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# Formation of Carcinogenic 4(5)-Methylimidazole in Maillard Reaction Systems

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4(5)-Methylimidazole has received the attention of federal and state regulatory agencies because of its carcinogenicity and common presence in foods and beverages. In the present study, the formation of 4(5)-methylimidazole in Maillard reaction model systems consisting of D-glucose/$NH_3$, L-rhamnose/$NH_3$, methylglyoxal/$NH_3$, and methylglyoxal/formaldehyde/$NH_3$ was investigated. 4(5)-Methylimidazole was formed at levels ranging from 0.49 to 0.71 mg/mL in the D-glucose/$NH_3$ model system. The formation of 4(5)-methylimidazole was slightly higher in the L-rhamnose/$NH_3$ system (0.91 mg/mL) than in the D-glucose/$NH_3$ system (0.71 mg/mL) under the conditions used in the present study. A methylglyoxal/$NH_3$ system produced significantly higher levels of 4(5)-methylimidazole (5.70 mg/mL), suggesting that methylglyoxal is an important precursor of 4(5)-methylimidazole. Ammonolysis of methylglyoxal, which is one of the glucose degradation products, was proposed to form formamide, which subsequently reacted with 2-aminopropanal (α-aminocarbonyl intermediate) formed from methylglyoxal to give 4- or 5-methylimidazole. The levels of 4(5)-methylimidazole found in commercial cola soft drinks range from 0.30 μg/mL (brand 3) to 0.36 μg/mL (brands 1 and 5).

**KEYWORDS: Caramel color; cola soft-drinks; Maillard reaction; 4(5)-methylimidazole**

## INTRODUCTION

Recently, 4(5)-methylimidazole has attracted a great deal of attention among federal and state regulatory agencies because of its carcinogenicity and presence in foods and beverages. The National Toxicology Program (NTP) has identified 4-methylimidazole as causing cancer (*1*). The Office of Environmental Health Hazard Assessment (OEHHA) within the California Environmental Protection Agency plans to list 4-methylimidazole as known to the State to cause cancer (*2*). This NTP determination was based on results obtained from a 2-year study on male and female F344/N rats and B6C3F1 mice exposed to 4-methylimidazole, which concluded that there was clear evidence of the carcinogenic activity of 4-methylimidazole in male and female B6C3F1 mice based on increased incidences of alveolar/bronchiolar neoplasms (*3*). The same research group observed induction of thyroid lesions in 14-week toxicity studies of 4-methylimdazole in Fischer 344/N rats and B6C3F1 mice (*4*).

4(5)-Methylimidazole is used in many products such as drugs, dyes, agricultural chemicals, and rubber (*3*). 4(5)-Methylimidazole is a relatively simple compound with a molecular weight of 82.1. It is soluble in water and ethanol. Alkylimidazoles, including 4(5)-methylimidzole, were first synthesized from α-dicarbonyl compounds and methyl alkyl ketones with ammonia in the mid 19th century (*5*). The yield of this method is relatively low, but it is still used for creating C-substituted imidazoles. This reaction mechanism suggests that sugars and amino acids in foods and beverages are ideal precursors of imidazoles because sugars degrade into alkyl dicarbonyls and alkyl ketones (*6*) and amino acids form ammonia and alkyl carbonyls via Strecker degradation (*7*). In fact, some Maillard reaction systems consisting of a sugar and an amino acid produced imidazoles including 4(5)-methylimidazole (*8*). There have been many reports on the presence of 4(5)-methylimidazole in various sauces and beverages, such as soy sauce (*9*) and coffee, in levels ranging from 0.3 to 3 ppm (*10*−*12*).

Many studies indicate that imidazoles are formed by Maillard reaction in foods and beverages (*8*). Formation of 4(5)-methylimidazole from a reaction of D-glucose and ammonia—a typical caramel-color preparation method for beverages—was reported in the Maillard reaction system for the first time in the early 1960s (*13*). It is proposed that foods containing a caramel color produced by a Maillard reaction contain 4(5)-methylimidazole. 4(5)-Methylimidazole was subsequently identified in various caramel samples at levels ranging from 7 to 200 ppm (*14*−*16*). These studies suggest that the production of a caramel color produces carcinogenic 4(5)-methylimidazole, which eventually enters into caramel-colored foods and beverages.

In the present study, 4(5)-methylimidazole formation in the Maillard reaction model systems was investigated to determine its possible presence in foods and beverages.

## EXPERIMENTAL PROCEDURES

**Chemicals.** D-Glucose, L-rhamnose, methyl glyoxal solution (40%), ammonium hydroxide solution (29%), formaldehyde solution (37%), and

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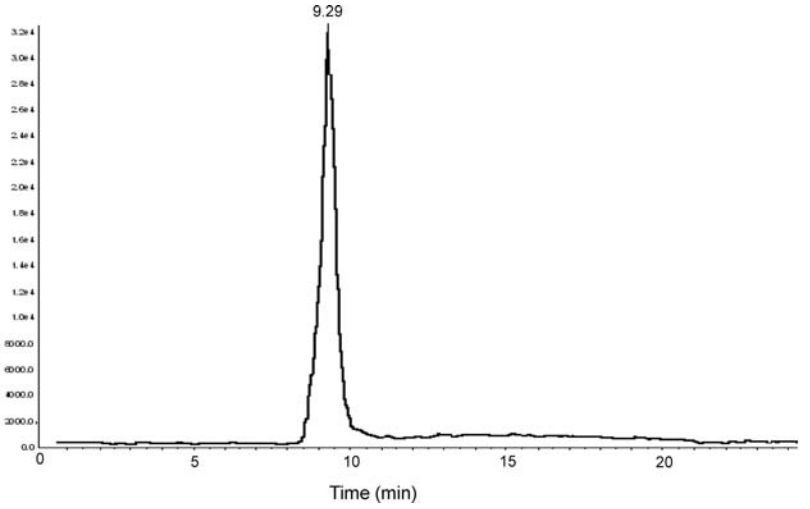


**Figure 1.** Typical chromatogram of a sample obtained from D-glucose and ammonia.

4(5)-methylimidazole were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Commercial cola soft drinks were bought from a local market.

Standard stock solution of 4(5)-methylimidazole (10 mg/L) was prepared by dissolving it into HPLC grade water. The solution was held stable for at least 1 week in the dark at 4 °C.

**Sample Preparations of Maillard Reaction Products.** A swing-top bottle containing 30 mL aqueous solutions of D-glucose (0.1 M) and ammonium hydroxide (0.1 M), L-rhamnose (1 M) and ammonium hydroxide (1 M), methyl glyoxal (1 M) and ammonium hydroxide (1 M), or methyl glyoxal (1 M), formaldehyde (1 M), and ammonium hydroxide (1 M) were cooled in an ice bath for 30 min. Solutions of D-glucose and ammonium hydroxide were heated at 70, 100, or 120 °C for 3, 6, or 12 h in an oven. Solutions with L-rhamnose and ammonium hydroxide, methyl glyoxal and ammonium hydroxide, or methyl glyoxal, formaldehyde, and ammonium hydroxide were heated at 100 °C for 2 h in an oven. After the samples had cooled to room temperature, the reaction samples were diluted with water 50−250-fold. Each sample (20 $\mu$L) was injected into LC-MS for 4(5)-methylimidazole analysis.

A commercial cola soft drink sample (5 mL) was passed through an Ansys SPEC SCX Disc 15 mg/3 mL cartridge (Varian, Walnut Creek, CA) after the addition of 30 $\mu$L of 0.1 N HCl, and then the cartridge was washed with 1 mL of methanol. Retained materials were eluted from the cartridge with 2 mL of methanol acidified with 5 M HCl (3:1, v/v). The extracts were condensed to dryness using a rotary vacuum evaporator at 39 °C. The residual materials were dissolved into 500 $\mu$L of Milli-Q water. Each sample (20 $\mu$L) was injected into LC-MS for 4(5)-methylimidazole analysis.

**Analysis of 4(5)-Methylimidazole.** 4(5)-Methylimidazole analysis was conducted with a Hewlett-Packard 1100 liquid chromatograph interfaced to an Applied Biosystems API 2000 MS/MS via an electrospray ionization (ESI) source operating in the positive ion mode at 400 °C with nitrogen gas. Chromatographic separation was accomplished with a 100 × 4.6 mm Varian Polaris RP column with a 3 $\mu$m particle size. The mobile phase was ACN (15 mmol of ammonia, solvent A) and water (15 mmol of ammonia, solvent B). A linear gradient that follows at 0−3 min, A/B = 2/98; at 10−15 min, 40/60; and at 17−20 min, 2/98, was used with a flow rate at 0.4 mL/min. Under these conditions, 4(5)-methylimidazole was eluted at 9.29 min. A typical chromatogram of a sample obtained from D-glucose and ammonia is shown in **Figure 1**.

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode to observe the transition of $m/z$ 83 to 56 for 4(5)-methylimidazole.

A standard curve for the quantitative analysis of 4(5)-methylimidazole was prepared by diluting the standard solution (10 mg/L) with HPLC grade water to prepare solutions of various concentrations (0.25, 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 $\mu$g/mL), and each solution (20 $\mu$L) was injected to LC-MS. A satisfactory standard curve was obtained ($y = 33879x + 14295$ and $R^2 = 0.9967$). The recovery efficiency of 4(5)-methylimidazole was examined using 10 $\mu$g/L aqueous solutions.

**Table 1.** Amount of 4(5)-Methylimidazole Formed in D-Glucose/Ammonium Hydroxide Maillard Model System

| sample | temperature (°C) | time (h) | amount (mg/mL) |
|---|---|---|---|
| 1 | 70 | 3 | 0.49 ± 0.03 |
| 2 | 70 | 6 | 0.62 ± 0.04 |
| 3 | 70 | 12 | 0.66 ± 0.01 |
| 4 | 100 | 3 | 0.71 ± 0.05 |
| 5 | 100 | 6 | 0.70 ± 0.04 |
| 6 | 100 | 12 | 0.66 ± 0.04 |
| 7 | 120 | 3 | 0.67 ± 0.03 |
| 8 | 120 | 6 | 0.67 ± 0.02 |
| 9 | 120 | 12 | 0.59 ± 0.03 |

## RESULTS AND DISCUSSION

The recovery efficiency of 4(5)-methylimidazole from aqueous solutions using the experimental processes described above was 102.5 ± 3.61% ($n = 3$), suggesting that the experimental processes used for 4(5)-methylimidazole in the present study were satisfactory.

**Table 1** shows the results of the D-glucose/ammonium hydroxide Maillard model system. 4(5)-Methylimidazole formed at levels ranging from 0.49 mg/mL (sample 1) to 0.71 mg/mL (sample 4). However, significant differences in the amount of 4(5)-methylimidazole formed in the D-glucose/ammonium hydroxide samples were not observed under the conditions used in the present study. Since the discovery of the Maillard reaction in 1912 (*17*), simple model systems consisting of a sugar and an amine compound, such as glucose and ammonia, have been widely used to investigate topics related to processed foods and beverages. A few years after Maillard's report, formation of brown color and glucosamine was reported in a D-glucose/$NH_3$ Maillard reaction system (*18*). Maillard reaction systems have been used for preparing caramel color for foods and beverages for over 100 years. Caramel color prepared by the controlled heat treatment of carbohydrate, including glucose, with ammonia was classified as Caramel Color III (synonyms: ammonia caramel, beer caramel) by the International Technical Caramel Association (ITCA) (*19*). In the 1990s, the presence of 4(5)-methylimidazole in caramel colors at levels ranging from 7.5 to 212.0 mg/kg was reported (*15*). More recently, 4(5)-methylimidazole was found in Caramel Color III samples at levels ranging from 73.3 to 187.8 $\mu$g/g (*10*).

**Table 2** shows the amount of 4(5)-methylimidazole formed in various Maillard model systems. In the present study, the L-rhamnose/$NH_3$ system was used because a previous paper

indicated that this system produced several imidazoles, including unsubstituted imidazole and 2-methyl-, 2,4-dimethyl-, 2-ethyl, and 2-formylimidazole (*20*). The formation of 4(5)-methylimidazole was slightly higher in the L-rhamnose/$NH_3$ system (0.91 mg/mL) than in the D-glucose/$NH_3$ system (0.71 mg/mL) under the conditions used in the present study. This may be due to the fact that L-rhamnose degrades more readily than D-glucose does to yield precursors (formaldehyde, acetaldehyde, glyoxal, methylglyoxal, etc.) of imidazole formation (*20*). These carbonyl compounds are also formed from amino acid via Strecker degradation (*21*). Therefore, a methylglyoxal/$NH_3$ system was also examined for 4(5)-methylimidazole formation in the present study. This system produced significantly higher levels of 4(5)-methylimidazole (5.70 mg/mL) compared with monosaccharide/$NH_3$ systems (0.49−0.91 mg/mL), suggesting that methylglyoxal is an important precursor of 4(5)-methylimidazole.

The results of a study using $^{13}C$-2-labeled amino acids showed that an α-amino carbonyl moiety—formed from α-dicarbonyl compounds or α-hydroxycarbonyl compounds with amino acid or ammonia—is the most important nitrogen-containing reactive intermediate in the formation of imidazoles (*21*). On the basis of this paper, formation mechanisms of 4(5)-methylimidazole from methylglyoxal were proposed and are shown in **Figure 2**. As mentioned above, methylglyoxal is formed from glucose degradation (*6*). To form an imidazole ring, it requires a −C−C− moiety with one amine and one =O moiety on adjacent carbon atoms. Ammonolysis of methylglyoxal gives formamide, which can be one of the precursors of imidazole ring formation. Formation of 4(5)-methylimidazole was not changed significantly by the addition of formaldehyde, suggesting that formamide did not form directly from formaldehyde with ammonia to participate in 4(5)-methylimidazole formation in significant amounts. Ammonolysis of methylglyoxal was proposed as the mechanism for forming formamide, which subsequently reacted with 2-aminopropanal (α-aminocarbonyl intermediate) to give 4- or 5-methylimidazole. The step from hydroxyacetone to 2-aminopropanal with $NH_3$ was adapted from a previous paper (*21*).

**Table 2.** Amount of 4(5)-Methylimidazole Formed in Various Maillard Model Systems

| model system | amount (mg/mL) |
|---|---|
| L-rhamnose/$NH_4OH$ | 0.91 ± 0.00 |
| methylglyoxal/$NH_4OH$ | 5.70 ± 0.01 |
| methylglyoxal/formaldehyde/$NH_4OH$ | 5.45 ± 0.29 |

**Table 3** shows the amount of 4(5)-methylimidazole analyzed in commercial cola soft drinks. The levels of 4(5)-methylimidazole found in commercial cola soft drinks range from 0.30 μg/mL (brand 3) to 0.36 μg/mL (brands 1 and 5). There are only a few reports on the levels of 4(5)-methylimidazole in commercial products. Commercial coffees and beers contain 4(5)-methylimidazole at levels ranging from 0.39 μg/g (Mexico) to 2.05 μg/g (Vitmelta) and from 1.58 ng/mL (Velkopopovicky kozel) to 28.03 ng/mL (Starobrno), respectively (*10*), indicating that the levels of 4(5)-methylimidazole in commercial cola soft drinks are similar to those in coffees.

It has long been known that some Maillard reaction systems consisting of sugar/amino acid produced mutagenic substances (*8*, *22*). In particular, there have been many reports on the toxicity of caramel color additives produced by Maillard reaction systems (*23*). For example, a commercial ammonia caramel coloring exhibited mutagenic activity toward *Salmonella*

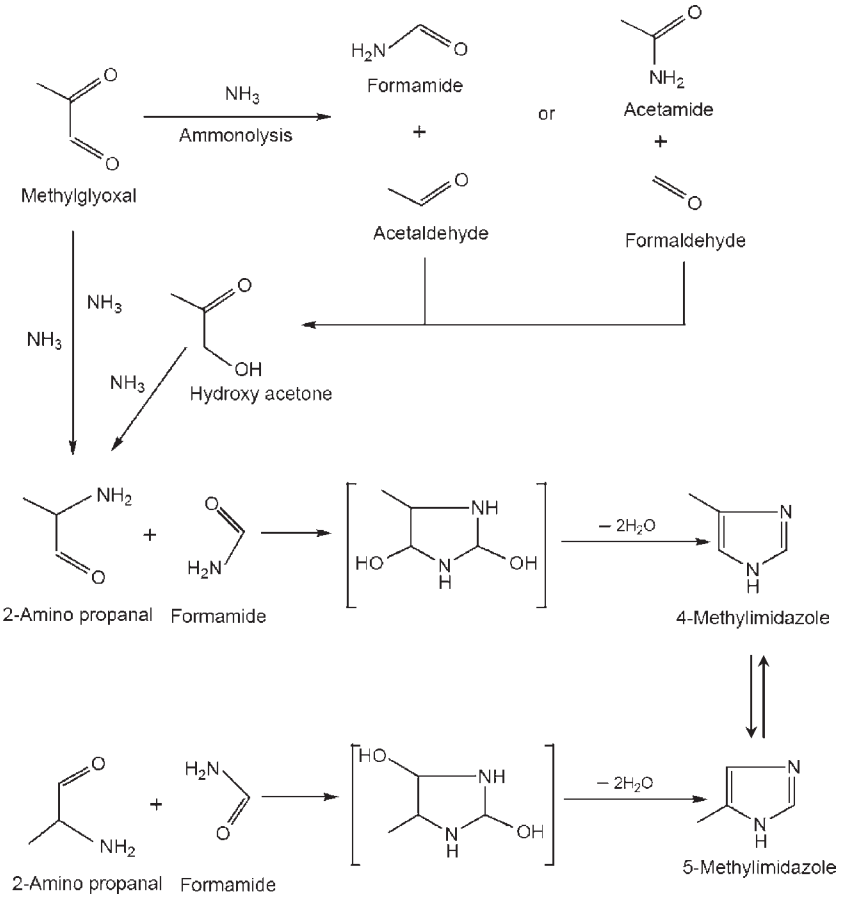


**Figure 2.** Proposed formation mechanisms of 4(5)-methylimidazole from methylglyoxal.

**Table 3.** Amount of 4(5)-Methylimidazole Found in Commercial Cola Soft Drinks

| cola soft-drink | amount ($\mu$g/mL) | in one bottle ($\mu$g/591 mL) |
|---|---|---|
| brand 1 | 0.36 ± 0.02 | 212.76 |
| brand 2 | 0.32 ± 0.01 | 189.12 |
| brand 3 | 0.30 ± 0.01 | 177.30 |
| brand 4 | 0.32 ± 0.02 | 189.12 |
| brand 5 | 0.36 ± 0.00 | 212.76 |

*typhimurium* TA 100 without metabolic activation (*24*). Caramel Color III, which is prepared for commercial beverages from D-glucose in the presence of $NH_3$, demonstrated immunotoxicity in rats, and the imidazole derivative 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole was found to be responsible (*23*). Therefore, many researchers have attempted to pinpoint the chemicals responsible for the toxicity of these Maillard reaction products. Now, toxicities of 4(5)-methylimidazole have been discovered. The study by NTP referred to above indicates that carcinogenic incidences were observed in experimental mice when they were fed diets containing 0, 312, 625, or 1250 ppm 4-methylimidazole for 2 years. The amounts of 4-methylimidazole in these diets were equivalent to average daily doses of approximately 4, 80, and 170 mg/kg of body weight to mice. If a person weighing 60 kg consumes one bottle of cola soft drink, only 3.3 $\mu$g/kg 4-methylimidazole is ingested. Therefore, the amounts ingested from these beverages may not be significant. However, discussion of possible cancer incidence in humans caused by 4(5)-methylimidazole present in foods and beverages is not within the scope of this study.

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