solution seven times, until a 0.035 ppb solution is achieved. A standard with just reduced BSA was used as a "0 ppb" blank solution. These standards were interspersed with the samples on the

ELISA plates as the data were collected, and the standard curve constructed using a quadratic regression.

Results of the Sepharose Column and ELISA Analysis

The results from the standard curve using the ELISA assay can be seen below. The curve shows good correlation (R^2=0.9984) over the concentration range of 0 ppb CML- 2.2 ppb CML, as seen in Figure 13.

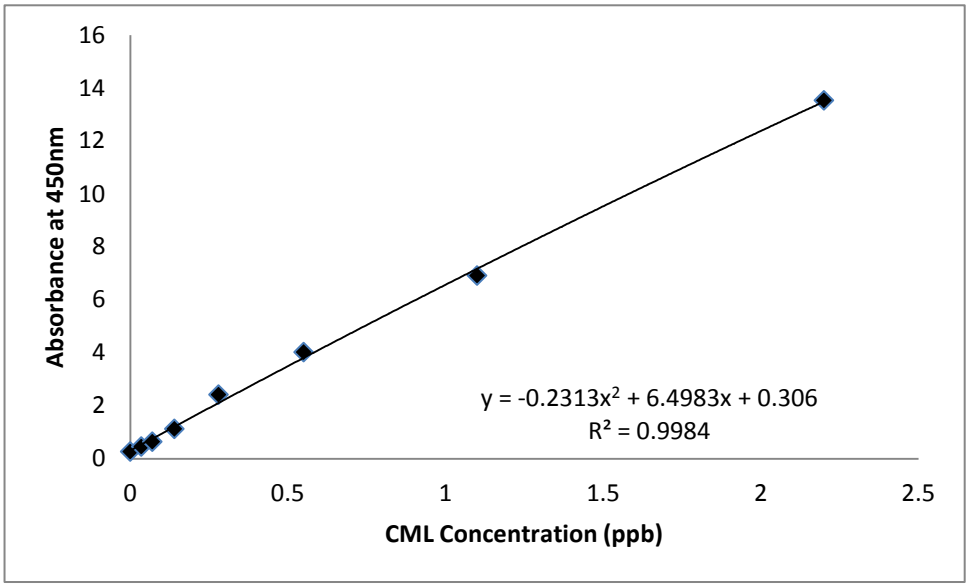

Figure 13: Absorbance versus CML concentration standard curve used during ELISA analysis of the percent recovery of the sepharose column

The results from the sepharose SPEs suggest that recovery on the Sepharose column is best when the concentration of the samples is 100 ppb CML or less, as seen in Table 10. This makes sense, because very high concentrations could saturate the available binding cites for CML on the sepharose matrix, while still having large amounts of CML in solution. The recovery improved, but was still less than 60% in samples that had concentrations of 100 ppm or less. Furthermore, there was a wide variation in the recovery between samples at any concentration, as seen in the percent recovery values in Table 10. The high variation suggests that the lysozyme matrix may have been inadequately regenerated, or that the flow rate during the load, wash, or elution steps varied from sample to sample. Both of these are possible, due to human error when using the vacuum manifold apparatus. Seals in the vacuum manifold are adjusted manually to keep a 1 drop/sec flow rate, and different concentrations of salts, molecular

size, and viscosities of the coupling buffer, acetate buffer, and the CML standards can cause variation of the flow rate. To minimize this variation, counter of flow rate meter could be used to help accurately measure the flow rate, to help stabilize the flow through the column.

Table 10: Summary of percent recovery data from the sepharose column

Sample	CML ppb in well	CML ppb in sample	CML (ng) recovered from column	CML (ng) loaded onto column	% Recovery	Average % Recovery
2 ppm CML Standard -#1	0.131	131	1960	10,000	19.6	7.26
2 ppm CML Standard -#2	0.043	42.7	640	10,000	6.40	
2 ppm CML Standard -#3	0.052	54.4	786	10,000	7.86	
100 ppm CML Standard-#1	0.033	3270	49,000	500,000	9.80	18.2
100 ppm CML Standard-#2	0.117	11700	176,000	500,000	35.2	
100 ppm CML Standard-#3	0.032	3160	47,400	500,000	9.47	
1 ppm CML Standard-#1	0.097	96.5	1450	5,000	29.0	37.9
1 ppm CML Standard-#2	0.156	156	2340	5,000	46.9	
100 ppb CML Standard-#1	0.214	21.4	321	500	64.1	57.3
100 ppb CML Standard-#2	0.168	16.8	252	500	50.4	
10 ppb CML Standard- #1	0.098	0.98	14.7	50	29.4	42.8
10 ppb CML Standard- #2	0.187	1.87	28.1	50	56.2	

During the recovery experiments, the Sepharose column began to show visible signs of degradation, which included shrinking bed volume, a slight increase in the transparency of the matrix, and "fouling" of the resin to the headspace above the bulk of the SPE matrix. The fouling and the decrease in bed volume are most likely linked; although some cloudiness was observed in the samples after extraction. Cloudiness in the extraction may result in the sepharose matrix leaking through the glass wool plug at the bottom of the column. The slight increase in transparency of the matrix could be from the loss of resin, or from the decoupling of the lysozyme from the matrix.

Conclusions - Sepharose Column

The recoveries from the columns varied too widely, and were too low even with a pure CML standard. Also, the column showed visible signs of degradation during the course of the experiment. This suggests that the column would have to be repacked roughly every 20 extractions, which is unacceptable in terms of time and cost. Every time the column would be re-packed, the percent recovery from the new column would have to be determined before any food samples could be analyzed. Furthermore, because the columns are hand packed one at a time, samples would also have to be extracted one at a time, slowing down the analysis process. From the recovery experiments using the Sepharose column, it can be concluded that this column was not suitable for the classification of CML in food stuffs.

Solid Phase Extractions using PRS Cartridges in acidic and basic solutions

The purpose of this experiment was twofold. One goal was to determine if a more accurate percent recovery was possible using the Bond Elut PRS SPE column. The other goal was to determine if eluting under acidic or basic conditions from the PRS column resulted in higher recoveries. The PRS SPE column is a sample preparation column produced by Agilent Technologies (Agilent Technologies 2010). The Bond Elut PRS column is a silica based cation exchange column with the structure depicted in Figure 15.

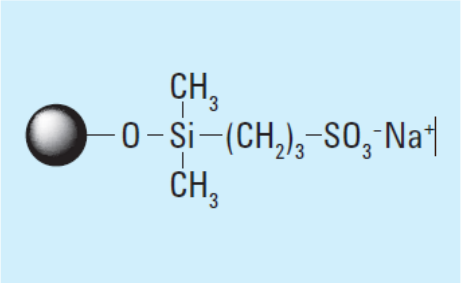

Figure 14: Structure of PRS resin as adapted from Agilent Technologies (2010)

Because of the ethyl group prior to the zwitterion, it can function in non-polar solvents, and is capable of extracting weak cationic species (Agilent Technologies 2010).

Carboxymethyllysine (CML) has two carboxyl groups and two amino groups, and is a pH sensitive compound. Under acidic conditions (pH >4.5), the amine groups are protonated, which should allow them to interact and displace sodium ion on the exchange resin. Previous work

suggested that CML is capable of binding to the PRS column, with a recovery higher than that of the lysozyme-linked cyanogens bromide-activated Sepharose 4B resin used in other studies (Mitsuhashi and others 1997). However, the data from these previous studies showed wide variability between the samples. Therefore, one of the major goals of this study was to retest the PRS column to determine if sample recovery is reproducible.

Agilent Technologies suggests elution of adsorbed compounds using a basic medium. Raising the pH of the column to pH 10 or more would deprotonate the carboxyl and amino groups on CML, allowing CML to be washed from the column. However, silica dissolves at pH's greater than 8, and a vacuum pump must be used to decrease the contact time of the silica with the basic media (Wang and Dunn 2012). Other methods have been proposed to elute from this type of cation exchange column by using a strongly acidic solution (Klejdus and others 2006). This elution mechanism would wash CML from the column by creating direct competition for the active sites with the protons from the elution media. However, the effectiveness of each method is unknown. Therefore, the secondary goal of this experiment was to use both the acidic and basic elution methods to determine which yields a higher percent recovery.

Materials and Methods

To perform the SPE's using the PRS column, the method developed by Klejdus and others (2006) was used, with some modifications. The PRS column was conditioned, under vacuum, with 5 mL ethyl acetate (EtAc), 5 mL methanol, and 5 mL of deionized water, in that order, at a flow rate less than 1 drop per second. A 100 ppb CML standard was prepared in a 1:1:3 (v:v:v) of methanol: acetonitrile:0.2% m-phosphoric acid in water. A 5 mL sample of this standard was further acidified with 33 µL of 0.1N HCl, and loaded onto the PRS column. After loading, the column was washed with 2 mL of methanol. Then the standard was eluted with 2 mL of either 1:3 5 M HCl: methanol, or 2 mL of 1 M NH_4OH. After extraction the samples were dried under a stream of nitrogen to complete dryness, then dissolved in 50 mL of 0.01 M phosphate buffer. After dissolving the sample, 1 mL was diluted to 10 mL with phosphate buffer. An OxiSelect^{TM} N^{ε}-(carboxymethyl) lysine ELISA kit from Cell Biolabs, Inc. (San Diego, CA) (San Diego, CA) was used to determine the amount of CML recovered from the column, using the assay protocol provided by the manufacturer, except the wash cycles were

increased to 6 times to reduce the background noise of the samples. The absorbance signals were recorded using data collection software and a Biotek microplate reader.

The mass loaded onto the column was determined by diluting 5 mL of the 100 ppb standard to 50 mL and then serially diluting 1 mL to 10 mL to reduce the concentration of the standard to within the range of the standard curve. The mass recovered was determined by converting the signal recorded on by the data collection software to a concentration by solving the quadratic equation for the standard curve, determined in previous work. The resulting concentrations were converted to mass by multiplying by 10 (to convert the 10 mL dilution to a ng mass), then multiplying by 50 to account for the mass of the entire reconstituted sample. The percent recovery was then determined using the following equation:

$$\%\ \boldsymbol{Recovery} = \mathbf{100} * \frac{\boldsymbol{mass\ CML\ recovered}}{\boldsymbol{mass\ of\ CML\ loaded\ on\ PRS\ column}} \qquad \textbf{Equation 1}$$

An average percent recovery and standard deviation for the percent recovery was determined for the acidic and basic elutions. A Student's T-test assuming unequal variances was conducted to determine if a significant difference existed between the two elution methods ($P < 0.05$).

Results and Discussion

During the drying process it was noted that the samples eluted with the acidic solution developed crystalline structures on the sides and bottom of the flask, as contrasted with the basic eluted samples. A summary of the data calculations is presented in the Table 11. From the table, the inconsistency in the data are clear. The theoretical mass loaded onto the column was 500 ng of CML. In comparison, the collected data suggests that only 57.5 ng of CML were loaded onto the column. However, the acid and base elutions show a recovered mass higher than the 100ppb standard, and therefore a recovery higher than 100%.

If the 57.48 ng CML mass recovered from the standard is discarded, and the theoretical mass of 500 ng is used in its place, the percent recoveries are drastically different, as seen in Table 12 . In this instance two outliers can be seen; the Acid 3 and Base 1 trials. They were determined to be outliers because their respective recoveries are more than double that of the other trials in their trial subset. It is also important to note that none of these trials are above 35% recovery, suggested as possible from previous work.

Table 11: Mass recovered and percent recovery of the acidic and basic elutions, using the calculated mass loaded from the 100 ppb standard.

Sample	Mass (ng) recovered from the column	Percent Recovery	Average % Recovery	Standard Deviation of % Recovery
Acid 1	33.16	57.70	142.56	102.53
Acid 2	65.24	113.50		
Acid 3	147.43	256.49		
Base 1	172.31	299.77	175.02	83.57
Base 3	81.03	140.96		
Base 2	70.22	122.15		
Base 4	78.85	137.17		
100 ppb standard	57.48	100.00		

When the outliers are discarded and the data recalculated, the data improve in average and standard deviation, as seen in Table 13. It is also of note to see that the acidic elution trials vary more widely than did the basic elution trials. This could possibly be due to the difference in the elution mechanism of the sample. Because the acidic elution works on a competitive basis, the data suggests that the CML bonded to the column out competed the protons from the elution acid, possibly through stearic hindrance, and is may not work as well as a basic elution medium. The trials that used a basic elution medium, only recovered between 14-16% of CML in the column. This could be because of incomplete deprotonation of the CML on the column, or that binding of CML to the column is genuinely 15%.

Table 12: Mass recovered and percent recovery of the acidic and basic elution techniques using the theoretical 500ng mass loaded onto the column.

Sample	Mass recovered from the column	% Recovery	Average % Recovery	Standard Deviation of % Recovery
Acid 1	33.16	6.63	16.39	11.79
Acid 2	65.24	13.05		
Acid 3	147.43	29.49		
Base 1	172.31	34.46	20.12	9.61
Base 3	81.03	16.21		
Base 2	70.22	14.04		
Base 4	78.85	15.77		
100 ppb standard	500.00	100.00		

The t-Test comparison of the acid and base trials showed no significant difference for any of the data sets used in calculation, suggesting that acidic and basic elution from the column should be the same. However, considering the wide variation among the acidic samples within the data set, it suggests that the basic elution media is more consistent, even with a low recovery, and is preferable to the acidic elution media.

Table 13: Mass recovered and percent recovery of acidic and basic elutions, excluding outliers and using the theoretical 500ng mass loaded onto the PRS column.

Sample	Mass recovered from the column	Percent Recovery	Average % Recovery	Standard Deviation of % Recovery
Acid 1	33.16	6.63	9.84	4.54
Acid 2	65.24	13.05		
Base 3	81.03	16.21	15.34	1.14
Base 2	70.22	14.04		
Base 4	78.85	15.77		
100 ppb standard	500.00	100.00		

Conclusions

From these data, it can be suggested that the percent recovery of CML from the PRS is 13.14% ± 3.86%, assuming that the mass loaded onto the PRS column was near the 500ng theoretical mass. The data also suggests that a basic elution media is preferable over an acidic elution media because the data produced are more precise. Future work would include adjusting the PRS procedure to increase the percent recovery, checking calibrations on equipment to reduce any instrumental error, and exploring alternative SPE columns.

Mass Loss of CML in the Load Wash and Elution Steps of SPE using PRS Extractions Columns

The PRS column is a cation exchange SPE column manufactured by Agilent Technologies. The Bond Elut PRS column is silica based resin with the following structure, as seen in Figure 14 above. Because of the ethyl group prior to the zwitterion, it can function in non-polar solvent, and is capable of extraction weak cationic species (Agilent Technologies 2010).

Carboxymethyllysine (CML) has two carboxyl groups and two amino groups, and is a pH sensitive compound. Under acidic conditions (pH <1.5), the amine groups are protonated, and displace sodium ion on the exchange resin. Previous work suggested that CML is capable of binding to the PRS column, but the percent recovery of CML is relatively low. Because of these low recoveries, it was necessary to test the load, wash, and elution fractions as they are passed through the column to determine where mass is lost through the SPE process.

Materials and Methods

After communications with Agilent Technologies, the procedure in the previous experiment (elutions under acidic and basic conditions) was modified to make the load and elute pH more extreme. Instead of preparing the 100 ppb standards in a 1:1:3 solution of methanol (MeOH): acetonitrile (AcN); 0.2% meta-H_3PO_4 solution, the standards were prepared in a 1:1:3 MeOH: AcN: 3.75% meta-H_3PO_4 solution, so that the final pH was 1.5. After samples were prepared, a vacuum was applied to the PRS column, and the column was conditioned with 5 mL ethyl acetate (EtAc), 5 mL MeOH, and 5 mL H_2O. Before the liquid level dropped below the top of the exchange resin, 5 mL of the 100 ppb standards were applied to the column. The fluid collected from the 5 mL loaded onto the column was collected and saved for analysis. After the standards were loaded onto the column, and the load fractions collected the columns were allowed to dry under vacuum conditions for 5minutes prior to washing. Columns were washed with 2 mL of MeOH, and the wash fractions were collected and saved for analysis. After washing the columns were again allowed to dry for 5 minutes under vacuum, as per the advice of the Agilent Technologies technical support‘s instructions. The standards were eluted with 2 mL of 29.47% (m/m) ammonium hydroxide, pH 12.8, and the fractions collected and saved for analysis.

All of the collected fractions that were saved for analysis were dried under a stream of nitrogen or under vacuum to dryness, then redissovled in 50 mL of 0.01 M phosphate buffer, pH 7.4 (1XPBS). Then, 1 mL of each fraction was diluted to 10 mL in 1XPBS. These samples were then analyzed for CML content using the Oxi-Select CML ELISA kit, according to the manufacturer’s instructions. The extractions were performed in quadruplicate. In addition 2 mL of the 100 ppb standard were diluted to 50 mL in 1XPBS, diluted again 1/10 in 1XPBS, and analyzed in quadruplicate with the extracted samples. The data from the ELISA kit were

recorded using data collection software and a Biotek 96-well microplate reader. A schematic of the procedure is presented in Figure 15.

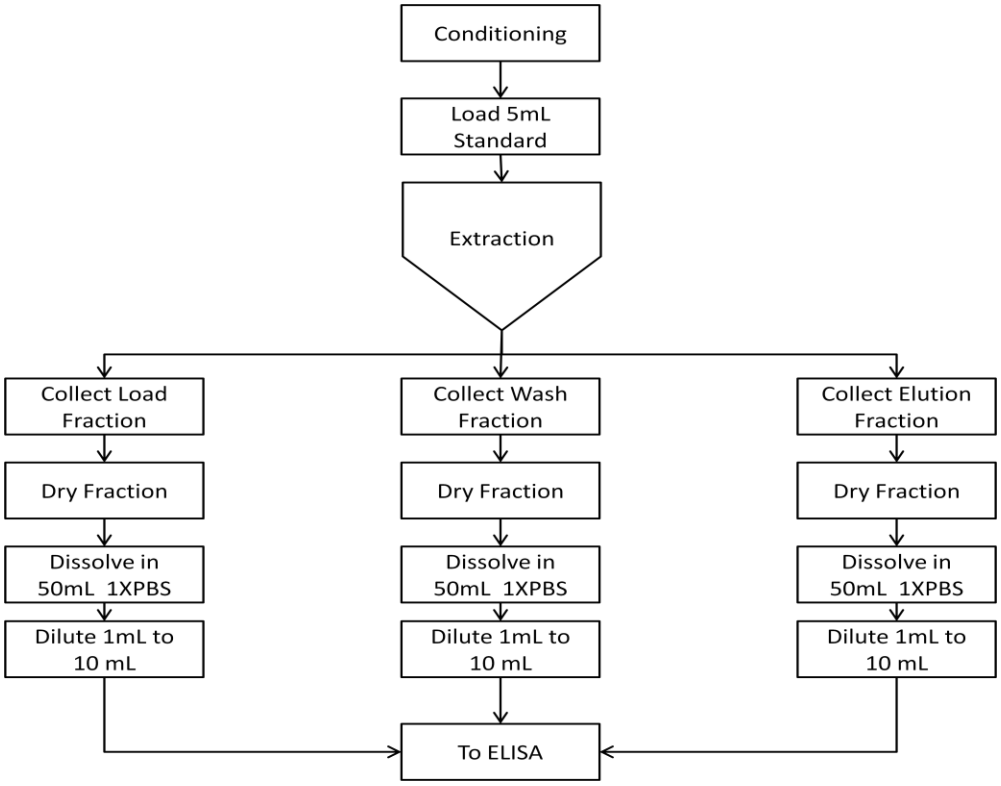

Figure 15: Schematic of SPE Extraction Process

Once the data were collected from the data collection software, the output was exported to Microsoft Excel. In Excel, the data were transformed to concentration in parts per billion (ppb, a.k.a ng/mL) by solving the quadratic equation from the standard curve, as established in previous experiments. The ppb concentration as then transformed to mass by multiplying the 10 mL that were in the analyzed 10 mL. This was then multiplied by 50, to account for the fact that only 1 mL of the original 50 mL of redissolved sample was diluted. An example calculation is presented in APPENDIX A- Sample Calculations attached at the end of the document. This was repeated for every fraction and every replicate, save the 100 ppb standard. An additional correction factor of 5/2 was included to account for the fact that only 2 mL of standard were diluted and tested on the ELISA, while 5 mL were loaded onto the column during the extraction process. The average and standard deviation of each fraction—load, wash, elute—was

determined, as well as the average and standard deviation of the 100 ppb standard. Furthermore, the total mass out of the column was determined by summing the average masses for the load, was and elute fractions. The total mass out was calculated to determine the mass balance of the column. Statistical analyses were performed on the average mass of the 100 ppb standard, and the total mass recovered from the extraction fraction to determine if the mass into and out of the column were statistically different (α=0.05).

Data and Results:

The data, similar to the previous experiment, showed some inconsistencies in the mass balance. As seen in Table 14, the average mass of CML loaded onto the column is only 137.25 ng, while the mass recovered is 215.73 ng CML. This suggests that CML was created in the column, because the two values are statistically different ($p<0.05$).

Table 14: Data and calculations table for mass of load (L), wash (W) and elution (E) fractions recovered from the PRS column, as well as the 100 ppb standard (std)

Sample	Signal	Concentration (ppb)	Ng in 10 mL Dilution	Ng recovered at each step	% Recovery	Avg. ng recovered in each step	Std Dev	Sum of L, W, E Steps
E1	0.913	0.0937	0.937	46.86	34.14	39.84	7.42	215.73
E2	0.784	0.0738	0.738	36.88	26.87			
E3	0.653	0.0535	0.535	26.75	19.49			
E4	0.939	0.0978	0.978	48.88	35.61			
L1	1.54	0.1912	1.912	95.60	69.65	105.62	42.89	
L2	0.889	0.0900	0.900	45.00	32.79			
L3	1.539	0.1910	1.910	95.52	69.60			
L4	2.696	0.3727	3.727	186.37	135.79			
W1	2.019	0.2661	2.661	133.06	96.95	70.27	32.59	
W2	0.699	0.0606	0.606	30.30	22.08			
W3	1.197	0.1378	1.378	68.89	50.20			
W4	0.938	0.0976	0.976	48.80	35.55			
std1	0.909	0.0931	0.931	116.38	84.79	137.25	32.30	
std2	1.325	0.1577	1.577	197.12	143.62			
std3	1.038	0.1131	1.131	141.38	103.01			
std4	0.794	0.0753	0.753	94.12	68.58			

However, L4 and W1 are outliers. Removal of these data points decreases the standard deviation of the load fractions by 9.37, and decreases the wash fraction standard deviation by

11.26, as shown in Table 15 below. After removal of the outliers, the 137.25 ng CML in the 100 ppb standard and the 167.88 ng CML recovered in the L, W, and E steps are no longer statistically different (p= 0.15577). However, the 137.25 ng CML is significantly less than the theoretical 500ng CML that should have been loaded into the column.

More importantly, of the 167.88ng CML that was recovered from the Bond Elut PRS column, 78.71 ng (46.88%) of that mass was recovered in the load fraction, and 39.84ng (29.38%) was recovered in the wash step, as seen in Table 15. This clearly indicates that CML does not, or only very weakly, associates with the PRS silica resin.

Table 15: Data and calculations table for mass of load (L), wash (W) and elution (E) fractions recovered from the PRS column, as well as the 100 ppb standard (std), with the outliers removed.

Sample	Signal	Concentration (ppb)	Ng in 10 mL Dilution	Ng recovered at each step	% Recovery	Avg. ng recovered in each step	Std Dev	Sum of L, W, E Steps
E1	0.913	0.0937	0.937	46.861	34.14	39.84	7.42	167.879
E2	0.784	0.0738	0.738	36.876	26.87			
E3	0.653	0.0535	0.535	26.750	19.49			
E4	0.939	0.0978	0.978	48.875	35.61			
L1	1.54	0.1912	1.912	95.599	69.65	78.71	33.52	
L2	0.889	0.0900	0.900	45.002	32.79			
L3	1.539	0.1910	1.910	95.520	69.60			
W2	0.699	0.0606	0.606	30.304	22.08	49.33	21.33	
W3	1.197	0.1378	1.378	68.894	50.20			
W4	0.938	0.0976	0.976	48.798	35.55			
std1	0.909	0.0931	0.931	116.378	84.79	137.25	32.30	
std2	1.325	0.1577	1.577	197.119	143.62			
std3	1.038	0.1131	1.131	141.375	103.01			
std4	0.794	0.0753	0.753	94.123	68.58			

Discussion Load, Wash, and Elute Fraction of Analysis of PRS SPE

Separation and recovery of CML from a food matrix is very important for the accurate quantification of CML. In previous experiments, the PRS Bond Elut column by Agilent Technologies showed promise in providing a high percent recovery of CML. However, with only a 23.7% recovery, the current data show that either the procedure or the exchange resin is

inadequate for retention and separation of CML. Also, there is over 300ng of difference between the actual and theoretical values of CML in the 100 ppb standard. This was attributed to using unsilanized glassware. Because of the polar nature of CML, it is probable that CML adsorbed to the surface where hydroxyl groups are present, and was thereby removed from the sample prior to separation.

For future work, it is suggested that only silanized glassware be used when handing the CML samples. The purpose of this is to remove interference of the glassware, particularly since the concentrations of CML in these samples is very low, and a large fraction of the mass could be lost. Also, because the recovery on the PRS column continues to be very low, changing the procedure by acidifying the water and methanol used in column conditioning might be recommended, as per the instruction of the Agilent Technologies Technical Support Staff. Also, different column could be used to to perform the SPE's of CML from a food matrix. One such alternate column is the C-18 cartridges used by Hull and others (2012), in the protein hydrolysate clean up section of their paper.

Solid Phase Extractions using C-18 Cartridges

Solid Phase Extraction (SPE) is one way of removing impurities from samples. Many SPE cartridges exist on the market that have a wide variety analyte specificities and can separate a wide variety of compounds. For carboxymethyllysine (CML), extraction has been done using several different SPE cartridges, including a Lysozyme-linked Cyanogen Bromide Activated Sepharose Gel column (Sepharose column), and the PRS column produced by Agilent technologies (Goldberg and others 2005; Agilent Technologies 2010).

However, another column has also been used—the C_{18} cartridge . Assar and others (2009), used a C_{18} cartridge to clean up protein hydrolysates before analysis of CML. In the experiment, we tried to replicate their procedure, and determine the percent recovery of CML on the C_{18} cartridge.

Materials and Methods

Chemicals

HPLC grade methanol (MeOH) and acetonitrile (AcN) were obtained from Fischer Scientific. Trifluoroacetic acid (TFA), phosphate buffered saline single use packets (pH 7.4, 0.01 M in 1L), and the Oxi-Select CML ELISA kit were obtained from Cell Biolabs (San Diego, CA). SPE cartridges (3 mL C_{18} Varian Bond Elut) were obtained from Agilent Technologies (Santa Clara, CA). Carboxymethyllysine (CML) was purchased from PolyPeptide Group (San Diego, CA).

Methods

One thousand µg/mL (ppm) CML stock solution was prepared by dissolving 50 mg of CML in 50 mL of 25:75 (v:v) MeOH:H_2O. This stock solution was separated into 5 screw-top vials, and stored at -20 °C until just prior to use. CML standards, 100 ng/mL (ppb), were prepared by serially diluting the 1000 ppm stock solution 1/10 four times with 1% (v/v) TFA in H_2O in silanized screw-topped vials. A 1% (v/v) TFA was prepared by diluting 250 µL of TFA to 25 mL with deionized H_2O. A 20:80 MeOH: H_2O (v:v) with 1% TFA was prepared by mixing 5 mL of MeOH with 20 mL of H_2O, then removing 250 µL of the total volume and adding 250 µL of TFA in its place. 0.01 M phostphate buffered saline (1XPBS), pH 7.4 was prepared by dissolving one packet in 1L of deionized water

C_{18} cartridges were attached to a vacuum manifold and vacuum was applied to 8mmHg. Cartridges were conditioned with 3 mL of MeOH then 3 mL of 1%TFA, at a flow rate of < 1 drop / second. Next 1 mL of the 100 ppb standard was loaded onto each column and the load fraction was collected in a 5 mL silanized vial with a Teflon seal. After loading fractions were collected, the columns were allowed to dry for 5 minutes. The standards were then eluted from the column with 3 mL of 20:80 MeOH: water with 1%TFA, and the elution fraction was collected in a 5 mL silanized screw top vial with a Teflon seal.

After all the fractions were collected, the samples were dried under vacuum at room temperature to dryness, and stored at 4 °C until sampling. In addition, two 1 mL samples of the 100 ppb standard were also dried under vacuum to remove the TFA. In one of the standard samples, some splashing did occur, and a small amount of mass was lost. However, once all of

the fractions and standard were dried, the samples were reconstituted in 1 mL of 1XPBS, and serially diluted 1/10 two times, or 1/400 in 1XPBS in 2 mL silanized screw-top vials with a Teflon seal, to make the CML concentration within the detection range of the ELISA kit. The samples were then analyzed on the ELISA kit according to the manufacturer's instructions. Absorbance data were collected using a Biotek microplate reader and data collection software. Once the data were recorded with the data collection software, they were exported to Microsoft Excel for further data analysis.

Collected absorbencies were transformed into ppb concentrations by solving the quadratic standard curve equation that was generated in previous work, as seen in Equation 2.

$$\frac{(-\boldsymbol{b})+\sqrt{\boldsymbol{b^2-4*a*(c-signal)}}}{\boldsymbol{2*a}} \qquad \textbf{Equation 2}$$

where a, b, and c are coefficients from the quadratic standard curve equation, and signal is the corrected absorbance of the sample at 450nm. With the final volume of the second serial dilution being 1 mL, the value calculated in that step is also the mass in ng of the sample. To correct for the fact that the sample was diluted 100 or 400 fold from the original reconstituted samples, the ng of the 2nd or 3rd serial dilution were multiplied by a correction factor of 100 or 400 to yield the mass in ng recovered from the C_{18} cartridge.

Outliers to the data were calculated using standardized residuals (Kuehl 2000). The standardized residuals were calculated using Equation 3:

$$\boldsymbol{w_{ij}} = \frac{\hat{e}_{ij}}{\sqrt{MSE}} \qquad \textbf{Equation 3}$$

where w_{ij} is the standardized residual, $\hat{e}_{ij}$ is the experimental error, and MSE is the mean square for error. The experimental error is calculated as follows:

$$\hat{e}_{ij} = \boldsymbol{y_{ij}} - \bar{y}_i \qquad \textbf{Equation 4}$$

where y_{ij} is the ng of a single trial, and $\bar{y}_i$ is the average of the signals within that same row of data. The MSE is calculated in the following way:

$$MSE = \frac{\sum_{i=1}^{t}\sum_{j=1}^{r}(\hat{e}_{ij})^2}{N-t} \qquad \textbf{Equation 5}$$

where N is the total number of recorded absorbencies, t is the total number of "treatments," in this case t=3 because there are three different fractions.

Once the standardized residuals were calculated, the average and standard deviation of the residuals were calculated. A data point was considered an outlier if the standardized residual for that data point was beyond two standard deviations of the residual average.

The two dried 100 ppb standards were not applied to the C_{18} cartridges before drying, and therefore the average mass was determined to be the mass in ng applied to each of the columns. The average and standard deviation of the mass recovered from the load and elution fractions was calculated using default equations in Microsoft Excel. A student's T-test was used to determine if the mass applied to the column and the total mass recovered from the column were statistically different ($p<0.05$). The percent recovery of each fraction was also calculated by dividing the mass recovered from the fraction by the average mass of the two 100 ppb standards, and converting that fraction to a percent. To be an acceptable extraction method, the mass recovered in the elution step needs to be 75%-100% of the total mass applied to the column.

Results

The C_{18} recovery data collected from samples diluted 1/100 is an average of the seven replicate readings of the microplate. Plate readings from these samples produced an "overflow error", indicating that the color in the samples was too intense for any light to be transmitted through the samples. However, later readings were able to be collected for data analysis. These data are under scrutiny because during that time, the microwells developed a black, string-like protein precipitate, as seen in Figure 16. During the subsequent seven replicates used for analysis the data values fluctuated due to the formation of the precipitate.

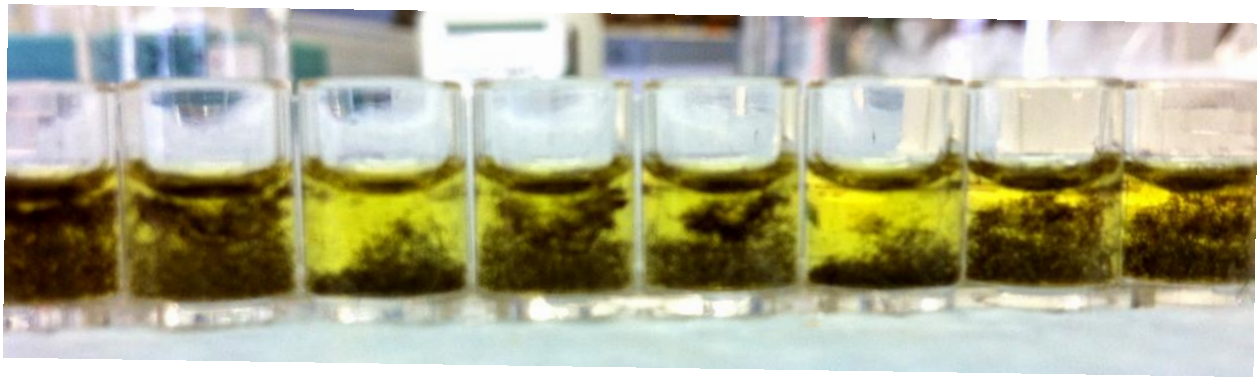

Figure 16: String-like protein precipitate observed in microplate wells at the end of seven data collection replications.

The summary of the data analysis and calculations can be seen in Table 16 below. The total mass recovered from the column was 421.823 ng CML, when the average mass recovered in the load and elution fraction were added together. This is a significantly higher mass than recovered on previous experiments. However, the Student's T-test also showed that the mass of the standards are statistically different ($P<0.05$) than the total mass recovered, and the average mass of the analyzed standards is closer to the mass of the load and elute fractions. Looking at percent recoveries in the individual replications also show that in only replication three had a recovery that was above 50% (data not shown). It is also very interesting to note that replication 3 had the slowest flow rate during the load step of the SPE.

Table 16: Summary of percent recovery calculations for SPE on the C_{18} cartridge during first analysis

Sample	Mass (ng) recovered from cartridge	Standard Deviation of Mass Recovered	Mass Recovered (%)	Total Mass recovered from column	Standard Deviation of total recovered mass
Elute	226.322	103.324	53.65%	421.823	147.180
Load	195.501	104.814	46.35%		
Standards	142.440	27.455	33.77%		

With the data from 1/100 extractions under scrutiny, the SPEs were conducted again, with the samples being diluted 1/400 instead to correct the overflow error. When the samples were re analyzed and diluted 1/400, the only observation was that the elution step of the second extraction, E2, was darker than the other two elution steps. The residual calculations for outliers

showed, however, that the only outlier in the data was Read 1/L1. The residual calculations can be seen in APPENDIX A- Sample Calculations below. Once the outlier had been removed, Equation 2 was applied to the data and the mass in ng was calculated. The average mass of the L, E and standards were calculated, and the percent recovery determined. A summary of the data can be seen in Table 17. The data show that of the 416ng of mass that were applied to the column, 69.3% were recovered in the elution, which is an acceptable percent recovery.

Table 17: Summary of percent recovery data from C18 SPEs diluted 1/400.

	Average Signal	**Average ppb**	**Average ng**	**Percent Recovery**
Standard	6.819	1.041	416.35	100.0%
E	4.874	0.721	288.57	69.3%
L	3.337	0.475	189.80	45.6%

Discussion and Conclusion

First, with the dramatic increase of mass that was recovered in this experiment compared to the previous experiment, (over a 100% increase), it is clear that use of silanized vials is critical to retaining mass throughout the drying and dilution process. The increase was so high, in fact, that the resulting overflow error in the microplate reader was unexpected. Also, the standard once again showed a low mass compared with the samples. This could be from the precipitate that formed during the plate reading, incomplete silanization of the vial, incomplete removal of the TFA during drying, and therefore a pH that was too acidic for the kit, or the TFA pH was extreme enough during drying that is dissolved the silane coating and CML adhered to the glass.

Second, with the protein precipitate forming in the plate while collecting data, the readings of this experiment are under extensive scrutiny. With that being said, the data suggest that the percent recovery using the C_{18} cartridge is reliably 50%. However, noting that the E3/L3 had the lowest flow rate and the highest recovery, it is probable that slowing the flow rate through the column during loading to less than 1 drop per second may increase the retained mass. While these data do not meet the original goal of greater than 75% recovery, the great improvement on the mass recovered may make this method viable for sample clean up.

The SPEs that were conducted with a 1/400 dilution show that the C_{18} cartridges can be used to achieve an acceptable recovery of CML standards. One thing of note, however, is that

the theoretical mass loaded onto the column was 100ng, not the 400ng recovered. However, this may be explained by the equipment used during the ELISA extractions. Two orbital shakers exist in the laboratory, and one has a much faster rotation rate around a smaller rotation radius. This could result in more vigorous shaking during the ELISA kit, and could inflate the results. It is of note that the standard curve was prepared using the slower of the shakers, and this could account for the inflation of the mass from 100 to 400ng. To prevent this error from occurring in future experiments, it is advised that a series of standards be run, possibly in duplicate, in addition to the samples to correct this error.

Conclusions from HPLC and SPE Experiments

From the experiments using HPLC analysis, it is clear that a method of detection with low limit of detection is necessary to analyze the presence of CML in foodstuffs. The ELISA kit used in the SPE experiments proved to be an adequate detection method. The SPE experiments showed that the percent recovery of CML reached its maximum of 69.3% when a C-18 extraction column is used. Control of the flow rate in the vacuum manifold will improve the recovery of CML from SPE columns. Furthermore, use of silanized glassware will prevent the adhesion of CML to the surface of the glassware, which also will improve the percent recovery in the column.

CHAPTER 3- Effect of Spices on CML Levels in Biscuits

Carboxymethyllysine (CML) is a Maillard browning product that can have an inflammatory effect in the body. Spices and their antioxidants may decrease the levels of CML in foods. The purpose of this study was to determine the effect of black pepper, cinnamon, rosemary, onion, and garlic at 0.5%, 1% and 2% levels on CML in quick bread biscuits. Biscuits were baked using each spice and spice level, as well as a control and a spiked sample. Color measurements were taken prior to CML analysis which included grinding; reduction of sugars with sodium borohydride, defatting with chloroform methanol, digestion with hydrochloric acid, neutralization and serial dilution, with detection being an ELISA kit with a CML specific antibody. The data show that CML was significantly inhibited ($P<0.05$) by all spices at each spice level. Chroma trends also show that increasing the spice level decreases the chroma in the black pepper, cinnamon and rosemary spices. Future work could look at the effect of lower levels of spice, or combinations of spices on CML levels.

Introduction

Advanced glycation end products (AGEs) are commonly occurring compounds that form in food matrices during the Maillard browning reaction. Maillard browning is the reaction of amino acids with reducing sugars (Ruttkat and Erbersdobler 1995). However, these compounds can form through a variety of mechanisms and pathways, such as free radical mechanisms (Nagai and others 2002), Schiff bases that dehydrate and tautomerize through Amadori rearrangements (Kasper and Schieberle 2005), and polymerization (Hofmann 1998). Carboxymethyllysine (CML) is an early stage Maillard browning product, that is a precursor of final browning products, such as melanoidins, and can be formed through "traditional browning" (Hodge and Rist 1953) as well as through lipid peroxidation reactions (Fu and others 1996).

AGEs in the body have been shown to aggravate diseases associated with inflammatory characteristics, such as diabetes (Uribarri and others 2007b), Alzheimer's (Candela and others 2010), fibromyalgia (Ruster and others 2005), and some cancers (Jiao and others 2011). Particularly with diabetes, it has been shown that AGEs in the body can change inflammation pathways, and increase oxidative stress markers (Schmidt and others 2000;Hermani and others 2005). Furthermore, AGEs can damage tissues in the body by forming irreversible crosslinks in

protein structures (Zieman and others 2007; Biemel and others 2001b). It is the thought that reducing dietary levels of the AGEs may help mitigate some of the symptoms of these diseases.

Reduction of AGEs in the body have been achieved using artificial AGE crosslink breakers, and through the use of natural antioxidants such as carnosic acid (Zieman and others 2007; Wang and others 2012). However, levels of AGEs in the body are not introduced exclusively through endogenous reactions, and are also introduced through foods ingested. Studies have shown that CML is present in meat, dairy, bread, sweets; in most foods that are cooked (Goldberg and others 2004; Assar and others 2009; Peng and others 2010a; Srey and others 2010; Hull and others 2012). Maillard browning can be controlled through the cooking method, with frying and broiling producing the most AGE's, followed by baking, then steaming and boiling (Goldberg and others 2004). However, palatability of food must be considered when selecting a cooking method, and controlling browning through cooking method alone is insufficient.

Maillard browning can also be controlled through the introduction of natural antioxidants into the food matrix. Most spices and herbs that are incorporated into foods have some natural antioxidant abilities that, in some cases, provide more antioxidant capacity than vitamin C, or synthetic antioxidants such as BHA or BHT (Shon and others 2004; Kortenska-Kancheva and others 2005; Iqbal and Bhanger 2007; Kapoor and others 2009; Wang and others 2011). Some studies have shown that incorporation of the antioxidants found in rosemary, cinnamon, garlic, onion, black pepper, and grapeseed extract, have lowered the levels of AGEs in foods such as frankfurters, and beef patties (Smith and others 2008; Oz and Kaya 2011a; Oz and Kaya 2011b; Ozcan and Arslan 2011; Wang and others 2011; Gibis and Weiss 2012; Ozvural and Vural 2012).

However, in baked goods, particularly breads, less research has been conducted on the effect that spices play on the formation of CML. This study attempts to observe the effect of black pepper, cinnamon, garlic, onion, and rosemary at 0, 0.5%, 1%, and 2% levels on the CML levels in quick-bread biscuits.

Materials and Methods

Chemicals

Betty Crocker Bisquick Original Pancake and Baking Mix (Bisquick), Dillons Brand 2% lowfat liquid milk (milk), McCormick Gourmet Collection onion powder, and McCormick Gourmet Collection 100% Organic Garlic powder were purchased from the local supermarket. All of the Bisquick mix used for baking was purchased at one time and spices were purchased from a single lot of each spice. A single lot each of black pepper-blend, cinnamon, and rosemary analytical standards were provided by the McCormick Science Institute (Hunt Valley, MD). Sodium borate decahydrate ACS reagent, potassium hydrogen phthalate, Biotech Performance Certified Phosphate buffered saline pouches, and sodium borohydride powder (98+ %) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide ACS certified pellets, trace metal grade hydrochloric acid, 0.2µm filtered methanol, acetonitrile Optima, and ACS certified chloroform were purchased from Fisher Scientific (Rockford, IL). Oxi-Select N^{ε}-(carboxymethyl) lysine (CML) ELISA kits were purchased from Cell Biolabs, Inc. (San Diego, CA) Liquid nitrogen was purchased from the Kansas State University chemical storeroom. Compressed nitrogen tanks were purchased from Linweld. Carboxymethyllysine (CML) standard (used in the spiked samples) was purchased from Polypeptide Group (San Diego, CA).

Formulation and Baking

A schematic of the experimental design for one replicate of baking is shown in Figure 17. Three replicates of baking were performed in total. In each replicate, a batch for control, each spice at 0.5%, 1%, and 2% levels, and a spiked batch were baked, for a total of 17 batches of biscuits. Three subsamples biscuits from each batch were collected (for a total of 53 samples per replicate), individually stored in a quart size freezer bag, and saved at -20 °C until grinding.

Each sample label was coded with a four part code for ease of reference. Because this is how individual samples are referred to in later sections, the coding system will be explained. The first part of the label was the replicate number, i.e. replicate one was labeled “R1.” The second part of the label code was the spice; BP for black pepper, Cinn for Cinnamon, Gar for Garlic, Oni for Onion, and Rose for Rosemary. Control and Spike samples were labeled

accordingly. Thirdly, was the spice level, either 0.5%, 1%, 2%, or "--"whether the sample was a spice, or a control sample. Lastly was the "trial," or the individual label for which of the three subsamples in a particular spice level. For example, if a sample was the third subsample for a 0.5% black pepper biscuit from replicate two, the code would read, "R2-BP-0.5%-#3."

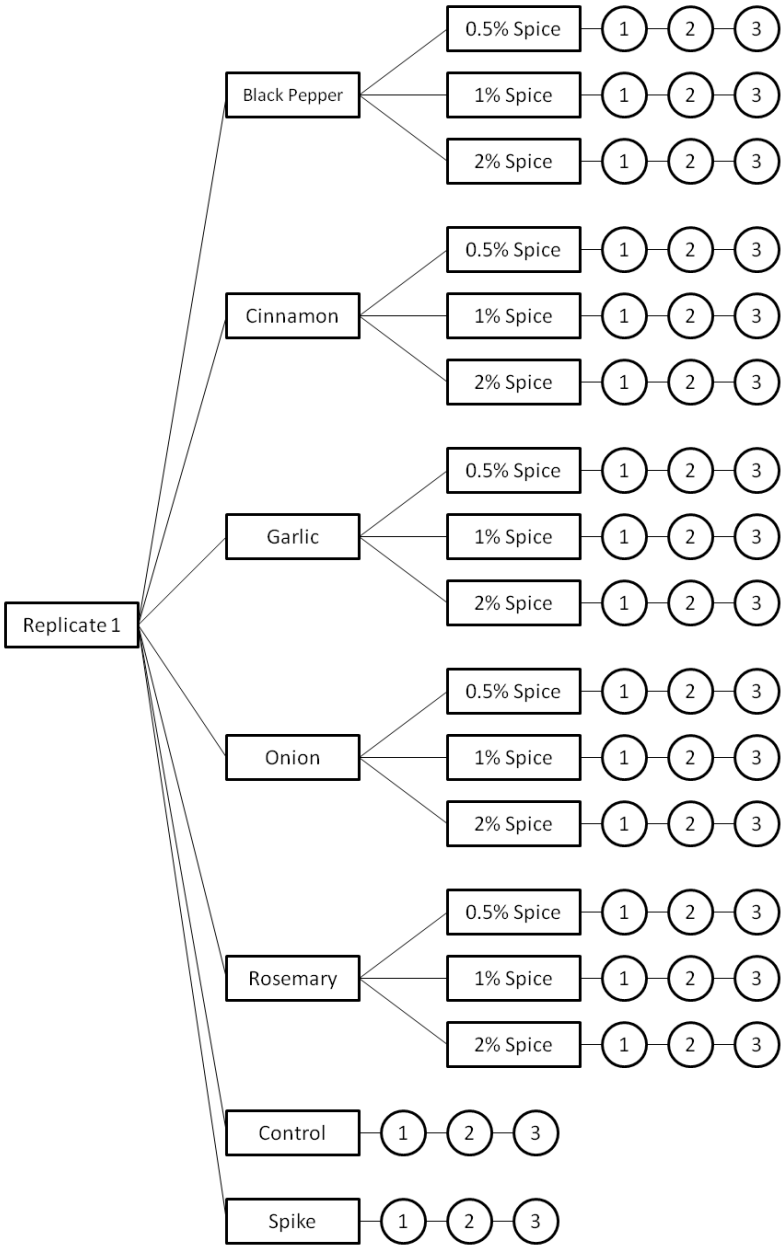

Figure 17: Schematic of baking of Replicate 1

To make each batch, Bisquick, milk, and spices were incorporated using Table 18, adjusting the formula to 0.5%, 1%, or 2% (w/w) spice level as the batch required. The flour and spices were weighed and stirred to homogeneity in a mixing bowl prior to the addition of the milk. Homogeneity was determined by visual inspection. Then the milk was incorporated and the mixture was mixed in a Rival 12-speed stand mixer for 1 minute at speed 4, or until the dough pulled away from the bowl and up onto the mixing blades. The dough was then turned out onto a rolling mat, which had been lightly dusted with additional Bisquick to prevent sticking, and kneaded 15-20 times until a stiff, cohesive dough formed. After kneading, the dough was rolled out to a thickness of 4mm using a rolling pin and 4mm guides. Biscuits were then cut using a 2.5 inch (6.35cm) cookie cutter, weighed for "pre-bake weight", and placed on a food grade stainless steel baking sheet lined with parchment paper. The remaining dough after cutting the biscuits was discarded. The biscuits were then baked for 9 minutes in standard GE domestic oven, 8" from the oven bottom that had been pre-heated to 450 °F (232.2 °C). After baking, the biscuits were removed from the oven, allowed to cool to room temperature, and re-weighed for a "post-bake" weight. After being re-weighed, each batch was placed into a pre-labeled, gallon size, plastic freezer bag, and stored at -20 °C until grinding.

Table 18: Baking formulation for biscuits and spices

	0% Spice (Control)	**0.5% Spice**	**1% Spice**	**2% Spice**	**Spike**
Baking Mix (g)	290.25	145.14	144.41	142.95	290.25
2% Milk (g)	150	75.01	74.63	73.88	150.00
Spice (g)	0	1.11	2.21	4.43	553 µL of 100 ppm Standard

Grinding

Prior to grinding, each batch of biscuits was removed from the freezer, and three biscuits were removed for analysis. Each biscuit was photographed, and three color measurements were made using a Hunter Lab Mini Scan EZ 4500L colorimeter. A representative photograph can be seen in Figure 18. Each sample was then cut into roughly 8 pieces, submersed in liquid nitrogen for 20 seconds, and ground to a fine powder on high speed using a Waring blender. The samples were then placed individually into pre-labeled, quart size freezer bags and frozen at -20 °C until further analysis.

Figure 18: Representative picture of a standard biscuit sample. A biscuit from replicate three, 1% garlic is shown here.

Reduction of Reducing Sugars

The purpose of addition of sodium borohydride solution to each sample is to convert reducing sugars found in the sample to sugar alcohols and thereby inhibit the formation of additional CML during the acid digestion step (Abdel-Akher and others 1951). An example of this reaction can be seen in Figure 19, illustrating the reduction of glucose to D-glucitol with sodium borohydride.

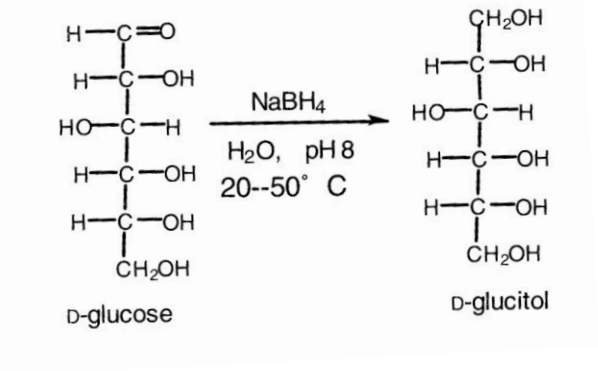

Figure 19: Chemical reaction of glucose with sodium borohydride, as seen in Essentials of Carbohydrate Chemistry (Robyt 1998)

Chemical Preparations

Prior to sample reduction, a 0.5 molar (M) sodium borate buffer (borate buffer) was made by dissolving 9.54 grams (g) of sodium borate decahydrate into 50 milliliters (mL) of deionized water. The pH of the resulting borate buffer slurry was adjusted to 9.2 with hydrochloric acid, as adapted from Assar and others (2009).

In addition to the borate buffer, a 0.1 M sodium hydroxide (NaOH) solution was made by diluting a 1 M NaOH solution 1:9 with deionized water. The 0.1 M NaOH solution was then standardized by titration with potassium hydrogen phthalate (KHP). To standardize with KHP, the NaOH solution was added to a clean burette, and the initial volume in mL was recorded. Then 0.7-0.9g of KHP was weighed to the nearest 0.1 mg into a 125 mL Erlenmeyer flask, and 50 mL of deionized water was added. Next, a stir-bar was added to the solution and allowed to completely dissolve on a stir-plate. When the KHP was completely dissolved, 5-6 drops of phenolphthalein indicator was added to the flask, and positioned under the burette containing the base to be standardized. The stopcock on the burette was opened and the NaOH solution was added drop wise, with continuous stirring on the stir-plate until a pale pink color persisted for 30 seconds. The stop-cock was then closed and the final volume in mL of the burette was recorded. The molarity (M) of the NaOH was then calculated in the following way:

$$\frac{g\ KHP}{204.23} = moles\ of\ KHP \qquad \textbf{Equation 6}$$

$$moles\ KHP = moles\ of\ NaOH \qquad \textbf{Equation 7}$$

$$\frac{moles\ NaOH}{(final\ volume\ NaOH\ recorded - inital\ volume\ NaOH\ recorded/1000} = M\ NaOH \qquad \textbf{Equation 8}$$

If the molarity of the solution was too low 1 M NaOH was added, and if the molarity of the solution of was too high, deionized water was added. After the addition of the NaOH or water the burette was emptied of the previous solution, and rinsed with the new NaOH, and the standardization process with KHP was repeated. Once the solution had reached 0.1 M NaOH, the standardization process was repeated two more times, and the average of the three trials was calculated and used as the molarity of the NaOH solution. Once the 0.1 M NaOH solution had been prepared, a 2 M sodium borohydride ($NaBH_4$) solution was made by dissolving 3.79g of $NaBH_4$ in 50 mL of the standardized 0.1 M NaOH solution (Assar and others 2009) .

Borohydride Reduction Procedure

Samples being analyzed were removed from the freezer and allowed to thaw to room temperature. Biscuit powder (99-101 mg) was accurately weighed to one tenth of a milligram into a pre-labeled 5 mL or 10 mL Kimex reaction vial using an Ohaus GA200 scale. Once each sample was weighed, 1.2 mL of the borate buffer and 600 microliters (µL) of the NaBH4 solution were pipetted into each vial using a 1-5 mL and a 100-1000 µL Fisher Scientific Finnpippette II pipette, respectively. Each vial was then fitted with a solid top black phenolic cap with a polytetrafluoroethylene (PTFE) faced rubber liner, and mixed with a Fisher Scientific vortex mixer until the biscuit powder was thoroughly incorporated, roughly 10 seconds. The cap on each sample was then loosened slightly to allow for diffusion of hydrogen gas away from the sample. All samples were then incubated overnight at 4 °C or for 4 hours at room temperature (roughly 23 °C) to allow for the reaction to complete (Assar and others 2009).

Defatting and Protein Precipitation

After the samples were incubated overnight in the sodium borohydride solution, they were removed from the refrigerator and 3 mL of 2:1 chloroform: methanol solution was added to each vial, and the lid replaced (Assar and others 2009). The chloroform: methanol solution was prepared by mixing 500 mL of chloroform and 250 mL of methanol to homogeneity in a 1L Erlenmeyer flask.

The sample was then inverted several times to mix the two liquids, and to facilitate dissolution of fat into the chloroform: methanol solution. The samples were then centrifuged at 4500 rpm for 10 minutes in a Thermo IEC Marathon 21000R centrifuge from Fisher Scientific. All of the liquid was then removed using a Pasteur pipette. This extraction process was repeated two additional times, for a total of three extractions with the chloroform: methanol solution. If a vial was damaged in the centrifuge, the procedure to this point was repeated with the exception that incubation of the sample in the sodium borohydride was conducted for 4 hours at 23 °C. After extractions, the samples were allowed to dry in a fume hood overnight at room temperature to ensure that the chloroform: methanol was removed.

Acid Digestion

CML can be formed on free lysine, or on a lysine bound in a protein (Goldberg and others 2004). The acid digestion step was to hydrolyze the proteins in the biscuit samples to amino acids and liberate any CML bound in the proteins. Chemical preparation and procedural steps are explained below.

Chemical Preparation

The HCl solution was prepared by diluting 53.4 mL of 11.242 M trace metal grade HCl to 100 mL with deionized water. The acid concentration was standardized by adding 50 mL of deionized water to a 5 mL aliquot of the acid and adding 5 drops of phenolphthalein as an indicator. Next the previously standardized 1 M NaOH was added to a clean burette, and the initial volume was recorded. NaOH was added drop wise to the HCl with constant stirring until a pale pink color persisted for 30 seconds, and the final volume of NaOH was recorded. Then the molarity of the acid was calculated in the following way:

$$\boldsymbol{M\ NaOH} * (\boldsymbol{final\ volume\ NaOH - inital\ volume\ NaOH}) = \boldsymbol{mol\ NaOH\ used} \quad \textbf{Equation 9}$$

$$\boldsymbol{mol\ NaOH\ used = mol\ HCl\ titrated} \quad \textbf{Equation 10}$$

$$\frac{\boldsymbol{mol\ HCl\ titrated}}{\boldsymbol{5mL\ HCl\ aliquot\ tested}} = \boldsymbol{M\ HCl} \quad \textbf{Equation 11}$$

If the molarity of the HCl solution was too concentrated deionized water was added to further dilute the solution, and if the HCl was too dilute, additional 11.242 M HCl was added. The titration procedure was repeated until the acid molarity was calculated to be between 5.9-6.1 three times successively. The average of the three consecutive trials was used as the final molarity of the HCl.

Acid Digestion Procedure

To each of the dried, defatted samples, 3 mL of the 6 M HCl was added. Then, the threading on each vial was lined with PTFE tape to prevent leaking while the samples were in the oven. The headspace of each vial was flushed with nitrogen to prevent additional oxidation of the samples due to oxygen in the air; either 10 seconds for a 5 mL Kimex vial, or 20 seconds for a 10 mL Kimex vial (Peng and others 2010a). The cap was then tightly fitted to each vial, and

the samples were placed in a plastic vial rack in a pre-heated Fisher Scientific Iso-Temp incubator at 110 °C for 24 hr (Assar and others 2009a).

Neutralization and Dilutions

After 24 hours of acid digestion, samples had to be neutralized and diluted to the recommended µg protein per mL solution of 10 µg/mL. To neutralize the sample, a 1 M NaOH solution was prepared and standardized similar to the preparation of the 0.1 M NaOH solution described in Acid Digestion section above, with one modification. Instead of a dilution of 1.0 M solution, the starting NaOH solution was created by dissolving 4.0g of NaOH pellets in 1.0L of deionized water. Then, if the concentration of the NaOH solution was too low during standardization, concentrated NaOH solution (molarity unknown, but roughly 8 M), was added to adjust the pH. To neutralize the samples, they were first inverted several times to homogenize the sample, then the lid was removed and a 200 µL aliquot was placed in a 2 mL polyethylene micro centrifugation vial, and to that 1100 µL of 1.0 M NaOH were added, which was then closed and inverted several time to facilitate mixing and neutralization. This was called "D0."

Prior to serial dilution of the samples, 0.1 M phosphate buffered saline, pH 7.4 (1XPBS) was prepared by adding 1 foil packet of 1XPBS powder to the bottom of a 1.0L volumetric flask, and diluting the solution to the 1.0L mark and stirring to homogeneity. To dilute the samples to the 10 µg/mL protein concentration, 800 µL of the 1XPBS were added to a 2 mL polyethylene microcentrifuge vial, and 150 µL of the D0 were added, the vial closed and inverted several time to facilitate even mixing. This was then termed "D1." To another 2 mL microcentrifuge vial, 800 µL of 1XPBS were placed, and 150 µL of D1 were added to it, closed and inverted to homogeneity. This was the final dilution, termed "D2" that was used in the ELISA assays. A diagram of the dilution scheme can be seen in Figure 20.

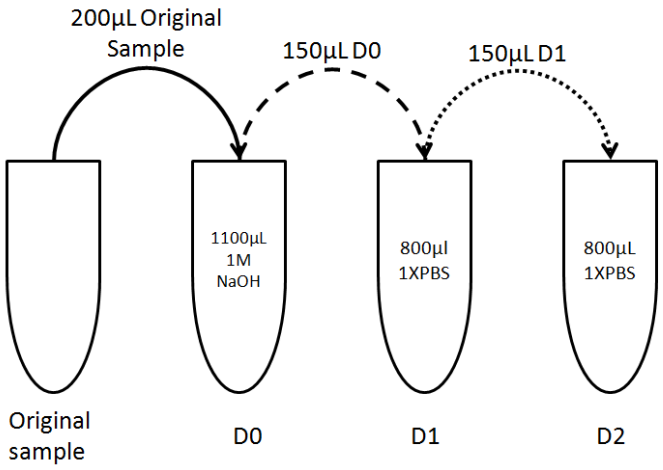

Figure 20: Diagram of dilution scheme used to dilute samples

ELISA Assays

The ELISA kit was provided by Cell Biolabs, Inc, and the provided instructions were followed to conduct the analysis. From each sample D2, 100 µL aliquots were transferred to their corresponding well on the ELISA microwell plate. The plate design for each of the kits used in these experiments can be seen Appendix B- Chapter 3 Supporting Data. These were then covered and allowed to incubate overnight at 4 °C. The next day, the microwell plate was inverted and shaken to remove the samples, tapped on an absorbent towel to remove excess liquid, and 200 µL of the provided assay diluent were added to each well and incubated for 2 hours on an orbital shaker, at 500 rpm. During this incubation, a 1X wash buffer (1XWash) was made by adding 20 mL of the provided 10X wash buffer to a 200 mL volumetric flask, diluting to the 200 mL mark with deionized water, and inverting several times to achieve homogeneity. Also during this incubation, the 1000X secondary anti-CML antibody provided by the kit was diluted to 1X by adding 7 µL of the 1000X antibody to 6993 µL of assay diluent in a silanized vial, as per the instructions. Two of these 1X- secondary antibodies were made to ensure that enough liquid was available for each microwell.

After 2 hr, the microwell plate was inverted over a sink, shaken to remove the bulk of the liquid, and dried by tapping the plate on an absorbent towel. Next each well was washed wit 250 µL of 1XWash buffer, then inverted, shaken and dried by tapping on a towel. The wash process was repeated a total of three times, then 100 µL of the 1X-secondary antibodies were added to

each well, and the plate incubated for one hour on an orbital shaker at 500 rpm. During the 1hr incubation, the conjugated antibodies were diluted 1000X in assay diluent as described above.

When the 1 hr secondary antibody incubation was complete, the wash process was completed 3 times, then 100 µL of the conjugated antibodies were added to each well, and the plate was again incubated for 1 hr on the orbital shaker at 500 rpm. During this incubation, the provided substrate stop solutions were removed from storage at 4 °C and allowed to warm to room temperature.

After the last 1 hr incubation was complete, the plate was washed a total of five times with the 1XWash buffer, and 100 µL of the substrate solution was added to each well, and allowed to incubate for 20 minutes at 500 rpm on the orbital shaker, to develop the color. Then without removing any of the substrate solution, 100 µL of the stop solution was added to each well to arrest color development. The absorbance of each well on the plate was then immediately read on a Biotek microplate reader and recorded for later analysis on the data collection software. Each plate was read three times on the Biotek microplate reader, and the average absorbance was used to calculate the levels of CML in the vial. A standard curve was constructed by making CML-BSA standards with the materials and instructions included with the kit. Briefly, a 2.2 ppb CML solution was made by diluting a provided CML-bovine serum albumin (BSA) stock solution, then serially diluting the 2.2 ppb solution 1:1 with reduced BSA solution seven times, until a 0.035 ppb solution is achieved. A standard with just reduced BSA was used as a "0 ppb" blank solution. These standards were interspersed with the samples on the ELISA plates as the data were collected, and the standard curve constructed using a quadratic regression.

Data were collected over three months, and samples were kept frozen unless analyzed. CML levels in the biscuits samples were analyzed using three different ELISA kits. "Kit 1" and "kit 2" were used to collect the original data, and samples were split between the two kits as described in the experimental plate designs in APPENDIX B- Chapter 3 Supporting Data below. Additional samples were rerun on "Kit 3," as the data required.

Proximate Analyses of Biscuits

Previously ground biscuits were removed from frozen storage and allowed to thaw to room temperature. Samples were sent to the Kansas State University Soils Testing Lab for nitrogen content analysis on a LECO analyzer (Sweeney and Rexroad 1987) . Fat and moisture were determined using a CEM Smart Trac 5 moisture and fat analyzer (Huettner and others 2010). The CEM system uses microwave heating to dry the sample to constant weight, and uses nuclear technology to determine the percentage of fat in a sample. Moisture and fat analyses were performed in duplicate unless otherwise stated.

Data Calculations

The percent nitrogen was converted to percent protein by multiplying the LECO data produced from the Soils Testing Lab by a conversion factor of 5.70. The average mass loss of each batch of biscuits was calculated by subtracting the average post-bake weight of a batch from the average pre-bake weight. The mass loss was used as a quality check of the biscuits, to ensure that no one batch differed too greatly in mass and mass loss than another batch.

The L, a*, b* data were average by "measurement" and then by "trial," meaning that each spice level of each spice had a total of nine measurements that were averaged to create the one L, a*, b* reading. Using the averaged L, a*,b* measurements, the "total color" was calculated using the following formula (Wrolstad and Smith 2010):

$$\boldsymbol{E = \sqrt{L^2 + a^{*2} + b^{*2}}} \qquad \textbf{Equation 12}$$

where E is the "total color", L is the lightness measurement, a * is the red/greed value, and b* is the blue/yellow value (Wrolstad and Smith 2010). In addition to the total color, the chroma values, or the color saturation, were calculated using the following equation and the same a* and b* values (Bal and others 2011):

$$\boldsymbol{C = \sqrt{a^{*2} + b^{*2}}} \qquad \textbf{Equation 13}$$

where C is the chroma value. These data calculations were also used as quality check in the biscuits, as well as a way to determine if there was a correlation between the surface browning and the CML content in the biscuits.

To calculate the ppb CML in the individual microplate wells, the regression correlation coefficients from the standard curve and the absorbance of the sample were used in Equation 2 as described above. Once the ppb in each well was calculated, the ppb in each vial was calculated by multiplying by the dilution factors from the dilutions during neutralization in the following way:

$$\boldsymbol{CML\ ppb\ in\ each\ well} * \frac{\mathbf{950}}{\mathbf{150}} * \frac{\mathbf{950}}{\mathbf{150}} * \frac{\mathbf{1300}}{\mathbf{200}} = \boldsymbol{CML\ ppb\ in\ orignal\ sample} \quad \textbf{Equation 14}$$

where each of the multipliers are the dilution factors are for D2, D1, and D0, respectively. After the ppb in each vial were calculated, the mg of CML in each vial were calculated by multiplying by the sample volume of 3 mL and converting from ng to mg by dividing by a conversion factor. From mg CML, mg CML/kg biscuit was calculated by dividing by the sample mass converted to kg. The mg CML/kg protein by dividing the mg CML by the kg of protein the sample, which was calculated my multiplying the sample weight, converted to kg, by the percentage of protein from the average protein from the proximate analyses.

Statistical Analysis

Outliers in the data were calculated using the Grubbs' test, which is recommended by the ISO (Miller and Miller 2005). Outliers were determined by first calculating a G-statistic using the following equation:

$$G = \frac{|x - \bar{x}|}{s} \quad \textbf{Equation 15}$$

where x is the suspected CML value, $\bar{x}$ is the average CML values for that particular spice level, and s is the standard deviation of the spices calculated with the suspect value included (Miller and Miller 2005). This G-statistic was then compared to the critical G-statistic of 2.215. If the G-statistic calculated for the sample exceeded the 2.215 threshold, then that data point was discarded as an outlier with 95% confidence.

For the mass loss data, a randomized complete block design (RCBD) was used, using replicate as a block, and assuming equal variances. For the L, a*, b*statistics, a RCBD assuming equal variances was used for the L values, and a complete randomized design (CRD) was an assumed unequal variances was used for the a* and b* statistics. For the average CML levels, a

RCBD or a CRD was used for statistical analysis, dependent upon which data were used for analysis, as discussed in further detail below.

Data with a P-value of 0.05 or smaller were considered statistically significant. All statics were calculated using SAS statistical analysis software, with the help of the Kansas State University Statistical Consulting Laboratory.

Results

Proximate Analyses

Biscuit powder remaining after analyses were collected according to section 0 above, and analyzed for moisture fat and protein. During analysis the 1% onion sample was lost. Also not enough 2% black pepper powder, and 0.5% rosemary biscuit powder remained in the sample to run the entire analysis. However, the remaining samples were analyzed, and the data are summarized in Table 19. The overall average for fat, moisture, and protein were 8.51, 16.50, and 7.39 percent, respectively.

Mass Loss During Baking

Each biscuit from each batch was weighed before and after baking, and an average mass loss per batch was calculated for each batch. A total of 17 batches were baked in each of the three replicates, as seen in Figure 18. The raw data and calculations for the mass loss can be seen in APPENDIX B- Chapter 3 Supporting Data. Mass loss per batch varied from 3.04-4.03 g, and was assumed to be mostly water loss from the sample during baking. When looking at the mass loss of individual batches, it seems that R2-Oni-0.5% lost more mass than other batches, clearly having the highest mass loss at 4.03g, as seen in Figure 21 below. Other non-statistically significant trends are that control batches in general retained more mass than did batches with spices or spiked batches.

Table 19: Average fat, moisture and protein content of representative biscuit samples. 'R' Represents the replicate number.

Sample	% Added Spice	Average% Fat	Average% Moisture	% Protein
Control R2#1	0%	8.94	16.17	7.36
Control R3#1	0%	8.20	17.53	7.53
Control R1#2	0%	8.45	16.33	7.17
Black Pepper R1#1	0.5%	8.58	15.77	7.74
Black Pepper R2#1	1%	9.26	15.30	7.54
Black Peppe R3#2	2%	8.56	14.28	7.60
Cinnamon R3#1	0.5%	7.86	18.45	7.28
Cinnamon R1#1	1%	9.03	14.20	7.72
Cinnamon R2#1	2%	7.78	19.23	7.36
Garlic R1#1	0.5%	8.71	15.65	7.07
Garlic R2#2	1%	7.94	18.93	6.97
Garlic R3#2	2%	8.08	15.81	7.47
Onion R2#3	0.5%	8.90	15.55	7.65
Onion R3#1	1%			7.16
Onion R1#1	2%	8.17	16.51	7.37
Rosemary R3#3	0.5%		14.90	7.51
Rosemary R1#3	1%	8.71	17.86	7.00
Rosemary R2#1	2%	9.11	16.64	7.41
Overall Average		8.51	16.50	7.39

However, when mass loss between spice levels and the control are compared, there are very few spice levels which show statistical significance, as seen in Table 20 below. Only garlic 2%, onion 0.5%, and onion 2% showed a higher mass loss than the controls. Also, when comparing these statistical differences, it bears noting that Oni 0.5% may only be significant because of the R2 batch, which had the highest mass loss. When comparing mass loss of the five spices to each other, only BP and Oni differ significantly in mass loss, where onion has a higher mass loss at 3.46g than the 3.30g mass loss in BP. Observations made during baking showed Onion and Garlic samples to have sticky texture, so it is possible that the increase in mass loss could be due to decreased gluten elasticity from the addition of the spices.

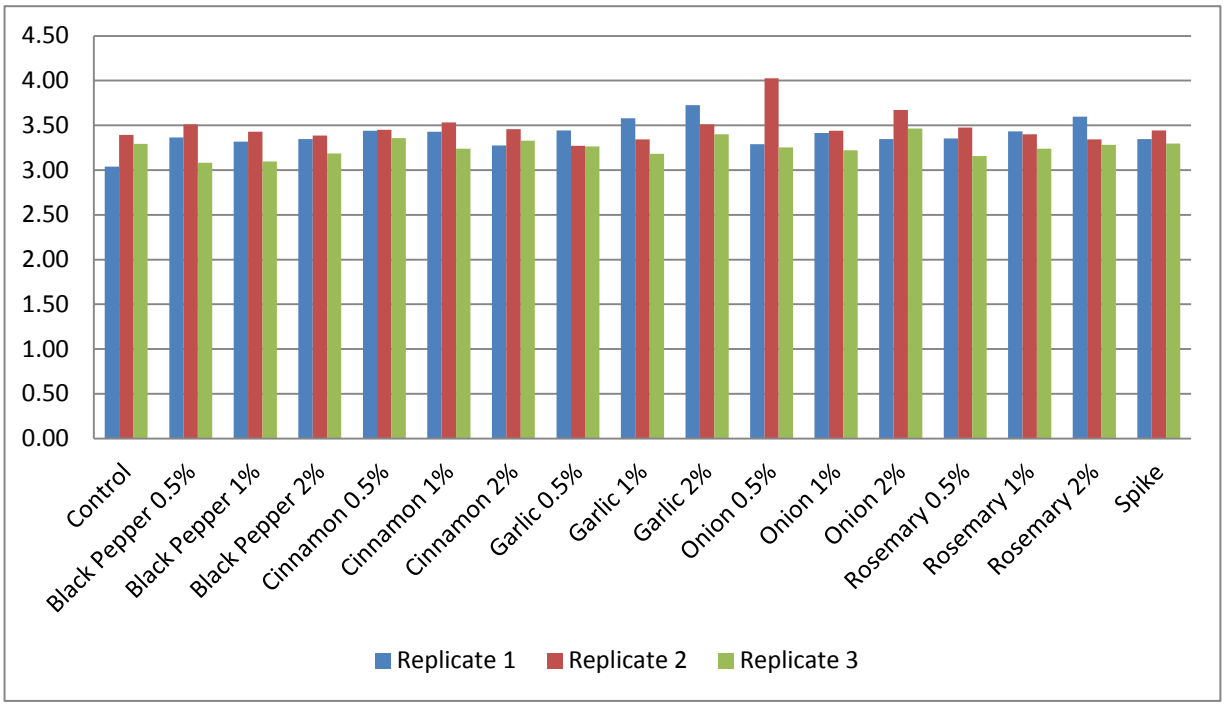

Figure 21: Mass loss in each batch of biscuits

Table 20: Mass loss by spice and level and spice alone (± standard Deviation. Samples with an 'a' are marks spice levels that are statistically different (P<0.05) from the control. Samples with a 'b' are statistically different than each other

	Average Mass Loss by Spice and Level	Average Mass Loss by Spice
Control	3.24 ± 0.18	3.24 ± 0.18
Black Pepper 0.5%	3.32 ± 0.22	3.30 ± 0.15[b]
Black Pepper 1%	3.28 ± 0.17	
Black Pepper 2%	3.31 ± 0.11	
Cinnamon 0.5%	3.42 ± 0.05	3.39 ± 0.10
Cinnamon 1%	3.40 ± 0.15	
Cinnamon 2%	3.35 ± 0.09	
Garlic 0.5%	3.33 ± 0.10	3.41 ± 0.17
Garlic 1%	3.37 ± 0.20	
Garlic 2%a	3.55 ± 0.17[a]	
Onion 0.5%a	3.52 ± 0.44[a]	3.46 ± 0.25[b]
Onion 1%	3.36 ± 0.12	
Onion 2%a	3.50 ± 0.16[a]	
Rosemary 0.5%	3.33 ± 0.16	3.36 ± 0.13
Rosemary 1%	3.36 ± 0.10	
Rosemary 2%	3.41 ± 0.17	
Spike	3.36 ± 0.07	3.36 ± 0.07

L, a, b* Color Measurements*

Prior to grinding, three L, a*, b* measurements were taken of each biscuit that would be later used for analysis. A total of 153 biscuits were used in all according to the design scheme in Figure 16 above. The raw data for the L, a*, b* measurements are available in APPENDIX B-Chapter 3 Supporting Data.

A summary of all L, a*, b*, total color, and chroma values by spice, and spice by level can be seen in Tables 21 and 22. Each category of color data, discussed individually by spice and by spice*level below, will referenced back to these two data tables.

Table 21: Summary of Average L, a*, b*, total color and chroma values by spice. Data with matching letters within a row are not statistically significant (P< 0.05). Note: Control and spike are only included as reference values, but were not included in statistical analysis.

Spice	Average L	Average a*	Average b*	Average Total Color	Average Chroma
Control	57.281	17.704	39.456	71.823	43.306
Black Pepper	54.745[a]	16.090[a]	36.226 [a,c,d]	67.636[a,c]	39.682[a]
Cinnamon	55.190 [a,b]	16.207 [a,b]	35.953 [a,c]	67.861 [a]	39.454 [b]
Garlic	57.935 [b,c]	17.459[c]	39.083 [b,d]	72.072 [b,c]	42.828 [c]
Onion	55.772 [a,b,c]	17.646[c,d]	37.887 [a,b,c,d]	69.874 [a,b,c]	41.824 [c, d]
Rosemary	57.730[b,c]	14.611 [e]	37.507 [a,b,c]	70.470 [a,b,c]	40.300 [d, e]
Spike	61.801	16.842	41.223	76.216	44.546

The average lightness/darkness (L) value of each batch of biscuits was 56.37, with the darkest being R1-BP-1% with an L value of 33.42 and the lightest being R3-Gar-1% with an L value of 65.16. When comparing the L values among the spices, black pepper batches are statistically darker (P<0.05) than onion or rosemary batches, as can be seen in Table 21 above. Cinnamon batches were also statistically darker than rosemary batches.

When comparing L values by spice*level to the controls, no statistical significance was found. However, the spiked batches were the lightest in color, and were statistically lighter in color than any of the black pepper or cinnamon spice levels, as well as the Gar-0.5%, Oni-1%, Oni-2%, and Rose 1% batches, as seen in Table 22 above.

Table 22: Summary of Average L, a*, b*, total color and chroma values by spice*level. Data with matching letters are not statistically significant (P<0.05).

Spice	Spice Level	L	a*	b*	Total Color	Chroma
Control	0.0%	57.281[a,b]	17.704[a]	39.456[a,c]	71.823 [a]	43.306[a]
Black Pepper 0.5%	0.5%	54.784 [a]	17.082[a]	36.800 [a]	68.194 [a,c]	40.587 [b,c]
Black Pepper 1%	1.0%	55.713 [a]	15.783 [a]	36.731 [a,c]	68.628 [a,c]	40.035 [a,c]
Black Pepper 2%	2.0%	53.737 [a]	15.407 [a]	35.148 [a]	66.086 [b,c]	38.425 [b,c]
Cinnamon 0.5%	0.5%	56.275 [a]	16.865[a]	38.059 [a]	70.027 [a,c]	41.647 [a,c]
Cinnamon 1%	1.0%	56.601 [a]	15.812 [b]	36.469, [a]	69.188 [a,c]	39.758 [b,c]
Cinnamon 2%	2.0%	52.683 [a]	15.944 [b]	33.332 [b]	64.369 [b,c]	36.957 [b,c]
Garlic 0.5%	0.5%	56.588 [a]	17.724[a]	38.658 [a,c]	70.821 [a,c]	42.553[a,c]
Garlic 1%	1.0%	60.361 [a,b]	16.701[a]	39.159 [a,c]	73.907 [a]	42.598[a]
Garlic 2%	2.0%	56.854 [a,b]	17.954[a]	39.432 [a]	71.488 [a]	43.333[a]
Onion 0.5%	0.5%	54.975 [a,b]	17.271 [a]	38.318 [a]	70.797 [a]	42.055 [a,c]
Onion 1%	1.0%	57.251 [a]	17.908 [a]	38.304 [a,c]	70.181 [a]	42.322[a]
Onion 2%	2.0%	54.975 [a]	17.760 [a]	37.040 [a,c]	68.643 [a,c]	41.096[a]
Rosemary 0.5%	0.5%	57.251 [a,b]	16.022 [a]	39.133 [a,c]	71.220 [a,c]	42.340[a]
Rosemary 1%	1.0%	56.073 [a]	15.554 [b]	36.993 [a]	68.976 [a]	40.144 [a,c]
Rosemary 2%	2.0%	59.867 [a,b]	12.257 [b,c]	36.395 [a]	71.215 [a,c]	38.418 [b,c]
Spike	0.0%	61.801[a,b]	16.842[a,c]	41.223[a]	76.216 [a]	44.546[a]

When looking at a* values, or the levels of red/green, the biscuits tend to be more red than green, with all of the samples having a positive a*value. When comparing the red/green coloring among the different spices (regardless of spice level) there is a high level of statistical difference among the different spices, with only garlic and onion batches being *not* statistically different. Rosemary batches have the highest amount of green than other batches, with an a* value of 14.61, as seen in Table 21 above. The highest a* values were seen in the control batches, where the average value was 17.70.

When comparing the a* values of different spices levels to the control biscuits, there is still some statistical difference among the different spice levels, as seen in Table 22 above. However, they tend to be in the higher levels of spice, where the spice may impart its own color to the biscuit, especially in the cinnamon and rosemary batches. Both of the 1 and 2% batches in the cinnamon, black pepper, and rosemary spices have lower a* values than do the controls. A general decrease in a* value can be seen as spice level increases can be seen among the black pepper, cinnamon, and rosemary spice levels. However, this trend is not statistically significant.

B* values look at the blue/yellow values of the biscuit, with a negative value indicating a blue color, and a positive value indicating a yellow color. Examining the biscuits by spice alone, the highest b* value was found was 41.22 in the spiked samples, indicating that the spiked biscuits had a more yellow appearance than other batches. The yellow color in the spiked samples was statistically more ($P<0.05$) than any of the spices. There were also differences between individual spices in yellow color. Garlic had the highest yellow color of the spices with a b* value of 37.887, while cinnamon had the least yellow color with a b* value of 35.953. There were also some statistically significant differences in the yellow color among the spices. As seen Table 21 above, rosemary was statistically different than any other spice, having a b* value of 35.507. Cinnamon biscuits were also, on average, statistically less yellow than garlic biscuits. While these small differences are statistically significant, they were not visible trends on the surface of the biscuit, and were not useful from a practical applications standpoint.

When examining the biscuits spice*level, as compared to the controls and spiked samples, differences in the yellowness of the biscuits can be observed. Compared to the 39.456 b* value of the control, only 2% black pepper, 2% cinnamon, and 2% rosemary are statistically less yellow, with b* values of 35.1478, 33.332, and 36.3948, respectively. The most yellow color was again seen in the spiked batches of biscuits, with an average b* value of 41.223, seen above in Table 22. On average, the yellow color in the spiked batches was statistically higher most of the other spice levels, save those where a higher level of yellow color was also present, such as the garlic 1% and 2%, onion 1% and rosemary 0.5%

When looking at total color by spice alone, the spiked samples had the highest average total color value of 76.21, with black pepper having the lowest average total color value at 67.64. However, total color did not statistically differ from the controls to the spices, as seen in Table 21 above. Among the spices black pepper and cinnamon did have statistically lower total color values of 67.64 and 67.86, respectively, compared to the total color value of garlic at 72.07. Rosemary spiced samples, when averaged, had higher total color values when compared with cinnamon. The spiked biscuits, when averaged, showed a higher total color value than any of the spices, but not any difference among the controls. Again, as with L and a* values, a decreasing b* value can be observed with an increasing spice level in the black pepper, cinnamon, onion and

rosemary spices. However, just as with the L and a* trends, this trend is also not statistically significant.

The color data thus far suggested that the addition of spice my darken the surface color of the biscuit, and spices that have a color to impart in the samples, such as the rosemary and the cinnamon, may induce a change in the a* and b* values as the level of spice increases.

When looking at total color by spice level, a general trend can be seen in the black pepper, cinnamon, and onion samples that the total color of the samples decreases as the spice level increase, as seen in Table 22 above. However, the trend is not statistically significant. Black pepper and cinnamon 2% samples did show a statistically lower total color value than the controls. Spiked samples again show the highest total color values, and were statistically different than ally of the black pepper and cinnamon samples, as well as 0.5% garlic, 1% and 2% garlic and 1% rosemary. After the L, a*, b*, we can look at the CML levels in the biscuits. Because there is very slight negative trend as the spice level increases in the L, a* and b* values, the same trend can be observed with total color. But, as with the other trends, this is also not statistically significant.

Finally, chroma values were calculated for each of the samples using and RCBD assuming unequal variances among the samples. Interesting trends are seen in the chroma data, particularly when analyzed by level of spice alone, as shown in Table 23. As the spice level increases, the chroma of each sample decreases, with spikes samples having the highest chroma average value at 44.55, then decreasing through the controls, 0.5%, 1% and 2% spice levels, with chroma values of 43.31, 41.84, 40.97 and 39.93, respectively. However, statistical analysis were between spice levels alone, and not between spike or control samples.

When the chroma data are grouped by spice alone, all of the spices also show statistically different chroma values, save onion versus garlic and rosemary. In terms of decreasing chroma values, the sequence of spices is: spike > control > garlic > onion > rosemary > black pepper > cinnamon, with chroma values ranging from 44.55 in the spiked samples, to 39.45 in the cinnamon samples, seen in Table 21 above.

Table 23 Average (plus-minus standard deviation) chroma values of the biscuits by spice level. Samples with the same letter are not statistically different (P<.05) from each other.

Level	Chroma
Spike	44.546 ± 0.897
Control	43.306 ± 2.229
0.5% [a]	41.836 ± 1.328
1% [b]	40.971 ± 1.947
2% [c]	39.925 ± 2.621

When data were analyzed spice by level, fewer statistically significant trends were seen between the different spices and controls, as seen in Table 22 above. Statistical significance was seen in between the control samples and 0.5% and 2% black pepper, 1% and 2% cinnamon, and 2% rosemary samples. However, some of the same trending can be seen in the spice by level samples as in the spice level alone analysis. In the black pepper, cinnamon, and rosemary samples, the chroma values decreased as the spice level increased, indicating that color became less saturated, or more gray, as the spice level increased.

CML Levels in Biscuits

CML data were collected over three collection periods, each lasting one week. Data collected during the first data collection periods was labeled as "kit 1"; data from the second data collection period was labeled as "kit 2"; data from the third data collection period was labeled as "kit 3". Raw data was collected as an absorbance at 450 nm which was converted to mg CML/kg biscuit as described in the Data Calculations section above. Raw data and data calculations for kit 1, kit 2, and kit 3 are included in APPENDIX B- Chapter 3 Supporting Data below.

Originally, kit 1 and kit 2 data were the only data to be statistically analyzed. CML calculations showed that black pepper, cinnamon, garlic and rosemary had an inhibitory effect, while addition of onion to samples had an oxidative effect, which can be seen in

Table 24 below. However, the variability of the data, particularly in the garlic, onion, and rosemary 2%, and in some of the controls subsamples, would make definitive conclusions very difficult to draw. Kit 3 was then purchased and the garlic, onion, 1% black pepper, 0.5%

and 2% rosemary, and samples that were calculated to be outliers among the controls (R1-Control-3, R2-Control 3, and R3-Control-1) were re analyzed with the objective of improving variability.

Table 24: Average (± standard deviation) mg CML/kg biscuit by spice level kit 1,2; kit 1,2,3 and "Tailored Data". Data with matching letters are not statistically significant (P<0.05)

	Kit 1, 2	Kit 1,2,3	"Tailored Data"
Spice*Level	CML (mg/kg biscuit)	CML (mg/kg biscuit)	CML (mg/kg biscuit)
Control	0.928 ± 0.457	0.665 ± 0.351[a]	0.545 ± 0.232[a]
Black Pepper 0.5%	0.183± 0.099	0.181 ± 0.098[b]	0.180 ± 0.100 [b]
Black Pepper 1%	0.339± 0.291	0.183 ± 0.078[b]	0.192 ± 0.044 [b]
Black Pepper 2%	0.148± 0.035	0.147 ± 0.034[b]	0.147 ± 0.034 [b]
Cinnamon 0.5%	0.140 ± 0.047	0.139 ± 0.047[b]	0.139 ± 0.047 [b]
Cinnamon 1%	0.148± 0.042	0.147 ± 0.042[b]	0.147 ± 0.042 [b]
Cinnamon 2%	0.159± 0.089	0.157 ± 0.088[b]	0.157 ± 0.088 [b]
Garlic 0.5%	0.447 ± 0.430	0.321 ± 0.291[a]	0.184 ± 0.051 [b]
Garlic 1%	0.384 ± 0.384	0.144 ± 0.042[b]	0.146 ± 0.037 [b]
Garlic 2%	0.612± 0.490	0.425 ± 0.402 [a]	0.191 ± 0.039 [b]
Onion 0.5%	1.235± 0.546	0.719 ± 0.642 [a]	0.212 ± 0.068 [b]
Onion 1%	0.889 ± 0.345	0.547 ± 0.420 [a]	0.212 ± 0.064 [b]
Onion 2%	1.071± 0.336	0.644 ± 0.490 [a]	0.226 ± 0.071 [b]
Rosemary 0.5%	0.397± 0.405	0.184 ± 0.091[b]	0.198 ± 0.074 [b]
Rosemary 1%	0.176 ± 0.116	0.174 ± 0.115 [b]	0.176 ± 0.116 [b]
Rosemary 2%	0.351± 0.466	0.149 ± 0.044 [b]	0.176 ± 0.033 [b]
Spike	0.864± 0.307	0.858 ± 0.305[a]	0.858 ± 0.305[c]

The new data were collected and analyzed two ways. Firstly, kit 1, 2, and 3 data were pooled as one data set and analyzed for outliers, which were then removed. The purpose of these analyses was to see if increasing the sample size would result in improved variability. Indeed, some improvement of variability was seen, particularly in the black pepper and rosemary samples (Table 24). However, there is a worsening of variability in onion and 2% garlic samples, even though only 0.5% onion shows an oxidative effect. Control samples showed some improvement in variability, but a reduction in the average CML values is also observed.

Statistical analysis was conducted on the pooled data, by spice and by spice*level. All of the black pepper, cinnamon, and rosemary spice levels showed reduced level of CML compared to the controls, as well as Garlic 1 (P<0.05). These data suggested that cinnamon appears to

have the largest inhibitory effect, followed by rosemary, black pepper, garlic, and onion (decreasing effect). The inhibitory effect of onion is, however, minimal with a CML decrease in of 0.028 mg CML/kg biscuit from onion to the controls.

The trends in the spice*level data are mirrored in the spice alone data. The pooled data does not show an inhibition of CML in onion samples, and each spice has a statistically lower CML value ($P<0.05$) than the spiked samples. The CML values for kit the pooled data do show, however, that onion CML values are higher ($P<0.05$) than the other spices, and that rosemary is statistically higher than cinnamon. A summary of these results can be seen in Table 25.

Table 25: Summary of Average (± standard deviation) mg CML/kg biscuit by spice only of kit 1,2,3 and "Tailored". Data with matching letters are not statistically different ($P<0.05$). Note: These data were only compared between spices only. Control and spike were not included in the analyses.

	Kit 1,2,3	**"Tailored" Data**
Spice	**CML (mg /kg biscuit)**	**CML (mg /kg biscuit)**
Control	0.665 ± 0.351	0.545 ± 0.232
Black Pepper	0.174 ± 0.077 [a,c]	0.174 ± 0.068 [a]
Cinnamon	0.148 ± 0.060 [a,c]	0.148 ± 0.060 [a]
Garlic	0.306 ± 0.312 [a]	0.172 ± 0.046 [a]
Onion	0.637 ± 0.520 [b,c]	0.217 ± 0.065 [a]
Rosemary	0.167 ± 0.080 [a]	0.183 ± 0.077 [a]
Spike	0.858 ± 0.305	0.858 ± 0.305

The second way that the kit 1, 2, and 3 data were analyzed was to replace kit 1 and kit 2, data with new data from kit 3, instead of analyzing the data collectively. Because of the high variability in kit 1 and kit 2, particularly kit 2, it was assumed that human/experimental error occurred, producing some of the variability observed in the data. If this is true, then replacing those data should improve variability.

Once the kit 3 data replaced kits 1 and 2 data, the "tailored" data set were tested for outliers and statistics were performed according to the Statistical Analysis section above. Improved variability can be seen across the board when the data are evaluated by spice or by spice and level, as seen in Figure 22 and Figure 23 below. These data also show slightly different trends than when the data are analyzed collectively. These data show that every spice at every spice level significantly ($P<0.05$) reduced the mg CML/kg biscuit. As with the collective

data, cinnamon produces the largest inhibitory effect, followed by garlic, black pepper, rosemary, and onion in descending order. However, the tailored data show that the onion inhibitory effect is much greater than in the collective data set, decreasing the mg CML/kg biscuit by 0.329 mg/kg.

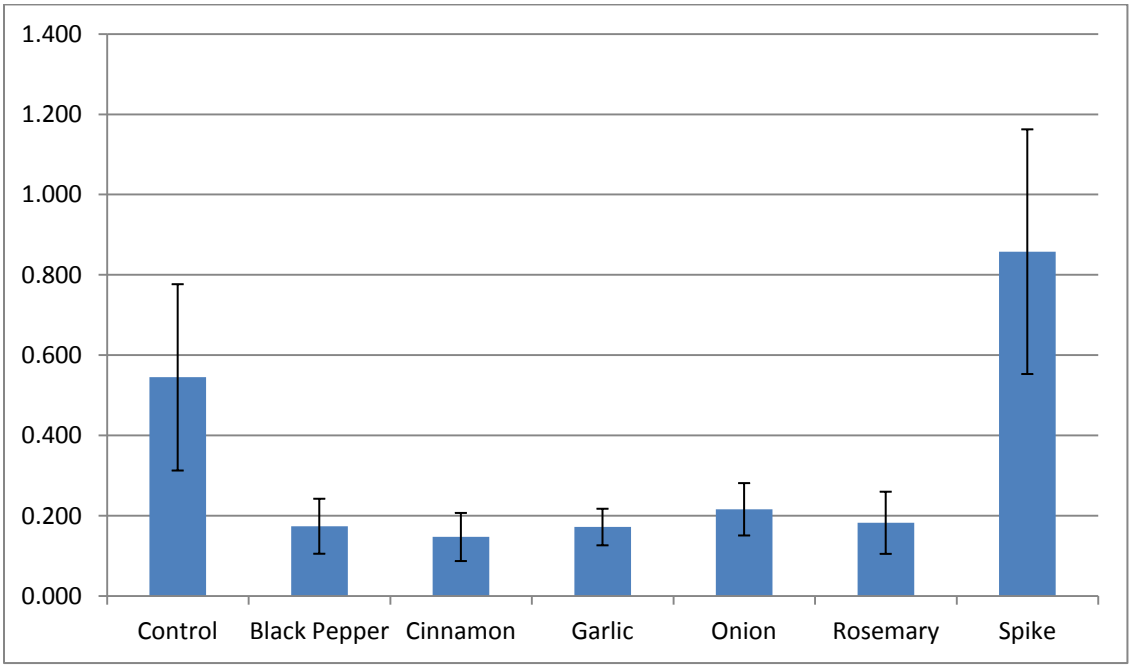

Figure 22: Average mg CML/kg biscuit by spice alone in the "tailored" data. All spices are statistically lower (P<0.05) than the control biscuits. Error bars represent ± standard deviation of the data.

These data also show slightly different trends than when the data are analyzed collectively. These data show that every spice at every spice level will significantly (P<0.05) reduced the mg CML/kg biscuit. As with the collective data, cinnamon produced the largest inhibitory effect, followed by garlic, black pepper, rosemary, and onion in descending order. However, the tailored data show an onion inhibitory effect is much greater than in the collective data set, decreasing the mg CML/kg biscuit by 0.329 mg/kg. The largest was determined to be in the 0.5% cinnamon spice level, although these data are not statistically different from that of other spices or spice levels.

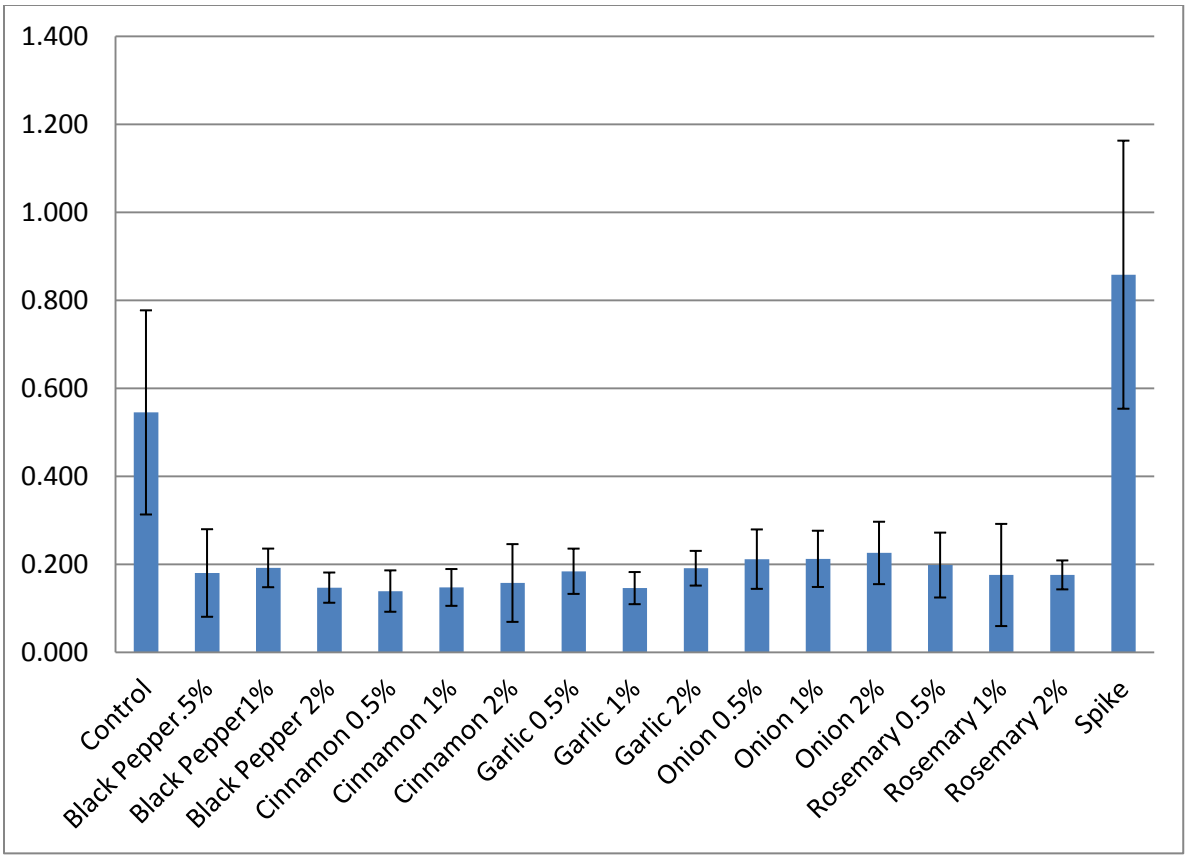

Figure 23: Average mg CML/kg biscuit by spice and level. All spice*level combinations are statistically lower (P<0.05) than the control and spiked samples. Error bars represent the ± standard deviation of the data.

Discussion

When considering the mass loss data, only the R2-Oni-0.5% batch lost more mass that the average. However, when comparing this mass loss to the CML data, no additional CML was formed, suggesting that this mass loss was not different than other batches. Some increases are seen in the L, and total color values (data not shown), suggesting that the color was more intense in this batch than the other onion batches with the same spice level, but again, not in such a way that differences in CML values were observed.

Interesting trends were also seen in the chroma values. The color saturation decreased as the spice level increased, and each spice level showed statistical significance. Studies have shown that addition of antioxidants to foods can help stabilize the color of breads and beef during storage (Bajaj and others 2006; Allen and Cornforth 2010). But these trends are slightly counter-intuitive, especially among cinnamon and rosemary spices which impart their own color

to the dough. It is possible that addition of the spices to the bread dough, may act to "muddy" the brown color of the biscuit, as exemplified in the change of both of the a* and b* values of the black pepper, rosemary, and cinnamon spices from the controls. The simplest explanation is that the biscuits tend to be a medium orange-red brown, and that the colors of these spices tend to make the brown a more "cool brown," especially in rosemary which tends to be a cool green. Cinnamon spice is also a cool dark brown, so the decrease that can be seen in the a* and b* values could be due to the cool nature of the red when it is added to the dough. With black pepper it is possible that the grey color would, instead of changing the levels of blue or green, increase the "level of grey" or dull the surface of the biscuit.

It is also possible that the levels of CML could affect color saturation, and that as the levels of CML decrease, the color saturation decreases. In addition, the Maillard reaction is so complex that addition of the spices could shift the amounts and composition of the late stage melanoidins, which could account for the change in chroma with the spice level. This might also account for the trend in the chroma data where the spiked samples have the most saturated or intense brown color, and from there the chroma values decrease because CML formation is inhibited more as the spice level increase. With the current data, these trends are not completely supported, because the inhibition of CML is not different from 0.5% to 2% levels of spice. However, future work could try to verify this trend.

Looking at the average mg CML/kg biscuit levels in the data, particularly the tailored data, addition of spices show a clear inhibitory effect on CML formation in the biscuits. This could either be due to the antioxidant capacity of the spices acting to stabilize free radicals produced during browning or possibly by acting as a hydrogen donor to prevent/inhibit the tautomerization and dehydration of the Maillard browning products in the mechanism proposed by Kasper and Schieberle (Kasper and Schieberle 2005). Cinnamon showed the highest inhibition followed by rosemary, black pepper, then onion and garlic. Cinnamon contains cinnamic acid and coumarin; and, rosemary contains carnosic acid, rosemarinic acid and carnosol (Omri and others 2012; Wang and others 2012). Garlic contains allyl-sulfides (Iciek and others 2011), and onion contains quercetin (Khiari and Makris 2012) All of these compounds have antioxidant activity that can inhibit Maillard browning.

Black pepper showing an inhibition is interesting, because its free radical scavenging activity is relatively low compared to other spices (Kim and others 2012). Yet in the data reported here, CML was inhibited by black pepper, and showed a more protective effect than did garlic and onion, in the case of the collective data. This is supported by studies that have shown black pepper to inhibit other carcinogenic Maillard browning products in meat, specifically heterocyclic amines (Oz and Kaya 2011a). In addition, mouse studies have shown that piperine, a compound in black pepper, has effects in the body similar to other AGE inhibitors, suggesting that piperine may have antioxidant type effects (Sabina and others 2010). Therefore, despite the low radical scavenging activity, black pepper can inhibit Maillard browning.

One drawback to the data collected here, is that these values are lower than literature values reported by other studies. A summary of average mg CML/ kg food values for other studies in bread and pastry items can be seen in Table 26. These data range from 1.16 mg/kg in sponge cake to 47.42 mg/kg in soda bread, which are much larger than the 0.047-0.3046 mg/kg seen in the data.

Experimental error, here could be the source of the difference. Spice distribution in throughout the dough could be one source of error, because homogeneity was determined by visual inspection alone. Future work could use a different means of verification of spice distribution. The extremeness of the pH coupled with the time needed for neutralization could decrease the recovery of CML from the food system. It is also possible that any free lysine, and therefore non protein-bound fractions of CML could have been lost during defatting and protein precipitation. Loss of pigmentation in the solids was observed during the first addition of chloroform/methanol to the sodium borohydride solution. After centrifugation, the sodium borohydride (top) layer was yellowed compared to the chloroform layer. It is possible that melanoidins, and non-protein bound CML could have been preferentially separated into the aqueous borohydride layer, and lost when the borohydride layer was removed. Future work could investigate the differences in CML levels when chloroform defatting is performed prior borohydride reduction versus borohydride reduction followed by defatting.

Table 26: Summary of mg CML/ kg food values in other bread/cereal studies.

Food	CML mg/ kg food	Reference
White bread crust	37.1	(Assar and others 2009)
Wholemeal bread crust	46.1	(Assar and others 2009)
White bread crumb	2.58	(Assar and others 2009)
Wholemeal bread crumb	4.45	(Assar and others 2009)
Breads and savory biscuits	12.9	(Hull and others 2012)
Cereals	25.5	(Hull and others 2012)
Sweets and snacks	18.1	(Hull and others 2012)
White bread	6.6	(Hull and others 2012)
Wholemeal bread	14	(Hull and others 2012)
Water biscuit	17.6	(Hull and others 2012)
Brown rolls, crusty	5.2	(Hull and others 2012)
Brown rolls, soft	4.5	(Hull and others 2012)
Soda bread	47.42	(Hull and others 2012)
Blank bread crust	35	(Peng and others 2010a)
Sponge cake with refined sucrose	1.16	(Srey and others 2010)

Another source of CML loss could be due to the use of non-silanized glassware. As discussed in Chapter 2 above, use of silanized glassware can improve the recovery of CML in the samples, because the nitrogen and hydroxyl groups in the CML are capable of adsorbing to the glassware. In this study silanized glassware was not used because of the acid digestion, with the pH of the solution being too extreme, and capable of degrading silanization on glassware. However, recovery of CML could be improved upon, possibly by using an enzyme digestion, at a more moderate pH.

The overall trend, that addition of spices in biscuits inhibits CML formation, is evident in both the pooled and tailored data. However, the large losses of CML through adsorption onto the glassware may suppress trends in the data, especially spice level dependent trends. For example, if 0.5% cinnamon inhibited CML formation better than 1% cinnamon, it may be important from a product development perspective because that would potentially affect the cost.

Conclusions and Future Work

The data collected here, show that the addition of spices to a biscuit matrix will reduce the levels of CML Maillard browning products produced. Cinnamon produced the greatest reduction, followed by rosemary and black pepper. The L, a*, b* color data showed that color

saturation decreased as the spice level increased. This could be due either to changes in the Maillard browning profile, or to the color of the spices themselves. Reported levels of CML reported here were lower than literature values. This could be due to adhesion of CML to the glassware, the time effect of the neutralization step, or the preferential extraction of Maillard products into the borohydride layer during the chloroform extractions.

Future work could focus on repeating the procedure to increase the sample size of each spice and level, to determine if a 0.5% spice level is more or less effective than a 1% or 2% level. A smaller level, such as 0.25% could also be used to see if there is a threshold level that needs to be reached to see an inhibitory effect. Furthermore, future work could focus on determining if the CML levels can be correlated with the chroma values of the biscuits. Other work could focus on determining if CML levels are increased if the borohydride reduction of the reducing sugars is performed after the chloroform defatting, by decreasing potential loss through preferential extraction to the aqueous borohydride layer. Future work could also repeat these procedures with a less harsh digestion of the proteins, so silanized glassware could be used, to see if CML levels in the biscuits would be found closer to reported literature values.

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APPENDIX A- Sample Calculations

Sample calculation of mass CML recovered from PRS SPE

Below is a sample calculation for the determination of the mass recovered on the elution step from replicate 1 of the PRS extractions.

The signal from the Biotek microplate reader for that 1E sample was 0.913, as recorded by the data collection software. The standard curve equation is:

$$\mathbf{y = -0.2313x^2 + 6.4983x + 0.306} \qquad \textbf{Equation 16}$$

as determined in a previous experiment, where y is the signal produced by the software and x is the concentration in parts per billion (ppb). Using this equation, and the recorded signal, the concentration was calculated as follows:

$$\boldsymbol{x = \frac{-6.4983+\sqrt{6.4983^2-[4*(-0.2313)*(0.306-0.913)]}}{2*(-0.2313)}} \qquad \textbf{Equation 17}$$

$$\boldsymbol{x = 0.0937\ ppb}$$

Next, to determine the mass in the 10 mL that were analyzed using the ELISA kit, the concentration was converted to mass in the following way:

$$\boldsymbol{\frac{0.0937 ng\ CML}{1 mL} * 10 mL = 0.937\ ng\ CML} \qquad \textbf{Equation 18}$$

To account for the fact that the 10 mL was only 1/50th of the original 50 mL of the reconstituted sample, the mass was multiplied by a unitless dilution factor (D.F.) of 50 in the following way:

$$\boldsymbol{0.937 ng\ CML * 50\ (D.F.) = 46.86\ ng\ CML} \qquad \textbf{Equation 19}$$

These calculations were repeated for every replicate of every fraction, except for the 100 ppb standard. For those replicates, an additional correction factor of 5/2, was introduced, because 5 mL of standard were loaded onto the column, while only 2 mL of standard were diluted and tested on the ELISA kit.

Determine of outliers of SPE Extraction of CML using C-18 cartridges, Diluted 1:399

The following tables are the Excel data used to determine outliers in the data. Table 27 below used equation 1 and the previously determined quadratic coefficients to convert the absorbencies to concentration in ppb. To convert to ng, the concentration was multiplied by a correction factor of 400, to account for the dilutions and the 1 mL reconstituted volume.

Table 27: Table converting absorbance to ng.

	Absorbance from data collection			Concentration in ppb			Mass in ng of each fraction		
Sample	Read 1	Read 2	Read 3	Read 1	Read 2	Read 3	Read 1	Read 2	Read 3
std 1	7.809	8.022	7.349	1.206	1.242	1.129	482.6	496.9	451.7
std 2	5.964	5.978	5.794	0.899	0.901	0.872	359.8	360.7	348.6
E1	2.076	2.084	2.092	0.275	0.276	0.278	110.0	110.5	111.0
E2	8.294	8.308	8.011	1.288	1.291	1.240	515.3	516.3	496.2
E3	4.336	4.362	4.300	0.634	0.639	0.627	253.8	255.5	251.5
L1	3.906	3.483	3.229	0.565	0.498	0.457	226.1	199.1	182.9
L2	3.671	3.655	3.63	0.528	0.525	0.521	211.1	210.1	208.5
L3	2.986	3.052	2.993	0.419	0.429	0.420	167.5	171.7	167.9

Table 28 below used the masses from Table 27 and calculated the experimental error, ehatij, for each data point collected. The experimental error was then used to calculate the MSE value, which was then used to calculate the standardized residual.

Table 28: Table used to calculate MSE. MSE is later used to determine the standardized residuals.

	ehatij for outlier test			ehatij^2				
Sample	Read 1	Read 2	Read 3	Read 1	Read 2	Read 3	SSE	MSE
std 1	5.506	19.870	-25.376	30.321	394.797	643.941	2394.138	114.007
std 2	3.416	4.337	-7.752	11.668	18.806	60.101		
E1	-0.502	0.000	0.502	0.252	0.000	0.252		
E2	6.065	7.014	-13.078	36.781	49.190	171.042		
E3	0.215	1.891	-2.106	0.046	3.576	4.434		
L1	23.436	-3.626	-19.811	549.248	13.144	392.457		
L2	1.215	0.192	-1.407	1.476	0.037	1.979		
L3	-1.544	2.645	-1.100	2.385	6.994	1.211		

Table 29 below lists the standardized residuals for each data point, as calculated using Equation 3. The average and standard deviation of the standardized residuals are -4.86E-17 and 0.956, respectively. To be considered an outlier, a standardized residual would have to be beyond two standard deviations of the standardized residual average; i.e. > 1.91 or <-1.91. Of the standardized residuals, only one outlier was found, Read 1/L1. This datum point was then excluded from calculations when the percent recovery was calculated.

Table 29: Table of standardized residuals for each data point. Read 1/L1 is highlighted because it is the only residual that falls beyond two standard deviations from the average residual.

	Standardized residuals		
Sample	Read 1	Read 2	Read 3
std 1	0.516	1.861	-2.377
std 2	0.320	0.406	-0.726
E1	-0.047	0.000	0.047
E2	0.568	0.657	-1.225
E3	0.020	0.177	-0.197
L1	2.195	-0.340	-1.855
L2	0.114	0.018	-0.132
L3	-0.145	0.248	-0.103