FORMATION OF 4(5)-METHYLIMIDAZOLE IN PROCESSED MEAT AND IN GLUCOSE AMINO ACID MODEL SYSTEM

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

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ABSTRACT

The International Agency for Research on Cancer (IARC) has classified 4(5)-methylimidazole (4-MeI) as a possible human carcinogen. Also, the National Toxicology Program (NTP) has concluded that 4-MeI is a probable cancer causing agent. Even though 4-MeI has been detected in several processed foods no research has been conducted to measure levels of 4-MeI in processed meat. A method using isobutylchloroformate (IBCF) and gas chromatography mass-spectrometry (GC-MS) was developed to detect and quantify 4-MeI in processed meat products. Levels of 4-MeI ranged from 0.04 to 1.01 μg/g with recovery of 94.76 to 103.94%.

Formation of 4-MeI in glucose-amino acid model system was studied. D-Glucose (Glu) was mixed individually in equimolar concentrations of 0.05, 0.1, or 0.15 M and equal volume with L-Alanine (Ala), L-Arginine (Arg), Glycine (Gly), L-Lysine (Lys), and L-Serine (Ser); and the mixtures were heat treated at 60, 120, and 160°C for 1 h. Among all tested amino acids, Glu-Arg produced the highest level of 4-MeI. The Glu-Lys model system showed higher browning and lower concentrations of 4-MeI.

The effect of ascorbic acid (ASA), a strong antioxidant, was studied in the Glu-Arg model system. Four concentrations of ASA (0.0375, 0.075, 0.15, and 0.3 M) were mixed with 0.15 M equimolar of Glu-Arg and heat treated at 160°C for 1h. ASA inhibited formation of 4-MeI by 40.29, 69.94, 75.13, and 96.25% for the 0.0375, 0.075, 0.15, and 0.3 M concentrations. Contrarily, ascorbic acids increased the browning in all treatments compared to control treatment by10.67, 15.47, 18.4, and 28.8% for the 0.0375, 0.075, 0.15, and 0.3 M concentrations. Adding ASA to processed food may reduce formation of 4-MeI and increase the browning that is a desired attribute to processed meat products.

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Approved by:
Major Professor
J. Scott Smith

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DEDICATION

I would like to dedicate my work to my beloved wife Dr. Zainab and my daughters Rafa and Jena. Thank you for being my guardian angles and there for me throughout the doctoral program, without it I could not have managed to be a doctor.

CHAPTER 1. REVIEW OF LITERATURE

INTRODUCTION

Heat treatment of foods is desirable because it helps develop color, flavor, aroma, and texture; however, despite these benefits, applying heat to foods also produces some undesirable compounds like polycyclic aromatic hydrocarbon (PAHs), furan, aromatic amines, 4(5)-methylimidazole (4-MeI), and acrylamide, all of which can cause adverse health effects. Some of these compounds have even been categorized as cancer causing agents.

Food colors are important to consumers, guiding them in choosing food and finding that food acceptable. Colors have been used extensively in different food products like dairy, meat, bakery, and beverages. Among different colorants used in the food industry, caramel colors account for more than 80% (by weight) of all colorant added to food products (Kamuf and others 2003). Caramel colors are highly specific food additives used to improve the flavor and texture of food products and to enhance the colloidal system by working as an emulsifying agent. They are the oldest and the most widely used colorants in food (Chappel and Howell 1992).

Caramelization is the process by which caramel color is developed through the reaction of carbohydrates and a nitrogen containing compound like ammonia or amino acids. Commercially, caramel colors are made by heat treatment of food grade carbohydrates (glucose) using a catalyst (acids, alkalis, or salt). Depending on which reactants are used in manufacturing caramel colors, the colors are classified into four types: caramel color I, II, III, and IV. Caramel colors III and IV have attracted the attention of the scientific community and regulatory agencies because of a safety concern with 4-MeI. In 2007, the National Toxicology Program of the U.S. Department of Health and Human Services (NTP 2007) performed a study on 4-MeI in rats and mice; their results showed that 4-MeI is carcinogenic. 4-MeI is usually the main imidazole

compound found in these two types of caramels. 4-MeI is formed in caramel III and IV due to the reaction of dextrose and ammonia (caramel III) or dextrose and ammonium sulfite (caramel IV).

Formation of 4-MeI during Maillard reaction from glucose and ammonia was first reported in the early 1960s (Komoto 1962). 4-MeI forms in thermally processed foods because of the reaction of carbohydrates and amino acids naturally found in foods. As an illustration, carbohydrates degrade upon heat treatment to dicarbonyl (4-MeI precursor) and alkyl ketones (Hodge 1967), and amino acids form alkyl carbonyls and ammonia via Strecker degradation (Strecker 1862). Therefore, 4-MeI forms from the reaction of dicarbonyl and ammonia. Moreover, 4-MeI can actually be added to foods when caramel color III or IV are added. Total elimination of 4-MeI from processed food is difficult; however, reducing its levels in caramel III color and IV by investigating new manufacturing strategies is achievable.

4-MeI CHARACTERISTICS

Physical and chemicals properties

At room temperature, 4-MeI (CAS number 822-36-6) is a light yellow solid crystal with a relative molecular mass of 82.11, boiling point of 263°C, and a flash point of 157°C (IARC 2012). Its melting point is between 46 - 48°C, and its vapor pressure is 0.007 mm Hg at 25°C. It is soluble in water and most polar organic solvents like ethanol and chloroform.

4-MeI ($C_4H_6N_2$) is a polar basic heterocyclic organic compound. Its basic nature relates to the ability of the nitrogen in the pyridine ring to accept a proton, and the electron-releasing property of the methyl group tends to increase the electron density around the pyridine nitrogen (Hofmann 1953).

The imidazole ring in 4-MeI is amphoteric and can function as an acid or base. The acidic site is on nitrogen atom number one while the basic site is on nitrogen numbers three (see Figure 1).

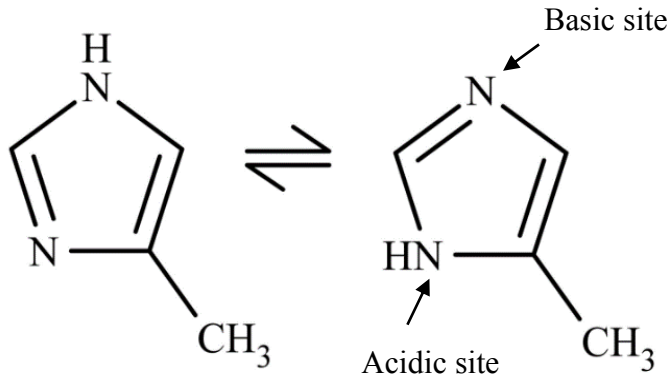

Figure 1. Tautomerism structures of 4-MeI molecule.

Different basic pK_a values for 4-MeI have been reported in the literatures. Hofmann (1953) and Klejdus and others (2006) reported a pK_a value of 7.52. A value of 7.6 was reported by Klejdus and others (2003). Petruci and others (2013) and Moretton and others (2011) noted a value for pK_a of 7.7 for 4-MeI. Both the basic pK_a (7.56) and the acidic pK_a (15.10) were reported by Grimmett (1980). The low vapor pressure of 4-MeI (0.007 mm Hg at 25°C) dictates it should not exist in the atmosphere.

PRODUCTION AND USE

Radzisewski (1882) synthesized 4-MeI from the reaction of formaldehyde and glyoxal with ammonia. In the middle of the 19th century, 4-MeI, an alkylimidazole was synthesized from the reaction of ammonia with either methyl alkyl ketones or α-dicarbonyl compounds (Debus

1858); this method has a low yield, but it is suitable for creating C-substituted imidazole (Moon and Shibamoto 2011). Moon and Shibamoto (2011) produced 4-MeI using a heated aqueous solution of ammonium hydroxide, methylglyoxal, and formaldehyde. 4-MeI can also be produced by photolysis of alkenyltetrazole derived from alkenes by sequential epoxidation, ring opening and dehydration (NTP 2007). Commercially, 4-MeI is produced by cyclocondensation of aldehyde and ammonia with methylglyoxal (Chan and others 2008).

4-MeI is widely used as a raw or intermediate material in pharmaceuticals, photographic chemicals, agricultural chemicals, dyes and pigments, cleaning products, and rubber products (OEHHA 2012). Specifically, 4-MeI is used as a raw material in synthesizing cardiovascular stimulants, disinfectants, aromatase inhibitors, epoxy resin anticholesteraemics, and neurotransmitter antagonists (NTP 2007). In addition, 4-MeI is a part of the imidazolephenoxyalkanal compound, which is used as a crosslinking agent for epoxy resin hardeners and corrosion inhibitors for cooling water in heat-exchange apparatus (Chan and others 2008).

4-MeI FORMATION

The Maillard reaction is the most studied non-enzymatic reaction occurring in processed foods, the reaction in which food flavor, color, and aroma are developed when reducing sugar reacts with amino acids in the presence of heat. In other words, the Maillard reaction occurs during condensation between the carbonyl compounds from reducing sugar and the amino acids of proteins (Nursten 2005).

4-MeI was first synthesized from an α-carbonyl compound and methyl alkyl ketones with ammonia (Debus 1858) and described in the Maillard model system representing caramel color

III in 1962 (Komoto 1962). Windaus and Knoop (1905) formed 4-MeI with a yield of 24% from a reaction of D-glucose, ammonia, and zinc hydroxide at room temperature for six weeks; 4-MeI was precipitated first as a zinc salt, and after applying hydrogen sulfite, precipitated later as an oxalate (see Figure 2).

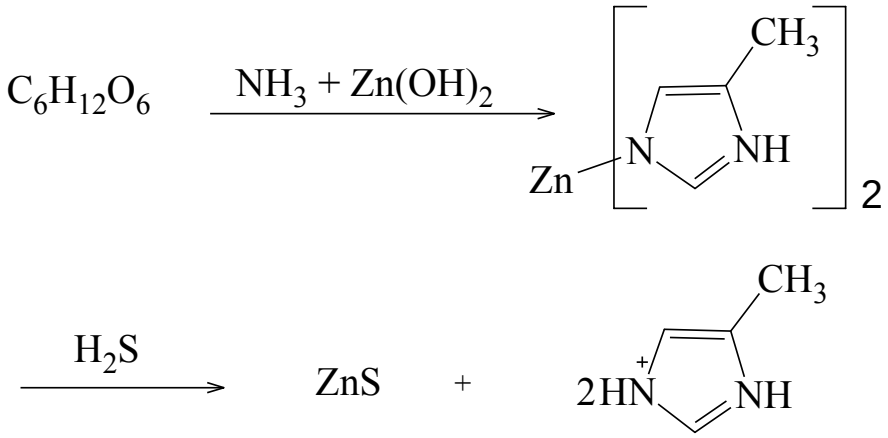

Figure 2. Formation of 4-MeI from zinc hydroxide as proposed by Windaus and Knoop (1905) (adapted from Kozlov and Vovk 1999).

In the past, imidazoles, including 4-MeI, lacked valuable flavors and produced certain off-flavors in cooked food and so did not attract much attention (Arctander 1969). Later, animal toxicity of 4-MeI was reported by Weiss and others (1986) and Nielsen and others (1993).

In 2007, the National Toxicology program reported 4-MeI as a suspected cancer causing agent. Because 4-MeI was widely found in foods and beverages, research and the regulatory agencies started to focus more on the formation mechanism, levels, enhancing factors, and reduction.

Both Seo and others (2014) and Moon and Shibamoto (2011) proposed formation of 4-MeI from the ammonolysis of methylglyoxal, but Moon and Shibamoto (2011) provided more detailed information on the formation pathway of 4-MeI (see Figure 3).

Moon and Shibamoto (2011) suggested that formamide did not form directly from formaldehyde with ammonia to form 4-MeI; ammonolysis of methylglyoxal produced formamide, which then reacted with 2-amino propanal to give 4- or 5-MeI.

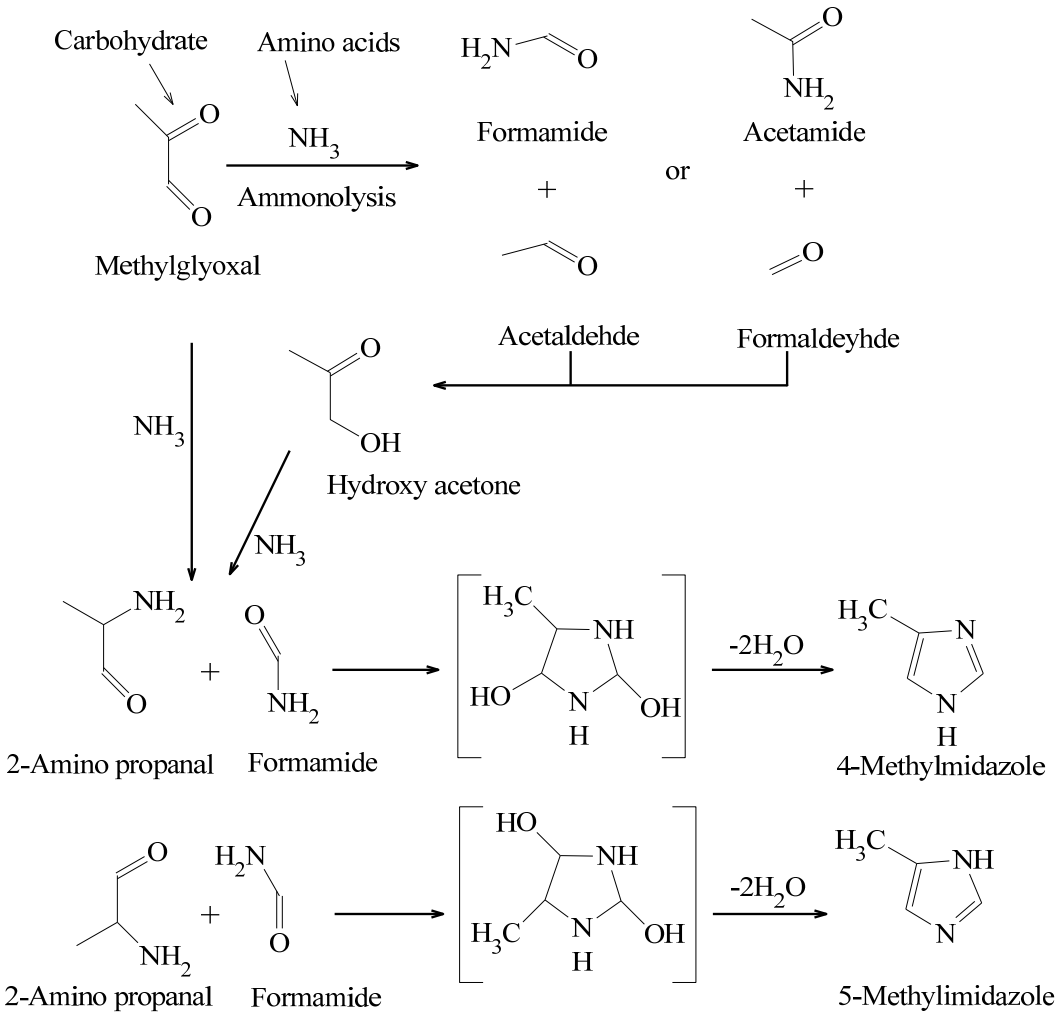

Figure 3. Formation mechanisms of 4-MeI (modified from Moon and Shibamoto 2011).

FACTORS AFFECTING 4-MeI FORMATION

Time and temperature

Both Jang and others (2013) and Seo and others (2014) reported levels of 1269 and 556 μg/g of 4-MeI in a 1 M 1:1 glucose/ammonium hydroxide Maillard model system; Jang and others (2013) applied a temperature of 150°C for 2 h while Seo and others (2014) applied 100°C for 2 h. Different in 4-MeI levels may be due to the applied temperature. In a D-glucose ammonium hydroxide Maillard model system, Moon and Shibamoto (2001) found neither temperature of heating time had significant effects on of 4-MeI formation when temperatures of 70,100, and 120°C were applied for 3, 6, or 12 h. In contrast, increasing the temperature by another 50°C increased level of 4-MeI by 2.3%.

Moisture

No data are available in the literature on the effects of water content on 4-MeI formation. In the Maillard reaction the rate of formation depends on how much free water is available. In foods with high a_w, the Maillard reaction is slow, possibly because the reactant is diluted. Moreover, in low a_w foods, mobility of the reactants is limited. Therefore, the products of the Maillard reaction are low even though the reactants concentration is higher. The best outcomes occur at intermediate a_w values. Wolfrom and Rooney (1953) showed maximum browning in the Maillard reaction occurs at 30% moisture (0.6-0.8 a_w).

Reactant concentrations

As more primary reactants like carbohydrates and compounds that contain nitrogen are present, more 4-MeI is formed. Lee and others (2013) stated that 4-MeI formation was dose

dependent on the reactants. In their results, levels of 4-MeI were 100.22, 69.78, and 7.18 μg/g in a 1.0, 0.5, and 0.1 M D-glucose/ammonium hydroxide system. In another study, D-glucose and ammonium hydroxide were mixed in equal portions (1, 0.5, and 0.1 M) and heat treated for 2 h at 150°C; levels of 4-MeI were incrassated according to reactants concentrations, the reported levels of 4-MeI were 28.56 μg/g (0.1 M), 666.69 μg/g (0.5), and 1269.71 μg/g (1.0 M) (Jang and others 2013).

Presence of other compounds

Along with carbohydrates and amino acids, lipids in food are another source of carbonyl compounds. Lipids produce many low molecular weight carbonyl compounds, including α-carbonyl compounds, upon oxidative degradation (Frankel 1982). Jiang and others (2013) used head space and GC-MS technique to demonstrate that, upon heat treatment of butter, margarine, or safflower oil, glyoxal, methylglyoxal, and diacetyl were formed.

Amino acids types

No data are available in the literature on the effect of type of amino acids on 4-MeI formation. Type of amino acid directly effects directly formation of 4-MeI in the Maillard reaction. In an experiment conducted by our group, aqueous solutions of Alanine (Ala), Arginine (Arg), Glycine (Gly), Lysine (Lys), and Serine (Ser) were individually mixed with aqueous solutions of D-glucose and heated to 60, 120, and 160°C for 1 h. Arg formed the highest amount of 4-MeI (1.0 μg/g), followed by Lys (0.23 μg/g), Ala (0.21 μg/g), Ser (0.16 μg/g), and Gly (0.15 μg/g), indicating that type of amino acid is important in forming 4-MeI.

Sugar types

Sugar type and sugar degradation rate directly effects formation of 4-MeI. In the Maillard model systems of D-glucose/ammonia and L-rhamnose/ammonia, the level of 4-MeI was slightly higher in the L-rhamnose/ammonia model (0.91mg/mL) than the D-glucose/ammonia model (0.71 mg/mL) (Moon and Shibamoto 2011). Shibamoto and Bernhard (1978) found that, L-rhamnose degrades more readily to yield precursors like formaldehyde, acetaldehyde, glyoxal, methylglyoxal than D-glucose. In addition, D-fructose, L-sorbose, D-xylose, L-arabinose, and D-mannose formed more 4-MeI than lactose, D-galactose, and maltose in a zinc hydroxide ammonia model system held at room temperature for 6 weeks (Windaus 1907; Inouye 1907).

In testing whether mono or polysaccharides can affect formation of 4-MeI, Klinger and others (2013) used D-glucose, D-xylose, cellulose, and xylan reacting with ammonia at different temperatures, to find that monosaccharides produced more 4-MeI than polysaccharides.

pH

Wong and Bernhard (1988) noticed the pH was reduced by three units in D-glucose/ammonium hydroxide, D-glucose/ammonium formate, D-glucose/ammonium acetate, D-glucose/glycine, and D-glucose/glutamate model systems. However, they did not report any relationship between pH and 4-MeI formation. No 4-MeI was detected in the glucose/ammonium hydroxide system according to their report. In general, the rate and the tendency of browning increases in the Maillard reaction as pH increases (Coca and others 2004). Increasing the pH increases the reactivity of amino acids due to acid base equilibrium (Bostan and Boyacıoglu 1997). In addition, pH plays an important role in formation of the end products types of Maillard reaction. Enolization of the Amadori product which is an intermediate compound in Maillard

reaction at pH 7 or below produces hydroxymethylfurfural (HMF). However, at pH above 7, the degradation of Amadori product occur due to 2,3 enolisation and result in formation of 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one (HMF^{one}) (Martins and others 2001).

4-MeI FORMATION MECHANISMS

Heinrich Debus and Bronislaw Leonard Radziszewski first proposed the alkylimidazoles (including 4-MeI) synthesis mechanism in 1858 that later become known as Debus-Radziszewski synthesis. Figure 4 shows the mechanisms of that synthesis.

In this figure, methylglyoxal, which is an α-dicarbonyl compound, reacts with ammonia by ammonolysis to form two intermediates: propane-1,2diimine and formaldehyde. The reaction between these compounds and the rearrangement of the chemical bonds lead to form (Z)-(holmiomethyl)(2-iminopropylidene)amine, and another rearrangement of the chemical bond gives a 4-methy-2H-imidazole. After chemical bonds are rearranged, 4-MeI is formed. Yield in this process is low, but the process is still used to synthesis C-substituted imidazoles (Moon and Shibamoto 2011).

According to the proposed Debus-Radziszewski synthesis procedure, 4-MeI forms in caramel colors III and IV when ammonia is added as a catalyst in the present of sugar and heat. In the caramelization process; sugars or lipids degrade upon heating into alkyl dicarbonyls and alkyl ketones (Moon and Shibamoto 2011) and amino acids or proteins into ammonia and alkyl carbonyls via Strecker degradation (Strecker 1862).

In Yaylayan and Haffenden (2003) 13C-2-labeled amino acids study, the α-amino carbonyl moiety from reaction between α -dicarbonyl compounds or α-hydroxycarbonyl compounds with amino acid or ammonia was a very important reactive intermediate in forming imidazoles.

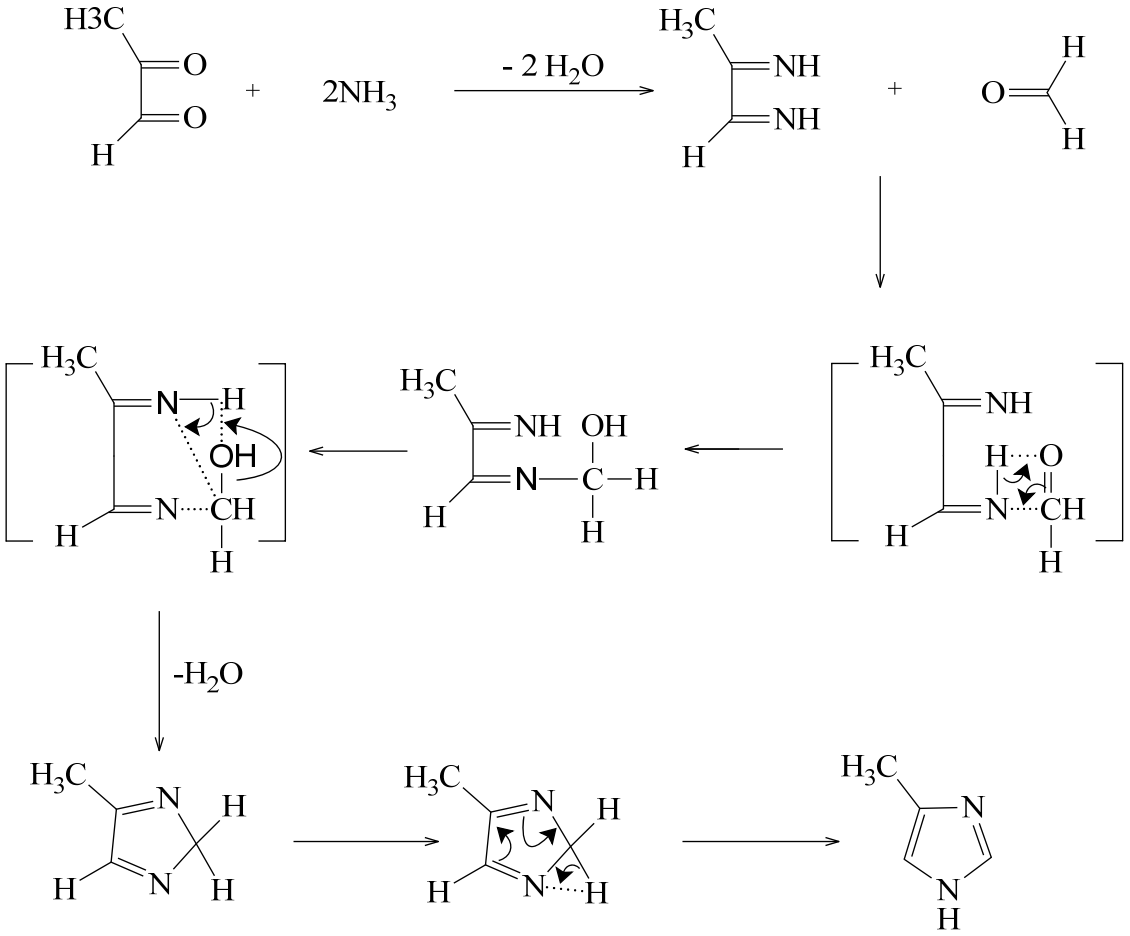

Figure 4. Debus-Radziszewski 4-MeI formation mechanism (modified from Hengel and Shibamoto 2013).

TOXICITY

Animal Toxicity

Inhaling 4-MeI vapor causes severe respiratory system irritation, and contact with 4-MeI can cause eye and skin burns. Nausea and vomiting may result if 4-MeI is ingested (Yuan and Burka 1995).

Animal studies have established the LD50 (dose required to kill half of the tested population within a certain time), LD100 (dose required to kill all the tested population within a certain time), and the CD50 (dose required to produce convulsions in half the tested population within a certain time). Nishie and others (1969) reported the LD50 of 4-MeI in different animals when 4-MeI was administered orally and intraperitoneally. For mice, doses were 370 mg/kg orally and 165 mg/kg intraperitoneally; for rabbits intraperitoneally the dose was 120 mg/kg; chicken was 210 mg/kg intraperitoneal and 590 mg/kg and orally. Hidaka (1976a) mentioned an LD50 oral dose of 173 mg/kg in rats. An LD100 of 4-MeI (500 mg/kg by weight) was noted in Bonfiglio (1988), and a CD50 value of 155 mg/kg by weight was reported by Nishie and others (1969).

In general, the toxicity symptoms of 4-MeI are neurological; low doses of 4-MeI caused convulsive effects like restlessness, bellowing, frothing at the mouth, and paralysis (Wiggins 1956) while death may occur at high doses. Other symptoms noted by Weiss and others (1986), were facial twitching, whole body tremors, and opisthotonos. Nielsen and others (1993) reported coughing, urination or defecation, and salivation as the toxicity effects of 4-MeI. Moreover, hypertrophy of the liver was also described by Hidaka (1976b).

Sivertsen and others (2009) investigated the convulsive effect of 4-MeI in mice; concentrations of 2 mM or more of 4-MeI inhibited the activity of the cerebral glutamate

decarboxylase (GAD) in brain tissue from mice B6D2. At concentrations of 5 mM to 50 mM, 4-MeI prevented binding of [^{3}H]GABA to cerebral GABA receptors. The convulsive effects of 4-MeI couldn't ultimately be excluded, suggesting that the convulsions could be related to the effect of 4-MeI on other receptor systems in the mammalian brain. In rabbit kidneys, 4-MeI stimulated phosphorylation (Na+ and K+)-ATPase (Schuurmans, Stekhoven and others 1988). In the liver of male Wistar rats, 4-MeI inhibits cytochrome P450, the enzyme that oxidizes toxic molecules in adult (Back and Tjia 1985).

Human Toxicity

Di Minno and others (1982) found that 4-MeI selectively inhibits thromboxane synthetase but not platelet-fibrin clot retraction induced by arachidonic acid *in vitro*. In contrast, 4-MeI did not significantly affect human platelet aggregations (Horton and others 1983). Back and others (1988) noted 4-MeI strongly inhibits human liver microsomal cytochrome P450. Wang and Schnute (2012) pointed out that exposure to 4-MeI should be thoroughly investigated and regulated in sensitive populations whose health may be susceptible to excessive exposure.

4-MeI CARCINOGENICITY

Because most human exposure to 4-MeI occurs throughout ingestion, 4-MeI carcinogenicity effects have been studied both *in vitro* and *in vivo* using animals as study subjects.

Chan and others (2005) fed male and female Fischer rats and B6C3F1 mice diets containing 4-MeI levels of 300, 800, or 2,500 μg/g for 15 days or levels of 625, 1,250, 2,500, 5,000, or 10,000 μg/g for 98 days. No change in biological activity was observed in the 15 day

studies. However, in the 98 day studies, 4-MeI caused tremors, ataxia, anemia, hepatocytic vacuolation, testicular degeneration, prostatic atrophy, liver cytoplasmic vacuolization, and renal degeneration and dilation.

Chan and others (2008) fed diets containing 4-MeI to both rats and mice. Rats (F344/N; 50 male and 50 female) were exposed to 625, 1,250, or 2,500 μg/g during a 2 year period. The average daily doses for males were 30, 55, or 115 mg 4-MeI per kg of body weight and 60, 120, or 250 mg 4-MeI per kg of body weight for females. Compared to control, Chan and others (2008) noted reduced body weight in both males and females when they were exposed to the higher doses. In all exposed groups, incidence of chronic inflammation, hepatic histiocytosis, and focal fatty change were significantly higher than control. Other symptoms, hyperactivity, excitability, clonic seizures, and impaired gait, were also noted in females exposed to the higher doses. There was clear evidence of mononuclear cell leukemia incidence in females exposed to the highest amount of 4-MeI. Rat males exposed to 2,500 μg/g and females exposed to 5,000 μg/g suffered from mixed cell foci hepatocellular eosinophilic.

Male and female B6C3F1 mice were fed diets containing 312, 625, or 1,250 μg/g of 4-MeI for 2 years (average daily doses of 40, 80, or 170 mg 4-MeI per kg of the body weight). Results showed both male and female mice lost weight and showed increased occurrence of alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, and alveolar/ bronchiolar adenoma or carcinoma. Females fed 1,250 μg/g 4-MeI showed clear evidence of alveolar epithelial hyperplasia, significantly higher than the control group. The survival rate of treated male and female rats and male and female mice was the same as for the control group. However, female rats exposed to the highest doses of 4-MeI ate less than the control group.

Recently, Krishna and others (2014) used three computational software programs to predict genotoxicity and tumorigenicity of 4-MeI and concluded that, genotoxicity activity can be predicted, but more animal studies are needed to effectively estimate tumorigenicity computationally. However, one software program, Osiris Property Explorer software (Actelion Pharmaceutical Ltd., Allschwil, Switzerland) available from (http:// www.organic-chemistry.org/prog/peo), indicated 4-MeI was tumorigenic (Krishna and others 2014).

4-MeI PHARMACOKINETICS

There is no data available on 4-MeI pharmacokinetics in humans, but, several researchers have investigated 4-MeI pharmacokinetics in animals. 4-MeI was recovered unchanged in urine 30 min after 216 mg/kg bw was administered intraperitoneally (IP) in rats; 90% of the compound was excreted within 8 h. After 5 min, the absorption rate was highest in the intestines, followed by the liver, blood, stomach, and kidneys (Hidaka 1976a).

Yuan and Burka (1995) noted that when Carbon-14 labeled 4-methylimidazole (^{14}C-4-MeI) was given orally to F344/N rats, peak plasma concentrations were reached after 0.5, 1.0, and 3.0 h of administration of 5, 50 or 150 mg/kg bw. They also noted that higher doses required more time to leave the plasma suggesting that the rate at which ^{14}C-4-MeI was eliminated from the body was dose dependent, although the approximate bioavailability of ^{14}C-4-MeI was 60 - 70% with negligible elimination through respiration, feces, and in bile materials.

When Karangwa and others (1990b) administered a single oral dose of 20 mg/kg bw to ewes, they found the level of 4-MeI in the plasma was 10 mg/kg after 27 min, and after 5 hours the level reached its maximum. 4-MeI remained unchanged in the urine at a level of 0.07 mg/kg; 69% of 4-MeI was bioavailable with a biological half-life of 9.38 h. Nielsen and others (1993)

had similar results with 4-MeI pharmacokinetics in heifers and lactating goats. In goats, most of the ^{14}C-4-MeI was eliminated from the plasma within 6 h of IV administration; oral dosage was the highest concentration in the plasma after 6 h with bioavailability ranging from 80 to 100%. The liver and the kidney had the highest concentrations of ^{14}C-4-MeI, and the total excreted amounts in the urine were 67 and 64% for IV and oral administration, 0.5% was found in the bile materials after IV exposure, and 3-4% was found in the goat milk after both IV and oral dosing; 17 and 9% were found in feces after IV and oral doses. Total recovery of ^{14}C-4-MeI was 88% for IV doses and 76% for oral doses.

Yuan and Burka (1995) described one minor hydrophilic metabolite found both in the urine and in plasma when ^{14}C-4-MeI was giving orally to F344/N rats. More detailed information about the metabolism of 4-MeI was presented by Cowgill (1955). After a dose of 4-MeI was administered to Long-Evans rats by intraperitoneal injection, urinary metabolites were isolated, analyzed, and identified. Figure 5 shows the suggested metabolic reaction of 4-MeI; oxidation of 4-MeI produces 2-hydroxymethylimidazole; oxidation of 2-hydroxymethylimidazole gives 1H-imidazole-4-carbaldehyde which oxidizes to 1H-imidzole-4-carboxlic acid.

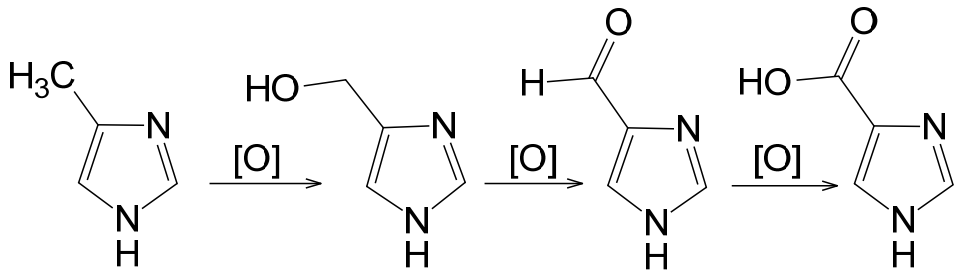

Figure 5. 4-MeI metabolism (Cowgill 1955).

In 2002, Dalvie and others reviewed the oxidative metabolism of 4-MeI, suggesting that oxidation of 4-MeI would create at least two forms of reactive intermediates: pyruvaldehyde and epoxide and dicarbonyl compounds (see Figure 6).

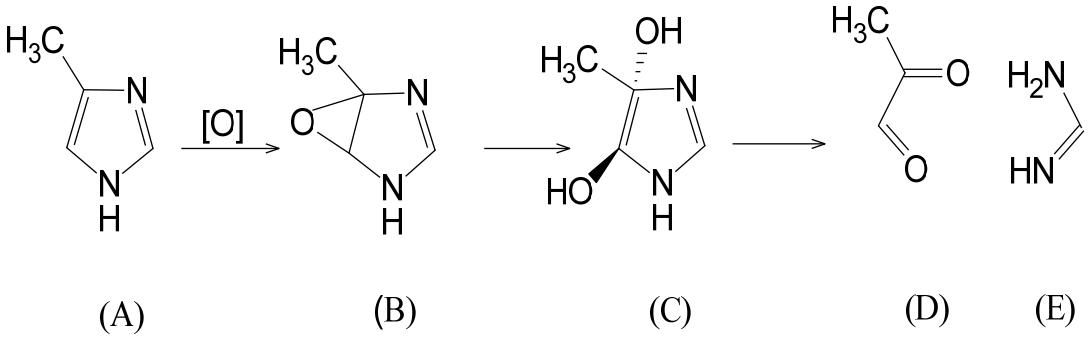

Figure 6. Oxidative metabolism of 4-MeI and formation of reactive intermediates (Dalvie and others 2002). (A) 4-MeI, (B) 1-methyl-6-oxa-2,4-diazabicyclo [3.1.0]hex-2-ene,(C) 4-methyl-1H-imidazole-4,5diol, (D) Methylglyoxal, (E) 2-aminoethanimidic acid.

LEVELS OF 4-MeI IN FOODS

Most human exposure to 4-MeI is through ingestion. 4-MeI can be found in foods either as a result of interaction between amino acids and reducing sugar in the presence of heat and moisture or by adding caramel colors that contain 4-MeI produced by either the ammonia or ammonia-sulfite process. Some research has reported different levels of 4-MeI in food matrices. Table 1 lists levels of 4-MeI in different food products. In coffee, the highest level reported in the roasted coffee was 2.05 mg/kg (Klejdus and others 2006), while in liquid coffee, as expected, because the solid coffee diluted, the highest level was 0.77 mg/kg. 4-MeI forms in coffee because of the roasting process. Dark beer contained higher levels of 4-MeI than other food

products; the highest level reported was 28.03 mg/kg (Klejdus and others 2006). In cola drinks, 4-MeI is present because caramel colors that contain 4-MeI are added to enhance the flavor, color, and taste.

EXPOSURE TO 4-MeI FROM NON-FOOD SOURCES

Other than ingestion, humans are exposed to 4-MeI through tobacco smoke (Moree-Testa and others 1984; Sakuma and others 1984). Two possible mechanisms of 4-MeI formation in tobacco were suggested by Moree-Testa and others (1984): (a) thermal degradation of histidine (pyrolysis), which is a 4-substituted imidazole amino acid and (b) reaction between ammonia and α-dicarbonyl compounds like methylglyoxal and aldehydes like formaldehyde.

The highest level of 4-MeI was found in dark, air-cured tobacco cigarettes without filters, and the lowest level in cigarettes with Virginia tobacco and low tar cigarettes, although, as Moree-Testa and others (1984) stated, "4-MeI is the most abundant imidazoles found in cigarette smoke".

Table 1. Reported levels of 4-MeI in different food products[a].

Product	Concentration (mg/kg)	Reference
Malt extract	ND	Fernandes & Ferreira (1997)
Coffee	Solid[b]: 0.39–2.05	Klejdus and others (2006)
Coffee	Solid[b]: 0.77–1.45, Liquid: 0.35–0.77	Lojková and others (2006)
Coffee	Solid[b] 0.30–1.24	Casal and others (2002)
Dark beer	1.58–28.03	Klejdus and others (2006)
Soda	0.30-0.36	Moon and Shibamoto (2011)
Cola	0.17-0.70	Yoshikawa and Fujiwara (1981)
Grape	0.15-0.16	
Whisky	ND-0.14	
Black beer	ND	
Beer, wine brandy	ND	
Milk product	Trace	
Worcestershire sauce	1.6-3.4	
Food cooked in soya sauce	0.89-3.2	
Soya sauce	0.37-0.55	
Confectioneries	ND-0.78	
Cola	0.13-0.34	Xu and others (2015)
Soft drink	<LOD	
Carbonated beverage	ND-0.69	Wang and Schnute (2012)
Cola	ND-0.56	Schlee and others (2013)
Soft drinks	ND-0.43	Cunha and others (2011)
Dark beer	ND-0.42	
Cola	0.18-0.61	
Dark beer	0.017	Yamaguchi and Masuda (2011)
Canned coffee	0.04	
Worcestershire sauce	0.02	
Other sauces	<0.002-4.8	
Balsamic vinegars	ND-5.24	Cunha and others (2014)
Soy sauce	ND-0.95	
Worcestershire sauce	ND-0.74	
Tonkatsu sauce	ND	
Marinara sauce	0.68	
Oyster sauce	0.75	
Sauce	LOD-1.71	Kim and others (2013)
Balsamic vinegars	1.24 and 5.97	
Complex seasoning	2.1-5.8	
Curry	ND-5.1	
Soft drink	ND-0.46	
Dressing	0.61	

[a]Adapted and modified from IARC (2012).

[b]Roasted, ND = Not detected.

LOD = Limit of detection.

LEVELS OF 4-MeI IN ANIMAL FEEDS

To increase the level of non-protein nitrogen, make the feed is more digestible, and increase the overall animal feed quality, animal feeds are heated in an aqueous solution of ammonia for a certain period (Waagepetersen and Vestergaard 1997). As a result of this process, several toxic compounds like imidazoles (e.g., 4-MeI) and pyrazine are formed.

Several factors affect the amount of 4-MeI in the ammoniated forage. According to Bergstrom (1991), higher concentrations of sugar and ammonia, higher temperatures, higher water activity, and longer heating time increase 4-MeI levels. 4-MeI has been found in milk, plasma, and urine of sheep and cows after eating of thermos-ammoniated forage. Muller and others (1998) found 90 μg/g /dry matter concentrations of 4-MeI in ammoniated forage, and after analyzing the plasma, urine, and milk of ewes fed ammoniated forage, they reported concentrations of 0.07 μg/g, 21 μg/g, and 0.23- 0.031 μg/g in the plasma, urine, and milk.

In addition, levels of 0.28 μg/g (plasma), 2.7 μg/g (milk), 5.8 μg/g (urine) were reported by Sivertsen and others (1993) in dairy cows fed thermos-ammoniated forage containing 58 μg/g 4-MeI. An average level of 0.72 μg/g of 4-MeI was detected in sheep plasma after the sheep consumed tall fescue (*Lolium arundinaceum*) with a level of 64.36 μg/g 4-MeI (Karangwa and others 1990a).

ANALYTICAL METHODS TO IDENTIFY AND QUANTIFY 4-MeI

Detection methods

4-MeI has been detected and quantified in foods using several different analytical techniques: high performance liquid chromatography (HPLC) (Thomsen and Willumsen 1981; Bergstrom 1991; Sivertsen and others 1993), liquid chromatography mass-spectrometry (LC-

MS) (Klejdus and others 2003; Lojkova and others 2006; Klejdus and others 2006), liquid chromatography tandem mass-spectrometry (LC-MS-MS) (Moon and Shibamoto 2011; Yamaguchi and Masuda 2011; Lee and others 2013; Kim and others 2013; Schlee and others 2013), ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS-MS) (Wang and Schnute 2012), pulsed amperometric detection (PAD) (Xu and others 2015), liquid chromatography-time-of-flight mass spectrometry (LC-TOFMS) (Yamaguchi and Masuda 2011), gas chromatography with flame ionization detector (GC-FID) (Wilks and others 1973; Wilks and others 1977; Buckee and Bailey 1978; Nielsen and others 1993), gas chromatography mass-spectrometry (GC-MS) (Fuchs and Sundell 1975; Fernandes and Ferreira 1997; Casal and others 2002; Cunha and others 2011; Moretton and others 2011; Cunha and others 2014; Seo and others 2014), gas chromatography with nitrogen phosphorus detector (GC-NPD) (Jang and others 2013), indirect competitive immunosorbent assay (ic-ELIZA) (Wu and others 2015), capillary isotachophoresis (Kvasnicka 1989), paper spray mass spectrometry (Li and others 2013), thin-layer chromatography (TLC) (Wilks and others 1973; Fuchs and Sundell 1975), Hydrogen-1 nuclear magnetic resonance (^{1}H NMR) (Monakhova and others 2013), and fluorometric detection (Gutierrez and others 1986).

Extraction methods

The literature suggests a variety of extraction methods to extract 4-MeI from different food matrices. Wang and Schnute (2012) and Schlee and others (2013) analyzed carbonated beverage samples directly without any extraction process. Kim and others (2013) used a one-step extraction with acetonitrile. Ion-pair extraction and solid phase extraction (SPE) were the most commonly used extraction techniques among different researchers. SPE using a strong cation

exchanger was the method of choice for many researchers because 4-MeI is a polar basic compound that can be protonated by adjusting its pH to less than 7.5 (usually around 5 pH).

Ion-pair extraction was developed by Thomsen and Willumsen (1981) and used after modification by Fernandes and Ferreira (1997), Casal and others (2002), Cunha and others (2011), and Seo and others (2014). In general, food samples or caramel color that contains 4-MeI is mixed with a phosphate buffer (pH 6). A chloroform solution containing bis-2-ethylhexylphosphate, an ion-pairing and adduct forming agent (Modin and Schill1975), is added, and after mixing and centrifugation, the chloroform layer is mixed with an aqueous solution of hydrochloric acid where 4-MeI is back-extracted in the hydrochloric acid solution. Ion-pair extraction has been shown to be a good method for extracting 4-MeI, not only from ammoniated caramel color, but from a variety of matrices like plasma, urine, milk, and fodder (Fernandes and Ferreira 1997).

SPE offers several advantages: good recovery, accuracy, high sensitivity, minimal solvent, safety, and fewer steps than ion-pair extraction. Klejdus and others (2006) extracted 4-MeI using three different SPE cartridges: Waters Oasis MCX mixed-mode cation exchange cartridge (Waters, Milford, U.S.A.), Applied Separations Spe-ed ^{TM} benzenesulphonic SCX strong cation exchange cartridge (Applied Separations, Allentown, U.S.A.), and Ansys SPEC SCX Disc benzenesulphonic acid silica based strong cation exchange cartridge (Ansys Technologies, Torrance, U.S.A.). Klejdus and others (2006) concluded that the highest recovery (98%) was obtained using the sorbent SPEC SCX Disc. Similarly, Moon and Shibamoto (2011) achieved a recovery of 102 ± 3.61% using the SPEC SCX Disc benzenesulphonic acid silica based strong cation exchange cartridge to extract 4-MeI from aqueous solutions. SPEC SCX Disc was also used by Xu and others (2015) to isolate 4-MeI in cola (recovery ranged from 95.1

to 103.4%) and soft drinks (recovery ranged from 99.6 to 102.2%). Yamaguchi and Masuda (2011) used C18 cartridges to extract 4-MeI from different food samples. Their reported recovery rate ranged from 95.6 to 103.6%.

Lojkova and others (2006) used supercritical fluid extraction (SFE) to extract 4-MeI from solid coffee samples and concluded that SFE had sufficient precision and recovery rate to be considered an alternative extraction method. Lojkova and others (2006) also indicated SPE was faster than SFE, which had an extraction time of 45 min but the advantage of SFE was the ability to extract analytes from solid food matrices where washing the analytes off with common organic solvents would be difficult.

DERIVATIZATION OF 4-MeI

Derivatization can be defined as a procedure that modifies an analyte's properties to enable chromatographic separation (Orata 2012). For example, to detect an analyte using GC, the analyte should be volatile. To make a chemical compound volatile usually most of the active hydrogen atoms are removed. Removing the hydrogen atoms will also improve the chromatographic process by preventing hydrogen-bonding (Husek and Simek 2001).

Derivatization of a high polarity compound makes it sufficiently volatile to be detected at a suitable temperature without thermal decomposition (Knapp 1979). 4-MeI is a basic polar compound, and because of its polarity, separation of 4-MeI using ordinary techniques is difficult. In fact, direct analysis of 4-MeI using GC is not recommended because of its high polarity and tendency for hydrogen-bonding. This makes obtaining a symmetrical peak shape impossible; peak tailing is always present (Moree-Testa and others 1984). To reduce the polarity and make 4-MeI more volatile, derivatization using different chemical compounds is used.

Acylation, silylation, and carbamate formation (alkyl chloroformate reagents) can be used to derivatize imidazoles. However, acylation and silylation have disadvantages; they required a moisture free environment, time, and multiple preparation steps. Moreover, the derivatives are unstable. Moretton and others (2011) used acetic anhydride to form 1-acetyl-4-MeI in caramel color, and Klinger and others (2013) used silylation with BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) containing 10% trimethylchlorosilane to derivatize 4-MeI in a D-glucose/ammonium hydroxide model system.

Alkyl chloroformate can be used to derivatize primary, secondary, and tertiary amines, phenol, thiol, and imidazole groups (Hiroyuki 1996). Isobutylchloroformate (IBCF) has several advantages as a derivatizing agent: it does not require certain conditions like drying or heating that may reduce recovery or repeatability; moisture does not affect the derivatization process, so it has wide application in food and biological sample; it is fast (4 - 5 sec); imidazoles derivatives are stable at room temperature; sample cleanup is not necessary before derivatizing; and it improves the imidazole chromatographic behavior. Therefore, many researcher have chosen to use IBCF to derivatize 4-MeI (Fernandes and Ferreira1997, Casal and others 2002, Cunha and others 2011, Moretton and others 2011, Cunha and others 2014, Seo and others 2014). Figure 7 illustrates the reaction between 4-MeI and IBCF. In the reaction, IBCF reacts with the nitrogen in the imidazole ring, and because 4-MeI exists in two forms, two derivatives result from derivatization process: 4-methylpropyl 1H-imidaozle-1-carboxlate and 5-methylpropyl 1H-imidaozle-1-carboxlate. Fernandes and Ferreira (1997) were the first to describe this phenomenon when they analyzed caramel color for the presence of 4-MeI by derivatization with IBCF. They reported two peaks after derivatization with IBCF and stated that the presence of the two peaks might be due to the tautomerization of 4-MeI.

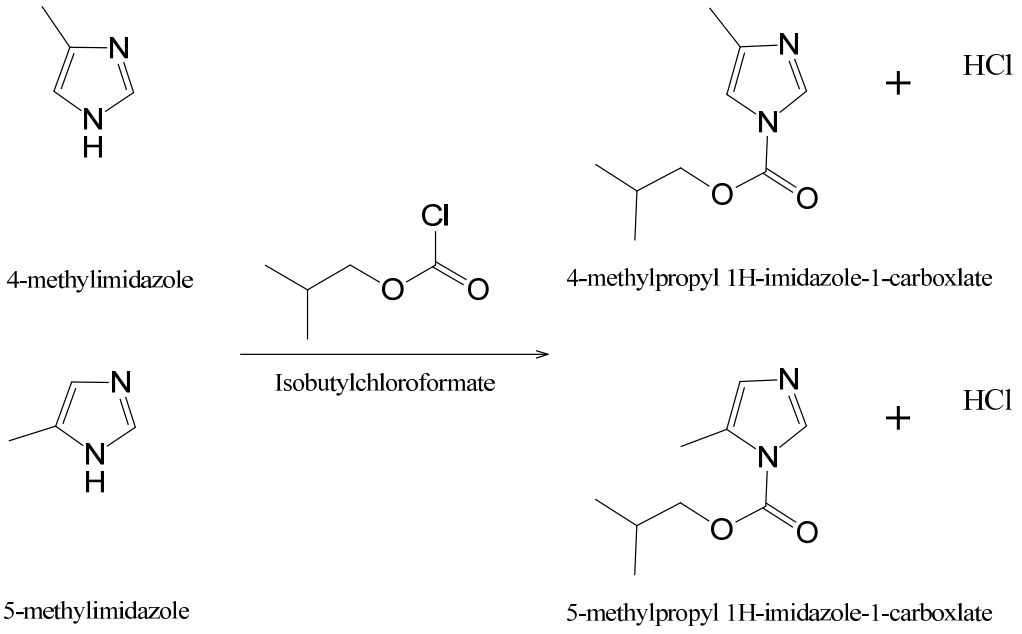

Figure 7. Reaction of 4-MeI with isobutylchloroformate (modified from Hengel 2013).

CARAMEL COLORS

Caramelization is the process through which caramel color forms, a non-enzymatic browning, occurring when sugars are heated to temperatures over 80°C with a pH between 3 and 9 without a source of nitrogen such as amino acids (Davies and Labuza 2003; Kroh 1994)

According to Kasim (2010), caramelization includes changes in the ring size of monosaccharides, breaking and reforming of glycosidic bonds, dehydration, and introducing double bonds and forming of anhydro rings.

Since sugar was first discovered, people have burned sugar to add color and flavor to their foods. In the 19th and 20th century, caramelization received significant attention because caramel colors were widely used in the brewing industry and later in soft drinks (Sethness 2014). In 1963, caramel colors were commercially produced in the U.S (Lee and others 2013).

Title 21 of the United States Code of Federal Regulations, part 73 section 73.85, defines caramel colors as food additives that are dark-brown, existing either in liquid or solid form and are made from a controlled heating and pressure process that uses different carbohydrates, food-grade acids, alkalis, and salts (CFR 2014). Carbohydrates like dextrose (glucose), invert sugar(glucose and fructose), lactose, malt syrup, molasses, sucrose, and starch hydrolysates are all permitted in manufacturing caramel color, along with acetic, citric, phosphoric, and sulfurous acids and the alkalis ammonium hydroxide, calcium hydroxide, potassium hydroxide, and sodium hydroxide. The only salts permitting in caramel colors are carbonate or bicarbonate, monobasic phosphate or dibasic, and sulfates or sulfites of ammonium, potassium, or sodium (CFR 2014).

Caramel colors are totally miscible with water, they have a burnt sugar odor, and a pleasant bitter taste (Kamuf and other 2003). In addition to coloring foods, caramel colors are colloidal agents and emulsifiers and are widely used in the food industry. Caramel color accounts for 95% by weight of permitted additive food color (IARC 2012). They are generally recognized as safe (GRAS) and can be added to foods according to good manufacturing practice (GMP). One restriction should be considered when adding caramel colors to food: caramel color should not be used to color foods if color is standard to the food's identity (CFR 2014).

The four types of caramel colors are classified according to their production process: caramel I (plain or caustic caramel color), caramel II (caustic-sulfite caramel), caramel III (ammonia caramel), and caramel IV (ammonia-sulfite caramel). Caramel color I carries a neutral to slightly negative ionic charge and is used in high proof alcohols; caramel color II possesses negative ionic charges and is commonly used in tea and brandy. Caramel color III carries a positive ionic charge and is used in bakery products, soya-bean sauces, gravies, soups, vinegars,

and dark beers; its use accounts for 20 - 25% of the total caramel colors in the U.S.A. and about 60% in Europe (IRAC 2012). Class IV caramel colors have a negative ionic charge and are used in soft drinks, pet foods, and soups (Houben and Penninks1994), accounting for approximately 70% of the caramel colorings produced worldwide (Licht and others 1992a).

The reactant charge in the caramel color is responsible for color stability; reactants with a negative charge make more stable caramel colors while reactants with both positive and negative charges make less stable caramel color (Kamuf and others 2003). Moreover, the ability of the caramel colors to slowly absorb oxygen is important in stabilizing the colloidal system, which in turns prevents the formation of haze and facilitates flavor retention (Chappel and Howell 1992).

The most important property of caramel colors is being miscible with the food matrix without flocculation, precipitation, and/or haze (Pintea 2008). This property also depends on the color stability. Stability of caramel color in the food matrix depends in part on the caramel color charge as well as the food matrix charge. Coagulation or precipitation may occur if the food matrix and the caramel color have opposite charges. In particular, soft drinks are usually negatively charged, requiring use of negatively charged caramel color IV.

4-MeI LEVELS IN CARAMEL COLORS

Caramel colors III and IV have drawn considerable attention because both have been shown to contain 4-MeI. Caramel color III also has 2-acetyl-4(5)-1,2,3,4-tetrahydroxybutyl imidazole (THI), an immune suppressing agent (Gugasyan and others 1995). Table 2 lists reported levels of 4-MeI in caramel colors III and IV. Licht and others (1992a) found the highest 4-MeI level of 1276 μg/g in caramel IV. The lowest was 0.26 μg/g in a caramel color (Kim and

others 2013). These different concentrations in levels of 4-MeI in caramel colors III and IV may be due either to different production processes or to the techniques used to detect 4-MeI.

Table 2. 4-MeI levels in caramel colors[a].

Product	Concentration (mg/kg)	Reference
Ammonia process caramel	1.74 - 3.7	Wu and others (2015)
Ammonia-sulfite caramel	0.74 - 2.53	
Caramel color	0.26 - 24.4	Kim and others (2013)
Ammonia process caramel	< 5 - 184	Licht and others (1992b)
Ammonia process caramel	105.9 - 163.1	Petruci and others (2013)
Ammonia process caramel	ND - 463	Allen and others (1992)
Ammonia process caramel	85.6 -187.8	Klejdus and others (2006)
Ammonia process caramel	34 -463	Brusick and others (1992)
Ammonia process caramel	Liquid: 14 - 24, Powder: 50	Ciolino (1998)
Ammonia process caramel	6.6 - 351 per 20 000 EBC units[b]	Thomsen & Willumsen (1981)
Ammonia caramel	7.5 - 210	Fernandes & Ferreira (1997)
Ammonia caramel	122 - 414	Kvasnicka (1989)
Ammonia caramel	25 - 303	Fuschs & Sundell (1975)
Ammonia-sulfite caramel	112 - 1276	Licht and others (1992a)
Ammonia-sulfite caramel	100.4 - 146.8	Petruci and others (2013)
Ammonia-sulfite caramel	146 - 215	Allen and others (1992)
Ammonia-sulfite caramel	ND - 387	Brusick and others (1992)
Ammonia-sulfite caramel	Liquid 130 - 300, Powder 480	Ciolino (1998)
Ammonia-sulfite caramel	62 - 341 per 20 000 EBC units	Thomsen & Willumsen (1981)

[a]Adapted and modified from IARC (2012).

[b]EBC units: caramel color intensity unit of the European Brewery Convention.

ND = Not detected.

4-MeI REGULATIONS IN FOODS AND CARAMEL COLORS

The USFDA has set no limits on the amount of allowable 4-MeI in processed foods. However, in January 2011, the Office of Environmental Health Hazard Assessment (OEHHA) has set a human ingestion limit to 16 μg/day for the No Significant Risk Level (NSRL); they increased the NSRL in October 2011 to 29 μg/day (OEHHA 2011a). For caramel colors III (ammonia processed) and IV (ammonia-sulfite processed), the Food and Agriculture Organization of The United Nations established a limit of $\leq$ 200 and 250 mg/kg of 4-MeI based on color intensity (JECFA 2011). It should be noted that, on March 2011, the European Food Safety Authority (EFSA) reduced the lower limit of Acceptable Daily Intake (ADI) of caramel color III (ammonia-processed) from 300 to 100 mg/kg bw/day; for caramel color IV (ammonia-sulfite processed), the ADI was set to 300 mg/kg bw/day (EFSA 2011a).

4-MeI RISK ASSESMENT BY REGLUATION AGENCIES

In 2011, the European Food Safety Authority (EFSA) evaluated human exposure risk from caramel colors that contain 4-MeI and concluded that exposure levels of 4-MeI in Europe were below the levels of the National Toxicology Program NTP study (NTP 2007), and exposure to 4-MeI from caramel color was not a concern (EFSA 2011b). In addition, EFSA re-evaluated consumer exposure to 4-MeI in caramel color in 2012, confirming its 2011 conclusion. EFSA also pointed out that 4-MeI may not be the cause of DNA mutations (genotoxicity), stating the type of tumors reported in mice in the NTP study can occur spontaneously in these animals; thus, EFSA stated that Europeans suffered no exposure risk from caramel colors that contain 4-MeI (FDA 2014).

In 2013, the FDA stated that "Based on the available information, FDA has no reason to believe that there is any immediate or short-term danger presented by 4-MeI at the levels expected in food from the use of caramel coloring" (FDA 2013). Also, the FDA pointed out that the administered doses of 4-MeI in the NTP carcinogenicity study far exceeded levels that a human can obtain from consuming caramel coloring used in processed foods and drinks. In 2014, the FDA considered reviewing current exposure levels to 4-MeI, but did not recommend any changes in consumer diets (FDA 2014). Similar to the FDA recommendations, Health Canada also reported that foods with low levels of 4-MeI do not represent a risk to humans (Global News 2013).

REDUCTION OF 4-MeI IN CARAMEL COLORS

Totally eliminating of 4-MeI from processed foods is impossible. However, 4-MeI levels can be reduced in caramel colors (caramel color III and IV) by modifying the manufacturing processes or by adding specific reactants known to influence the Maillard reaction. Thus investigating potential mitigation strategies is very important (Jang and others 2013). In fact, several companies have either used caramel colors that have low levels of 4-MeI (caramel colors I and II) or have used new strategies to reduce 4-MeI in processed foods. As an illustration, in January 2011, California listed 4-MeI as a probable carcinogen and set the NSRL intake as 29 μg per day (OEHHA 2011b); in march 2012, both Coca-Cola and Pepsi companies declared that they had directed their caramel color supplier to modify their manufacturing process to reduce the levels of 4-MeI to comply with the new California standard (The Guardian 2012). However, as of April 2015, in states other than California, Pepsi and Coca-Cola products still contain 4-MeI levels considered safe by the FDA but exceeding the California NSRL of 29 μg per day.

Seo and others (2014) studied the effect of food additives on 4-MeI formation in the caramel color (glucose/ammonia) model system. Maximum reduction (80.2%) was achieved when iron sulfate (0.1 M) was added to the glucose (1 M) and ammonia (1 M) model system. Seo and others (2014) also reported reduction values for magnesium sulfate (13.1%), zinc sulfate (17.2%), tryptophan (26.4%), and cysteine (28.5%); however, they also reported adding sodium sulfate sulfite increased 4-MeI levels by 4.8% and adding calcium sulfate increased 4-MeI levels by 18.0%. In the same experiment, Seo and others (2014) also noted that the degree of darkness (L*) in the model system decreased when sodium sulfate was added while the degree of redness (a*) increased when magnesium sulfate or cysteine were added, b* values did not change significantly in any treatment. Seo and others (2014) stated that L*, a*, and b* values did not correlate significantly with 4-MeI formation.

The role of sulfite in the formation of 4-MeI was investigated by Lee and others (2013) in a glucose/ammonia/sulfite model system. Sodium sulphite was added to a glucose:ammonium hydroxide solution and heated. The results showed that the lowest amount of sodium sulphite actually increased production of 4-MeI, while higher amounts reduced the production of 4-MeI. They concluded that adding specific amount of sulfite may reduce 4-MeI formation in caramel color. They also tested the effects of sodium sulfite on the intensity of the color itself and noted that the lowest amount of sodium sulfite increased the color intensity significantly more than treatment with higher amount of sodium sulfite.

Jang and other (2013) also investigated inhibition 4-MeI formation. Sodium sulfite forms an adduct with aldehyde compounds, including formaldehyde, which is an important precursor for 4-MeI formation. They suggested that adding sulfite during manufacture of caramel color IV

may suppress formation of 4-MeI (see Figure 8). The reduction percentages increased as more sodium sulfite was added to the D-glucose ammonium hydroxide Maillard model system.

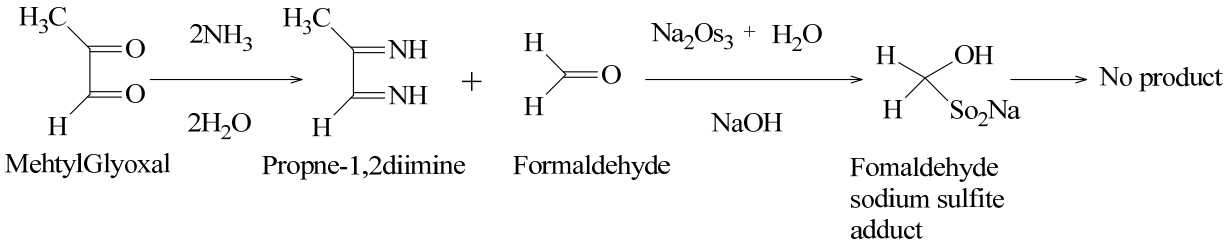

Figure 8. Inhibition of 4-MeI formation (adapted from Jang and others 2013).

SUMMARY

4-MeI belongs to imidazole compounds; a group of chemicals contain an imidazole ring with two nitrogens, it is widely used by different industries as a raw material, intermediate or component.

4-MeI is formed in the Maillard reaction due to the reaction between sugar and amino acids it is also present in ammoniated caramel colors III and IV. 4-MeI is a neurotoxic and can cause bovine bonkers syndrome in ruminants.

Research had reported 4-MeI as a suspected carcinogen and efforts are under way to eliminate its present in processed foods. No limits of 4-MeI have been established in foods by the regulatory agencies and 4-MeI can be added to food according to good manufacturing practice. However, 29 μg/day has been set by the Office of Environmental Health Hazard Assessment (OEHHA) as a No Significant Risk Level (NSRL). Total elimination of 4-MeI from processed food is impossible but reducing its level in caramel colors is achievable.

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CHAPTER 2. DETECTION AND QUANTIFICATION OF 4(5)-METHYLIMIDAZOLE IN COOKED MEAT[1, 2]

ABSTRACT

4(5)-methylimidazole (4-MeI) is a nitrogen-containing heterocyclic compound found in class III and IV ammoniated caramel colors, a group of additives widely used in the food industry. A suspected carcinogen and neurotoxin and efforts are underway to limit its presence in foods. Several methods have been developed to detect and quantitate 4-MeI in different food matrices, including roasted coffee, beer, soft drinks, and soy sauce; however, no methods are available to measure 4-MeI in cooked meat and meat products containing lipids and high levels of interfering nitrogen compounds such as amino acids and peptides. A rapid method using 0.1 M sodium acetate buffer (pH 5) as an extraction solvent followed by derivatization with isobutylchloroformate (IBCF) and gas chromatograph mass-spectrometry (GC-MS) was developed to quantify 4-MeI in cooked meat products with added caramel colors containing 4-MeI. Selected Ion Monitoring mode (SIM) was used to monitor 4-MeI ions fragments. In the eight commercial meat products tested, 4-MeI levels ranged from 0.041-1.015 mg/kg, with recovery of 94.76-103.94%. In addition, a matrix-matched calibration performed by analyzing a spiked cooked meat sample indicated no significant difference ($P < 0.05$), which means the meat matrix had no effect on the developed method. This method proved useful in analyzing 4-MeI in meat products with added caramel color containing 4-MeI.

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INTRODUCTION

Food colors improve appearance by compensating for color lost during processing and storage, protecting original color in foods, and adding color to colorless foods. Color also can convince consumers to choose a specific product. Caramel colors account for more than 80% of all colorants added to foods, with annual global consumption exceeding 200,000 tons (Kamuf and others 2003). Caramel colors are produced by controlled heat treatment of food-grade carbohydrates with the aid of catalysts such as salts, ammonia, sulfites, ammonium salt, and alkalis. According to the synthesis method and the catalyst used, caramel colors are classified into four types (JECFA 2006): Caramel color I (plain or caustic caramel), caramel color II (caustic sulfite caramel), caramel color III (ammonia caramel), and caramel color IV (ammonia-sulfite caramel). They are generally recognized as safe (GRAS) and can be added to foods according to good manufacturing practice (GMP) without limits (CFR 2013).

Caramel color types III and IV have received significant attention due to the presence of 4-MeI, a byproduct formed during the reaction between ammonia and reducing sugar in the presence of heat. It is a heterocyclic nitrogen-containing compound with a molecular formula of $C_4H_6N_2$. The compound is widely used as raw or intermediate material in many applications, such as pharmaceuticals, photography chemicals, agricultural chemicals, dyes, and pigments (OEHHA 2012). 4-MeI is a neurotoxin and convulsion agent in cattle, rabbits, chickens, and mice (Karangwa and others 1990) and inhibits cytochrome P450, the enzyme that metabolizes the oxidation of many low-mass carcinogens in human liver (Hargreaves and others 1994). In addition, 4-MeI is a carcinogen; in 2007, the National Toxicology Program (NTP) conducted an animal study to investigate the toxicity and carcinogenicity of 4-MeI and showed clear evidence of increased incidences of mononuclear cell leukemia in female rats and alveolar and bronchiolar

carcinomas in male and female mice (NTP 2007). However, the mechanisms of carcinogenesis were not known (Grosse and others 2011). As a result of NTP findings, 4-MeI was listed on January 7, 2011, as a chemical known to the state of California to cause cancer under Proposition 65 (the Safe Drinking Water and Toxic Enforcement Act of 1986; California Health and Safety Code 25249.5 *et seq*.) (OEHHA 2011). Moreover, the International Agency for Research on Cancer (IARC) listed 4-MeI in the 2B group, "possibly carcinogenic to humans" in 2012 (IARC 2013).

4-MeI has been identified in caramel colors (Buckee and Bailey 1978; Fernandes and Ferreira 1997; Klejdus and others 2006; Moretton and others 2011; Petruci and others 2013; Schlee and others 2012; Wilks and others 1973; Yamaguchi and Masuda 2011) and in processed foods, such as coffee (Casal and others 2002; Klejdus and others 2006; Lojkoval and others 2006; Yamaguchi and Masuda 2011), soy sauce (Yamaguchi and Masuda 2011), beer (Cunha and others 2011; Klejdus and others 2006), carbonated beverages (Cunha and others 2011; Lim and Shin 2013; Schlee and others 2012; Wang and Schnute 2012; Yamaguchi and Masuda 2011), and milk (Muller and others 1998).

Although 4-MeI has been detected and quantified in different foods, no research has been conducted to estimate the levels of 4-MeI in cooked meat or cooked meat with added caramel color. Due to the physical/chemical characteristics of 4-MeI and the nature of the cooked meat matrix, isolation and quantification of 4-MeI is challenging. Cooked meat has a complex matrix that includes proteins, amino acids, lipids, and low-mass chemicals as well as added ingredients such as salts and preservatives. Several analytical methods have been described in the literature to detect/quantitate 4-MeI. Lim and Shin (2013), Schlee and others (2012), Moon and Shibamoto (2011), and Wang and Schnute (2012) used liquid chromatography-tandem mass spectrometry

(LC-MS-MS). Moretton and others (2011) applied heat-cutting two-dimensional liquid chromatography (LC-LC). Hydrophilic interaction chromatography (HILIC) was used by Lojkova and others (2006). GC-MS was used by Lim and shin (2013), Casal and others (2002), Cunha and others (2011), and Fernandes and Ferreira (1997). Rabe and others (1998) used a thin-layer chromatography to determine 4-MeI, whereas Petruci and others (2013) used capillary electrophoresis. Hydrogen-1 Nuclear Magnetic Resonance (^{1}H NMR) was used by Monakhova and others (2012). The purpose of the present study was to develop a rapid method to detect and quantitate 4-MeI in cooked meat products with added caramel color containing 4-MeI using GC-MS and to investigate the matrix effects of the cooked meat on the method.

MATERIALS AND METHODS

Chemicals and reagents

Pyridine, 4-methylimidazole (≥ 98%), isobutylchloroformate, and isobutanol were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Isotope deuterium labeled internal standard 4-methylimidazole-d_6 (4-MeI-d_6) was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Acetonitrile, hexane, sodium bicarbonate, acetic acid, and sodium acetate were obtained from Fisher Scientific Corp. (Pittsburgh, Pa., U.S.A.). Sodium acetate buffer (0.1 M and pH 5) was prepared by dissolving 0.82 g in 100 mL of deionized water, and the pH was adjusted by drop-wise addition of concentrated acetic acid. Caramel color made with ammonia and ammonia-sulfite was obtained from D. D. Williamson (D.D.W. Corp., Louisville, Ky., U.S.A.) and Sethness (Sethness Products Corp., Skokie, Ill., U.S.A.). All reagents and solvents used to conduct experiments were analytical-grade. Carrier gas for the GC-MS (ultra-high purity helium) was obtained from Matheson Tri-Gas (Manhattan, Kans., U.S.A.).

Sampling

Different cooked meat products with added caramel color were purchased from local supermarkets. No further sample preparation process was applied for liquid-based products such as beef broth or caramel color products. For solid products such as beef patties, turkey sausage patties, and pork sausage patties, two or three patties were selected from each bag and ground using a KitchenAid food processor (model KFP600BU; Whirlpool Corp., St. Joseph, Mich., U.S.A.). For canned/packed meat products such as beef pot roast, pork gravy, beef gravy, and beef stew, the contents of container were mixed and a portion submitted for analysis. Three samples of each meat product were analyzed in duplicate. Ammoniated caramel color products were analyzed directly in triplicate.

Sample Preparation

The internal standard, 4-MeI-d_6 (Figure 9), was added to 3 g of each sample, transferred to a 50 mL polypropylene centrifuge tube (Corning Inc., Corning. N.Y., U.S.A.), and mixed with 9 mL of the sodium acetate buffer. The sample was vortexed for 1 min and homogenized for another 1 min using an Omni TH homogenizer (Omni International, Kennesaw, Ga., U.S.A.), with the speed dial set to 2. Samples were kept in a refrigerated centrifuge (model Marathon 21000R; Thermo Scientific Corp., Pittsburgh, Pa., U.S.A.) at 4°C and centrifuged afterward at 9150 × g for 15 min, and a portion of the supernatant was filtered through a 30 mm PTFE 0.45 μm syringe filter (Agilent Technology Inc., Santa Clara, Calif., U.S.A.).

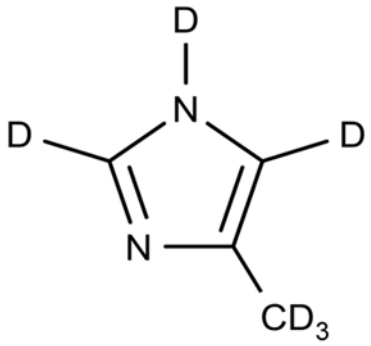

Figure 9. Chemical structure of 4-MeI-d6.

Sample derivatization

Samples were derivatized according to a method previously described by Fernandes and Ferreira (1997), with some modification. A 125 μL aliquot of the sample filtrate was first mixed with 125 μL of acetonitrile:isobutanol:pyridine (50:30:20; v/v) in a silane-treated vial (Agilent Technology Inc., Santa Clara, Calif., U.S.A.). Then 15 μL (10 + 5 μL) of IBCF were added to the mixture and the vial was shaken briefly by hand, and 250 μL of 1.0 M sodium bicarbonate solution (8.4 g in 100 mL of deionized water) were added, followed by 250 μL of hexane. Finally, 1 μL from the hexane layer (upper) was injected manually into the GC–MS. The injection needle was equipped with a RAX repeating adaptor (SGE Inc., Austin, Tex., U.S.A.) to ensure reproducibility. 4-MeI and 4-MeI-d_6 standards were prepared in sodium acetate buffer and derivatized as the samples.

Samples fat determination

Sample fat percentages were determined according to AOAC (2012) (# 2008.6) using

microwave drying and nuclear magnetic resonance (NMR) analysis; CEM SMART Trac system (CEM Corp., Matthews, N.C., U.S.A.) was used to measure the fat percentage for the samples.

GC-MS system

Determination of 4-MeI was conducted using an HP 5890 Series II *Plus* gas chromatograph (Agilent Technology Inc., Santa Clara, Calif., U.S.A.) fitted with a split/splitless injection port containing a 4 mm internal diameter liner (ultra-inert single goose neck containing deactivated glass wool). The GC instrument was equipped with an HP 5972 mass spectral detector. Chemstation software (G1701BA Version B.01.00) was used for data acquisition.

The GC-MS separation was performed on DB-35 column (30 m × 0.25 mm I.D. × 0.25 μm (35%-phenyl)-methylpolysiloxane film thickness) (Agilent Technology Inc., Santa Clara, Calif., U.S.A.). A guard column (DB-35) was connected prior to the GC column with an ultra-inert union (Agilent Technology Inc., Santa Clara, Calif., U.S.A.). The sample was injected in splitless mode with the injector temperature set to 250°C and purge-off time set to 1 min. The oven temperature program was set as follows: 60°C held for 1 min, ramped to 150°C at 25°C/min, held for 5 min and ramped again to 300°C at 25°C/min and held for 4.40 min. The total run time was 21 min. The transfer line temperature was 280°C, and the solvent delay time for the MS detector was 7.0 min. The carrier gas was set to a consistent flow at 1 mL/min. The MS system was routinely calibrated using the autotune calibration function of the mass selective detector. Selective Ion Monitoring (SIM) mode was used to quantitate 4-MeI and 4-MeI-d_6 based on peak area using the precursor ion(s) and three product ion(s).

CALCULATION OF 4-MeI LEVELS

Levels of 4-MeI in cooked meat products were calculated according to the following equations:

$$\text{4-MeI } (\mu\text{g/kg}) = \frac{\text{Added 4-MeI-d}_6\text{ internal standard (ng)} \times \text{sample peak areas of 4-MeI (a + b)}}{\text{Sample weight} \times \text{sample peak area of 4-MeI-d}_6\text{ (a + b)} \times \text{GC-MS response factor}}$$

$$\text{GC-MS response factor} = \frac{\text{Added 4-MeI standard } (\mu\text{g/kg}) \times \text{peak area of 4-MeI-d}_6\text{ (a + b)}}{\text{Added 4-MeI-d}_6\text{ standard } (\mu\text{g/kg}) \times \text{peak area of 4-MeI (a + b)}}$$

STATISTICAL ANALYSIS

SAS (Version 9.3, SAS Institute Inc., Cary, N.Y., U.S.A.) was used for data analysis. Sample fat content effects on 4-MeI recovery rates were evaluated where recovery rates were treated as response variables, and fat contents were treated as explanatory variables; simple linear regression models were generated using general-purpose regression procedure (PROC REG).

Sample matrices effects were assessed where the response variable was the integrated area. The treatment structure was two-way crossed. The first treatment was 4-MeI levels of 0.04, 0.2, and 1.0 mg/kg; the second treatment had two levels (generated by a standard curve in sodium acetate buffer or in beef gravy). Based on the observed data, the block effect was negligible, so the design was a complete randomized design. Analysis of variance (ANOVA) was performed to analyze the data using general linear regression procedure (PROC GLM), and a P value of < 0.05 was considered a significant outcome in all statistical analysis conducted.

RESULTS AND DISCUSSION

GC-MS conditions for identification and quantification of 4-MeI

Although GC-FID and GC-MS were used to quantify 4-MeI in caramel color and food matrices, direct determination of 4-MeI using GC is difficult because of its chemical characteristics. 4-MeI has low volatility (its boiling point is 263°C) and high polarity, which make its peak shape asymmetrical. Lim and Shin (2013) concluded that direct detection of 4-MeI by GC-MS using the SIM mode without derivatization is not sensitive enough. Reducing the polarity and increasing volatility requires a derivatization reaction, but a moisture- free environment (silylation derivatization), heating, and multiple reaction steps may affect derivatization result. Derivatization with IBCF offers several advantages; it is robust, rapid, can be applied to aqueous samples, and doesn't require special treatment such as heating or drying, and stable (compared with methylchloroformate or ethylchloroformate). The only documented disadvantage is column degradation (Fernandes and Ferreira 1997; Casal and others 2002).

Several techniques were used to extract 4-MeI from the samples. Casal and others (2002) and Fernandes and Ferreira (1997) used ion-pair extraction with bis-2-ethylhexylphosphate to extract 4-MeI from the sample, derivatized 4-MeI with IBCF, extracted 4-MeI derivative in chloroform, and used GC-MS to identify and quantitate the compound. Cunha and others (2011) used the same sample extraction and derivatization but extracted 4-MeI derivative in isooctane. In this method, we derivatized the sample directly without an extraction step and removed 4-MeI derivative after derivatization in hexane. We compared the abilities of different solvents to extract 4-MeI after the derivatization step, and no difference was noticed in peak areas between hexane, isooctane, chloroform, and cyclohexane; in fact, hexane gave better peaks shape. Cunha and others (2011) found no differences in peak areas and shapes of 4-MeI between chloroform

and isooctane when it was extracted after derivatization.

The full scan mode using the GC-MS (from *m/z* 20 *to m/z* 500) was first performed for 4-MeI and 4-MeI-d_6 to identify precursor and product ions. Table 3 shows selected *m/z* ions for 4-MeI and 4-MeI-d_6. For 4-MeI, ion *m/z* 182 was selected as a precursor ion and *m/z* 109, 82, and 81 were selected as product ions. For 4-MeI-d_6, ion *m/z* 187 was selected as a precursor ion and *m/z* 114, 87, and 85 as product ions. Figures 10 and 11 show the *m/z* ions profile for 4-MeI and 4-MeI-d_6 after derivatization with IBCF.

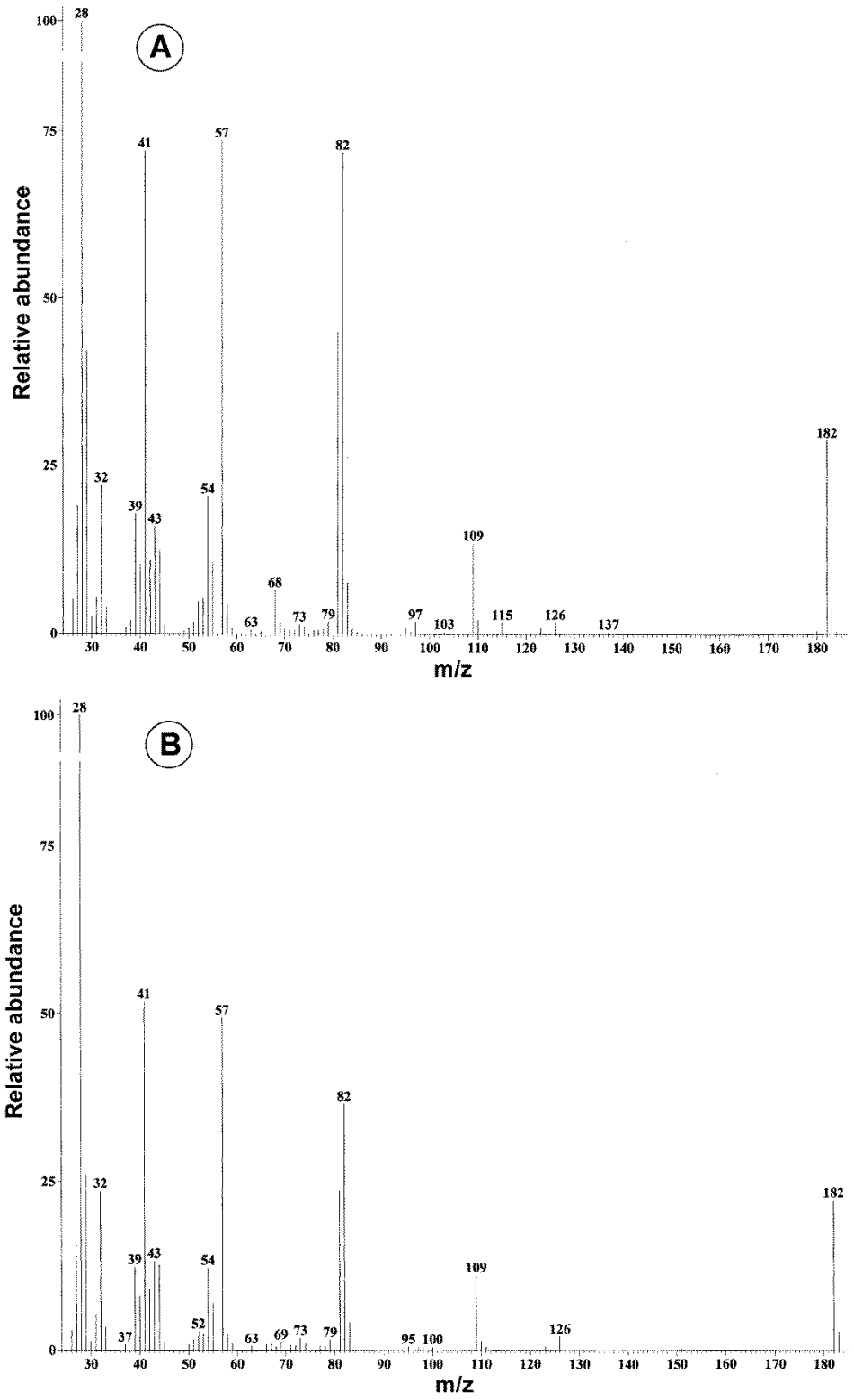

Figure 10. Mass spectrum of relative ion *m/z* fragments abundance for tautomeric derivatives (A) 4-MeIa, and (B) 4-MeIb obtained in SCAN mode of the GC-MS.

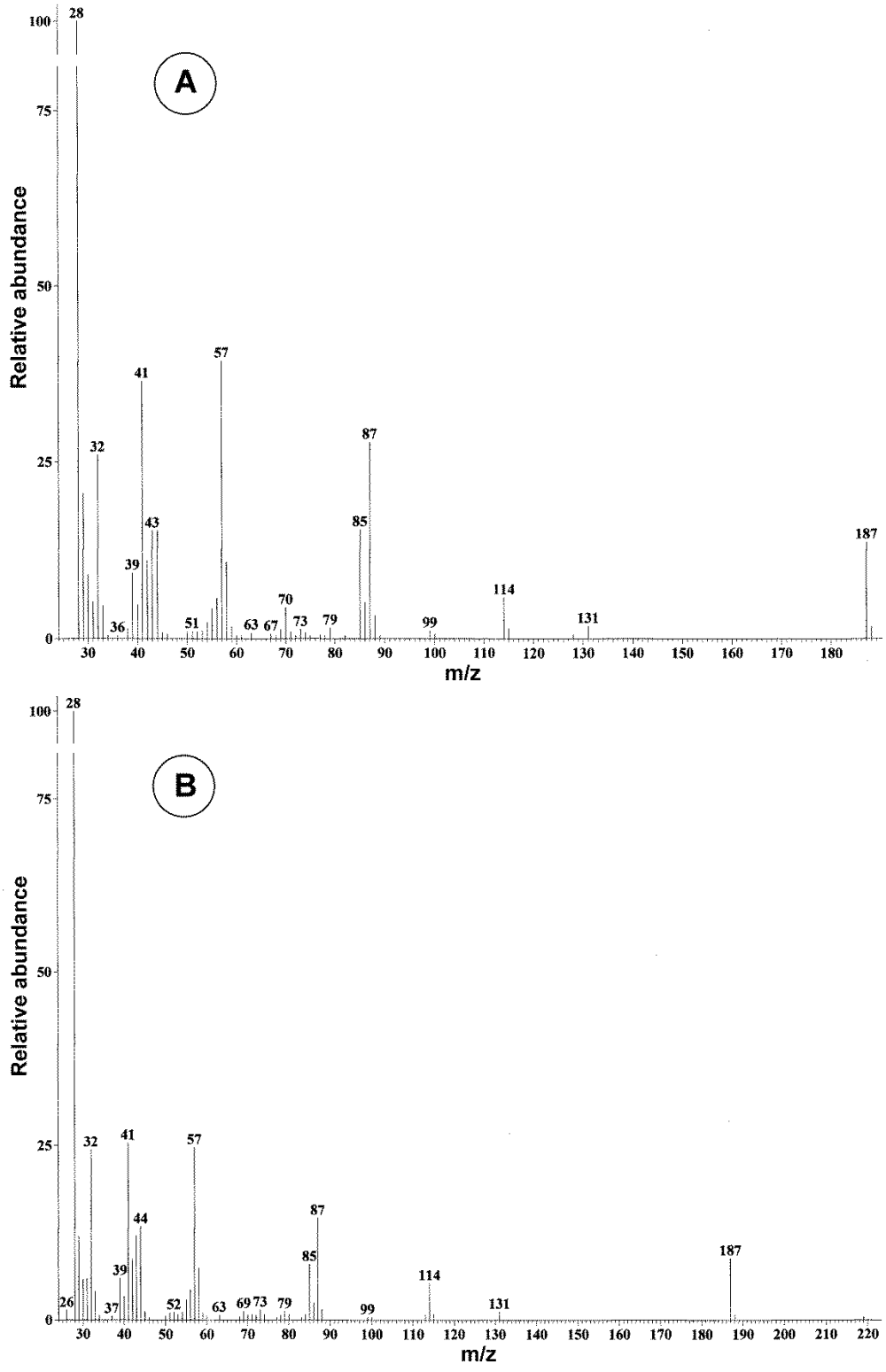

Figure 11. Mass spectrum of relative ion *m/z* fragments abundance for (A) 4-MeI-d6a, and (B) 4-MeI-d6b obtained in scan mode of the GC-MS.

Table 3. Selected ions to identify 4-MeI and 4-MeI-d6 by GC-MS SIM.

Compound ID	Precursor ion (*m/z*)	Conformation ions (*m/z*)
4-MeI	182	109, 82, 81
4-MeI-d_6	187	114, 87, 85

Both compounds gave two peaks after derivatization, 4-MeIa and 4-MeIb for 4-MeI, and4-MeI-d_6a and 4-MeI-d_6b for 4-MeI-d_6 (Figure 12).

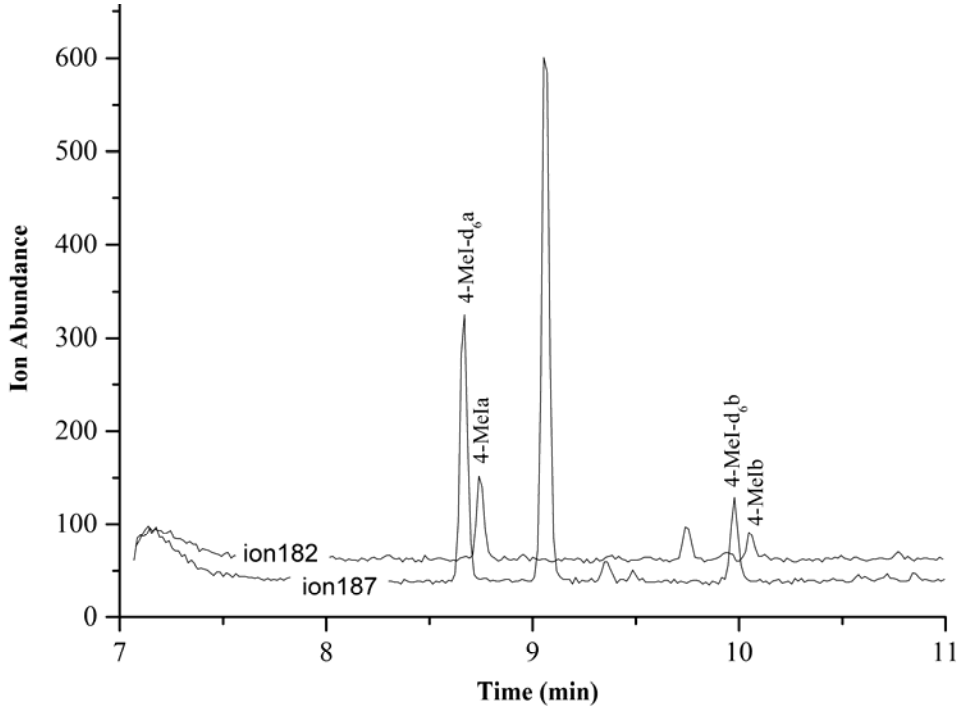

Figure 12. Reconstructed GC-MS chromatogram of beef gravy in merge format for ion *m/z* 182 for 4-MeI (0.06 mg/kg) and ion *m/z* 187 for 4-MeI-d6 (0.1 mg/kg).

Fernandes and Ferreira (1997) pointed out the presence of these two peaks after derivatization of 4-MeI with IBCF. Fernandes and Ferreira (1997) also stated that "the presence of these two peaks is probably due to tautomerism equilibrium in 4-MeI molecule, a well-known

phenomenon which consists in a proton chemical shift between the two nitrogens of the imidazole ring". In our method, peak areas of the parent ions *m/z* 182 and *m/z* 187 for 4-MeI and 4-MeI-d_6 were used for the detection and quantification process.

During sample preparation and after buffer addition and homogenization, samples were allowed to stay in the centrifuge at 4°C for about 30 min before centrifugation. This step was important to physically separate the fat from the sample and helped with sample filtration. Also, three pH values of the sodium acetate buffer were evaluated (1.5, 3, and 5) for 4-MeI extraction; samples prepared in low pH (1.5 and 3) prevented elimination of fat from the samples. Samples extracted with pH 5 buffer were clearer but had the same recovery rates.

To our knowledge, no research has been performed to detect and quantitate 4-MeI in cooked meat products with added caramel color. Al-Sahal (2003) analyzed cooked meat contaminated with ammonia gas for the presence of 4-MeI and detected no trace of 4-MeI. Table 4 shows 4-MeI levels in eight different cooked meat products with different fat contents and added caramel color purchased from supermarkets. Levels of 4-MeI ranged from 0.041 to 1.015 mg/kg.

Two commercial caramel colors were evaluated: caramel color A (ammonia processed) and caramel B (sulfite-ammonia processed). Their 4-MeI levels were 21.85 (100.94% recovery) and 309.17 (101.78% recovery) mg/kg (Table 5).

Table 4. 4-MeI levels, recovery, and fat content of different cooked meat.

Sample type	4-MeI (mg/kg)[1]	Recovery %	Fat %[1]
Beef patty	1.015 ± 0.021	97.89	10.74 ± 0.18
Turkey sausage patty	0.280 ± 0.007	102.19	10.85 ± 0.13
Beef pot roast	0.176 ± 0.013	94.76	4.04 ± 0.94
Pork gravy	0.161 ± 0.015	99.49	0.64 ± 0.01
Beef broth[2]	0.140 ± 0.019	99.60	0.00 ± 0.00
Beef gravy	0.064 ± 0.003	96.87	1.14 ± 0.01
Pork sausage patty	0.046 ± 0.007	97.73	36.78 ± 0.30
Beef stew	0.041 ± 0.007	97.73	0.90 ± 0.18

[1]Values are means ± standard error (n=3); each sample was analyzed in duplicate.

[2]Fat-free products.

Table 5. 4-MeI levels and recoveries of two different caramel colors.

Sample ID	4-MeI (μg/g)[1]	Recovery %
Carmel A[2]	21.85 ± 0.50	100.94
Carmel B[3]	309.17 ± 5.54	101.78

[1]Values are means ± standard error for each samples analyzed in triplicate.

[2]Ammonia-processed caramel color.

[3]Ammonia-sulfite processed caramel color.

Other researchers have found various levels of 4-MeI in caramel colors. Yamaguchi and Masuda (2011) reported a value of 121 mg/kg for sulfite-ammonia processed caramel color. Schlee and others (2012) found levels from175 to 658 mg/kg in different sulfite-ammonia

processed caramel color. Fernandes and Ferreira (1997) found levels of 211.96 and 41.79 mg/kg in two samples of ammonia caramel color. Monakhova and others (2012) showed levels of 401 to 662 mg/kg in caramel color. These differences may be a result of different manufacturing processes and conditions.

A preliminary study was conducted by our group to investigate the present of 4-MeI in an aqueous model system of D-glucose and different selected amino acids when heat was applied. 4-MeI was detected in the model system indicating that, the levels of 4-MeI in the tested cooked meat samples could be resulted from adding caramel colors containing 4-MeI as well as formation of 4-MeI due to the reaction between the sugar and the nitrogen containing molecules in the meat (proteins, amino acids, and peptides) in the presence of heat and moisture. Yamaguchi and Masuda (2011) reported different levels of 4-MeI in different naturally brewed soy sauces.

METHOD VALIDATION

The recovery efficiency of 4-MeI was examined by spiking a beef gravy sample with 0.04, 0.20, and 1.00 mg/kg (n=3). The average recovery rates were 95.55 ± 0.05, 93.47 ± 0.02, and 98.12 ± 0.18%. Limit of detection (LOD) was calculated to be 25 μg/kg with a signal-to-noise ratio of 3:1, whereas the limit of quantification (LOQ) was estimated to be 40 μg/kg with a signal-to-noise ratio of 10:1. In calculation of both the LOD and LOQ, samples were spiked with a decreasing amount of 4-MeI until the signal-to-noise ratios were achieved. The sum of the areas of the two peaks for each compound 4-MeI (a + b) and 4-MeI-d_6 (a + b) were used in the calculation; however, the smaller peaks (4-MeIb and 4-MeI-d_6b) were the limitation in our method. The LOD and the LOQ for the proposed method were lower than those of Fernandes

and Ferreira (1997; 0.250 and 1 mg/kg) but higher than those of Cunha and others (2011; 0.6 and 2.2 μg/kg); however, the LOD of the proposed method is higher than Casal and others (2002; 5 μg/kg) but the LOQ is similar (40 μg/kg). The linearity of the proposed method was tested by spiking a beef gravy sample with three different concentrations of 4-MeI-d_6 (0.1, 1, 10 mg/kg); after generating the standard curve, the calculated correlation coefficient (r^2) was 0.9998 indicating the proposed method has a good linearity. The repeatability of the overall method was also tested by analyzing three replicates of a spiked beef gravy sample with two different concentrations of 4-MeI-d_6 (0.1 and 1 mg/kg) during three days periods. The proposed method showed a good repeatability with % RSD value of <7%.

The ratios of ions *m/z* 182 to 82 for 4-MeI and *m/z* 187 to 87 for 4-MeI-d_6 were estimated to be 1:2. Consequently, the sensitivity of the proposed method could be improved if quantification with ions *m/z* 82 and *m/z* 87 were used for 4-MeI and 4-MeI-d_6, respectively. Attempts to use *m/z* 82 and *m/z* 87, however, were not successful for some of the analyzed samples because of interfering peaks.

We used 4-MeI-d_6 as an internal standard instead of 2-methylimidazole (2-MeI) or 2-ethylimidazole (2-EI) as in previously published research, because some of the analyzed samples showed shared retention time and ion spectrum profiles similar to the 2-MeI and 2-EI peaks. Moreover, using a high polarity column (DB-35) and slower temperature program helped to better separate 4-MeI and 4-MeI-d_6; Fernandes and Ferreira (1997) concluded, using a high polarity column and slower temperature program may help to separate 4-MeI from other imidazole derivatives.

We investigated the effects of sample fat content on the recovery of 4-MeI, but we found no significant effects ($P < 0.05$) of fat content. Due to different food additives as well as the

presence of proteins, lipids, amino acids, and low-mass-containing nitrogen molecules formed during meat cooking or processing, the overall meat matrix effects was investigated; three concentrations of 4-MeI, 0.04, 0.2, and 1.0 mg/kg (three replicates of each concentration), were prepared in the sodium acetate buffer or spiked into commercial cooked beef gravy. After generating the standard curves, no significant differences ($P < 0.05$) were found between the two standard curves slopes, indicating the absence of matrix effects on the extraction or derivatization process.

4-MeI INTAKE

4-MeI intake level through cooked meat consumption

Humans can be exposed to 4-MeI through skin contact or inhalation, but ingestion remains the major pathway of human exposure. Figure 13 shows the level of 4-MeI in μg per serving size of the analyzed samples and the No Significant Risk Level (NSRL) of California's Proposition 65 (29 μg/day). Out of the eight products tested, three showed levels higher than 29 μg: beef patties (70.70 μg), beef broth (33.60 μg), and beef pot roast (29.92 μg). However, tested samples with low 4-MeI levels per serving size may be considered safe but can provide an intake of more than 29 μg if more than one serving is consumed per day.

Different levels of 4-MeI were reported in food products other than meat in the literature. Levels of 0.307 to 1.241 mg/kg in roasted coffee were reported by Casal and others (2002). Cunha and others (2011) found levels of 3 to 613 μg/kg in different soft drinks and dark beer, and Yamaguchi and Masuda (2011) reported levels of 0.34 to 4.8 mg/kg in soy sauce and other food products. A research group recently analyzed different soft drinks and syrup and found levels of 4-MeI from 3.4 to 352.5 and 0.7 to 38.0 μg/serving sizes, respectively (Consumer

Report 2014a, b). Because 4-MeI is widely used in the food industry, 4-MeI intake should be estimated from different food products.

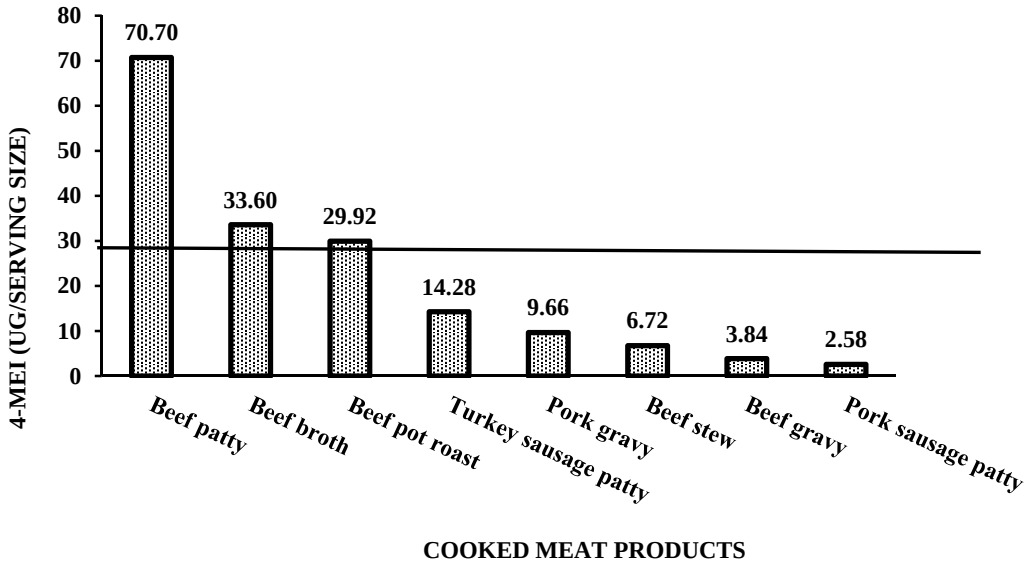

Figure 13. Average microgram levels of 4-MeI levels per serving size in different cooked meat products. The horizontal line represents the "No Significant Risk Level" of 29 microgram/day of 4-MeI (OEHHA 2011).

CONCLUSION

Caramel colors are widely used in the food products such as meat, beverages, bakery, and dairy products, and daily human exposure to 4-MeI from different foods with added caramel color is important because 4-MeI may pose health hazards. Therefore, a GC-MS method was developed to detect and quantify 4-MeI in cooked meat with added caramel color. The method is based on adjusting the pH of the sample using sodium acetate buffer followed by acetylation

with isobutylchloroformate and GC-MS analysis. The method was used to quantify commercial cooked meat products with different fat levels with added caramel color. The samples varied from solid (ground meat) to liquid (beef broth). The developed method proved suitable to quantify 4-MeI in cooked meat product and does not require heating or drying that may result in significant loss of 4-MeI from the sample.

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CHAPTER 3. FORMATION OF 4(5)-METHYLIMIDAZOLE IN AQUEOUS D-GLUCOSE-AMINO ACIDS MODEL SYSTEM[1]

ABSTRACT

The International Agency for Research on Cancer (IRAC) has classified 4(5)-methylimidazole (4-MeI) as a group 2B possible human carcinogen. Thus, how 4-MeI forms in a D-glucose (Glu) amino acids (AA) model system is important, as it is how browning is affected. An aqueous solution of Glu was mixed individually in equimolar at three concentrations (0.05, 0.1, and 0.15 M) with aqueous solutions of L-Alanine (Ala), L-Arginine (Arg), Glycine (Gly), L-Lysine (Lys), and L-Serine (Ser). The Glu-AA mixtures were reacted at 60, 120, and 160°C for 1 h. How temperature, reactant concentrations, pH, and water activity before heat treatment affect the formation of 4-MeI was examined; the effect of each treatment on browning was also examined. Gas chromatography-mass spectrometry after derivatization with isobutylchloroformate was used to measure 4-MeI levels. No 4-MeI was formed at 60°C for any treatment combination; however, at 120°C and 0.05 M, Glu-Arg and Glu-Lys produced 0.13 and 0.14 μg/g of 4-MeI. At 160°C and 0.05 M all treatment combinations formed 4-MeI. At 160°C and 0.15 M, the observed levels of Glu-Ala, Glu-Arg, Glu-Gly, Glu-Lys, and Glu-Ser were 0.21, 1.00, 0.15, 0.22, and 0.16 μg/g. The AA type, reactant concentrations, and temperature significantly affected ($P < 0.0001$) formation of 4-MeI as well as browning. Glu-Lys treatment in all combinations produced the most browning, but Glu-Arg produced the most 4-MeI. This method showed that foods processed using low temperature may have reduced levels of 4-MeI.

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INTRODUCTION

Louis-Camille Maillard, a French chemist, described in 1912 the reaction between reducing sugar and amino acid in the presence of heat as responsible for the distinctive flavor and browning color in foods. Later, Hodge (1953) was the first to suggest a three stage mechanism for the Maillard reaction: (1) sugar-amine condensation and Amadori rearrangement (colorless; no near-ultraviolet absorption), (2) sugar dehydration, sugar fragmentation, and amino acid degradation (colorless or yellow; strong near-ultraviolet absorption), and (3) aldol condensation and aldehyde-amine polymerization to form heterocyclic nitrogen compounds (highly colored). The Maillard reaction has been widely used in the food industry to design foods with specific sensory attributes to attract consumers (Ames 1990; Zeng and others 2011). The Maillard reaction occurs between reducing sugar and nitrogen-containing compounds such as proteins, amino acids, or ammonia.

Color development is the main characteristic of the Maillard reaction. On the other hand, color can be developed in food in absence of amino acids in a process called caramelization. Caramelization occurs when carbohydrates are heated to a specific temperature, a process used for producing caramel colors. Caramel colors are the most common food colors used in the food industry, produced by controlled heat and pressure of food grade carbohydrates in the presence of chemical promoters. The formation of caramel color is affected by the concentration of the reactants, pH, temperature, time, moisture content, and the nature of the reactants (Pintea 2008; Benhura and others 1999).

Despite the benefits of adding caramel colors, toxic health effects are undesirable. Caramel color III has been associated with decreased numbers of blood lymphocytes in rats (Evans and others 1977) and these effects were stronger in animals fed diets low in vitamin B_6

(Gobin and Paine 1989). In a rat experiment conducted by Houben and others (1992), changes in thymus morphology and changes in macrophage subpopulation as well as lymphopenia and reduced numbers of nucleated cells in the peripheral organs were noted. 2-Acetyl-4(5)-(1,2,3,4-tetrahydroxy)butyl-imidazole (THI), present only in caramel color III, caused changes in immune system function, including severe impairment in response to mitogenic stimulation of splenic cells in rat (Sinkeldam and others 1988; Kroplien and others 1985).

4-MeI can be found in caramel colors III and IV; it has been categorized as a group 2B possible human carcinogen by the International Agency for Research on Cancer (IRAC 2012). The National Toxicology Program of the U.S. Department of Health and Human Services found that it caused cancer (NTP 2007). In addition, 4-MeI is a neurotoxin that can cause convulsions in mice and rabbits (Nishie and others 1969) or cattle (Fairbrother and others 1987).

4-MeI forms in foods during sugar and ammonia reaction upon heating (Moon and Shibamoto 2011) or can be added to food as caramel colors III and IV. Even though 4-MeI was reported in the Maillard reaction of Glu-Gly and Glu-Glt (Glt = glutamate) model (Wong and Bernhard 1988), no research has further assessed how 4-MeI forms in the sugar-amino acid model system, because 4-MeI does not contribute flavor to food.

Ammonia is important in formation of 4-MeI (Moon and Shibamoto 2011) and, ammonia forms during heat degradation of amino acids (Sohn and Ho 1995). Thus, although 4-MeI contributes nothing to the flavor of food, it can form in food, and as a possible carcinogen, it should be investigated. Therefore, this study was set up to investigate formation of 4-MeI in the Maillard model system consisting of sugar and amino acids and to study what actually affects both 4-MeI formation and browning.

MATERIALS AND METHODS

Chemicals and reagents

Glucose (99.5%), alanine (98.5%), arginine (98%), glycine (98.5%), lysine (98%), and serine (99%), isobutylchloroformate (98%), and isobutanol came from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Isotope deuterium labeled 4-methylimidazole-d_6 (4-MeI-d_6) was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Acetonitrile, hexane, and sodium bicarbonate were obtained from Thermo Scientific Inc. (Waltham, Mass., U.S.A.). All solvents and reagents used to conduct the analysis were analytical grades. Carrier gas (ultra-high purity helium) for the GC-MS was supplied by Matheson Tri-Gas (Manhattan, Kans., U.S.A.). Water used to prepare reagents or model systems was double deionized prepared with a Barnstead PCS filtration system (Thermo Scientific Inc., Waltham, Mass., U.S.A.). Sodium bicarbonate solution (1.0 M) was prepared by dissolving 8.4 g in 100 mL of double deionized water. Hydrochloric acid (0.1 N) was prepared by mixing 0.82 mL of concentrated hydrochloric acid (36.5 - 38%) with 100 mL of double deionized water.

Model system preparation

Glu solution was mixed in equimolar individually with solutions of Ala, Arg, Gly, Lys, or Ser in equal volume with 0.25 mL of each solution in three different concentrations of 0.05, 0.1, 0.15 M. All solutions were prepared at room temperature and subjected to heat treatments.

Model system reaction procedure

The Glu-AA acid mixture was transferred to a 1 ml reaction vial (Wheaton Corp., Millville, N.J., U.S.A.). Each vial was inserted into a heating vessel consisting of a threaded

body (cylindrical; outer diameter of 15 mm), and two screw caps (interior diameter of 15 mm) one on each end. The heating vessel body and the caps were brass (zinc 33% and copper 66% alloy; Watts Corp. North Andover, Mass. U.S.A.) (see Figure 14). To seal the reaction vial inside the heating vessel, a silicone septum (15 mm; Sun Sri, Thermo Scientific Rockwood, Tenn., U.S.A.) was inserted into the cap on the end used to open the vial. The heating vessels were hand tightened and placed into an oven (HP 5890; Agilent Technology Inc., Santa Clara, Calif., U.S.A.). The vessels were exposed to three temperatures: 60, 120, or 160°C for 1 h. The oven temperature during the experiment was monitored using a USB-TC thermocouple and Tracer DAQPro software (Measurement Computing™, Waltham, Mass., U.S.A.). pH and water activity (a_w) were measured before heat treatment, and pH, browning, and 4-MeI levels were recorded after heat treatment.

Model system pH measurements

Before heating the vessels, the pH of the Glu-AA mixture was measured at room temperature (25°C) using an Accumet pH meter (model AP 115) equipped with Accumet flat surface single junction pH probe. Both the probe and the pH meter were from (Thermo Scientific Corp. Pittsburgh, Pa., U.S.A.). After heat treatment, vessels were immediately immersed in an ice bath to minimize further reaction. After a short time, samples were removed from the ice bath and brought to room temperature; pH was then measured again.

Figure 14. Heating vessel used in model system thermal treatment.

Model system water activity measurements

The a_w of the Glu-AA mixtures was measured using an AquaLab series 3 water activity meter with a 47 mm diameter chamber (Decagon Devices, Inc., Pullman, Wash. U.S.A.). The instrument was calibrated using 0.760 and 0.920 a_w standards purchased from AquaLab. All a_w measurements were conducted at room temperature (25°C).

Model system browning measurements

Degree of browning intensity among the Glu-AA mixtures was measured according to Lee and others (2013). A portion of the Glu-AA mixture was transferred into a 1 cm polystyrene cuvette (Thermo Scientific Inc., Waltham, Mass., U.S.A.), and readings were recorded at a wavelength of 420 nm using a Genesys 10vis spectrophotometer (Thermo Scientific Inc., Waltham, Mass., U.S.A.). Double deionized water was used as the blank and also to dilute dark samples exceeding the instrument limit.

4-MeI sample measurements

Derivatization

After adding the internal standard 4-MeI-d_6, samples were derivatized according to Fernandes and Ferreira (1997) as modified by Karim Smith (2015). A 125 μL aliquot of the sample was first mixed with 125 μL of acetonitrile:isobutanol:pyridine (50:30:20; v/v) in a silane treated vial (Agilent Technology Inc., Santa Clara, Calif., U.S.A.). Then 15 μL (10 + 5 μL) of IBCF were added, and after shaking briefly by hand (5-10 sec), 250 μL of 1.0 M sodium bicarbonate solution was added, followed by 250 μL of hexane. Finally, 1 μL from the hexane layer (upper) was injected into the GC-MS. 4-MeI, 4-MeI-d_6 standards were prepared in 0.1 N hydrochloric acid and derivatized using the same procedure.

GC-MS system operation conditions

4-MeI was detected and quantified using an HP 5890 Series II *Plus* gas chromatograph (Agilent Technology Inc., Santa Clara, Calif., U.S.A.) equipped with an HP 5972 mass spectral detector. The GC was fitted with a split/splitless injection port containing a 4 mm internal diameter liner (ultra-inert single gooseneck with deactivated glass wool). Samples were injected automatically using an HP 7673b autosampler (Agilent Technology Inc., Santa Clara, Calif., U.S.A.). Chemstation software (G1701BA Version B.01.00) was used to control the GC-MS and the autosampler as well as for data acquisition.

The GC-MS separation was performed with a DB-35 column (30 m × 0.25 mm I.D. × 0.25 μm (35%-phenyl)-methylpolysiloxane film thickness). A guard column (DB-35) was connected with the GC-MS column using an ultra-inert union. The GC-MS column, the ultra-inert union, and the guard column were from Agilent Technology (Agilent Technology Inc.,

Santa Clara, Calif., U.S.A.). Samples were injected in splitless mode. The injector temperature was 250°C, and purge off time was set to 1 min. The temperature program for the GC was set to 60°C, held for 1 min, ramped to 150°C at 25°C/min, held for 5 min, ramped again to 300°C at 25°C/min, and held for 4.40 min. The total run time was 21 min. The transfer line temperature was 280°C, and the solvent delay time for the MS detector was 7.0 min. The carrier gas flow was 1 mL/min of consistent flow. The autotune calibration function of Chemstation software was used to calibrate the MS system whenever calibration was needed. Selective Ion Monitoring (SIM) mode was performed to qualify 4-MeI and 4-MeI-d_6 based on peak area using the precursor ion(s) and three product ion(s).

STATISTICAL ANALYSIS

4-MeI and browning levels were analyzed independently. Because detection limit is too low, only certain temperature/amino acid combinations achieved detectable levels for 4-MeI. To correct for incomplete data, only data from specified temperatures (See Table 6) were used when analyzing the response variables for each amino acid.

Table 6. Temperatures used in the statistical analysis for each amino acid.

	Amino acids				
Response	**Ala**	**Arg**	**Gly**	**Lys**	**Ser**
4-MeI	160°C	120 & 160°C	160°C	120 & 160°C	160°C
Browning	120 & 60°C	60, 120, & 160°C	120 & 160°C	60, 120, & 160°C	120 & 160°C

The general experimental design was a completely randomized design (CRD) with three replications at each temperature/concentration combination. For the response variable of browning, the treatment structure was a 3 (temperature) x 3 (concentration) factorial with Arg and Lys and a 2 (temperature) x 3 (concentration) factorial with the Ala, Gly, and Ser. For the response variable of 4-MeI, the treatment structure was a 2 (temperature) x 3 (concentration) factorial with Arg and Lys and a one-way analysis of variance (concentration) with the Ala, Gly, and Ser.

All analyses were conducted using the GLM procedure inside SAS software version 9.4 (SAS Institute Inc., 2013). F-tests were calculated for main effects (concentration and/or temperature) and any interactions (concentration*temperature) if present. Multiple pairwise comparisons between temperature/concentration combinations were made with a Tukey adjustment to determine significant differences.

Additional covariates measuring initial pH and water activity levels were added individually to the prior analysis to investigate their association with levels of 4-MeI and browning. This resulted in an analysis of covariance (ANCOVA) that estimated individual (concentration or concentration*temperature) intercepts and a common slope for each specified covariate. Due to the few number of replications, limited power was available to find significant associations with the data.

Lastly, a multivariate analysis of variance (MANOVA) was performed to investigate the association between change in pH, amount of browning, and creation of 4-MeI. Data collected at 160°C was pooled across amino acids for analysis. By incorporating the design structure into the analysis, we avoid naïve Pearson's correlation coefficient estimates that are confounded with the significance of concentration levels inside the data.

RESULTS AND DISCUSSION

Change in the pH and effects of pH

pH reductions were observed in all treatment combinations after heat treatments (Table 7). Wong and Bernhard (1988) also reported lower pH levels after heat treatment in the Maillard model system using Glu and Gly. The initial pH before heat treatment for both Glu-Arg and Glu-Lys were higher than Glu-Ala, Glu-Gly, and Glu-Ser. Both Arg and Lys are basic amino acids; they have a basic side chain at neutral pH, and because of their high pKa, this side chain remains positive charged. Table 8 shows the pH values after heat treatments.

Several articles in the literature have pointed out the effect of the initial pH of reactants in the Maillard reaction. The degree of browning and formation of the end products depends on reactant pH. In general, increasing the pH of reactant medium increases the Maillard reaction rate (Ashoor and Zent 1984; Petriella and others 1985).

The two essential reactants in the Maillard reaction are reducing sugars and amino acids. The reactive form of sugar is the open-chain form, while the reactive form of the amino group is the non-protonated form. These two reactant forms are favored when the pH in the Maillard reaction is higher (Newton and others 2012). The protonation and non-protonation states of the amino group depend on the amino group pKa and the surrounding pH. For example, the pKa of Gly is 9.6; as the pH increases, the non-protonated amino group percentage increases (Figure 15). This is the most favorable conditions for the Maillard reaction (Martins and others 2001).

Sugar fragmentation occurs at pH values below neutral (O'Beirne 1986), increasing significantly at higher pH values and temperatures (Clarke and others 1997).

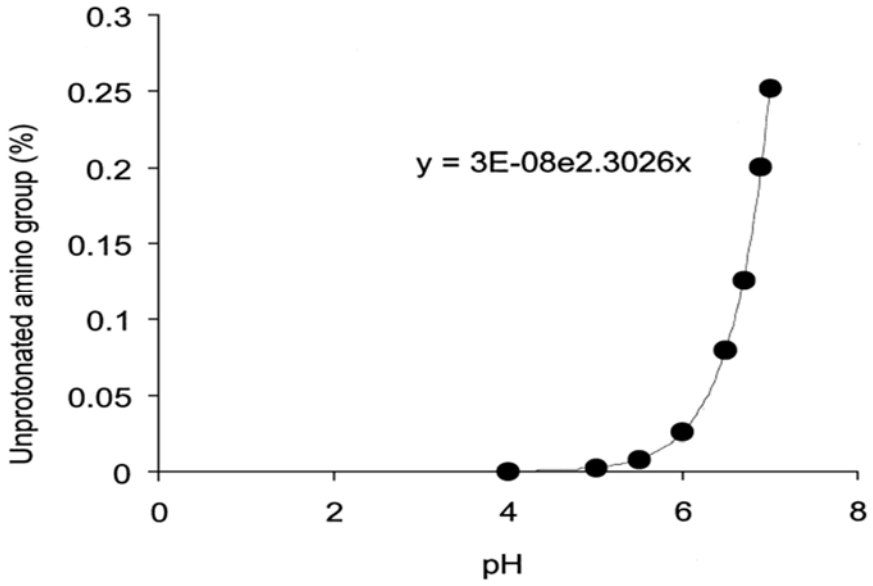

Figure 15. Effect of pH on the deprotonated Gly amino group (Martins and others 2001).

Table 7. pH values before heat treatment for all treatments combinations**.**

	60°C			120°C			160°C		
Glu-AA	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**
Glu-Ala	7.41 ± 0.01	7.02	6.32 ± 0.01	7.41 ± 0.01	7.01 ± 0.01	6.32 ± 0.01	7.23 ± 0.01	7.02	6.32 ± 0.01
Glu-Arg	10.52 ± 0.02	10.11	10.62 ± 0.01	10.52 ± 0.02	10.10 ± 0.01	10.62 ± 0.01	10.44 ± 0.01	10.11	10.62 ± 0.01
Glu-Gly	720 ± 0.01	6.52 ± 0.01	6.45 ± 0.03	7.20 ± 0.01	6.54 ± 0.02	6.45 ± 0.03	7.12 ± 0.01	6.52 ± 0.01	6.45 ± 0.03
Glu-Lys	9.94 ± 0.03	9.79 ± 0.01	10.02 ± 0.01	9.94 ± 0.03	9.84 ± 0.02	10.02 ± 0.1	9.87 ± 0.02	9.79 ± 0.01	10.02 ± 0.01
Glu-Ser	6.84 ± 0.02	6.55 ± 0.02	5.93 ± 0.01	6.84 ± 0.02	6.58 ± 0.01	5.93 ± 0.01	6.80 ± 0.03	6.55 ± 0.02	5.93 ± 0.01

Values represent the mean ± standard error (n = 3).

Standard errors equal to zero were deleted from the table.

Table 8. pH values after heat treatment for all treatments combinations**.**

	60°C			120°C			160°C		
Glu-AA	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**
Glu-Ala	7.17 ± 0.02	6.84 ± 0.04	6.87 ± 0.01	7.17 ± 0.01	6.08 ± 0.02	5.18 ± 0.01	5.89 ± 0.02	5.19 ± 0.01	5.10 ± 0.01
Glu-Arg	10.41 ± 0.01	9.96 ± 0.01	10.35 ± 0.01	9.00 ± 0.01	8.09 ± 0.01	8.08 ± 0.02	8.72 ± 0.01	7.47 ± 0.01	7.48 ± 0.03
Glu-Gly	7.10 ± 0.01	6.93 ± 0.02	6.79 ± 0.01	6.77 ± 0.01	6.11 ± 0.02	5.00 ± 0.02	5.61 ± 0.01	4.87 ± 0.01	4.75 ± 0.01
Glu-Lys	9.86 ± 0.01	9.54 ± 0.01	9.71 ± 0.01	8.84 ± 0.01	7.34 ± 0.02	7.06 ± 0.01	8.34 ± 0.01	5.58 ± 0.01	5.46
Glu-Ser	6.68 ± 0.01	6.50 ± 0.01	6.35 ± 0.02	6.54 ± 0.01	6.26 ± 0.02	5.07 ± 0.01	6.40 ± 0.03	5.21 ± 0.01	5.11 ± 0.02

Values represent the mean ± standard error (n = 3).

Standard errors equal to zero were deleted from the table.

Effect of temperature

Several researchers have noted the effect of temperature on Maillard reaction; the rate of the Maillard reaction increases as the temperature increases (Maillard 1912; Labusa and others 1994; O'Brien and others 1998; Coca and others 2004). An increase in temperature leads to an increase in the reactivity of the sugar and the amino group, resulting in more browning during the Maillard reaction (Martins and others 2001; Clarke and others 1997). As predicted, as the temperature increased, the rate at which 4-MeI formed increased. Temperature had a significant effect ($P < 0.001$) both on the formation of 4-MeI and on browning.

In two separate experiments conducted by Jang and others (2013) and Lee and others (2013), with Glu and ammonia in equimolar 0.1, 0.5, and 1.0 M mixtures, each were heat treated at 100°C or 150°C for 2 h. Lee and others (2013) reported levels of 4-MeI at 7.18 μg/g for the 0.1 M mixture, 67.78 μg/g for the 0.5 M mixture, and 100.22 μg/g for the 1.0 M mixture. Jang and others (2013) reported levels of 52.78 μg/g for the 0.1 M mixture, 666.69 μg/g for the 0.5 M mixture, and 1268.71 μg/g for the 1.0 M mixture, representing an increase in % 4-MeI levels of 7.3 time for the 0.1 M mixture, 9.8 for the 0.5 M mixture, and 12.69 for the 1.0 M mixture when the reaction temperature was increased from 100°C to 150°C. Our results also showed, levels of 4-MeI increased when the reaction temperature was increased. The 4-MeI levels for the Glu-Arg treatment at 120°C were 0.13, 0.26, and 0.39 μg/g for 0.05, 0.1, and 0.15 M mixture, but when the reaction temperature was increased to 160°C, the levels of 4-MeI increased to 0.20, 0.66, and 1 μg/g for the same concentrations. Browning intensity also increased as the heating temperature increased in all treatment combinations.

Water activity

Table 9 shows the a_w of the Glu-AA treatment combinations at 0.05, 0.1, and 0.15M. This table shows the lowest a_w for all treatments at 0.1 M. The a_w is important in forming caramel colors in food. The first stage of the Maillard reaction requires water. Therefore, the rate of Maillard reaction depends on the amount of free water available to the reactant. However, in high water content food, the Maillard reaction rate occurs less readily because the reactant is diluted, while in food with low water content, the mobility of reactants is limited even if they are present in high concentrations. Wolfrom and Rooney (1953) reported maximum browning occurred at 30% moisture, corresponding to an a_w between 0.6 - 0.8.

The overall a_w activity in all treatments was not significant ($P > 0.05$) indicating no effect of a_w on either 4-MeI formation or browning. Dennis and others (2015) also reported a_w had no significant effect on the formation of HCAs, although, they used diethylene glycol in their model system. We used a model system consisting of 100% water.

Table 9. a_w values for all treatment combinations before heat treatment.

	60°C			120°C			160°C		
Glu-AA	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**
Glu-Ala	0.996	0.981	0.994	0.996	0.981	0.994	0.996	0.981	0.994
Glu-Arg	0.997	0.981	0.994	0.997	0.981	0.994	0.995	0.981	0.994
Glu-Gly	0.996	0.979	0.995	0.996	0.979	0.995	0.995	0.979	0.995
Glu-Lys	0.996	0.980	0.991	0.996	0.980	0.991	0.995	0.980	0.991
Glu-Ser	0.995	0.978	0.994	0.995	0.978	0.994	0.995	0.978	0.994

Values represent the mean ± standard error (n = 3).

Standard errors equal to zero were deleted from the table.

4-MeI levels

4-MeI was detected using the precursor ion *m/z* 182 and three product ions *m/z* 81, 82, and 109. The ion *m/z* 82 was used to quantify 4-MeI (Figure 16). For 4-MeI-d_6, the precursor ion *m/z* 187 and three product ions *m/z* 85, 87, and 114 were used for detection purposes while ion *m/z* 87 was used to quantify the compound. After derivatization with IBCF, 4-MeI or 4-MeI-d_6 showed two compounds: 4-MeIa and 4-MeIb, and 4-MeI-d_6a and 4-MeI-d_6b. Similar results were reported by Fernandes and Ferreira (1997) and Karim and Smith (2015). Karim and Smith (2015) also reported that the sum of these two peaks was used to calculate the amount of 4-MeI in the samples; however, the limit of detection (LOD) or the limit of quantification (LOQ) were limited to the second peak (4-MeIb) due to its relatively small area compared to that of the first peak area (4-MeIa). In this study, the LOD was 0.05 ppm (signal-to-noise ratio of 3:1), and LOQ was 0.1 ppm (signal-to-noise ratio of 10:1). These two values were lower (0.25 and 0.4 ppm) than of Karim and Smith (2015). This difference could be due to the usage of the *m/z* 82 ion compared to the *m/*z 182 ion used by Karim and Smith (2015) to calculate the amount of 4-MeI in the samples. Karim and Smith (2015) also mentioned that the *m/z* 182 ion was used instead *m/z* 82 due to the occurrence of interfering peaks from the tested samples with m/z 82 ion in the meat extract.

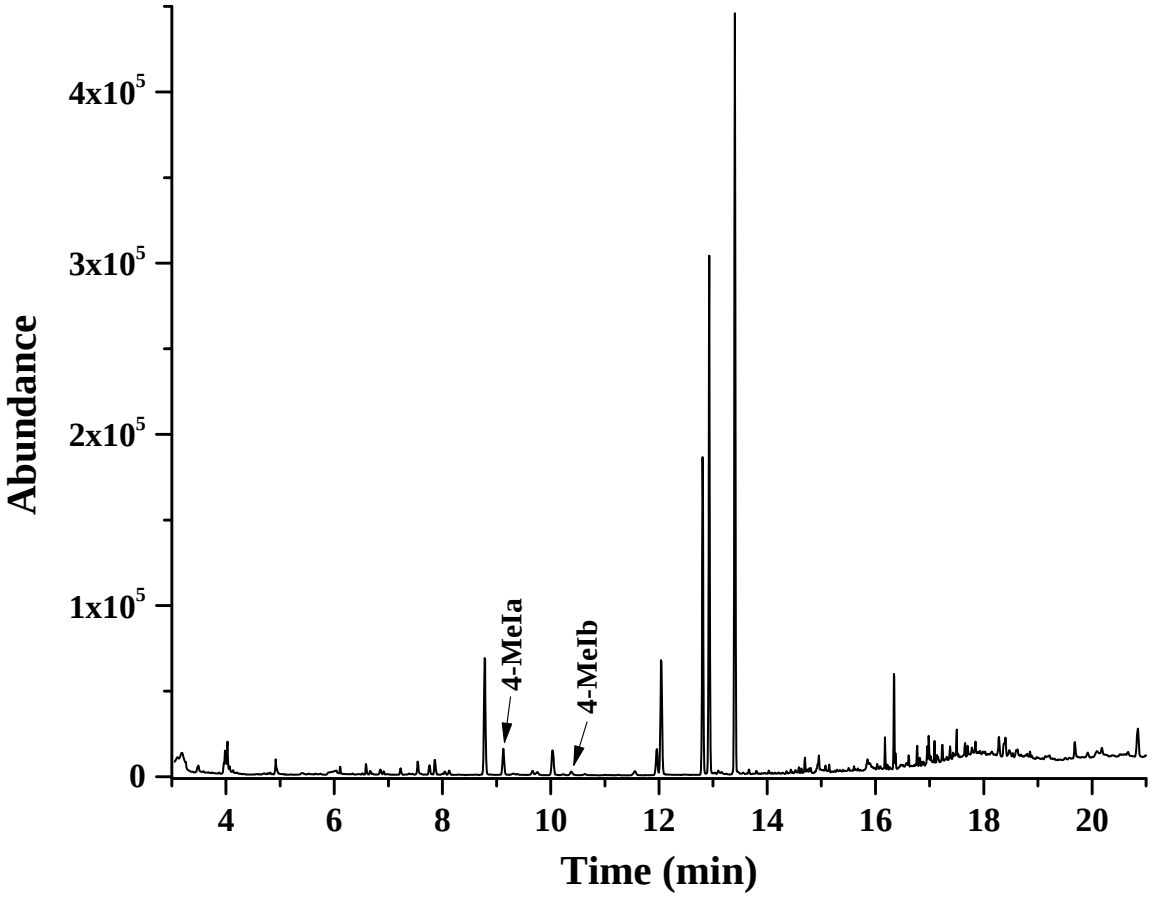

Figure 16. GC-MS chromatogram in SIM mode for *m/z* 81, 82, 109, and 182 ions showing two compounds (4-MeIa and 4-MeIb) after derivatization with isobutylchloroformate for Glu-Arg sample with 0.1 M concentration and heat treatment at 160°C for 1h.

In general, there was a significant relationship ($P < 0.001$) between type of AA and 4-MeI formation. Arg showed more formation of 4-Mel than Ala, Gly, Lys, and Ser. However, there were no significant differences ($P > 0.05$) among Ala, Gly, Lys, and Ser.

Derivatizing of 4-MeI with IBCF and GC-MS analysis has proven to be suitable and an accurate analysis (Fernandes and Ferreira 1997; Karim and Smith 2015). No levels of 4-MeI were detected at 60°C for 1 h in all treatment combinations. At 120°C and 0.05 M, levels of 0.013 μg/g and 0.014 μg/g for Glu-Arg and Glu-Lys were found; the amount of 4-MeI for Glu-Arg increased with increasing concentrations and higher heating temperatures; for Glu-Lys treatment, 4-MeI levels did not increase as rapidly as for Glu-Arg; 4-MeI formation was not detected in Glu-Ala, Glu-Gly, or Glu-Ser in all treatments combinations even when reactants were heated at 120°C for 1 h.

Table 10 shows 4-MeI levels in all treatment combinations at 160°C and 0.05 M. All treatments produced 4-MeI at 160°C and 0.05 M. The Glu-Arg treatment produced the most 4-MeI (1.0 μg/g); Glu-Ala and Glu- Lys treatments produced similar levels of 4-MeI (0.21 and 0.22 μg/g). Glu-Gly and Glu-Ser produced the least amount of 4-MeI (0.15 and 0.16 μg/g).

Shibamoto (1983) reported that 4-MeI formed in heat-processed foods and beverages that contain sugars and amino acids, which are an ideal precursor of imidazoles, including 4-MeI. Wong and Bernhard (1988) also detected 4-MeI in Glu and Gly model system. Sugars degrade to alkyl carbonyl and alkyl ketones (Moon and Shibamoto 2009), and amino acids degrade to ammonia and alkyl carbonyls via Strecker degradation (Strecker 1862). Several researchers have reported the formation of 4-MeI in ammonia and the Glu Maillard model system (Moon and Shibamoto 2011, Lee and others 2013, Seo and others 2014). In fact, preparing caramel colors III and IV involves food grade sugars reacting with ammonia.

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Table 10. Levels of 4-MeI (μg/g) in all treatment combinations.

	60°C			120°C			160°C		
Glu-AA	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**
Glu-Ala	N/D	N/D	N/D	N/D	N/D	N/D	0.12 ± 0.01	0.17 ± 0.01	0.21 ± 0.01
Glu-Arg	N/D	N/D	N/D	0.13 ± 0.01	0.26 ± 0.01	0.39 ± 0.01	0.20 ± 0.01	0.66 ± 0.03	1.00 ± 0.01
Glu-Gly	N/D	N/D	N/D	N/D	N/D	N/D	0.11 ± 0.01	0.13 ± 0.01	0.15 ± 0.01
Glu-Lys	N/D	N/D	N/D	0.14 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.18 ± 0.01	0.22 ± 0.01	0.22 ± 0.01
Glu-Ser	N/D	N/D	N/D	N/D	N/D	N/D	0.11 ± 0.01	0.13 ± 0.01	0.16 ± 0.01

Values represent the mean ± standard error (n = 3).

N/D = not detected.

Due to low detection limit significant differences between values haven’t been performed.

Formation of 4-MeI depends on the reactant concentration and its rate of degradation. Ammonia is released from the thermal degradation of amino acids from the amino group. Sohn and Ho (1995) conducted an experiment to measure the amount of ammonia from thermal degradation rate of 19 amino acids in a controlled pH buffer of 8; 19 amino acids were heated for 2 h at 180°C in a conventional drying oven. An ion selective electrode measured levels of ammonia released from the degraded amino acids during heat treatment, Arg had higher level of ammonia than Lys, Gly, Ala, and Ser during heat treatment; the reported ammonia levels for Ala, Arg, Gly, Lys, and Ser were < 5, ~ 28, < 5, < 5, and 5 - 6%. Both Arg and Lys contain two nitrogen atoms, so the amount of the released ammonia with these two amino acids should be higher than one nitrogen amino acids such as Ala, Gly, or Ser. Interestingly Lys (most reactive amino acids) did not release more ammonia than Arg, which might be due to the basic property of Lys and the extremely high pKa value (Sohn and Ho 1995). In this experiment, Lys also did not form 4-MeI; Arg did even though Lys produced more browning than Arg. This might be due to the amount of ammonia released by Lys. Moreover, 0.3 M concentrations of Glu, Ala, Arg, Gly, Lys, and Ser were prepared individually and heated to 160°C for 1 h to investigate color development and 4-MeI formation (Figure 17). Even though browning occurred in Glu, Lys, and Ser, no 4-MeI levels were detected in any of the tested solutions. The developed color in Glu is due to caramelization a well-known pheromone in heated carbohydrates; in Lys and Ser, the developed color is due to Strecker degradation.

As mentioned above, the amount of the released ammonia from the thermal degradation of the tested amino acid might be the responsible for formation of 4-MeI. The higher amounts of 4-MeI in the heat treated Glu-Arg model system (see Table 10) may be because of the effect of the initial pH; in our study, the Glu-Arg pH was 10.4, so the open-chain form of Glu is

predominant as is the non-protonated form of the Arg. In Glu-Lys model, even though the initial pH was10, levels of 4-MeI were lower than Glu-Arg, suggests another heterocyclic nitrogen-containing compounds formed instead 4-MeI. More studies are recommended to investigate the compounds produced in the Glu-Lys model under thermal treatment. For Glu-Gly, Glu-Lys, and Gly-Ser, the initial low pH and the low amount of released ammonia could be the reason levels of 4-MeI are low in those models.

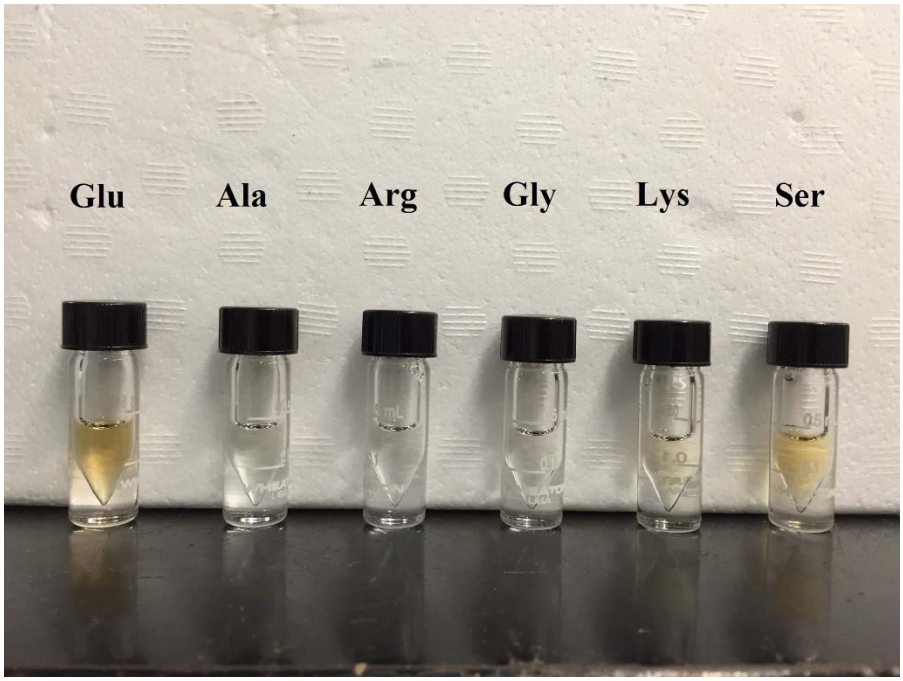

Figure 17. Browning development in individual solutions of Glu, Ala, Arg, Gly, Lys, and Ser.

Reactant concentrations

Both 4-MeI and browning increased as the concentration of Glu-AA increased. Lee and others (2013) noted formation of 4-MeI in a Glu and ammonium hydroxide model system had a dose response to reactant concentrations when heat was applied (100°C) for 2 h.

In another experiment, 4-MeI levels increased as the concentrations of the reactants increased in equimolar solutions of 0.1, 0.5, and 1.0 M of Glu and ammonium hydroxide model system when heated at 150°C for 2 h (Jang and others 2013).

Browning

In general, the type of AA had a significant relationship ($P < 0.001$) with browning. The Glu-Lys model showed the highest browning, followed by Glu-Arg. Both were significantly higher than other tested amino acids, possibly because both have high pH values that enhanced alkaline degradation of Glu and the subsequent reaction of the degradation products with both Lys and Arg to produce browning (Willits and others 1958). Yu and others (2012) noted similar results when Glu-AA models were heat treated at120°C for 1 h; they reported browning intensity follows: Glu-Lys > Glu-Gly > Glu-Ala > Glu-Arg > Glu-Ser. Compared to our results, the only two AA with browning were Glu-Lys and Glu-Arg when both heat treated at 120°C for 1h. The difference between our results and Yu and others (2012) occurred because of the pH of the reactants. In our method, the Glu-AA mixtures were prepared in water while in Yu and others (2012) the Glu-AA models were prepared in a phosphate buffer of pH 7. Phosphate buffers are known to increase the rate of the Maillard reaction (Newton and others 2012).

No visible color occurred in any treatment held at 60°C for 1 h (Figure 18); however, a slight browning was measured in Glu-Lys at a concentration of 0.05 M; this browning increased

at 0.1 M and 0.15 M (Table 11). At 120°C and 0.05 M, the browning was more intense for Glu-Lys and Glu-Arg but Glu-Lys produced more browning than Glu-Arg. The first step of the Maillard reaction requires protons to initiate. In the Glu-Lys reaction, additional protons are supplied by the ε-amino group, which may lead to more browning in the Glu-Lys solution (Ajandouz and Puigserver 1999). No visible browning was noted for Glu-Ala, Glu-Gly, or Glu-Ser (Figure 19) at 120°C and 0.05 M. At 120°C and 0.1M, we noted more browning development in Glu-Arg and Glu-Lys (see Figure 20).

At 120°C and 0.15 M concentration, all combination treatments showed visible browning, but browning was more intense in the Glu-Lys and Glu-Arg treatments (Figure 21). Figure 22 shows the browning development of all treatments at 160°C and 0.05 M concentration for 1 h. All treatment combinations showed browning, in the following order intensity: Glu-Lys > Glu-Arg > Glu-Gly > Glu-Ser > Glu-Ala.

Glu-Lys also had more browning than Glu-Arg, Glu-Gly, and Glu-Ser when 0.1 M equimolar aqueous solutions of glucose and different amino acids were heat treated at 130°C for 2 h (Hwang and others 2011). Consequently, more browning occurred in all treatment combinations when the temperature increased (Figure 23 and Figure 24).

Table 11. Browning formation in all treatments combinations measured at 420 nm.

	60°C			120°C			160°C		
Glu-AA	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**	**0.05 M**	**0.1 M**	**0.15 M**
Glu-Ala	0.00	0.003	0.00	0.03	0.05	0.14	2.68 ± 0.10	15.66 ± 0.02	27.55 ± 0.45
Glu-Arg	0.01	0.007	0.00	2.20	11.58 ± 0.04	21.40 ± 0.10	4.55 ± 0.10	25.58 ± 0.06	41.05 ± 0.29
Glu-Gly	0.00	0.003	0.00	0.01	0.08	0.28 ± 0.0	2.91 ± 0.23	15.61 ± 0.06	37.06 ± 0.46
Glu-Lys	0.02	0.03	0.07	7.07	27.45 ± 0.07	46.78 ± 0.11	12.01 ± 0.14	45.61 ± 0.16	73.06 ± 1.04
Glu-Ser	0.00	0.003	0.00	0.01	0.06	0.18	3.50 ± 0.10	21.23 ± 0.13	34.63 ± 0.31

Values represent the mean ± standard error (n = 3).

Standard errors equal to zero were deleted from the table.

Due to low detection limit significant differences between values haven't been performed.

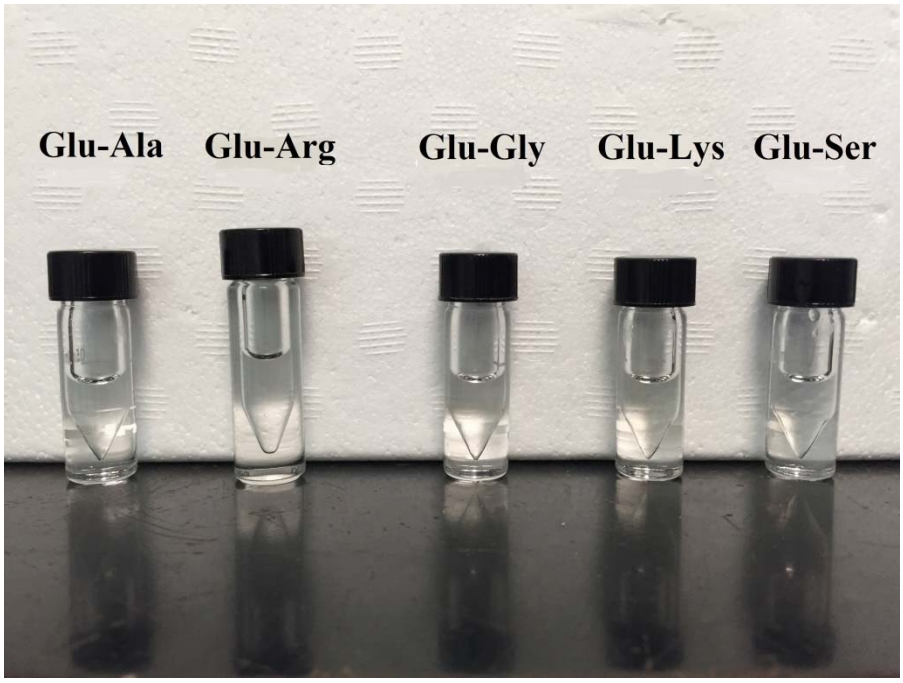

Figure 18. Browning development for all treatment combinations at 60°C for 1 h and 0.15. M.

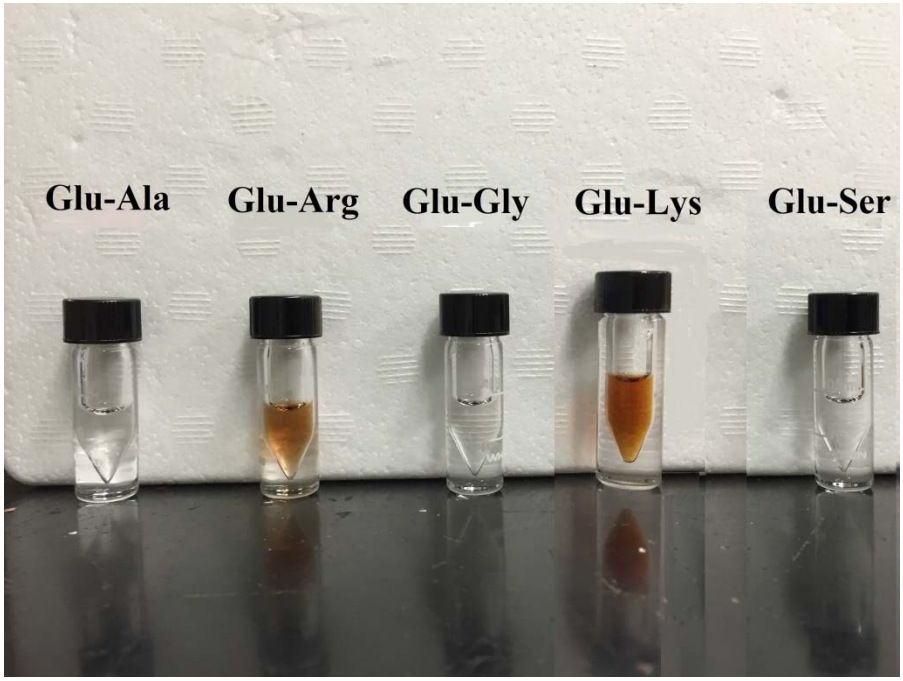

Figure 19. Browning development for all treatment combinations at 120°C for 1 h and 0.05 M.

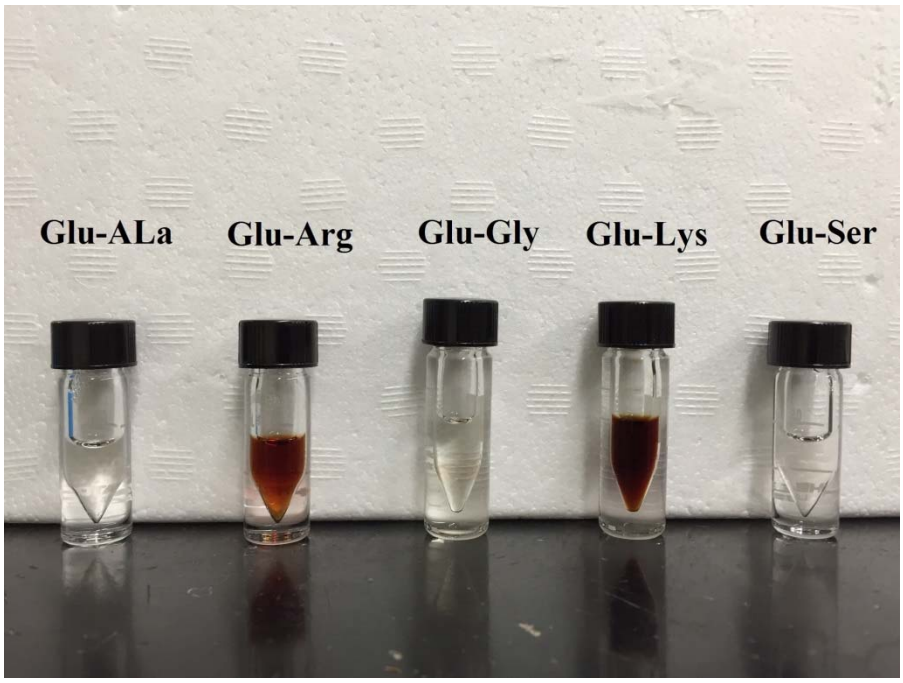

Figure 20. Browning development for all treatment combinations at 120°C for 1 h and 0.1 M.

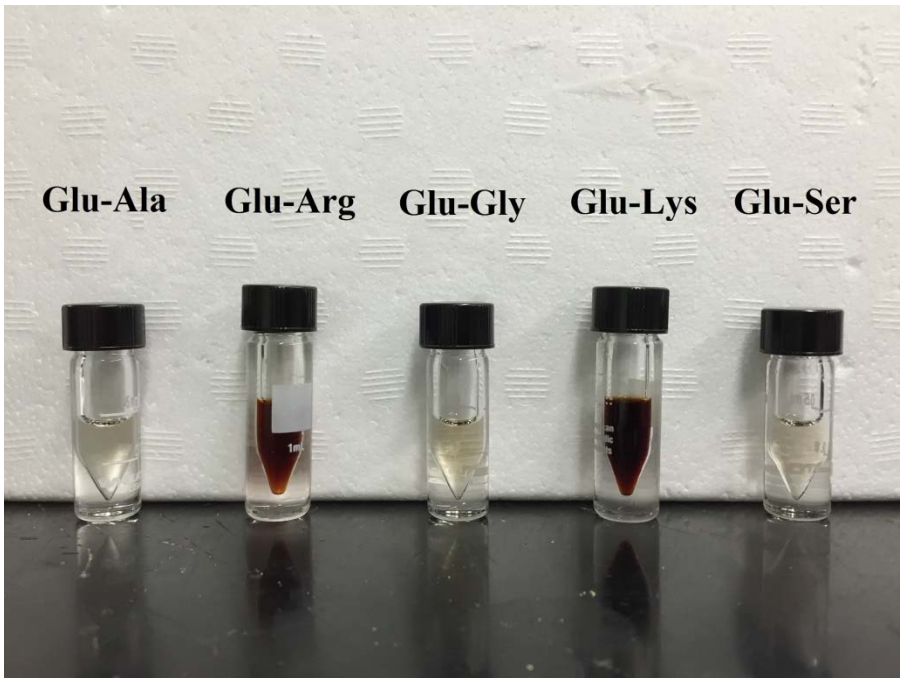

Figure 21. Browning development for all treatment combinations at 120°C for 1 h and 0.15 M.

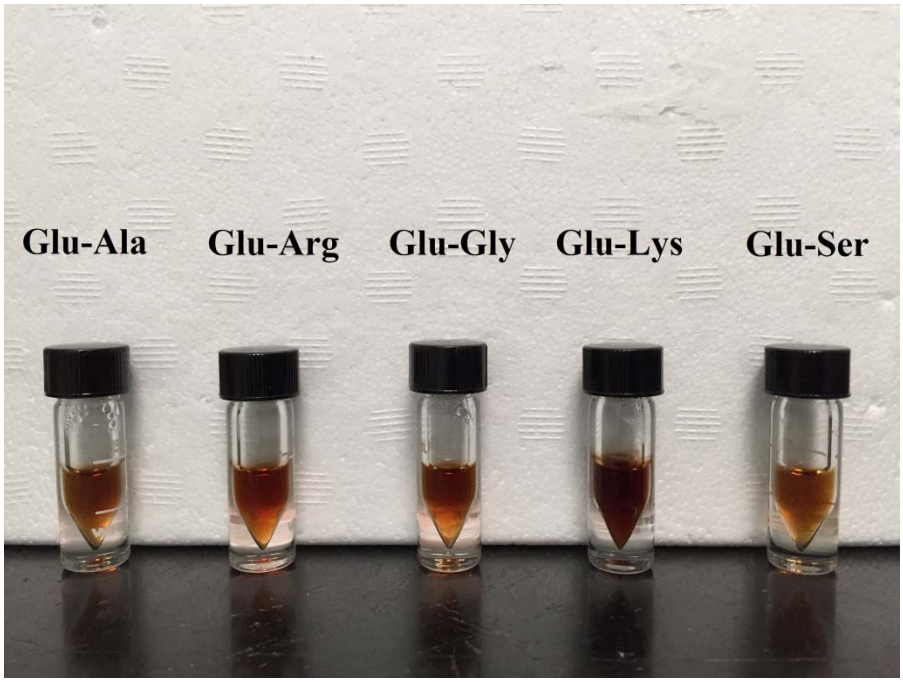

Figure 22. Browning development for all treatment combinations at 160°C for 1 h and 0.05 M.

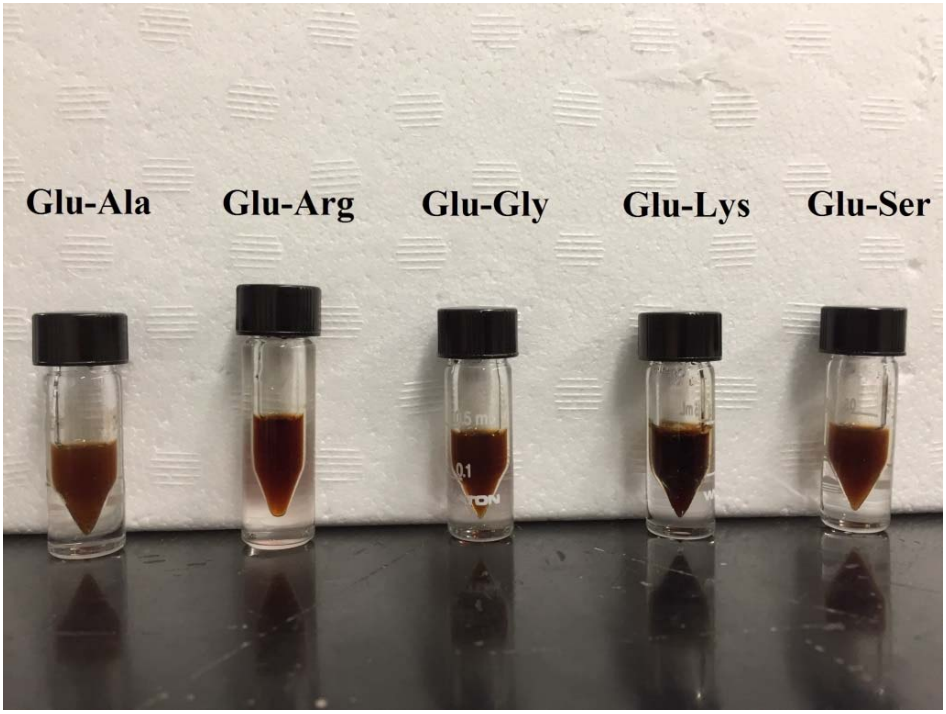

Figure 23. Browning development for all treatment combinations at 160°C for 1 h and 0.1 M.

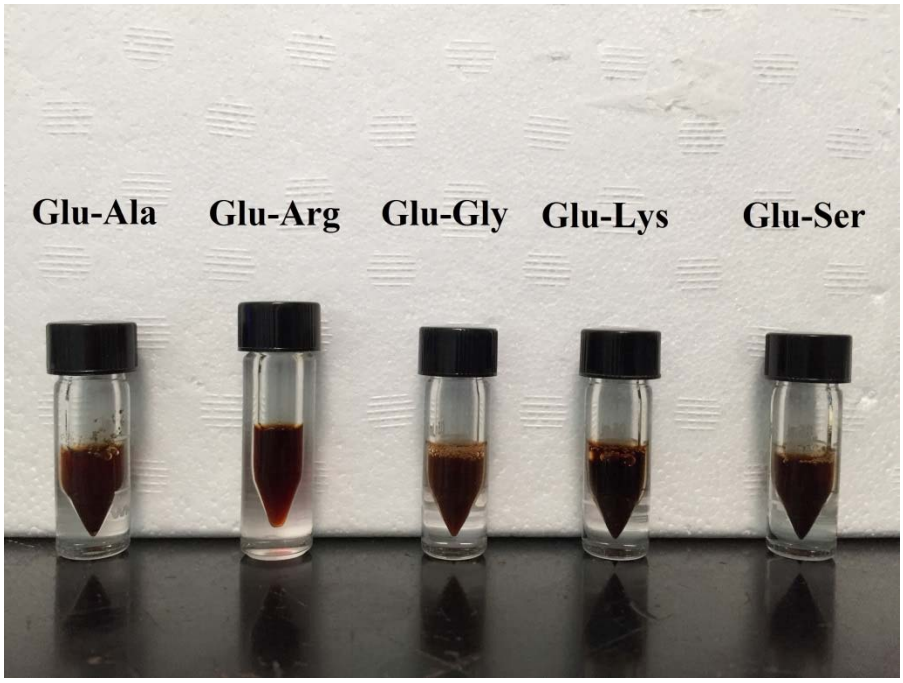

Figure 24. Browning development for all treatment combinations at 160°C for 1 h and 0.15 M.

CONCLUSION

4-MeI formation in an equimolar Maillard model system of Glu and 5 different amino acids was investigated. Arg showed the most 4-MeI formation of all tested amino acids. Lys, however, produced darker brown color with less 4-MeI formation than Arg. The temperature, concentration, and pH significantly affected 4-MeI formation, with no significant interaction between water activity and either browning or 4-MeI formation.

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CHAPTER 4. INHIBITION OF 4(5)-METHYLIMIDAZOLE FORMATION IN AN AQUEOUS D-GLUCOSE L-ARGININE MODEL SYSTEM

ABSTRACT

Eliminating harmful chemicals completely from processed foods is a difficult task but an important objective of both the food industry and regulatory agencies. Several studies have shown the toxicity of 4(5)-Methylimidazole (4-MeI) as a neurotoxin and possible carcinogen. Therefore, we investigated how to inhibit 4-MeI formation in the Maillard reaction model system. The effect of ascorbic acid (ASA) on the formation of 4-MeI and browning in the glucose-amino acid (AA) model system was studied. Four concentrations of ASA (0.0375, 0.075, 0.15, or 0.3 M) were added to an aqueous solution of D-glucose:L-arginine (Glu:Arg; 0.15:0.15 M). Mixtures were heated at 160°C for 1 h in a convection oven. Levels of 4-MeI after heat treatment were quantified using isobutylchloroformate (IBCF) as a derivatizing agent followed by gas chromatography mass-spectrometry (GC-MS). How ASA concentrations, initial pH of reactants, and water activity affected formation of 4-MeI and browning were studied. Compared to control, ASA inhibited formation of 4-MeI by 40.29, 59.94, 75.13, and 96.25% for 0.0375, 0.075, 0.15, and 0.3 M concentrations. ASA also increased browning in the model system; the levels increased by 10.67, 15.47, 18.4, and 28.8% for 0.0375, 0.075, 0.15, and 0.3 M concentrations of ASA. In addition, solid phase micro extraction (SPME) GC-MS analysis comparison between two samples of Glu-Arg with and without ASA showed adding ASA reduced volatile compounds in the sample. Addition of ASA was shown to inhibit 4-MeI formation and increased browning, a desirable quality in some food products.

INTRODUCTION

4-MeI is a heterocyclic compound with two nitrogen atoms derived from imidazole; it is widely used in the pharmaceuticals, chemical, and agricultural industries (OEHHA 2011). 4-MeI is also formed as a by-product in caramel coloring manufactured by controlled heat treatment of food grade carbohydrates with ammonia or ammonium salts (Moretton and others 2011). Caramel color (the main source of 4-MeI) is a common food additive used by the food industry, which means 4-MeI is widely present in a variety of foods including roasted meats, coffee, wine, carbonated beverages, and soy sauce (Yamaguchi and Masuda 2011). Moreover, 4-MeI is the main imidazole in the class III and IV caramel colorings (Buckee and Bailey 1978) and was also found in ammoniated forage and hay (Karangwa and others 1990).

Concentrations of 4-MeI in caramel colors can vary depending upon the manufacturing process, and the types of carbohydrates and nitrogen-containing compounds used (Mueller and Jork 1993; Fernandes and Ferreira 1997; Xiao and Liao 2005).

The toxicity and carcinogenicity of 4-MeI in foods and beverages have recently attracted an attention from regulatory agencies. In fact, the Center for Science in the Public Interest (CSPI) petitioned the FDA to ban colorings made with ammonia processing because of the presence of 4-MeI (CSPI 2011). Therefore, finding effective strategies to reduce formation of 4-MeI in foods and beverages and understanding the underlying mechanisms of 4-MeI formation (including precursors to 4-MeI) is important.

4-MeI was first isolated in 1962 (Komoto 1962)from mixture containing Glu and ammonium hydroxide after heat treatment, which produced a typical class III caramel color used in alcoholic beverages. Later, subsequent studies indicated that reducing sugars and AA in the

Maillard reaction system are the ideal precursors to imidazole formation, suggesting that 4-MeI can form in the Maillard reaction (Shibamoto 1983).

The Maillard browning reaction is responsible for the flavor and browning development in foods and beverages. It is also called the non-enzymatic carbonyl-amino reaction when reducing sugar is degraded into α-dicarbonyl compounds and ammonia is produced from AA via Strecker degradation. The reducing sugar and ammonia are then condensed into 2-,4-, or 5-substituted imidazoles (Hodge 1967; Strecker 1862; Moon and Shibamoto 2011), so the production of caramel coloring via the Maillard reaction contains 4-MeI, which is unavoidably formed and present in foods prepared by heat treatment.

Many studies on inhibiting 4-MeI formation have been published, and many focus on controlling the development of the Maillard reaction by reducing heating times, temperatures, and pH, or adjusting water activity and sulfur dioxide treatment (Nursten 2005). In addition, the effect of different chemicals on formation of 4-MeI was studied. A higher reaction rate during the Maillard reaction can be induced by copper and iron, but manganese and zinc can inhibit the Maillard reaction (Newton and others 2012). Calcium inhibits formation of the end products of Maillard reaction by forming complexes with certain sugars (Rizzi 2008). Seo and others (2014) demonstrated that certain food additives (for example, metal ions) and AA (tryptophan and cysteine, among others) can effectively inhibit formation of 4-MeI in the caramel model system at 100°C for 2 h.

Consumers prefer natural food additives, so ingredients containing natural antioxidants like plant extracts, spices, and herbs may be good alternatives. Rosemary (*Rosmarinus officialis* Labiatae) is commonly used a flavoring and antioxidant in foods. Several studies have shown that adding rosemary extracts to cooked meat could significantly reduce heterocyclic amines

(HCAs) because rosemary has high concentrations of antioxidants that can act as free radical scavengers (Puangsombat and Smith 2010). The main antioxidant compounds in rosemary are the phenolic diterpenes (carnosol, rosmanol and carnosic acid) and phenolic acids (rosmarinic acid and caffeic acid). Among these, rosmarinic acid is the most powerful antioxidant, and more importantly, it has higher thermal stability (Zhang and others 2012).

Another important water-soluble and free radical scavenger is ascorbic acid. ASA is the most common antioxidant used as a food additive to increase the nutritional values of foods. Many researchers have reported that adding ASA to meat was effective in preventing color changes as well as inhibiting the oxidative reaction in salted meats (Kim and others 1997; Okayama and others 1987; Sanchez-Escalante and others 2001). However, adding ascorbic acid to processed foods or foods with high levels of ASA (citrus juice or dried fruits, for instance) caused unwanted browning because of the reaction between ASA and dehydroascorbic acid (DHA; oxidized form of ASA) with the AA (Belitz and others 2004). Bharate and Bharate (2012) described brown pigment forming from the reaction of DHA with AA (see Figure 25).

Besides the lipid and color stability benefits of ASA, incorporating ASA into the Maillard reaction may also inhibit the formation of mutagenic HCAs. Wong and others (2012), using a Maillard reaction model system (130^{o}C for 2 h), showed that ASA could interact with Maillard reaction precursors and reduce the levels of 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP) by 70% and 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx) by 35% (both are compounds containing an imidazole ring). These results provide strong evidence that ASA effectively inhibit the formation of HCAs, and might significantly inhibit other types of the Maillard reaction products such as 4-MeI.

The Maillard reaction is responsible for producing aroma during cooking and thermal processing in the food industry (Cerny 2008). Thermal processing of foods that contain amino compounds (protein, peptides, and amino acids) and carbonyl groups like reducing sugars generates a number of volatile compounds. This gives processed food its distinct aroma of toasting, roasting, baking, nuts and frying, as well as contributing to the quality of finished food products. Many studies have also reported the formation of volatile compounds in Maillard reactions where ribose and cysteine (Cys; important meat aroma precursors) were studied (Gasser and Grosch 1990; Farmer and Mottram 1990; Hofmann and Schieberle 1995; Cerny and Davidek 2003).

Different heterocyclic volatile compounds have been identified in the Maillard reaction, including derivatives of furan, pyrrol, thiophenes, thiazoles, and thrithiolane, which are generally recognized as the main aromatic compounds in thermal-processed meat (Shahidi and others 1986).

ASA has been widely used as a preservative and stabilizer in processed food because of its strong antioxidant capacity and reducing power; however, ASA, another carbonyl compound, reacts with AA in the presence of heat and produces different compounds, including aroma.

To our knowledge, only a limited number of studies have been performed on aroma formation in the Glu-Arg model system using ASA, although in 2010, Yu and Zhang studied aroma formation in a Cys-ASA model using heat treatment. They identified 43 aroma compounds, including thiazoles, thiophenes, and pyrazines.

The objectives of our study were to: (1) examine ASA as a food additive and its ability to inhibit the formation of 4-MeI in a Glu-AA model and (2) investigate how ASA affects aroma formation in a Glu-Arg model system under heat treatment.

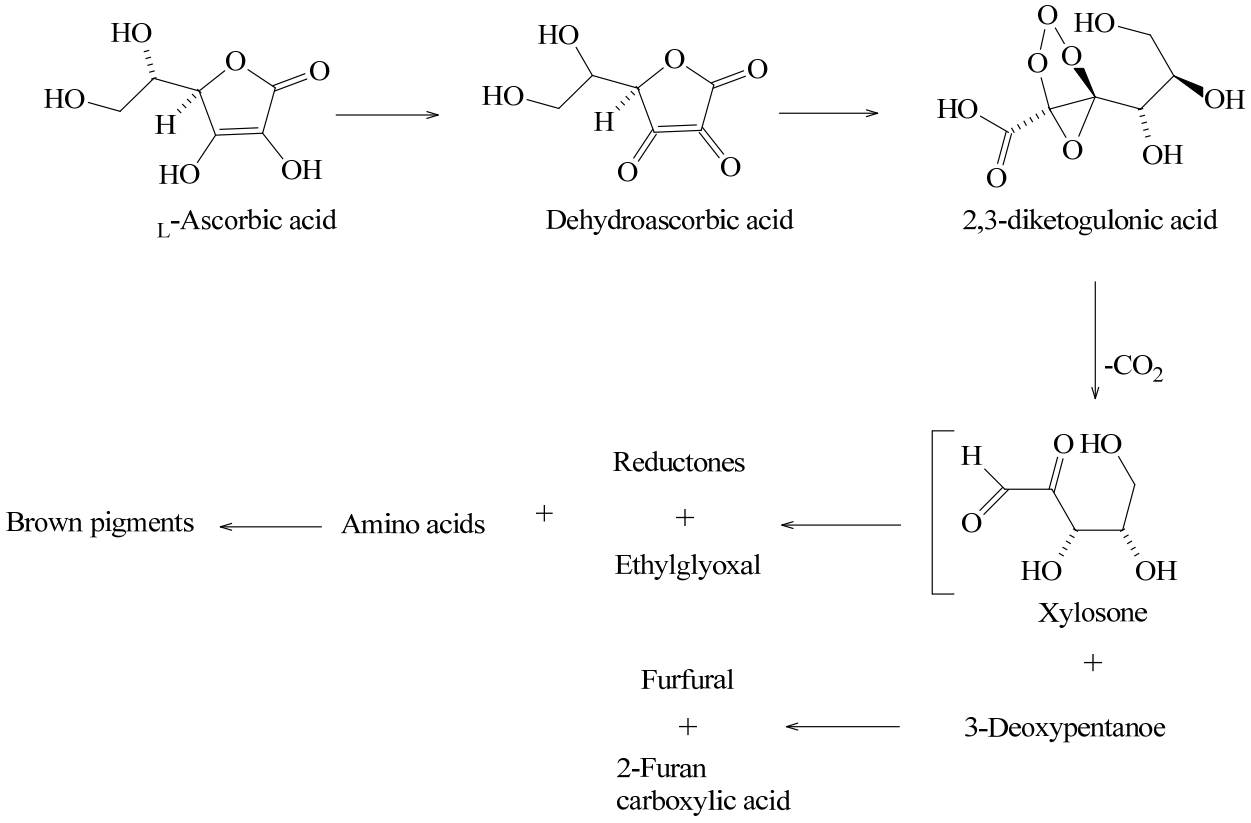

Figure 25. Browning formation in the reaction of dehydroascorbic acid with amino acid (Bharate and Bharate 2012).

MATERIALS AND METHODS

Chemicals and reagents

Isobutanol, pyridine, isobutylchloroformate, D-glucose (99.5%), L-arginine (98%), L-ascorbic acid, and 4-methylimidazole (≥ 98%) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Acetonitrile, hexane, and sodium bicarbonate, were obtained from Fisher Scientific Corp. (Pittsburgh, Pa., U.S.A.). The internal standard isotope deuterium labeled 4-methylimidazole-d_6 (4-MeI-d_6) was from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). All reagents and solvents used in the experiments were analytical-grade, and the doubled deionized

water, used to prepare all solutions, was prepared using Barnstead PCS filtration system (Thermo Scientific Inc., Waltham, Mass., U.S.A.). Sodium bicarbonate solution (1.0 M) was prepared by dissolving 8.4 g of sodium bicarbonate in 100 mL of double deionized water. Hydrochloric acid (0.1 N) was prepared by mixing 0.82 mL of concentrated hydrochloric acid (36.5 - 38%) with a 100 mL of double deionized water. The solid phase micro extraction (SPME) fiber was coated with 0.75 μm carboxen/polydimethylsiloxane (CAR-DPMS) purchased from Supelco Inc. (Bellefonte, Pa., U.S.A.). Carrier gas (ultra-high purity helium) for the GC-MS was obtained from Matheson Tri-Gas (Manhattan, Kans., U.S.A.).

Model system preparation

Aqueous solutions of Glu and Arg were first mixed in equimolar of 0.15 M of both compounds, and then four concentrations of aqueous solutions of ASA were added: 0.0375, 0.075, 0.15, or 0.3 M. The final volume of the Glu-Arg-ASA was 0.5 mL. All solutions were prepared using double deionized water at room temperature (25°C); the solutions were used for a reaction treatment of 1 h at 160°C.

Model system reaction procedure

The Glu-Arg-ASA mixtures were transferred to a 1 ml reaction vials (Wheaton Corp., Millville, N.J., U.S.A.) inserted into heating vessels. The vessel had three parts: a cylindrical threaded body (outer diameter of 15 mm) and two identical screw caps (interior diameter of 15 mm) on the top and bottom of the cylindrical body. The vessel parts were made of brass (zinc 33% and copper 66% alloy; Watts Corp. North Andover, Mass. U.S.A.). To seal the reaction vial inside the vessel, a silicone septum (15 mm; Sun Sri, Thermo Scientific Rockwood, Tenn.,

U.S.A.) was inserted into the top cap facing the opening end of the reaction vial. Vessels were hand tightened and inserted into an oven (HP 5890; Agilent Technologies Inc., Santa Clara, Calif., U.S.A.). A USB-TC thermocouple connected to a data logger and Tracer DAQPro software (Measurement Computing™, Waltham, Mass., U.S.A.) were used to record the oven temperature during heating time. The pH and water activity (a_w) were measured before the heat treatment; pH, browning, and 4-MeI levels were measured after heat treatment.

pH measurements of the model system

The pH values were detected using Accumet pH meter (model AP 115) equipped with an Accumet flat surface pH probe (single junction). Both the probe and the pH meter were obtained from Thermo Scientific Corp. (Pittsburgh, Pa., U.S.A.). For pH measurements before the heat treatment, vials were allowed to stabilize at room temperature before values were recorded. After heat treatment, the heated vessels were immediately immersed in an ice bath to arrest the reaction and then brought to the room temperature (25°C) before the pH was measured.

Model system water activity measurement

The a_w of the Glu-Arg-ASA mixtures was measured before heat treatment using an AquaLab series 3 water activity meter with a 47 mm diameter chamber (Decagon Devices, Inc., Pullman, Wash. U.S.A.). Two standards, a_w 0.760 and 0.920, were used to standardize the water activity meter before measuring the a_w of the mixtures. Measurements were performed at room temperature (25°C).

Model system browning measurement

The browning intensity after heat treatments was measured using the method of Lee and others (2013); we used a 1 cm polystyrene cuvette (Thermo Scientific Inc., Waltham, Mass., U.S.A.) with a Genesys 10vis spectrophotometer (Thermo Scientific Inc., Waltham, Mass., U.S.A.) at a wavelength of 420 nm. A 100-fold dilution of the model system mixtures was prepared with double deionized water and measured against a double deionized water blank.

4-MeI sample measurement

Derivatization

Model systems were derivatized using a previously published method (Fernandes and Ferreira 1997), modified by Karim and Smith (2015). A 125 μL of acetonitrile: isobutanol: pyridine (50:30:20; v/v) was mixed with a 125 μL aliquot of a sample of the model system in a silane treated vial (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.). The internal standard 4-MeI-d_6 was added to the mixture, and then IBCF at a volume of 15 μL (10 + 15 μL) was added. Vials were shaken by hand for 5-10 sec., and then 250 μL of 1.0 M sodium bicarbonate solution was added, followed by 250 μL of hexane. Finally, 40 μL from the upper layer (hexane) was transferred to a limited volume autosampler vials (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.); 1 μL was injected into the GC-MS. 4-MeI and 4-MeI-d_6 standards were prepared in 0.1 N hydrochloric acid and derivatized using the same methodology as for the reaction mixtures.

GC-MS system operation conditions

An HP 5890 Series II *Plus* gas chromatograph with an HP 5972 mass spectral detector and autosampler HP 7673b (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.) was used to analyze samples for 4-MeI. The GC was equipped with a split/splitless injection port and had a 4 mm internal diameter liner (ultra-inert single gooseneck with a deactivated glass wool) (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.). Chemstation software (G1701BA Version B.01.00) was used to control the GC-MS and the autosampler as well as to process the data.

4-MeI and 4-MeI-d_6 separation and quantification were achieved using a DB-35 column [30 m × 0.25 mm I.D. × 0.25 μm (35%-phenyl)-methylpolysiloxane film thickness]. A guard column (DB-35) was installed prior to the GC column using an ultra-inert union. The GC-MS column, ultra-inert union, and guard column were from Agilent Technologies Inc. (Santa Clara, Calif., U.S.A.). Samples were injected in splitless mode. The GC-MS operation parameters were: 250°C for the injector, with the transfer line temperature at 280°C, and the initial oven temperature at 60°C. The oven was held at 60°C for 1 min, ramped at 25°C/min to 150°C; then after waiting 5 min, the temperature was ramped again at 25°C/min to 300°C and held for 4.40 min. The total run time was 21 min. The data acquisition delay time for the MS detector was 8 to 11 min. 4-MeI and the 4-MeI-d_6 separation was achieved at 1 mL/min of consistent flow of helium gas. The auto-tune calibration function of the Chemstation software was used to calibrate the MS system periodically. Selective Ion Monitoring (SIM) mode was performed to qualify 4-MeI and 4-MeI-d_6 based on peak area. 4-MeI was identified using the precursor ion *m/z* 182 and three product ions *m/z* 81, 82, 109, and quantified using the *m/z* 82 ion. 4-MeI-d_6 was identified using the precursor ion *m/z* 187 and three product ions *m/z* 85, 87, 114, and quantified using *m/z* 87 ion.

Determining volatile compounds in Glu-Arg and Glu-Arg-ASA treatments

Volatile compounds were identified using solid phase micro extraction (SPME) and GC-MS. An amount of 0.5 mL of Glu-Arg (control; 0.15 M each) or 0.5 mL of Glu:Arg:ASA (0.15:0.15:0.3 M) were placed in 2 mL sealed vials. Each vial was incubated at 50°C for 10 min in a heating block (Lab-line model 2050; Thermo Scientific Inc., Waltham, Mass., U.S.A.). A Carboxen/DPMS SPME fiber was used to extract the volatile compounds from the reactant mixtures. The fiber was inserted in the headspace of the vial at 50°C for 15 min; the fiber was then removed and inserted into the injection port of the GC-MS for 8 min. The injector port was fitted with a 2 mm internal diameter ultra-inert liner. The GC-MS used to perform the analysis was an HP 5890 Series II *Plus* gas chromatograph (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.) equipped with an HP 5972 mass spectral detector (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.) fitted with a DB-35 column [30 m × 0.25 mm I.D. × 0.25 μm (35%-phenyl)-methylpolysiloxane film thickness]. GC-MS operation conditions were as follows: the oven temperature was 50°C and held for 3 min. the temperature was ramped up at 7°C/min to 180°C and maintained for 5 min. The total run time was 21 min. Injector temperature was 260°C, and detector temperature was 300°C. Full scan mode (*m/z* 28 to 700) was performed to analyze the volatile compounds based on peak area. Headspace-SPME samples were injected in the splitless mode. The carrier gas was (ultra-high) helium at a flow rate of 1 mL/min. Volatile compounds were identified by matching spectra with those in an NIST08 spectral library.

STATISTICAL ANALYSIS

4-MeI and browning levels were compared for five concentrations of ascorbic acid. ASA was mixed with Glu and Arg solutions, 0.15 M each, with no ASA in control, 0.0375 ASA,

0.075 ASA, 0.15 ASA, and 0.30 M ASA. Three replications for each set of Glu-Arg-ASA solutions were made.

The general experimental design was a completely randomized design (CRD) with a one-way analysis of variance (ASA concentration) as its treatment structure. All analyses were conducted using the GLM procedure in the SAS software version 9.4 (SAS Institute Inc., 2013). F-tests were calculated for the main effect of concentration. Multiple pairwise comparisons between concentration levels were made with a Tukey adjustment to determine significant differences.

Additional covariates measuring initial pH and water activity levels were added individually to the prior analysis to investigate their association with 4-MeI and browning levels. This resulted in an analysis of covariance (ANCOVA) that estimated individual concentration intercepts and a common slope for each specified covariate.

RESULTS AND DISCUSSION

Initially, 4-MeI formation was investigated using a heat treated model system with AA and Glu (see part 3 of the dissertation). In that model system, Glu:Arg in equimolar of 0.15 M formed the most 4-MeI. Thus, in this study, we used an aqueous solution in equimolar of Glu and Arg (0.15M) to investigate the inhibitory effects of ASA on 4-MeI formation. ASA was added to Glu-Arg at a ratio of 1:1:0 (control), 1:1:0.25, 1:1:0.5, 1:1:1, and 1:1:2 for 0.15:0.15:0, 0.15:0.15:0.0375, 0.15:0.15:0.075, 0.15:0.15:0.15, and 0.15:0.15:0.3 M.

Effect of ASA on 4-MeI formation

Derivatization of the model samples after heat treatment with IBCF resulted in two peaks forming for 4-MeI (4-MeIa and 4-MeIb) and for 4-MeI-d_6 (4-MeI-d_6a and 4-MeI-d_6b). The sum of both peaks (4-MeIa + 4-MeIb) for *m/z* 82 ion was used to calculate the amount of 4-MeI, the limit of detection (LOD), and the limit of quantification (LOQ). The LOD of the proposed method was 0.05 ppm (signal-to-noise ratio of 3:1), and the LOQ of the proposed method was 0.1 ppm (signal-to-noise ratio of 10:1).

An absolute standard curve was established (y = 274969x +10280) using a series of 4-MeI standard solutions (0.1, 0.5, 1.0, and 10 ppm), with an acceptable R^2 of 0.999, indicating a good linearity in the proposed method.

Adding ASA had a significant effect ($P < 0.001$) on the formation of 4-MeI. Figure 26 shows the effect of the ASA concentrations on 4-MeI formation. As the concentration of ASA increased, a decreased in the 4-MeI peak area occurred. Figure 27 shows the decreases in the peak areas of 4-MeI as more ASA was added. The extent of ASA concentrations contribution to reducing 4-MeI formation was: 0.3 M > 0.15 M > 0.075 M > 0.0375 M, with a corresponding mole ratio of Glu:Arg:ASA of 1:1:2 > 1:1:1 > 1:1:0.5 > 1:1:0.25 M.

The amount/percentage of 4-MeI levels compared to control (1.23 ppm; 100%) were 0.73 ppm (40.29%), 0.49 ppm (59.94%), 0.30 ppm (75.13%), and 0.5 ppm (96.25%) for 0.0375, 0.075, 0.15, and 0.3 M of ASA concentrations. These results clearly suggest that ASA as a traditional antioxidant and as a commonly used food additive, plays an important role in inhibiting of 4-MeI formation in Glu-Arg model system.

The lowest level of 4-MeI observed was 0.046 μg/g, which occurred when 0.30 M ASA was added. This represents a 1:1:2 ration of Glu:Arg:ASA where the amount of ASA was two

times higher than Glu and Arg. To our knowledge, this inhibitory effect of ASA on the carcinogen 4-MeI not only in food products but also in the Glu-AA model system has not been reported before now.

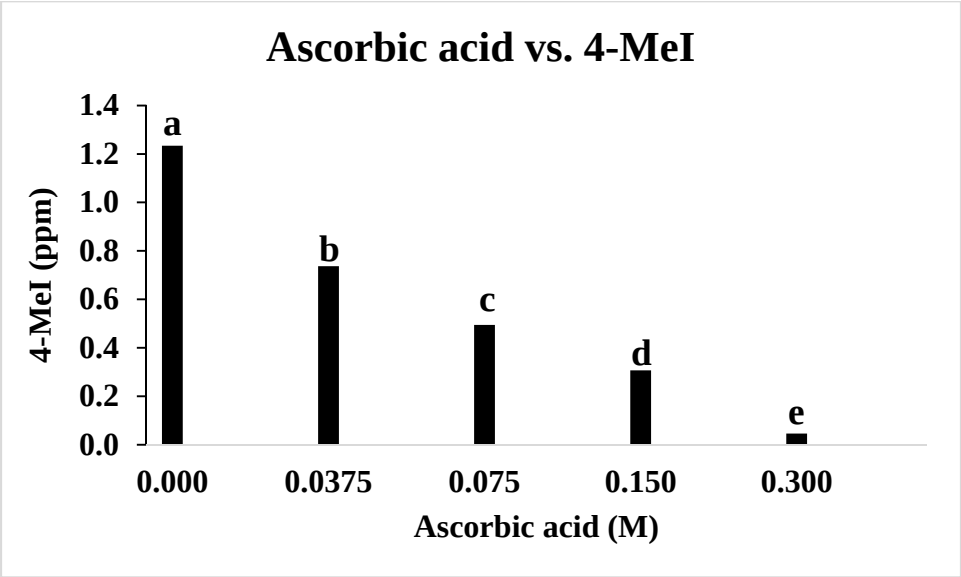

Figure 26. Effect of ASA on 4-MeI formation. Columns that do not share a letter are significantly different.

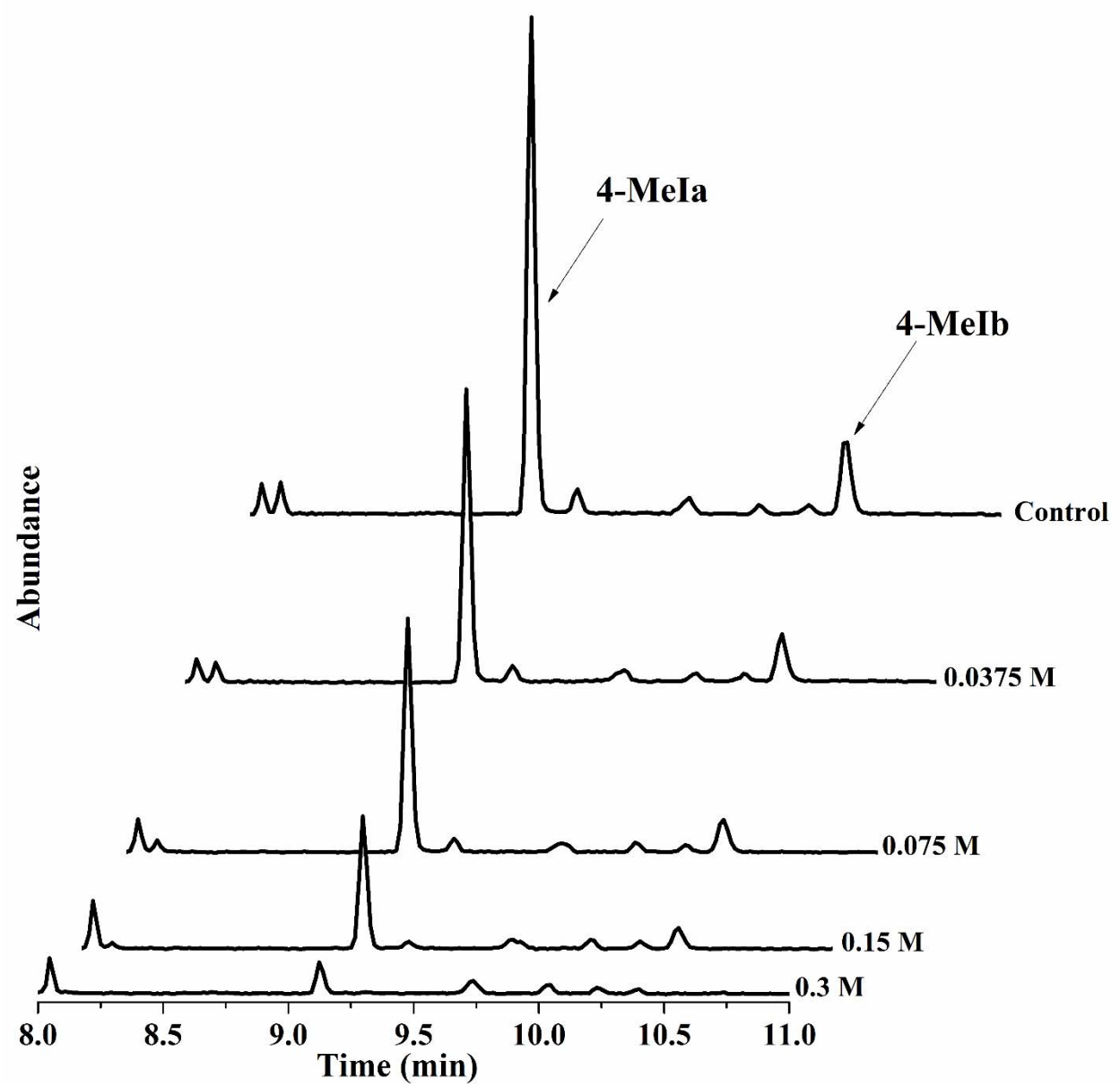

Figure 27. GC-MS SIM mode chromatograph of *m/z*182 ion showing reduction of the 4-MeI peaks related to the amount of added ASA (0.375, 0.75, 0.15, and 0.3 M).

Seo and others (2014) reported that various food additives like metal ions reduced the amount 4-MeI formed in a caramel model system heated at 100ºC for 2 h, with 0.1 M iron sulfate resulting in the greatest % reduction (~80%). They also stated that methylglyoxal (MGO), results

in the thermal degradation of sugar and a major radical intermediate in Maillard pathways, interacted with ammonia and aldehyde to produce 4-MeI in an aqueous solution, particularly when pH > 7. Moreover, previous research has only suggested that the strong antioxidant activity of ASA was not likely a key part of the mechanism inhibiting HCAs formation. The research further emphasized that ASA might also operate as a trap intervening in the formation pathway of HCAs like 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP) by forming intermediate-ASA adducts (Wong and others 2012). Thus, besides its powerful free radical scavenging capacity, ASA, added at the appropriate concentration, could affect the formation 4-MeI through a similar intermediate-trapping pathway, and simultaneously, reducing the optimal pH of the Maillard reaction to an acidic condition. However, more studies are required to understand the underlying inhibitory mechanism of 4-MeI in the Maillard reaction system.

Effect of ASA on the browning color development

Since browning is generally used as an indicator of Maillard reaction rates and is quantitatively related to formation of melanoidins as Maillard reaction products (MRPs) (Yeboah and others 1999), it is interesting that ASA enhanced browning. ASA concentration had a significant effect on browning. Figure 28 shows the absorbance levels of browning at a wavelength of 420 nm after heat treatment of the reactants. The browning intensity % was calculated by comparing the absorbance of each group with the control (Glu:Arg:ASA;1:1:0). ASA concentrations significantly increased ($P < 0.001$) the browning in all treatment combinations. Browning levels were 42.60, 53.27, 58.07, 61, and 71.4 for 0 (control), 0.0375, 0.075, 0.15 and 0.3 M ASA concentrations; this represents increasing in the browning by10.67, 15.47, 18.4, and 28.8%. The increase in the browning in all the treatments could be caused by the

degradation products of ASA, as ASA polymerized with Glu and AA yielding browning materials (Sawamura and others 1991). A similar effect was noted by Porter and others (2006) in Glu and glycine (Gly) model; adding ASA enhanced browning. They also stated that the enhanced browning effect is probably because of the transformation of ASA to dehydroascorbic acid, which produces strong browning when reacting with AA. It is possible also that ASA degraded to DHA during heat treatment and enhanced the browning through a reaction with Arg. Obviously, adding ASA to the Glu-Arg model system not only increased browning but also suppressed 4-MeI formation. Figure 29 shows browning development in Glu-Arg-ASA model system.

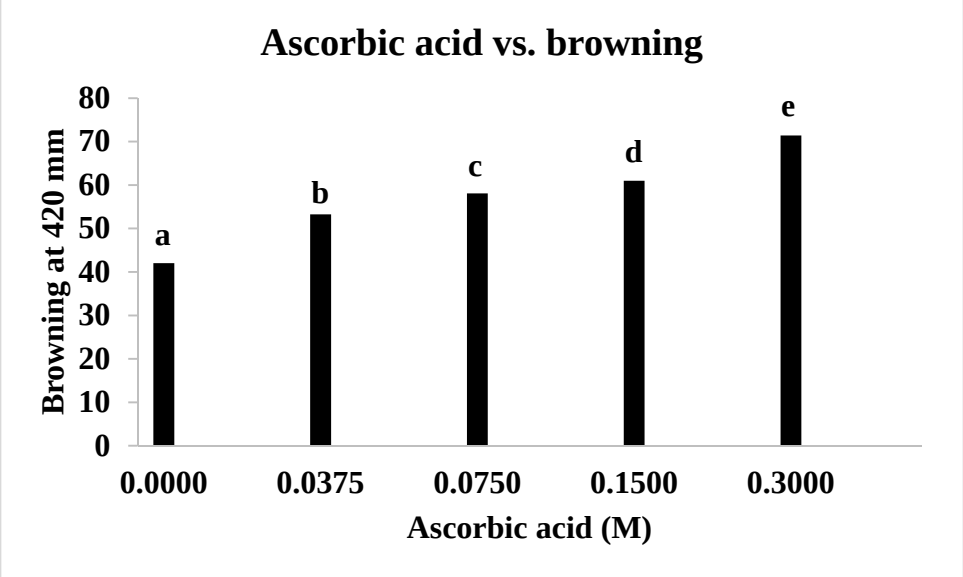

Figure 28. Effect of ascorbic acid concentrations on browning. Columns that do not share a letter are significantly different.

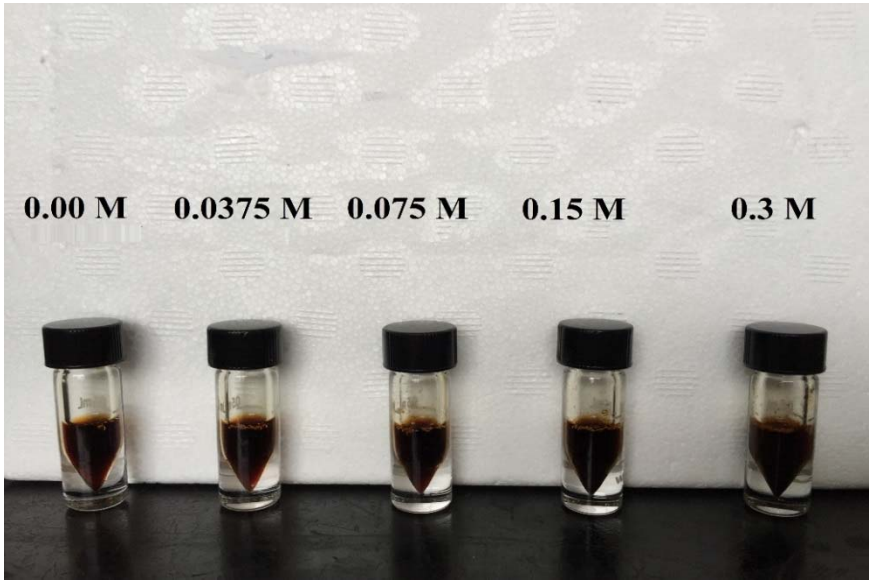

Figure 29. Browning in Glu-Arg-ASA model after heat treatment at 160ºC for 1 h.

Other factors affecting formation of 4-MeI and browning

Table 12 shows the influences of ASA on the model system pH before and after heat treatment at 160ºC for 1 h. As expected, compared to the control (pH of 10.41), the pH of the model system decreased as the concentration of ASA increased. The pH dropped after heat treatment in the control and all treatment combinations, except the treatment where the ratio of Glu:Arg:ASA was 1:1:2.

The decrease in pH after heat treatment was possibly associated with the thermal degradation of Arg and the instability of ASA under heat treatment. On the other hand, the increase in the pH after the heat treatment for Glu:Arg: ASA (1:1:2) could have been caused by ASA as it degraded, producing intermediate compounds preventing further declines in the pH.

The pH before the heat treatment showed a significant ($P < 0.049$) effect on the formation of 4-MeI. In addition, the pHs of both model systems with and without ASA before heat

treatment and the ASA concentration interacted significantly ($P < 0.001$). The significant interaction between ASA concentration and pHs of both model systems with ASA and without ASA before heat treatment suggests that both ASA concentrations and the initial pH affected 4-MeI levels.

Table 12. pH and a_w of the Glu-Arg-ASA model system.

ASA (M)	pH initial	pH after reaction	pH difference	a_w
0.00	10.42 ± 0.009	7.23 ± 0.037	-3.18 ± 0.035	0.990 ± 0.002
0.0375	9.48 ± 0.003	6.42 ± 0.103	-3.05 ± 0.102	0.992 ± 0.002
0.075	9.03 ± 0.003	5.80 ± 0.023	-3.23 ± 0.020	0.991 ± 0.001
0.15	6.11 ± 0.003	5.62 ± 0.007	-0.50 ± 0.009	0.989 ± 0.001
0.30	4.08 ± 0.001	4.68 ± 0.019	0.60 ± 0.019	0.988 ± 0.002

Values are means ± standard error (n = 3).

Taking into consideration the effects of pH on the Maillard browning reaction in systems containing Glu and AA (Ajandouz and others 2001), when the pH value is below neutral (pH < 7), the fragmentation of Glu might be significantly affected, inhibiting 4-MeI formation by altering the Maillard reaction pathway. Moreover, the a_w had no significant effect ($P > 0.51$) on 4-MeI formation, possibly because of the low concentrations of ASA used in this experiment.

The ASA concentration had a significant effect on browning as did initial pH of both treatments. In addition, ASA concentration and the initial pH showed a significant relationship indicating that both ASA concentration and the initial pH affected browning in the model system. Moreover, a_w had a significant effect ($P < 0.024$) on browning, suggesting that as ASA concentrations increased browning also increased.

Effect of ASA on aroma formation

Our results showed 12 volatile compounds 11 heterocyclic aromatic compounds and 1 alicyclic compound formed during the heat treatment; those compounds have been identified in both the Glu-Arg (control) and Glu-Arg-ASA (1:1:2) model systems where pyrazines and furan derivatives were the predominate heterocyclic aromatic compounds (see Table 13). Figure 30 and Figure 31 show the GC-MS chromatogram of the volatile compounds both in the sample with added ASA and in the control. The volatile compounds produced from model systems with and without ASA were very similar, indicating that both reactions produce the same types of aromatic compounds through thermal treatment. However, the total volatile compounds formed in the model system with ASA were lower than control. Bharate and Bharate (2012) indicated that ASA inhibits formation of pyrazine compounds, and adding of 1% ASA to a Glu-Gly model system reduced 2,5-dimethlpyrazine and 2,3,5-trimethylpyrazine by 52%. Adding ASA also appeared to inhibit levels of volatile compounds. For example, methylpyrazine decreased by as much as 100 times compared to control. Our results thus suggest that ASA may inhibit aroma formation in some processed foods.

Converting ASA concentrations to percentages

Lemon juice is usually used to marinate meat before cooking. To compare the concentration of ASA used in this experiment with amount of ASA in natural lemon juice, the molar concentrations of ASA were calculated as percentages. The percent of ASA used in our experiment is higher than the actual ASA in natural lemon juice (0.04 %; Igwe 2014). The calculated percentages for ASA were 5.28% (0.3 M), 2.64% (0.15 M), 1.32% (0.075 M), and 0.66% (0.0375 M).

CONCLUSION

We investigated the effect of ASA on 4-MeI formation and on browning in the Glu-Arg Maillard model system. While adding ASA inhibited 4-MeI formation, browning of the model system also increased. Adding ASA twice the concentration of sugar and amino acids in the model system inhibited 4-MeI formation by 96.25% and increased browning intensity by 28.8%. Statistical analysis of the results revealed that the reduction in the 4-MeI levels and the increase in browning are due to the effect of added ASA and the resulting drop in the initial pH of the reactant. However, adding ASA to the Glu-Arg model system reduced volatile compound formation. Therefore, adding ASA to food may reduce 4-MeI formation and enhance, browning which is a desired property in food, ASA may also reduce the off-flavor, another desired property in food products.

Table 13. The volatile compounds identified in the Glu-Arg and Glu-Arg-ASA model system determined by SPME with GC-MS analysis.

	Identified compounds	Retention time (min)	Molecular Weight (g/mol)	Peak area without ASA	Peak area with ASA
HACs	1,3-Diazine	4.67	80	1.600E + 09	1.090E + 09
	Pyrazine, methyl	6.67	94	1.300E + 09	0.010E + 09
	Furfural	7.00	96	0.160E + 09	0.110E + 09
	2-Furanmethanol	7.18	98	0.240E + 09	0.140E + 09
	Pyrazine, 2,5-dimethyl	8.29	108	0.080E + 09	0.043E + 09
	Ethanone, 1-(2-furanyl)-	8.82	110	0.040E + 09	0.027E + 09
	Pyrazine, ethenyl	9.08	106	0.007E + 09	0.003E + 09
	2-Furancarboxaldehyde, 5-methyl	10.28	110	0.280E + 09	0.016E + 09
	Acetylpyrazine	11.53	122	0.002E + 09	0.001E + 09
	Pyrazine, 2-ethyl-3,5-dimethyl	12.14	136	0.005E + 09	0.003E + 09
	Ethanone, 1-(1H-pyrrol-2-y1)-	12.79	109	0.005E + 09	0.003E + 06
AC	2-Cyclopenten-1-one, 2-hydroxyl-3-methyl	11.72	112	0.025E + 09	0.014E + 07

HACs = heterocyclic aromatic compounds.

AC = Alicyclic compound.

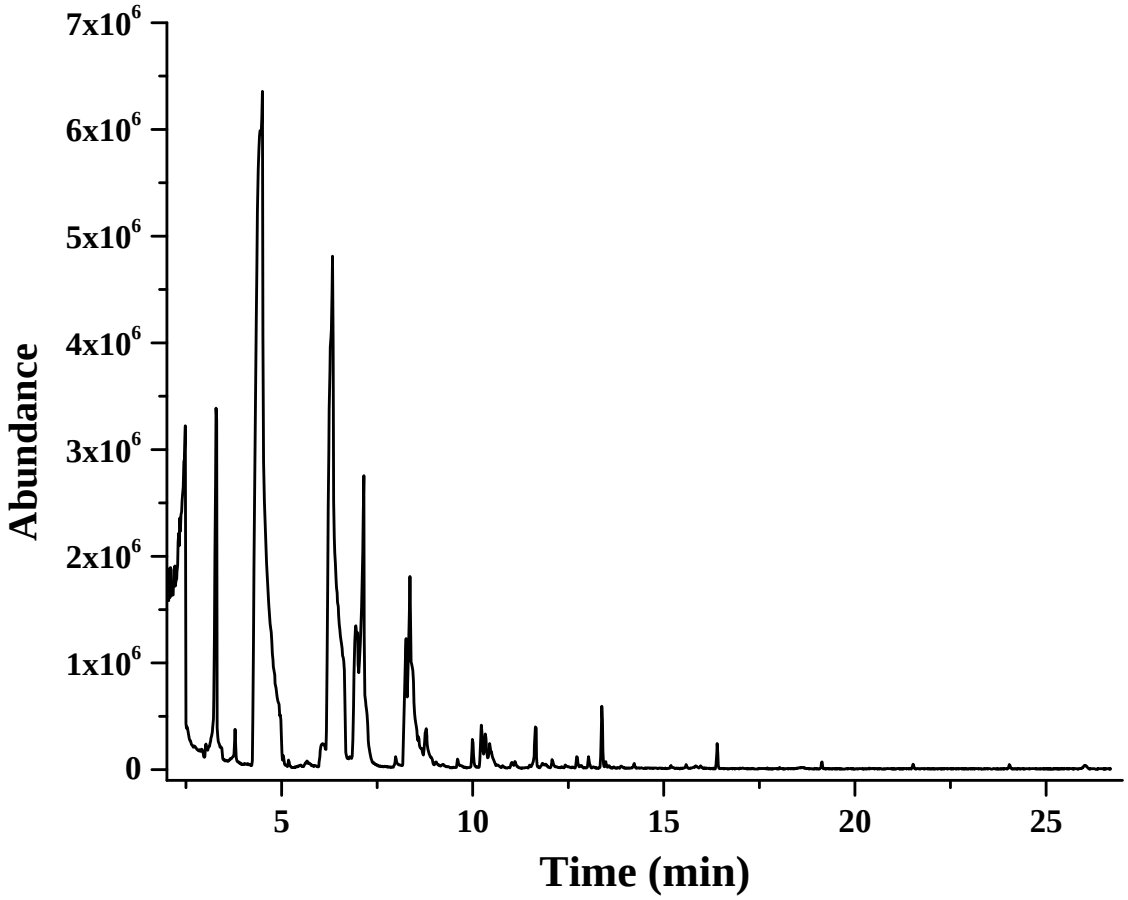

Figure 30. Total ion chromatogram SPME GC-MS for Glu-Arg-ASA (1:1:2 ratio) treatment.

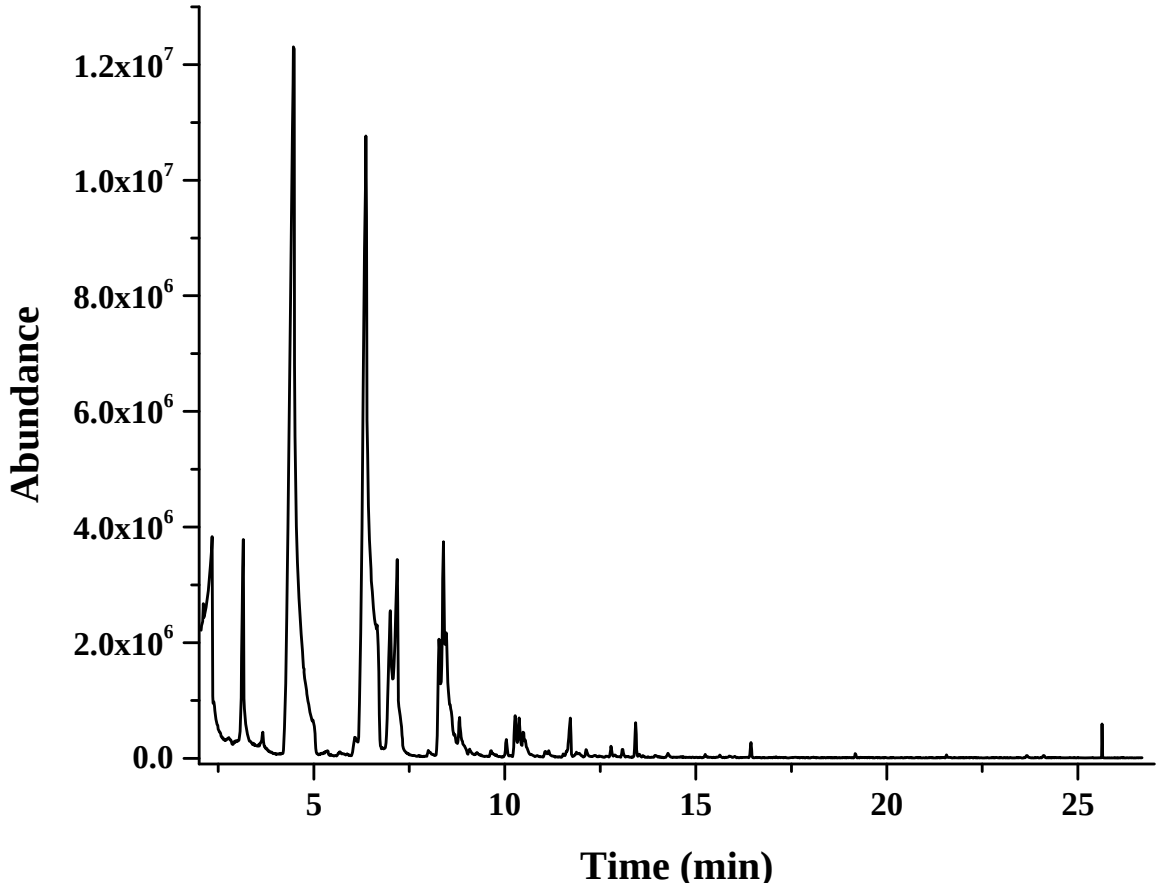

Figure 31. Total ion chromatogram SPME GC-MS for Glu-Arg (1:1 ratio) treatment.

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SUMMARY

4-MeI a carcinogenic compound found in cooked foods and caramel colors III and IV formed from the reaction of reducing sugar with either amino acids or ammonia.

In food products and in caramel colors, 4-MeI levels can be varied according to the manufacturing conditions. Concentration and type of the reactant as well as processing time and temperature are the major factors affecting formation of 4-MeI.

We concluded that human intake of 4-MeI is low, however, some food products showed levels of 4-MeI higher than the established no significant risk levels (29 μg/day), highest levels of 4-MeI were found in alcoholic beverages and because 4-MeI is widely present in food products, investigating the total intake of 4-MeI from different processed foods is necessary.

Because of the increasing evidence of the risk cancer, inhibition formation of 4-MeI and reducing its levels in processed food is desired. Ascorbic acids showed good inhibition property against formation of 4-MeI, but how ascorbic acid inhibits formation of 4-MeI, still a question needed to examine closely by the scientific community. In addition, adding natural antioxidant like rosemary may also inhibit formation of 4-MeI since rosemary is known by its antioxidant property against formation of heterocyclic amines other carcinogens found in heat treated food.

Our research data can be used by the food industry to modify the process conditions to reduce formation of 4-MeI in heat treated food or caramel color manufacturing. In addition, these data will help to estimate daily human exposure to 4-MeI, and will provide a better understanding of the role of 4-MeI as a cancer causing agent in the population of the U.S.

APPENDIX

LIST OF FIGURES

TITLE 27, CALIFORNIA CODE OF REGULATIONS

CHAPTER 1. SAFE DRINKING WATER AND TOXIC ENFORCEMENT ACT OF 1986

ARTICLE 7. NO SIGNIFICANT RISK LEVELS

Section 25705. Specific Regulatory Levels Posing No Significant Risk.

Amend Section 25705(b) as follows:

***

(b) Levels of exposure deemed to pose no significant risk may be determined by the lead agency based on a risk assessment conducted by the lead agency pursuant to the guidelines set forth in Section 25703, or a risk assessment reviewed by the lead agency and determined to be consistent with the guidelines set forth in Section 25703.

(1) The following levels based on risk assessments conducted or reviewed by the lead agency shall be deemed to pose no significant risk:

Chemical Name	*Level (micrograms/day)*
Acrylonitrile	
***	
Methylhydrazine sulfate	0.18
~~4-Methylimidazole~~	~~16~~
4-Methylimidazole	29
5-Morpholinomethyl-3-[(5-nitrofurfurylidene) -amino]-2-oxazolidinone	0.18

***

NOTE: Authority cited: Section 25249.12, Health and Safety Code. Reference: Sections 25249.5, 25249.6, 25249.9, 25249.10 and 25249.11, Health and Safety Code.

Figure A-1. No significant risk levels of 4-MeI changed from 16 to 29 mg/day.

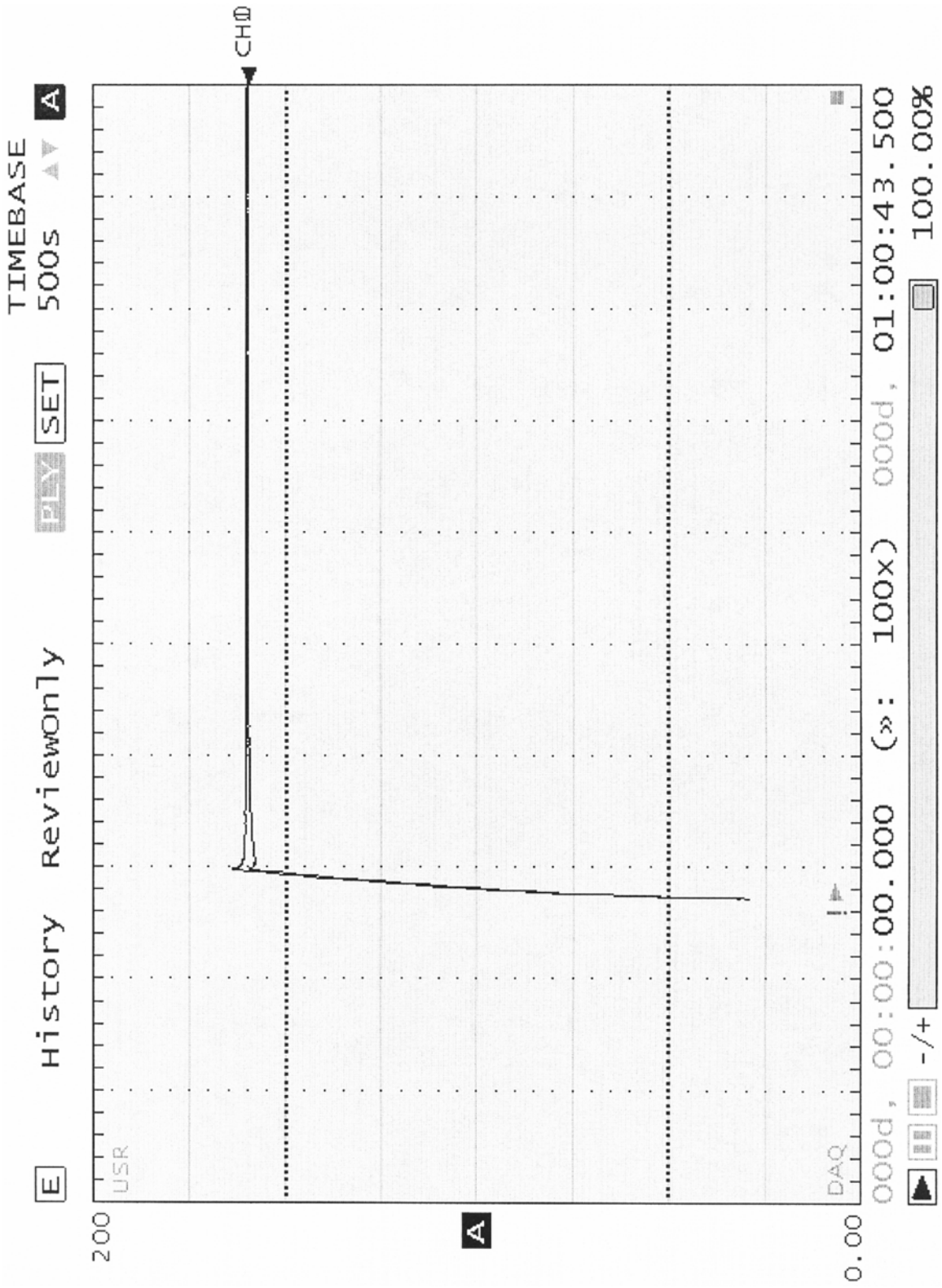
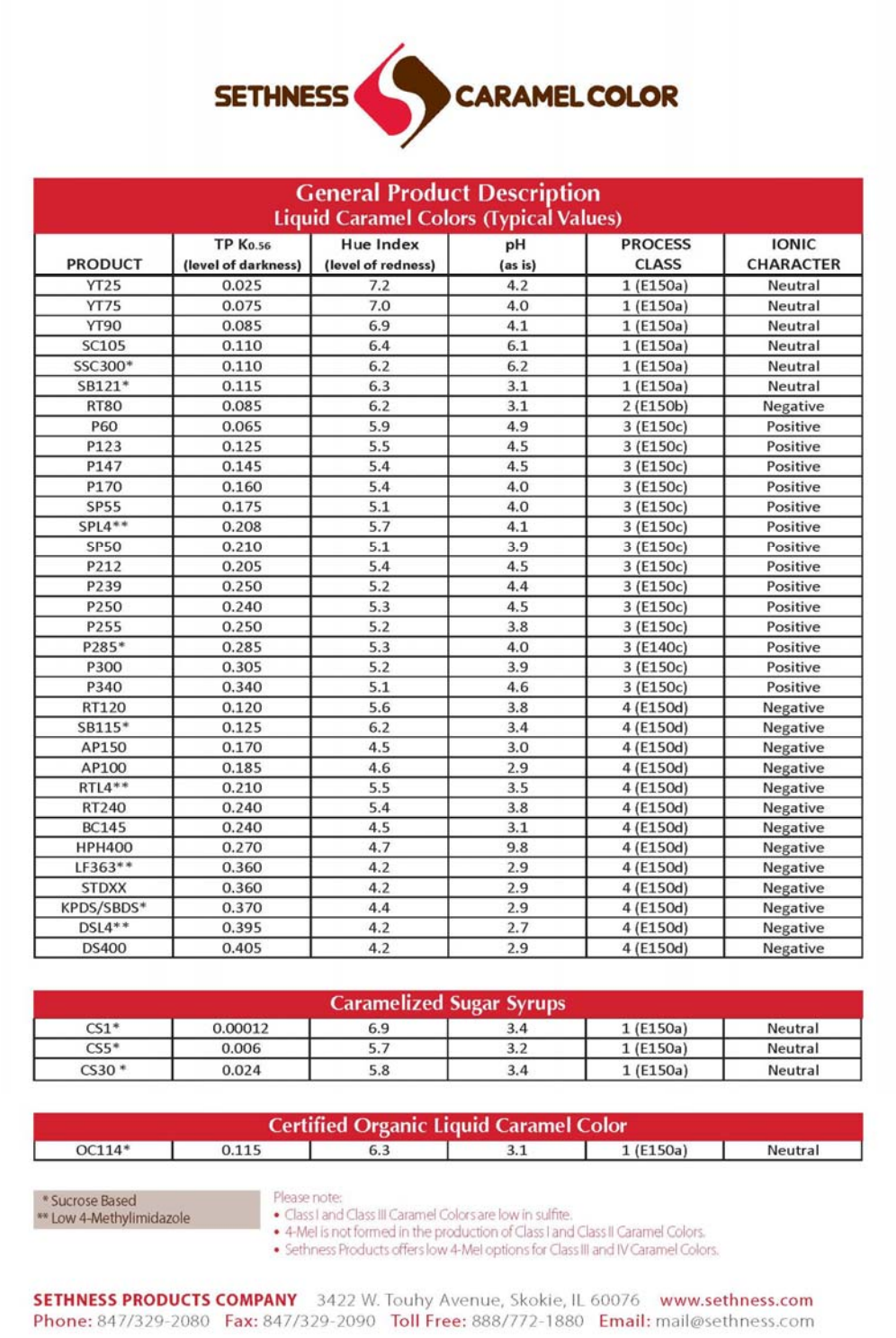


Figure A-2. Temperature reading recorded using the data logger during heat treatment of glucose and amino acid model system.

Agents Classified by the *IARC Monographs*, Volumes 1–109

CAS No	Agent	Group	Volume	Year
000822-36-6	4-Methylimidazole	2B	101	2013
000074-88-4	Methyl iodide	3	41, Sup 7, 71	1999
000108-10-1	Methyl isobutyl ketone	2B	101	2013
	Methylmercury compounds (NB: Evaluated as a group)	2B	58	1993
000080-62-6	Methyl methacrylate	3	60	1994
000066-27-3	Methyl methanesulfonate (NB: Overall evaluation upgraded to Group 2A with supporting evidence from other relevant data)	2A	7, Sup 7, 71	1999
000129-15-7	2-Methyl-1-nitroanthraquinone (uncertain purity)	2B	27, Sup 7	1987
000070-25-7	*N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (NB: Overall evaluation upgraded to Group 2A with supporting evidence from other relevant data)	2A	4, Sup 7	1987
000684-93-5	*N*-Methyl-*N*-nitrosourea (NB: Overall evaluation upgraded to Group 2A with supporting evidence from other relevant data)	2A	17, Sup 7	1987
000615-53-2	*N*-Methyl-*N*-nitrosourethane	2B	4, Sup 7	1987
090456-67-0	*N*-Methylolacrylamide	3	60	1994
000298-00-0	Methyl parathion	3	30, Sup 7	1987
000832-69-9	1-Methylphenanthrene	3	92	2010
085878-63-3	7-Methylpyrido[3,4-*c*]psoralen	3	40, Sup 7	1987
000493-52-7	Methyl red	3	8, Sup 7	1987
000144-34-3	Methyl selenac	3	12, Sup 7	1987
000098-83-9	α-Methylstyrene	2B	101	2013
000056-04-2	Methylthiouracil	2B	79	2001
000443-48-1	Metronidazole	2B	13, Sup 7	1987
000101-61-1	Michler's base [4,4′-methylenebis(*N*,*N*-dimethyl)-benzenamine]	2B	27, Sup 7, 99	2010
000090-94-8	Michler's ketone [4,4′-Bis(dimethylamino)benzophenone]	2B	99	2010
101043-37-2	Microcystin-LR	2B	94	2010
	Microcystis extracts	3	94	2010
	Mineral oils, highly-refined	3	33, Sup 7	1987
	Mineral oils, untreated or mildly treated	1	33, Sup 7, 100F	2012
002385-85-5	Mirex	2B	20, Sup 7	1987
000050-07-7	Mitomycin C	2B	10, Sup 7	1987
065271-80-9	Mitoxantrone	2B	76	2000
	Modacrylic fibres	3	19, Sup 7	1987
000096-24-2	3-Monochloro-1,2-propanediol	2B	101	2013
000315-22-0	Monocrotaline	2B	10, Sup 7	1987

Figure A-3. Listing 4-MeI as a 2B group possible human carcinogen by the International Agency for Research on Cancer.



SETHNESS CARAMEL COLOR

General Product Description
Liquid Caramel Colors (Typical Values)

PRODUCT	TP $K_{0.56}$ (level of darkness)	Hue Index (level of redness)	pH (as is)	PROCESS CLASS	IONIC CHARACTER
YT25	0.025	7.2	4.2	1 (E150a)	Neutral
YT75	0.075	7.0	4.0	1 (E150a)	Neutral
YT90	0.085	6.9	4.1	1 (E150a)	Neutral
SC105	0.110	6.4	6.1	1 (E150a)	Neutral
SSC300*	0.110	6.2	6.2	1 (E150a)	Neutral
SB121*	0.115	6.3	3.1	1 (E150a)	Neutral
RT80	0.085	6.2	3.1	2 (E150b)	Negative
P60	0.065	5.9	4.9	3 (E150c)	Positive
P123	0.125	5.5	4.5	3 (E150c)	Positive
P147	0.145	5.4	4.5	3 (E150c)	Positive
P170	0.160	5.4	4.0	3 (E150c)	Positive
SP55	0.175	5.1	4.0	3 (E150c)	Positive
SPL4**	0.208	5.7	4.1	3 (E150c)	Positive
SP50	0.210	5.1	3.9	3 (E150c)	Positive
P212	0.205	5.4	4.5	3 (E150c)	Positive
P239	0.250	5.2	4.4	3 (E150c)	Positive
P250	0.240	5.3	4.5	3 (E150c)	Positive
P255	0.250	5.2	3.8	3 (E150c)	Positive
P285*	0.285	5.3	4.0	3 (E140c)	Positive
P300	0.305	5.2	3.9	3 (E150c)	Positive
P340	0.340	5.1	4.6	3 (E150c)	Positive
RT120	0.120	5.6	3.8	4 (E150d)	Negative
SB115*	0.125	6.2	3.4	4 (E150d)	Negative
AP150	0.170	4.5	3.0	4 (E150d)	Negative
AP100	0.185	4.6	2.9	4 (E150d)	Negative
RTL4**	0.210	5.5	3.5	4 (E150d)	Negative
RT240	0.240	5.4	3.8	4 (E150d)	Negative
BC145	0.240	4.5	3.1	4 (E150d)	Negative
HPH400	0.270	4.7	9.8	4 (E150d)	Negative
LF363**	0.360	4.2	2.9	4 (E150d)	Negative
STDXX	0.360	4.2	2.9	4 (E150d)	Negative
KPDS/SBDS*	0.370	4.4	2.9	4 (E150d)	Negative
DSL4**	0.395	4.2	2.7	4 (E150d)	Negative
DS400	0.405	4.2	2.9	4 (E150d)	Negative

Caramelized Sugar Syrups

CS1*	0.00012	6.9	3.4	1 (E150a)	Neutral
CS5*	0.006	5.7	3.2	1 (E150a)	Neutral
CS30 *	0.024	5.8	3.4	1 (E150a)	Neutral

Certified Organic Liquid Caramel Color

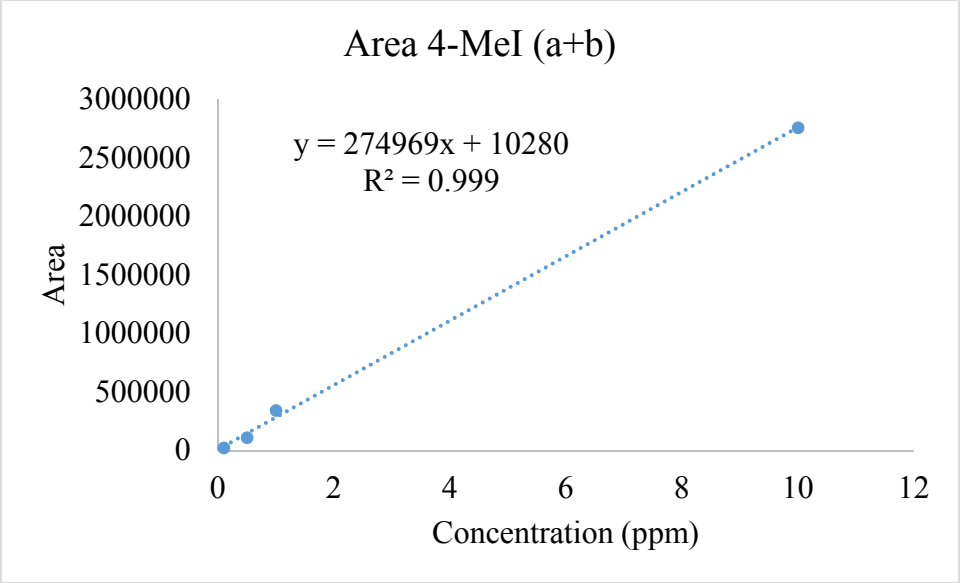
OC114*	0.115	6.3	3.1	1 (E150a)	Neutral

* Sucrose Based
** Low 4-Methylimidazole

Please note:
- Class I and Class III Caramel Colors are low in sulfite.
- 4-Mel is not formed in the production of Class I and Class II Caramel Colors.
- Sethness Products offers low 4-Mel options for Class III and IV Caramel Colors.

SETHNESS PRODUCTS COMPANY 3422 W. Touhy Avenue, Skokie, IL 60076 www.sethness.com
Phone: 847/329-2080 **Fax:** 847/329-2090 **Toll Free:** 888/772-1880 **Email:** mail@sethness.com

Figure A-4. Caramel color product specifications showing the ionic charges of different caramel colors.



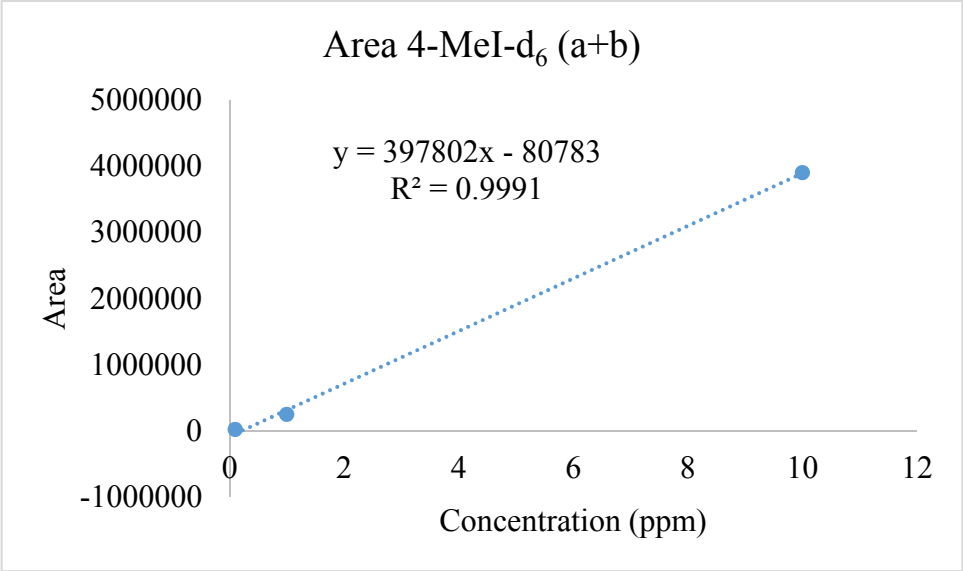


Figure A-5. Standard curves of 4-MeI and 4-MeI-d_6 by GC-MS.

Figure A-6. Stastical code used to analyze part 3 of the dissertation.

```
/*
Notes: Separate Analyses by Amino Acids (2/2/15)
       Pairwise comparisons between
*/

/* SAS Analysis */

ods rtf file = "C:\Users\bloedow\Documents\Consulting\Clients\Faris Hussain
(Fall 2014-Present)\Analysis\SAS Output\Statistical Analysis on 4-
Methylimidazole Project (02_25_15).doc";

title '4-Methylimidazole Project (Faris Hussain)';

*Imports the Full 4-Methylimidazole Dataset from Excel into SAS;
 proc import out=Browning
   datafile='C:\Users\bloedow\Documents\Consulting\Clients\Faris Hussain
(Fall 2014-Present)\Data\SAS Data Analysis (02_09_15).xlsx'
   dbms=xlsx
   replace;
   sheet="meidata";
   getnames=yes;
 run;

*Creates truncated 4-MeI variable from the Full 4-Methylimidazole Dataset;
 data Browning;
   set Browning;
   format Conc 4.2;
   _4_MeI=round(_4_MeI,0.000001);
   pH_Change=pHa-pHb;
 run;

*Sorts the Full 4-Methylimidazole Dataset by Temp, Conc, & AA;
 proc sort data=Browning;
   by Temp Conc AA;
 run;

*Print of the Full 4-Methylimidazole Dataset;
  proc print data=Browning;
    title2 'Print-out of the Full 4-Methylimidazole Dataset';
  run;

/* Statistical Graphics to visually inspect the raw data */

 *Scatter Plots: Browning*4-MeI by Temperature;
  proc sgscatter data=Browning ;
    plot Browning*_4_MeI / datalabel=Conc group=AA;
      by Temp ;
      title2 'Browning vs. 4-Methylimidazole';
  run;
```

```
 *Scatter Plots: 4-MeI*Browning by Temperature;
  proc sgscatter data=Browning ;
    plot _4_MeI*Browning / datalabel=Conc group=AA;
      by Temp ;
      title2 '4-Methylimidazole vs. Browning';
  run;

 *Lattice Scatter Plots: 4-MeI*Browning by Amino Acid & Temperature;
  proc sgpanel data=Browning ;
      panelby AA Temp / layout=lattice columns=5 rows=3;
    scatter x=Browning y=_4_MeI / group=Conc;
    colaxis label="Browning Level" Min=0 Max=50 Values=(0 to 80 by 20) ;
      rowaxis label="4-Methylimidazole Level" Min=0 Max=1 Values=(0 to 1 by
0.2) ;
      title2 '4-Methylimidazole vs. Browning';
  run;

 *Lattice Scatter Plots: 4-MeI*Concentration by Amino Acid & Temperature;
  proc sgpanel data=Browning ;
      panelby AA Temp / layout=lattice columns=5 rows=3;
    scatter x=Conc y=_4_MeI / group=Conc;
    colaxis label="Concentration" Min=0.05 Max=0.15 Values=(0.05 to .15 by
.05) ;
      rowaxis label="4-Methylimidazole Level" Min=0 Max=1 Values=(0 to 1 by
0.2) ;
      title2 '4-Methylimidazole vs. Concentration';
  run;

 *Lattice Scatter Plots: Browning*Concentration by Amino Acid & Temperature;
  proc sgpanel data=Browning ;
      panelby AA Temp / layout=lattice columns=5 rows=3;
    scatter x=Conc y=Browning / group=Conc;
    colaxis label="Concentration" Min=0.05 Max=0.15 Values=(0.05 to .15 by
.05) ;
      rowaxis label="Browning Level" Min=0 Max=50 Values=(0 to 80 by 20) ;
      title2 'Browning vs. Concentration';
  run;

 *Lattice Scatter Plots: aW*pHb by Amino Acid & Temperature;
  proc sgpanel data=Browning ;
      panelby AA Temp / layout=lattice columns=5 rows=3;
    scatter x=pHb y=aW / group=Conc;
    colaxis label="pH (Before)" Min=4 Max=12 Values=(4 to 12 by 1);
      rowaxis label="Water Activity" Min=0.975 Max=1 Values=(0.975 to 1 by
0.005) ;
      title2 'Water Activity vs. pH (Before)';
  run;

*Creates Dataset (pH) to compare the pH level measurements (Before & After);
 data pH;
   set Browning;
   array XX[2] pHb pHa;
   do Time=1 to 2;
   pH=XX[Time];
```

```
   output;
   end;
   drop pHb pHa;
 run;

*Creates categorical variable (Status) to timing of measurements with pH
Dataset;
 data pH;
   set pH;
   if Time=1 then Status="Before"; else Status="After";
 run;


 *Lattice Scatter Plots: pH*Status by Amino Acid & Temperature;
  proc sgpanel data=pH ;
      panelby AA Temp / layout=lattice columns=5 rows=3;
    scatter x=Status y=pH / group=Conc;
    colaxis label="Status" ;
      rowaxis label="pH" Min=4 Max=12 Values=(4 to 12 by 1) ;
      title2 'pH vs. Status';
  run;

/* Response Variable: Browning */

* Amino Acid: Alanine ;

 *Creates Dataset (ALA_Brown) for completely detectable Browning data with
Alanine;
   data ALA_Brown;
     set Browning;
     where AA="Alanine" & (Temp=120 | Temp=160);
     drop _4_MeI;
   run;

 *Print of the Full ALA_Brown Dataset;
   proc print data=ALA_Brown;
     title2 'Print-out of the Full ALA_Brown Dataset';
   run;

 *Correlation between Variables of Full ALA_Brown Dataset;
   proc corr data=ALA_Brown plots=matrix(hist nvar=all);
     var Browning Conc aW pHb pHa pH_Change;
       by Temp;
     title2 'Pearson Correlation (Alanine)';
   run;

 *Experimental Design Analysis (Classification Variables);
   proc glm data=ALA_Brown plots=all;
     class Temp Conc;
       model Browning = Temp|Conc / solution SS3 ;
       lsmeans Temp|Conc / cl ;
       lsmeans Temp*Conc / pdiff=all adjust=tukey;
       title2 'Response Variable: Browning (Alanine)';
       title3 'GLM Model (Experimental Design)';
       title4 'General Analysis' ;
   run;
```

```
   proc glm data=ALA_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp*Conc ;
     means Temp*Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: Browning (Alanine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Test of Equal Variance' ;
   run;

 *Experimental Design Analysis (Additional Covariates);
   proc glm data=ALA_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp|Conc aW / solution SS3 p ;
       lsmeans Temp|Conc / cl ;
       title2 'Response Variable: Browning (Alanine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=ALA_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp|Conc pHb / solution SS3 p ;
       lsmeans Temp|Conc / cl ;
       title2 'Response Variable: Browning (Alanine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Additional Covariates' ;
   run;

 *Regression-based Analysis (Continuous Variables);
   proc glm data=ALA_Brown plots=all;
       model Browning = Temp|Conc / solution SS3 p ;
       title2 'Response Variable: Browning (Alanine)';
       title3 'GLM Model (Regression)';
   run;

 *Regression-based Analysis (Additional Covariates);
   proc glm data=ALA_Brown plots=all;
       model Browning = Temp|Conc aW / solution SS3 p ;
       title2 'Response Variable: Browning (Alanine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=ALA_Brown plots=all;
       model Browning = Temp|Conc pHb / solution SS3 p ;
       title2 'Response Variable: Browning (Alanine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

* Amino Acid: Arginine ;

 *Creates Dataset (ARG_Brown) for completely detectable Browning data with
Arginine;
   data ARG_Brown;
     set Browning;
```

```
    where AA="Arginine";
    drop _4_MeI;
  run;

 *Print of the Full ARG_Brown Dataset;
  proc print data=ARG_Brown;
    title2 'Print-out of the Full ARG_Brown Dataset';
  run;


 *Correlation between Variables of Full ARG_Brown Dataset;
  proc corr data=ARG_Brown plots=matrix(hist nvar=all);
    var Browning Conc aW pHb pHa pH_Change;
      by Temp;
    title2 'Pearson Correlation (Arginine)';
  run;

 *Experimental Design Analysis (Classification Variables);
  proc glm data=ARG_Brown plots=all;
    class Temp Conc;
      model Browning = Temp|Conc / solution SS3 ;
      lsmeans Temp|Conc / cl ;
      lsmeans Temp*Conc / pdiff=all adjust=tukey;
      title2 'Response Variable: Browning (Arginine)';
      title3 'GLM Model (Experimental Design)';
      title4 'General Analysis' ;
  run;

  proc glm data=ARG_Brown plots=all;
    class Temp Conc ;
      model Browning = Temp*Conc ;
    means Temp*Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
      title2 'Response Variable: Browning (Argeine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Test of Equal Variance' ;
  run;

 *Experimental Design Analysis (Additional Covariates);
  proc glm data=ARG_Brown plots=all;
    class Temp Conc ;
      model Browning = Temp|Conc aW / solution SS3 p ;
      lsmeans Temp|Conc / cl ;
      title2 'Response Variable: Browning (Argeine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=ARG_Brown plots=all;
    class Temp Conc ;
      model Browning = Temp|Conc pHb / solution SS3 p ;
      lsmeans Temp|Conc / cl ;
      title2 'Response Variable: Browning (Argeine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;
```

```
 *Regression-based Analysis (Continuous Variables);
   proc glm data=ARG_Brown plots=all;
       model Browning = Temp|Conc / solution SS3 p ;
       title2 'Response Variable: Browning (Arginine)';
       title3 'GLM Model (Regression)';
       title4 'General Analysis' ;
   run;

 *Regression-based Analysis (Additional Covariates);
   proc glm data=ARG_Brown plots=all;
       model Browning = Temp|Conc aW / solution SS3 p ;
       title2 'Response Variable: Browning (Arginine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=ARG_Brown plots=all;
       model Browning = Temp|Conc pHb / solution SS3 p ;
       title2 'Response Variable: Browning (Arginine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

* Amino Acid: Glycine ;

 *Creates Dataset (GLY_Brown) for completely detectable Browning data with
Glycine;
   data GLY_Brown;
     set Browning;
     where AA="Glycine" & (Temp=120 | Temp=160);
     drop _4_MeI;
   run;

 *Print of the Full GLY_Brown Dataset;
   proc print data=GLY_Brown;
     title2 'Print-out of the Full GLY_Brown Dataset';
       title3 ;
       title4 ;
   run;

 *Correlation between Variables of Full GLY_Brown Dataset;
   proc corr data=GLY_Brown plots=matrix(hist nvar=all);
     var Browning Conc aW pHb pHa pH_Change;
       by Temp;
     title2 'Pearson Correlation (Glycine)';
   run;

 *Experimental Design Analysis (Classification Variables);
   proc glm data=GLY_Brown plots=all;
     class Temp Conc;
       model Browning = Temp|Conc / solution SS3 ;
       lsmeans Temp|Conc / cl ;
       lsmeans Temp*Conc / pdiff=all adjust=tukey;
       title2 'Response Variable: Browning (Glycine)';
       title3 'GLM Model (Experimental Design)';
       title4 'General Analysis' ;
```

```
   run;

   proc glm data=GLY_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp*Conc ;
     means Temp*Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: Browning (Glycine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Test of Equal Variance' ;
   run;

 *Experimental Design Analysis (Additional Covariates);
   proc glm data=GLY_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp|Conc aW / solution SS3 p ;
       lsmeans Temp|Conc / cl ;
       title2 'Response Variable: Browning (Glycine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=GLY_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp|Conc pHb / solution SS3 p ;
       lsmeans Temp|Conc / cl ;
       title2 'Response Variable: Browning (Glycine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Additional Covariates' ;
   run;

 *Regression-based Analysis (Continuous Variables);
   proc glm data=GLY_Brown plots=all;
       model Browning = Temp|Conc / solution SS3 p ;
       title2 'Response Variable: Browning (Glycine)';
       title3 'GLM Model (Regression)';
       title4 'General Analysis' ;
   run;

 *Regression-based Analysis (Additional Covariates);
   proc glm data=GLY_Brown plots=all;
       model Browning = Temp|Conc aW / solution SS3 p ;
       title2 'Response Variable: Browning (Glycine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=GLY_Brown plots=all;
       model Browning = Temp|Conc pHb / solution SS3 p ;
       title2 'Response Variable: Browning (Glycine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

* Amino Acid: Lysine ;
```

```
 *Creates Dataset (LYS_Brown) for completely detectable Browning data with
Lysine;
   data LYS_Brown;
     set Browning;
     where AA="Lysine";
     drop _4_MeI;
   run;

 *Print of the Full LYS_Brown Dataset;
   proc print data=LYS_Brown;
     title2 'Print-out of the Full LYS_Brown Dataset';
       title3 ;
       title4 ;
   run;

 *Correlation between Variables of Full LYS_Brown Dataset;
   proc corr data=LYS_Brown plots=matrix(hist nvar=all);
     var Browning Conc aW pHb pHa pH_Change;
       by Temp;
     title2 'Pearson Correlation (Lysine)';
   run;

 *Experimental Design Analysis (Classification Variables);
   proc glm data=LYS_Brown plots=all;
     class Temp Conc;
       model Browning = Temp|Conc / solution SS3 ;
       lsmeans Temp|Conc / cl ;
       lsmeans Temp*Conc / pdiff=all adjust=tukey;
       title2 'Response Variable: Browning (Lysine)';
       title3 'GLM Model (Experimental Design)';
       title4 'General Analysis' ;
   run;

   proc glm data=LYS_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp*Conc ;
     means Temp*Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: Browning (Lysine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Test of Equal Variance' ;
   run;

 *Experimental Design Analysis (Additional Covariates);
   proc glm data=LYS_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp|Conc aW / solution SS3 p ;
       lsmeans Temp|Conc / cl ;
       title2 'Response Variable: Browning (Lysine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=LYS_Brown plots=all;
     class Temp Conc ;
       model Browning = Temp|Conc pHb / solution SS3 p ;
       lsmeans Temp|Conc / cl ;
```

```
        title2 'Response Variable: Browning (Lysine)';
        title3 'GLM Model (Experimental Design)';
        title4 'Additional Covariates' ;
    run;

  *Regression-based Analysis (Continuous Variables);
    proc glm data=LYS_Brown plots=all;
        model Browning = Temp|Conc / solution SS3 p ;
        title2 'Response Variable: Browning (Lysine)';
        title3 'GLM Model (Regression)';
        title4 'General Analysis' ;
    run;

  *Regression-based Analysis (Additional Covariates);
    proc glm data=LYS_Brown plots=all;
        model Browning = Temp|Conc aW / solution SS3 p ;
        title2 'Response Variable: Browning (Lysine)';
        title3 'GLM Model (Regression)';
        title4 'Additional Covariates' ;
    run;

    proc glm data=LYS_Brown plots=all;
        model Browning = Temp|Conc pHb / solution SS3 p ;
        title2 'Response Variable: Browning (Lysine)';
        title3 'GLM Model (Regression)';
        title4 'Additional Covariates' ;
    run;

* Amino Acid: Serine ;

  *Creates Dataset (SER_Brown) for completely detectable Browning data with
Serine;
    data SER_Brown;
      set Browning;
      where AA="Serine" & (Temp=120 | Temp=160);
      drop _4_MeI;
    run;

  *Print of the Full SER_Brown Dataset;
    proc print data=SER_Brown;
      title2 'Print-out of the Full SER_Brown Dataset';
        title3 ;
        title4 ;
    run;

  *Correlation between Variables of Full SER_Brown Dataset;
    proc corr data=SER_Brown plots=matrix(hist nvar=all);
      var Browning Conc aW pHb pHa pH_Change;
        by Temp;
      title2 'Pearson Correlation (Serine)';
    run;

  *Experimental Design Analysis (Classification Variables);
    proc glm data=SER_Brown plots=all;
      class Temp Conc;
        model Browning = Temp|Conc / solution SS3 ;
        lsmeans Temp|Conc / cl ;
```

```
      lsmeans Temp*Conc / pdiff=all adjust=tukey;
      title2 'Response Variable: Browning (Serine)';
      title3 'GLM Model (Experimental Design)';
      title4 'General Analysis' ;
  run;

  proc glm data=SER_Brown plots=all;
    class Temp Conc ;
      model Browning = Temp*Conc ;
    means Temp*Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
      title2 'Response Variable: Browning (Serine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Test of Equal Variance' ;
  run;

 *Experimental Design Analysis (Additional Covariates);
  proc glm data=SER_Brown plots=all;
    class Temp Conc ;
      model Browning = Temp|Conc aW / solution SS3 p ;
      lsmeans Temp|Conc / cl ;
      title2 'Response Variable: Browning (Serine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=SER_Brown plots=all;
    class Temp Conc ;
      model Browning = Temp|Conc pHb / solution SS3 p ;
      lsmeans Temp|Conc / cl ;
      title2 'Response Variable: Browning (Serine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

 *Regression-based Analysis (Continuous Variables);
  proc glm data=SER_Brown plots=all;
      model Browning = Temp|Conc / solution SS3 p ;
      title2 'Response Variable: Browning (Serine)';
      title3 'GLM Model (Regression)';
      title4 'General Analysis' ;
  run;

 *Regression-based Analysis (Additional Covariates);
  proc glm data=SER_Brown plots=all;
      model Browning = Temp|Conc aW / solution SS3 p ;
      title2 'Response Variable: Browning (Serine)';
      title3 'GLM Model (Regression)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=SER_Brown plots=all;
      model Browning = Temp|Conc pHb / solution SS3 p ;
      title2 'Response Variable: Browning (Serine)';
      title3 'GLM Model (Regression)';
      title4 'Additional Covariates' ;
  run;
```

```
*Previous Analysis for Browning including Amino Acid;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA / outp=new;
      random  Rep(Temp Conc) ;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Default)';
      title4 ;
  run;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA  / outp=new;
      repeated AA / subject=Rep(Temp Conc) type=un;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Unstructured)';
  run;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA  / outp=new;
      repeated AA / subject=Rep(Temp Conc) type=cs;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Compound Symmetry)';
  run;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA  / outp=new;
      repeated AA / subject=Rep(Temp Conc) type=csh;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Heterogeneous CS)';
  run;

*Previous Analysis for Browning including Amino Acid & Additional Covariates
(pHb & aW);

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA pHb aW / outp=new;
      random  Rep(Temp Conc) ;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Default)';
      title4 ;
  run;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA pHb aW / outp=new;
      repeated AA / subject=Rep(Temp Conc) type=un;
```

```
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Unstructured)';
  run;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA pHb aW / outp=new;
      repeated AA / subject=Rep(Temp Conc) type=cs;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Compound Symmetry)';
  run;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA pHb aW / outp=new;
      repeated AA / subject=Rep(Temp Conc) type=csh;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Heterogeneous CS)';
  run;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA pHb / outp=new;
      repeated AA / subject=Rep(Temp Conc) type=un;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Unstructured)';
  run;

  proc mixed data=Browning covtest cl;
    class   Temp Conc Rep AA;
      model   Browning = Temp|Conc|AA pHb / outp=new ddfm=Satterth;
      repeated AA / subject=Rep(Temp Conc) type=un;
      lsmeans Temp|Conc|AA / cl;
      title2 'Response Variable: Browning';
      title3 'Mixed Model (Unstructured)';
  run;

/* Response Variable: 4-Methylimidazole */


* Amino Acid: Alanine ;

 *Creates Dataset (ALA_4MeI) for completely detectable 4-MeI data with
Alanine;
   data ALA_4MeI;
     set Browning;
     where AA="Alanine" & Temp=160;
   run;

 *Print of the Full ALA_4MeI Dataset;
   proc print data=ALA_4MeI;
     title2 'Print-out of the Full ALA_4MeI Dataset';
       title3 ;
```

```
      title4 ;
  run;

*Correlation between Variables of Full ALA_4MeI Dataset;
  proc corr data=ALA_4MeI plots=matrix(hist nvar=all);
    var _4_MeI Browning Conc aW pHb pHa pH_Change;
    title2 'Pearson Correlation (Alanine)';
  run;


*Experimental Design Analysis (Classification Variables);
  proc glm data=ALA_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc / solution SS3 ;
      lsmeans Conc / cl;
      lsmeans Conc / pdiff=all adjust=tukey;
      title2 'Response Variable: 4-MeI (Alanine)';
      title3 'GLM Model (Experimental Design)';
      title4 'General Analysis' ;
  run;

  proc glm data=ALA_4MeI plots=all;
    class Conc ;
      model _4_MeI = Conc ;
    means Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE HOVTEST=OBRIEN ;
      title2 'Response Variable: 4-MeI (Alanine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Test of Equal Variance' ;
  run;

*Experimental Design Analysis (Additional Covariates);
  proc glm data=ALA_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc aW / solution SS3 ;
      lsmeans Conc / cl;
      title2 'Response Variable: 4-MeI (Alanine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=ALA_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc pHb / solution SS3 ;
      lsmeans Conc / cl;
      title2 'Response Variable: 4-MeI (Alanine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

*Regression-based Analysis (Continuous Variables);
  proc glm data=ALA_4MeI plots=all;
      model _4_MeI = Conc / solution SS3 p ;
      title2 'Response Variable: 4-MeI (Alanine)';
      title3 'GLM Model (Regression)';
      title4 'General Analysis' ;
  run;
```

```
 *Regression-based Analysis (Additional Covariates);
   proc glm data=ALA_4MeI plots=all;
       model _4_MeI = Conc aW / solution SS3 p ;
       title2 'Response Variable: 4-MeI (Alanine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=ALA_4MeI plots=all;
       model _4_MeI = Conc pHb / solution SS3 p ;
       title2 'Response Variable: 4-MeI (Alanine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

* Amino Acid: Arginine ;

 *Creates Dataset (ARG_4MeI) for completely detectable 4-MeI data with
Arginine;
   data ARG_4MeI;
     set Browning;
     where AA="Arginine" & (Temp=120 | Temp=160);
   run;

 *Print of the Full ARG_4MeI Dataset;
   proc print data=ARG_4MeI;
     title2 'Print-out of the Full ARG_4MeI Dataset';
       title3 ;
       title4 ;
   run;

 *Correlation between Variables of Full ARG_4MeI Dataset;
   proc corr data=ARG_4MeI plots=matrix(hist nvar=all);
     var _4_MeI Browning Conc aW pHb pHa pH_Change;
       by Temp;
     title2 'Pearson Correlation (Arginine)';
   run;

 *Experimental Design Analysis (Classification Variables);
   proc glm data=ARG_4MeI plots=all;
     class Temp Conc;
       model _4_MeI = Temp|Conc / solution SS3 ;
       lsmeans Temp|Conc / cl;
       lsmeans Temp*Conc / pdiff=all adjust=tukey;
       title2 'Response Variable: 4-MeI (Arginine)';
       title3 'GLM Model (Experimental Design)';
       title4 'General Analysis' ;
   run;

   proc glm data=ARG_4MeI plots=all;
     class Temp Conc ;
       model _4_MeI = Temp*Conc ;
     means Temp*Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: 4-MeI (Arginine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Test of Equal Variance' ;
```

```
  run;

 *Experimental Design Analysis (Additional Covariates);
  proc glm data=ARG_4MeI plots=all;
    class Temp Conc;
      model _4_MeI = Temp|Conc aW / solution SS3 ;
      lsmeans Temp|Conc / cl;
      title2 'Response Variable: 4-MeI (Arginine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=ARG_4MeI plots=all;
    class Temp Conc;
      model _4_MeI = Temp|Conc pHb / solution SS3 ;
      lsmeans Temp|Conc / cl;
      title2 'Response Variable: 4-MeI (Arginine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

 *Regression-based Analysis (Continuous Variables);
  proc glm data=ARG_4MeI plots=all;
      model _4_MeI = Temp|Conc / solution SS3 p ;
      title2 'Response Variable: 4-MeI (Arginine)';
      title3 'GLM Model (Regression)';
      title4 'General Analysis' ;
  run;

 *Regression-based Analysis (Additional Covariates);
  proc glm data=ARG_4MeI plots=all;
      model _4_MeI = Temp|Conc aW / solution SS3 p ;
      title2 'Response Variable: 4-MeI (Arginine)';
      title3 'GLM Model (Regression)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=ARG_4MeI plots=all;
      model _4_MeI = Temp|Conc pHb / solution SS3 p ;
      title2 'Response Variable: 4-MeI (Arginine)';
      title3 'GLM Model (Regression)';
      title4 'Additional Covariates' ;
  run;

* Amino Acid: Glycine ;

 *Creates Dataset (GLY_4MeI) for completely detectable 4-MeI data with
Glycine;
  data GLY_4MeI;
    set Browning;
    where AA="Glycine" & Temp=160;
  run;

 *Print of the Full GLY_4MeI Dataset;
  proc print data=GLY_4MeI;
    title2 'Print-out of the Full GLY_4MeI Dataset';
      title3 ;
```

```
      title4 ;
  run;

*Correlation between Variables of Full GLY_4MeI Dataset;
  proc corr data=GLY_4MeI plots=matrix(hist nvar=all);
    var _4_MeI Browning Conc aW pHb pHa pH_Change;
    title2 'Pearson Correlation (Glycine)';
  run;


*Experimental Design Analysis (Classification Variables);
  proc glm data=GLY_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc / solution SS3 ;
      lsmeans Conc / cl;
      lsmeans Conc / pdiff=all adjust=tukey;
      title2 'Response Variable: 4-MeI (Glycine)';
      title3 'GLM Model (Experimental Design)';
      title4 'General Analysis' ;
  run;

  proc glm data=GLY_4MeI plots=all;
    class Conc ;
      model _4_MeI = Conc ;
    means Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE HOVTEST=OBRIEN ;
      title2 'Response Variable: 4-MeI (Glycine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Test of Equal Variance' ;
  run;

*Experimental Design Analysis (Additional Covariates);
  proc glm data=GLY_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc aW / solution SS3 ;
      lsmeans Conc / cl;
      title2 'Response Variable: 4-MeI (Glycine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=GLY_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc pHb / solution SS3 ;
      lsmeans Conc / cl;
      title2 'Response Variable: 4-MeI (Glycine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

*Regression-based Analysis (Continuous Variables);
  proc glm data=GLY_4MeI plots=all;
      model _4_MeI = Conc / solution SS3 p ;
      title2 'Response Variable: 4-MeI (Glycine)';
      title3 'GLM Model (Regression)';
      title4 'General Analysis' ;
  run;
```

```
 *Regression-based Analysis (Additional Covariates);
   proc glm data=GLY_4MeI plots=all;
       model _4_MeI = Conc aW / solution SS3 p ;
       title2 'Response Variable: 4-MeI (Glycine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=GLY_4MeI plots=all;
       model _4_MeI = Conc pHb / solution SS3 p ;
       title2 'Response Variable: 4-MeI (Glycine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

* Amino Acid: Lysine ;

 *Creates Dataset (LYS_4MeI) for completely detectable 4-MeI data with
Lysine;
   data LYS_4MeI;
     set Browning;
     where AA="Lysine" &  (Temp=120 | Temp=160);
   run;

 *Print of the Full LYS_4MeI Dataset;
   proc print data=LYS_4MeI;
     title2 'Print-out of the Full LYS_4MeI Dataset';
       title3 ;
       title4 ;
   run;

*Correlation between Variables of Full LYS_4MeI Dataset;
   proc corr data=LYS_4MeI plots=matrix(hist nvar=all);
     var _4_MeI Browning Conc aW pHb pHa pH_Change;
       by Temp;
     title2 'Pearson Correlation (Lysine)';
   run;

*Experimental Design Analysis (Classification Variables);
   proc glm data=LYS_4MeI plots=all;
     class Temp Conc;
       model _4_MeI = Temp|Conc / solution SS3 ;
       lsmeans Temp|Conc / cl;
       lsmeans Temp*Conc / pdiff=all adjust=tukey;
       title2 'Response Variable: 4-MeI (Lysine)';
       title3 'GLM Model (Experimental Design)';
       title4 'General Analysis' ;
   run;

   proc glm data=LYS_4MeI plots=all;
     class Temp Conc ;
       model _4_MeI = Temp*Conc ;
     means Temp*Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: 4-MeI (Lysine)';
       title3 'GLM Model (Experimental Design)';
       title4 'Test of Equal Variance' ;
```

```
  run;

 *Experimental Design Analysis (Additional Covariates);
  proc glm data=LYS_4MeI plots=all;
    class Temp Conc;
      model _4_MeI = Temp|Conc aW / solution SS3 ;
      lsmeans Temp|Conc / cl;
      title2 'Response Variable: 4-MeI (Lysine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=LYS_4MeI plots=all;
    class Temp Conc;
      model _4_MeI = Temp|Conc pHb / solution SS3 ;
      lsmeans Temp|Conc / cl;
      title2 'Response Variable: 4-MeI (Lysine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

 *Regression-based Analysis (Continuous Variables);
  proc glm data=LYS_4MeI plots=all;
      model _4_MeI = Temp|Conc / solution SS3 p ;
      title2 'Response Variable: 4-MeI (Lysine)';
      title3 'GLM Model (Regression)';
      title4 'General Analysis' ;
  run;

 *Regression-based Analysis (Additional Covariates);
  proc glm data=LYS_4MeI plots=all;
      model _4_MeI = Temp|Conc aW / solution SS3 p ;
      title2 'Response Variable: 4-MeI (Lysine)';
      title3 'GLM Model (Regression)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=LYS_4MeI plots=all;
      model _4_MeI = Temp|Conc pHb / solution SS3 p ;
      title2 'Response Variable: 4-MeI (Lysine)';
      title3 'GLM Model (Regression)';
      title4 'Additional Covariates' ;
  run;

* Amino Acid: Serine ;
 *Creates Dataset (SER_4MeI) for completely detectable 4-MeI data with
Serine;
  data SER_4MeI;
    set Browning;
    where AA="Serine" & Temp=160;
  run;

 *Print of the Full SER_4MeI Dataset;
  proc print data=SER_4MeI;
    title2 'Print-out of the Full SER_4MeI Dataset';
      title3 ;
      title4 ;
```

```
  run;

*Correlation between Variables of Full SER_4MeI Dataset;
  proc corr data=SER_4MeI plots=matrix(hist nvar=all);
    var _4_MeI Browning Conc aW pHb pHa pH_Change;
    title2 'Pearson Correlation (Serine)';
      title3 ;
      title4 ;
  run;

  proc glm data=SER_4MeI plots=all;
    class Conc AA;
    model _4_MeI Browning pH_Change = Conc ;
      MANOVA H=Conc / printe ;
      title2 'Temperature = 120 C';
      title3 'GLM Model (MANOVA)';
      title4 'Conditioned on Amino Acid*Concentration' ;
  run;

*Experimental Design Analysis (Classification Variables);
  proc glm data=SER_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc / solution SS3 ;
      lsmeans Conc / cl;
      lsmeans Conc / pdiff=all adjust=tukey;
      title2 'Response Variable: 4-MeI (Serine)';
      title3 'GLM Model (Experimental Design)';
      title4 'General Analysis' ;
  run;

  proc glm data=SER_4MeI plots=all;
    class Conc ;
      model _4_MeI = Conc ;
    means Conc / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE HOVTEST=OBRIEN ;
      title2 'Response Variable: 4-MeI (Serine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Test of Equal Variance' ;
  run;

*Experimental Design Analysis (Additional Covariates);
  proc glm data=SER_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc aW / solution SS3 ;
      lsmeans Conc / cl;
      title2 'Response Variable: 4-MeI (Serine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;

  proc glm data=SER_4MeI plots=all;
    class Conc;
      model _4_MeI = Conc pHb / solution SS3 ;
      lsmeans Conc / cl;
      title2 'Response Variable: 4-MeI (Serine)';
      title3 'GLM Model (Experimental Design)';
      title4 'Additional Covariates' ;
  run;
```

```
 *Regression-based Analysis (Continuous Variables);
   proc glm data=SER_4MeI plots=all;
       model _4_MeI = Conc / solution SS3 p ;
       title2 'Response Variable: 4-MeI (Serine)';
       title3 'GLM Model (Regression)';
       title4 'General Analysis' ;
   run;

 *Regression-based Analysis (Additional Covariates);
   proc glm data=SER_4MeI plots=all;
       model _4_MeI = Conc aW / solution SS3 p ;
       title2 'Response Variable: 4-MeI (Serine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

   proc glm data=SER_4MeI plots=all;
       model _4_MeI = Conc pHb / solution SS3 p ;
       title2 'Response Variable: 4-MeI (Serine)';
       title3 'GLM Model (Regression)';
       title4 'Additional Covariates' ;
   run;

*Issue: The censored data prevents us from being able to complete a regular
analysis using the experimental design;
        * Previous sub-analysis on 4-Methylimidazole at the Temperature of
160 deg. Celcius (Complete Detection) ;

 *Creates Dataset (Temp_160) for 4-MeI data completely detectable at
Temperature of 160 deg. Celcius;
   data Temp_160;
     set Browning;
     where Temp=160;
   run;

 *Print of the Full Temp_160 Dataset;
   proc print data=Temp_160;
     title2 'Print-out of the Full Temp_160 Dataset';
       title3 ;
       title4 ;
   run;

 *Performs preliminary Analysis using the experimental design (Removal of
Temp);
   proc mixed data=Temp_160 covtest cl;
     class   Conc Rep AA;
     model   _4_MeI = Conc|AA / outp=new;
       random  Rep(Conc) ;
       lsmeans Conc AA Conc*AA / cl;
       title2 'Response Variable: 4-MeI (Temp=160 C)';
       title3 'Mixed Model';
       title4 ;
   run;

 *Checks the Normality of overall residuals;
   proc univariate normal plot data=new;
```

```
    var resid;
      title3 'Checking Normality of Overall Residuals';
  run;

 *Checks the Normality of overall residuals by Amino Acid;
  proc sort data=new;
    by AA;
  run;

  proc univariate normal plot data=new;
    var resid;
      by AA;
      title3 'Checking Normality of Overall Residuals by Amino Acid';
  run;

  proc mixed data=Temp_160 plots=all;
    class Rep AA;
    model _4_MeI = Conc AA AA*Conc / noint solution ;
  run;

  proc glm data=Temp_160 plots=all;
    class Rep AA;
    model Browning = Conc AA AA*Conc / noint solution ;
  run;

/* MANOVA */

*Creates Dataset (Temp_160) for 4-MeI data completely detectable at
Temperature of 160 deg. Celcius;
  data Temp_160;
    set Browning;
    where Temp=160;
  run;

 *Print of the Full Temp_160 Dataset;
  proc print data=Temp_160;
    title2 'Print-out of the Full Temp_160 Dataset';
      title3 ;
      title4 ;
  run;

 *Correlation between 4-MeI, Browning, & pH Change Variables of the Full
Temp_160 Dataset;
  proc corr  data=Temp_160 plots=matrix(hist nvar=all) ;
    var _4_MeI Browning pH_Change ;
      title2 'Temperature = 160 C';
      title3 'Pearson Correlation Coefficient';
      title4 '4-Methylimidazole vs. Browning';
  run;

 *MANOVA Analysis between 4-MeI, Browning, & pH Change Variables with the
Full Temp_160 Dataset (Conditioned on Amino Acid*Concentration);
  proc glm data=Temp_160 plots=all;
    class Conc AA;
    model _4_MeI Browning pH_Change = AA Conc AA*Conc ;
      MANOVA H=AA Conc AA*Conc / printe ;
      title2 'Temperature = 160 C';
```

```
      title3 'GLM Model (MANOVA)';
      title4 'Conditioned on Amino Acid*Concentration' ;
   run;

 *MANOVA Analysis between 4-MeI, Browning, & pH Change Variables with the
Full Temp_160 Dataset (Conditioned on Amino Acid);
   proc glm data=Temp_160 plots=all;
     class AA;
     model _4_MeI Browning pH_Change = AA ;
       MANOVA H=AA / printe ;
       title2 'Temperature = 160 C';
       title3 'GLM Model (MANOVA)';
       title4 'Conditioned on Amino Acid (Disregards Concentration)' ;
   run;

 *Creates Dataset (Temp_120) for 4-MeI data completely detectable at
Temperature of 120 deg. Celcius;
   data Temp_120;
     set Browning;
     where Temp=120;
   run;

 *Print of the Full Temp_120 Dataset;
   proc print data=Temp_120;
     title2 'Print-out of the Full Temp_120 Dataset';
       title3 ;
       title4 ;
   run;

 *Correlation between Browning & pH Change Variables of the Full Temp_120
Dataset;
   proc corr  data=Temp_120 plots=matrix(hist nvar=all) ;
     var Browning pH_Change ;
       title2 'Temperature = 120 C';
       title3 'Pearson Correlation Coefficient';
       title4 '4-Methylimidazole vs. Browning';
   run;

 *MANOVA Analysis between Browning & pH Change Variables with the Full
Temp_120 Dataset (Conditioned on Amino Acid*Concentration);
   proc glm data=Temp_120 plots=all;
     class Conc AA;
     model Browning pH_Change = AA Conc AA*Conc ;
       MANOVA H=AA Conc AA*Conc / printe ;
       title2 'Temperature = 120 C';
       title3 'GLM Model (MANOVA)';
       title4 'Conditioned on Amino Acid*Concentration' ;
   run;

 *MANOVA Analysis between Browning & pH Change Variables with the Full
Temp_120 Dataset (Conditioned on Amino Acid*Concentration);
   proc glm data=Temp_120 plots=all;
     class AA;
     model Browning pH_Change = AA ;
       MANOVA H=AA / printe ;
       title2 'Temperature = 120 C';
       title3 'GLM Model (MANOVA)';
```

```
        title4 'Conditioned on Amino Acid (Disregards Concentration)' ;
    run;
ods rtf close;
```

Figure A-7. Stastical code used to analyze part 4 of the dissertation.

```
/*
Notes: Testing the effect of Ascorbic Acid on Glucose_Arginine Mixure @ 160
deg. Celsius  (2/18/15)
*/

/* SAS Analysis */

ods rtf file = "C:\Users\bloedow\Documents\Consulting\Clients\Faris Hussain
(Fall 2014-Present)\Analysis\SAS Output\Statistical Analysis on Ascorbic Acid
Project (03_09_15).doc";

title 'Ascorbic Acid Project (Faris Hussain)';

*Imports the Full Ascorbic Acid Dataset from Excel into SAS;
 proc import out=Ascorbic
   datafile='C:\Users\bloedow\Documents\Consulting\Clients\Faris Hussain
(Fall 2014-Present)\Data\SAS Data Analysis_Ascorbic Acid (02_18_15).xlsx'
   dbms=xlsx
   replace;
   sheet="SAS";
   getnames=yes;
 run;

*Creates new and truncated variables from the Full Ascorbic Acid Dataset;
 data Ascorbic;
   set Ascorbic;
   format Ascorbic_Acid 6.4;
   pH_Change=pHa-pHb;
   log_browning=round(log10(Browning),0.000001);
   log_4_MeI=round(log10(_4_MeI),0.000001);
   ln_browning=round(log(Browning),0.000001);
   ln_4_MeI=round(log(_4_MeI),0.000001);
 run;

*Sorts the Full Ascorbic Acid Dataset by Ascorbic_Acid;
 proc sort data=Ascorbic;
   by Ascorbic_Acid;
 run;

*Print of the Full Ascorbic Acid Dataset;
  proc print data=Ascorbic;
    title2 'Print-out of the Full Ascorbic Acid Dataset';
  run;

/* Statistical Graphics to visually inspect the raw data */

 *Scatter Plots: 4-MeI*Browning by Ascorbic Acid Concentration;
  proc sgscatter data=Ascorbic ;
    plot _4_MeI*Browning / datalabel=Ascorbic_Acid group=Ascorbic_Acid;
      title2 '4-Methylimidazole vs. Browning';
  run;
```

```
 *Scatter Plots: 4-MeI by Ascorbic Acid Concentration;
  proc sgscatter data=Ascorbic ;
    plot (_4_MeI log_4_MeI ln_4_MeI)*Ascorbic_Acid / group=Ascorbic_Acid
columns=3 rows=1;
      title2 '4-Methylimidazole vs. Ascorbic Acid Concentration';
  run;

 *Scatter Plots: Browning by Ascorbic Acid Concentration;
  proc sgscatter data=Ascorbic ;
    plot (Browning log_Browning ln_Browning)*Ascorbic_Acid /
group=Ascorbic_Acid columns=3 rows=1;
      title2 'Browning vs. Ascorbic Acid Concentration';
  run;

*Creates Dataset (pH) to compare the pH level measurements (Before & After);
 data pH;
   set Ascorbic;
   array XX[2] pHb pHa;
   do Time=1 to 2;
   pH=XX[Time];
   output;
   end;
   drop pHb pHa;
 run;

*Creates categorical variable (Status) to timing of measurements with pH
Dataset;
 data pH;
   set pH;
   if Time=1 then Status="Before"; else Status="After";
 run;

 *Lattice Scatter Plots: pH*Status by Ascorbic Acid Concentration;
  proc sgpanel data=pH ;
      panelby Ascorbic_Acid / columns=5 rows=1 novarname ;
    scatter x=Status y=pH / group=Status;
    colaxis label="Status" ;
      rowaxis label="pH" Min=2 Max=12 Values=(2 to 12 by 2) ;
      refline 7 / axis=y lineattrs=(color=purple thickness=1);
      title2 'pH vs. Status';
  run;

/* Statistical Analysis */

* Response Variable: Browning;

 *Experimental Design Analysis (Classification Variables);
   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model Browning = Ascorbic_Acid / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
     means Ascorbic_Acid / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: Browning';
       title3 'GLM Model (Experimental Design)';
```

```
   run;

   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model log_Browning = Ascorbic_Acid / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
     means Ascorbic_Acid / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: Log10(Browning)';
       title3 'GLM Model (Experimental Design)';
   run;

   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model ln_Browning = Ascorbic_Acid / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
     means Ascorbic_Acid / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: Ln(Browning)';
       title3 'GLM Model (Experimental Design)';
   run;

 *Experimental Design Analysis (Additional Covariates);
   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model Browning = Ascorbic_Acid aW / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
       title2 'Response Variable: Browning';
       title3 'GLM Model (Experimental Design)';
   run;

   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model Browning = Ascorbic_Acid pHb / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
       title2 'Response Variable: Browning';
       title3 'GLM Model (Experimental Design)';
   run;

 *Regression-based Analysis (Continuous Variables);
   proc glm data=Ascorbic plots=all;
       model Browning = Ascorbic_Acid / solution SS3 p ;
       title2 'Response Variable: Browning';
       title3 'GLM Model (Regression)';
   run;

   proc glm data=Ascorbic plots=all;
       model Browning = Ascorbic_Acid Ascorbic_Acid*Ascorbic_Acid / solution
SS3 p ;
       title2 'Response Variable: Browning';
       title3 'GLM Model (Regression)';
   run;

   proc glm data=Ascorbic plots=all;
       model log_Browning = Ascorbic_Acid / solution SS3 p ;
       title2 'Response Variable: Log10(Browning)';
       title3 'GLM Model (Regression)';
```

```
   run;

   proc glm data=Ascorbic plots=all;
       model log_Browning = Ascorbic_Acid Ascorbic_Acid*Ascorbic_Acid /
solution SS3 p ;
       title2 'Response Variable: Log10(Browning)';
       title3 'GLM Model (Regression)';
   run;


   proc glm data=Ascorbic plots=all;
       model ln_Browning = Ascorbic_Acid / solution SS3 p ;
       title2 'Response Variable: Ln(Browning)';
       title3 'GLM Model (Regression)';
   run;

   proc glm data=Ascorbic plots=all;
       model ln_Browning = Ascorbic_Acid Ascorbic_Acid*Ascorbic_Acid /
solution SS3 p ;
       title2 'Response Variable: Ln(Browning)';
       title3 'GLM Model (Regression)';
   run;

* Response Variable: 4-Methylimidazole;

 *Experimental Design Analysis (Classification Variables);
   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model _4_MeI = Ascorbic_Acid / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
     means Ascorbic_Acid / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: 4-Methylimidazole';
       title3 'GLM Model (Experimental Design)';
   run;

   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model log_4_MeI = Ascorbic_Acid / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
     means Ascorbic_Acid / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: Log10(4-MeI)';
       title3 'GLM Model (Experimental Design)';
   run;

   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model ln_4_MeI = Ascorbic_Acid / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
     means Ascorbic_Acid / HOVTEST=BARTLETT HOVTEST=BF HOVTEST=LEVENE
HOVTEST=OBRIEN ;
       title2 'Response Variable: Ln(4-MeI)';
       title3 'GLM Model (Experimental Design)';
   run;

 *Experimental Design Analysis (Additional Covariates);
```

```
   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model _4_MeI = Ascorbic_Acid aW / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
       title2 'Response Variable: 4-Methylimidazole';
       title3 'GLM Model (Experimental Design)';
   run;

   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
       model _4_MeI = Ascorbic_Acid pHb / solution SS3 ;
       lsmeans Ascorbic_Acid / cl pdiff adjust=Tukey ;
       title2 'Response Variable: 4-Methylimidazole';
       title3 'GLM Model (Experimental Design)';
   run;

 *Regression-based Analysis (Continuous Variables);
   proc glm data=Ascorbic plots=all;
       model _4_MeI = Ascorbic_Acid / solution SS3 p ;
       title2 'Response Variable: 4-Methylimidazole';
       title3 'GLM Model (Regression)';
   run;

   proc glm data=Ascorbic plots=all;
       model _4_MeI = Ascorbic_Acid Ascorbic_Acid*Ascorbic_Acid / solution
SS3 p ;
       title2 'Response Variable: 4-Methylimidazole';
       title3 'GLM Model (Regression)';
   run;

   proc glm data=Ascorbic plots=all;
       model log_4_MeI = Ascorbic_Acid / solution SS3 p ;
       title2 'Response Variable: Log10(4-MeI)';
       title3 'GLM Model (Regression)';
   run;

   proc glm data=Ascorbic plots=all;
       model log_4_MeI = Ascorbic_Acid Ascorbic_Acid*Ascorbic_Acid / solution
SS3 p ;
       title2 'Response Variable: Log10(4-MeI)';
       title3 'GLM Model (Regression)';
   run;

   proc glm data=Ascorbic plots=all;
       model ln_4_MeI = Ascorbic_Acid / solution SS3 p ;
       title2 'Response Variable: Ln(4-MeI)';
       title3 'GLM Model (Regression)';
   run;

   proc glm data=Ascorbic plots=all;
       model ln_4_MeI = Ascorbic_Acid Ascorbic_Acid*Ascorbic_Acid / solution
SS3 p ;
       title2 'Response Variable: Ln(4-MeI)';
       title3 'GLM Model (Regression)';
   run;

* Measuring Association between 4-MeI, Browning, & pH Change;
```

```
 *Correlation between 4-MeI, Browning, & pH Change Variables of the Full
Ascorbic Acid Dataset;
   proc corr  data=Ascorbic plots=matrix(hist nvar=all) ;
     var _4_MeI Browning pH_Change ;
       title2 'Pearson Correlation Coefficient';
       title3 '4-Methylimidazole, Browning, & pH Change';
   run;

 *MANOVA Analysis between 4-MeI, Browning, & pH Change Variables with the
Full Ascorbic Acid Dataset (Conditioned on Ascorbic Acid Concentration);
   proc glm data=Ascorbic plots=all;
     class Ascorbic_Acid;
     model _4_MeI Browning pH_Change = Ascorbic_Acid ;
       MANOVA H=Ascorbic_Acid / printe ;
       title2 'GLM Model (MANOVA)';
       title3 'Conditioned on Ascorbic Acid Concentration' ;
   run;


ods rtf close;
```