JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Review

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# Carcinogenic 4(5)-Methylimidazole Found in Beverages, Sauces, and Caramel Colors: Chemical Properties, Analysis, and Biological Activities

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**ABSTRACT:** Since the National Toxicology Program (NTP) identified 4(5)-methylimidazole [4(5)-MI] as a cancer causing chemical in 2007 and the State of California added it to the Proposition 65 list of compounds as a carcinogen on January 7, 2011, many researchers and regulatory agencies have become focused on the presence of 4(5)-MI in foods and beverages. 4(5)-MI has been known to form in the Maillard reaction system consisting of a sugar and ammonia—a typical caramel-color preparation method for beverages. 4(5)-MI is identified in various beverages and sauces, which are colored with caramel, as well as in caramel color itself. Analysis of 4(5)-MI is extremely difficult due to its high water solubility, but the analytical method for 4(5)-MI has progressed from conventional paper chromatography, gas chromatography, and gas chromatography−mass spectrometry to the most advanced high-performance liquid chromatography−mass spectrometry. Various studies indicate that caramel colors and carbonated beverages contain 4(5)-MI in levels ranging from 0 to around 1000 ppm and from 0 to about 500 ppm, respectively. Reports of the toxicity of 4(5)-MI at relatively high levels suggest that it may cause some adverse effects on human consumers.

**KEYWORDS:** *beverages, caramel colors, carcinogens, Maillard reaction, 4(5)-methylimidazole*

## INTRODUCTION

In the mid 19th century, alkylimidazoles, including 4(5)-methylimidazole [4(5)-MI], were first synthesized from α-dicarbonyl compounds and methyl alkyl ketones with ammonia.[1] The formation of imidazoles in Maillard browning model systems consisting of a sugar and an amino acid has also been known since the mid 1900s.[2] The Maillard model system used to study products formed in processed or cooked foods also originated around the same time. In particular, analyses of volatile flavor chemicals formed in the Maillard model systems were among the most intensively pursued. Among the volatile chemicals formed in the Maillard model systems, heterocyclic compounds have received much attention as flavor chemicals. By the 1980s, the number of volatile heterocyclic compounds found had reached over 1000.[3] Figure 1 shows typical heterocyclic compounds, which are analogues of imidazole with a five-membered ring system, found in the Maillard model systems. Homologues of some of these chemicals have received much attention as the chemicals that give characteristic cooked flavors. For example, pyrroles possess characteristic roasted or toasted flavors in diluted solutions, and some thiazole derivatives give a pleasant coffee flavor.[4]

Imidazoles have not received the same level of attention among flavor chemists due to the lack of interesting flavors or tastes and the production of certain off-flavors in cooked foods.[4] Imidazoles, particularly 4(5)-MI, are formed by Maillard reaction in heat-processed foods and beverages.[3] Research demonstrated the formation of 4(5)-MI in the Maillard reaction system consisting of D-glucose and ammonia, producing the typical caramel-color in beverages.[7] This suggests caramel colors contain 4(5)-MI, which was eventually identified in various classes of caramel colors.[8]

Additional toxicology research further suggested that the preparation of a caramel color produces carcinogenic 4(5)-MI. Toxicity of caramel color was expected when the formation of mutagens in a Maillard reaction system consisting of D-glucose and ammonia was reported in 1979,[9] followed by a study using a D-glucose/ammonia/sulfide browning model system.[10] A column chromatographic methanol fraction obtained from a reaction mixture of this Maillard reaction system exhibited potent mutagenicity, and GC-MS analysis of this fraction identified 18 imidazole derivatives, such as 2-methylimidazole.[10] Another Maillard model system consisting of ammonia and maltol, which has been reported to be a mutagen,[11] also produced mutagens, and imidazoles were found to be major products of this reaction mixture.[12]

In 1975, the World Health Organisation (WHO) summarized and discussed toxicological studies on caramel colors.[13] A review reported that caramel color III disturbed immune functions and changed resistance in infection models on rodents. Imidazole derivative 2-actyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole (THI) was determined as the responsible principle.[14] However, there was no description of 4(5)-MI in these publications. The biological activities of THI have been reported in some papers; for example, THI caused a selective lymphopenia in mice at relatively high doses.[15] Finally, in 2007, reports from the National Toxicology Program (NTP) identified 4(5)-MI as a cancer-causing chemical,[5] thus causing many researchers and regulatory agencies to focus on the presence of 4(5)-MI in foods and beverages. Subsequently, on January 7, 2011 the State of California listed 4(5)-MI in Proposition 65 as a carcinogen and adopted a final "Safe

Received: November 14, 2012
Revised: January 6, 2013
Accepted: January 7, 2013
Published: January 7, 2013





dx.doi.org/10.1021/jf304855u | J. Agric. Food Chem. 2013, 61, 780−789

| Compound | Structure | MW | BP (°C) | Water solubility | Sensory nature |
|---|---|---|---|---|---|
| Furan |  | 68.08 | 32.0 | insoluble | spicy, smoky, cinnammon-like |
| Pyrrole |  | 67.09 | 131.0 | slightly | sweet ether-like, slightly smoky |
| Thiophene |  | 84.14 | 84.4 | insoluble | weak sulfurous |
| Oxazole |  | 69.06 | 69.5 | slightly | pyridine-like, sweet in a diluted solution |
| Thiazole |  | 85.13 | 116.5 | slightly | slightly smoky, coffee-like |
| Imidazole |  | 68.08 | 257.0 | soluble | odorless |

**Figure 1.** Profile of imidazole analogue heterocyclic compounds.

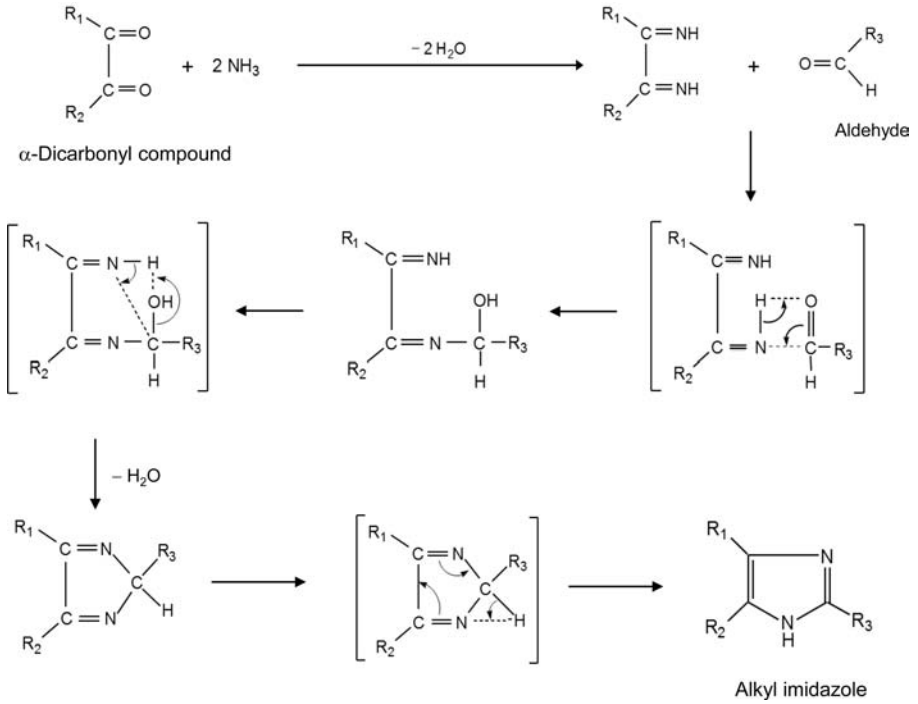


**Figure 2.** Proposed mechanisms of Debus–Radziszewski imidazole synthesis.

Harbor" level = 29 μg/day in February 2012, which they raised from an earlier proposal of 16 μg/day.[6]

## CHEMICAL AND PHYSICAL PROPERTIES OF 4(5)-MI

Various imidazoles were synthesized in the mid 1800s. 4(5)-MI (CAS Registry No. 822-35-6) is a relatively simple compound with a molecular weight of 82.1. Its melting point is 46–48 °C, and the boiling point is 263 °C. It is a colorless to slightly yellow solid that is soluble in water and ethanol.[16] Unsubstituted imidazole is a simple five-membered heterocyclic compound. This ring system is present in various biological substances, such as amino acid, histidine, and related proteins and enzymes. An imidazole ring is also present in many bioactive compounds, including antifungal drugs.[17] The synthetic method of producing alkylimidazoles, including 4(5)-MI, was first determined in 1858.[1] Later, this method came to be known as the Debus–Radziszewski imidazole synthesis.[18] Figure 2 shows the proposed mechanisms of this synthesis. In this figure, when methylglyoxal ($R_1$ = $CH_3$ and $R_2$ = H) is reacted with formaldehyde ($R_3$ = H), 4(5)-MI is produced. The yield of this method is relatively low, but it is still used for creating C-substituted imidazoles. Later, a method that offered a 49% yield of 4(5)-MI from a reaction between $HCONH_2$ and $RCHClCHClOCH_2CH_2R$ (R = H or $CH_3$) was developed.[19]

The Debus–Radziszewski synthesis method suggests that sugars or lipids and amino acids or proteins in foods and


dx.doi.org/10.1021/jf304855u | J. Agric. Food Chem. 2013, 61, 780–789

beverages are ideal precursors of imidazoles because sugars and lipids degrade into alkyl dicarbonyls and alkyl ketones[20,21] and amino acids form ammonia and alkyl carbonyls via Strecker degradation.[22] In fact, some Maillard reaction systems consisting of a sugar and an amino acid have produced imidazoles, including 4(5)-MI.[3] For example, a Maillard reaction system consisting of diacetyl (2,3-butanedione) and ammonia produced 2,4,5-trimethylimidazole as the major product.[23] Studies with $^{13}$C-2-labeled glycine and alanine have demonstrated that imidazoles can be formed from diacetyl reacting with these amino acids.[24]

Also, a recent study showed Maillard model systems consisting of methylglyoxal and $NH_4OH$ or methylglyoxal/formaldehyde and $NH_4OH$ produced 5.70 and 5.45 mg/mL 4(5)-MI, respectively.[25] In this study, the formation mechanisms of 4- or 5-methylimidazole suggested that methyglyoxal reacted with $NH_3$ to form an intermediate, 2-aminopropanal, which subsequently reacted with formaldehyde to produce 4(5)-MI.

Various aspects of the chemical properties of imidazoles have been reported. A lipid oxidation study indicated that 4(5)-MI exhibited significant prooxidative activity.[26] Additionally, a kinetic study on the iodination of imidazole demonstrated the role of a methyl substituent in the rate of iodination, which was, in decreasing order, 4(5)-MI > 2-methylimidazole > imidazole.[27]

There are three methylimidazole isomers: 2-, 4-, or 5-methylimidazole. Among those, 4- and 5-methylimidazoles are present as tautomers in an aqueous solution at neutral-to-basic pH as shown in Figure 3. The neutral imidazole ring exists as

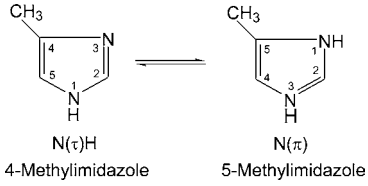


**Figure 3.** Structures of 4- and 5-methylimidazole tautomers in an aqueous solution at neutral-to-basic pH.

two tautomers that differ from each other in the position of protonation, and the N$\tau$-protonated (N$\tau$-H) tautomer is more stable than the N$\pi$-protonated (N$\pi$-H) one.[28] The tautomer ratio of 4- and 5-methylimidazoles in a solution was calculated by using a combination of quantum mechanical calculations and molecular dynamics simulations as 1.3.[29] Therefore, the presence of 4- and 5-methylimidazoles in an aqueous solution is approximately 57 and 43%, respectively. A methyl group stays on the same carbon atom in an imidazole ring. The carbon number changes from 4 to 5 due to the proton transfer. Thus, 4(5)-MI is the most commonly used name for these isomers.

In biological systems, 4(5)-MI reportedly possesses proton donor activity. Human carbonic anhydrase has been shown to be activated by 4(5)-MI.[30] It has been reported that electrons on an imidazole moiety of histidine in a dentrimer enzyme model participate in the ester hydrolysis of pyrene trisulfonate esters.[31] Dentrimers, which are treelike molecules containing histidine residues, have been used for various purposes, including as catalysts, in drug delivery, and in artificial vaccines as well as for gene delivery into cells.[32−35] These papers indicate that the imidazole moiety plays an important role in biological systems.

## ANALYSIS OF 4(5)-MI IN CARAMELS, SAUCES, AND BEVERAGES

4(5)-MI is used in many products such as drugs, dyes, agricultural chemicals, photographic chemicals, and rubber.[36] As mentioned above, there is also strong evidence that imidazoles, including 4(5)-MI, are formed in foods and beverages by the Maillard reaction.[3] The presence of 4(5)-MI in a reaction mixture of glucose and ammonia was reported in 1905, even before the concept of the Maillard reaction was advanced in 1912.[37] Maillard reaction systems have been used to study various processes associated with the formation of chemicals in foods and beverages, such as flavors, toxicants, and antioxidants.[38] However, imidazoles, including 4(5)-MI, have not received much attention from food chemists due to the lack of a characteristic flavor. Therefore, there are only a few studies on 4(5)-MI in Maillard reaction systems, as shown in Table 1. In the Maillard reaction systems utilized in those studies, $NH_4OH$ was commonly used as the ammonia source in an aqueous solution.

**Table 1. 4(5)-MI Reported in Maillard Reaction Systems**

| system | analytical method | year reported | ref |
|---|---|---|---|
| D-glucose/ammonia | paper chromatography | 1952 | 39 |
| molasses/ammonia | polarography | 1955 | 40, 41 |
| invert sugar/ammonia | paper chromatography | 1956 | 42 |
| sugar cane bagasse/ammonia | paper chromatography | 1961 | 43 |
| D-glucose/ammonia | paper chromatography | 1962 | 7 |
| DL-glyceraldehyde/ammonia | paper chromatography−spectrophotometry | 1964 | 44 |
| molasses/ammonia | column chromatography | 1966 | 45 |
| pine/$(NH_4)_2SO_3$ | column chromatography | 1966 | 46 |
| methylpentose/ammonia | IR | 1966 | 2 |
| L-ascorbic acid/ammonia | paper chromatography | 1975 | 47 |
| D-glucose/ammonium formate | gas chromatography | 1988 | 48 |
| D-glucose/ammonium acetate | gas chromatography | 1988 | 48 |
| D-glucose/glycine | gas chromatography | 1988 | 48 |
| D-glucose/glutamate | gas chromatography | 1988 | 48 |
| D-glucose/ammonia | LC-MS | 2011 | 25 |
| L-rhamnose/ammonia | LC-MS | 2011 | 25 |
| D-glucose/ammonia/sulfite | LC-MS | 2012 | 49 |

The primary analytical method used in the 1950s and 1960s was paper chromatography, even though gas chromatography (GC) was readily available. It was only after the advancement and common use of gas chromatography−mass spectrometry (GC-MS) that the study of flavor chemicals formed in Maillard reaction systems became popular. Despite the rise in popularity of such studies, however, there are virtually no reports on 4(5)-MI formation in Maillard reaction systems from the mid 1970s through 2011. In addition to the lack of interesting flavor characteristics, imidazoles, including 4(5)-MI, are water-soluble and have not been extracted with organic solvents for use in flavor analysis. For the investigation of flavor chemicals using Maillard reaction systems, the extraction of an aqueous reaction mixture with an organic solvent is essential to recover volatile or flavor chemicals for GC analysis. Therefore, most early


dx.doi.org/10.1021/jf304855u | J. Agric. Food Chem. 2013, 61, 780−789

studies on 4(5)-MI were done using paper chromatography instead of GC, as shown in Table 1.

Identification of 4(5)-MI was also achieved using a conventional comelting point method with a synthesized authentic compound.[7] The quantitative estimation was performed using a spectrophotometric method at 500 nm.[44] 4(5)-MI was also determined by paper chromatography after being derivatized into imidazolium picrate.[47] Later, details of the structure and the other physical properties of a picrate derivative were reported (see Figure 4).[50] In addition,

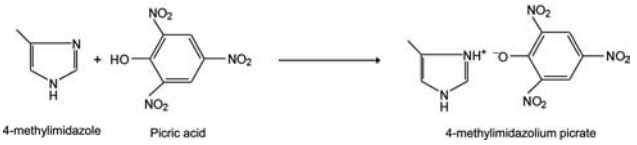


**Figure 4.** Reaction formula of 4-methylimidazole with picric acid to form a derivative.

imidazoles were extracted satisfactorily from an aqueous solution from a Maillard reaction using a liquid−liquid continuous extractor.[48] In this study, a D-glucose/ammonium formate Maillard model system yielded the highest amount of 4(5)-MI among Maillard reaction systems consisting variously of D-glucose/$NH_4OH$, ammonium formate, ammonium acetate, glycine, or glutamate.

Since its toxicity was recently reported in 2007, studies on 4(5)-MI formation in Maillard reaction systems have resumed.[5] One study reported that D-glucose/ammonia Maillard reaction systems produced 4(5)-MI ranging from 0.49 mg/mL (heated at 70 °C for 3 h) to 0.70 mg/mL (heated at 100 °C for 6 h).[25] The same study also found 0.91 mg/mL 4(5)-MI in a reaction mixture of L-rhamanose and ammonia heated at 100 °C for 2 h. In another recent study, a Maillard model system consisting of D-glucose/ammonia/sulfite reacted under conditions simulating caramel-color manufacturing yielded amounts of 4(5)-MI ranging from 7.0 to 155.0 ppm.[49] The same study observed that the addition of sulfite reduced 4(5)-MI formation significantly.

4(5)-MI was proposed to be present in caramel colors, and it has been identified in various commercial caramel colors. There are four classes of caramel color (classes I, II, III, and IV) based on the application and the reactants used in their manufacture.[49] Class I compounds is prepared by heating carbohydrates with alkali or acid. Class II colors are manufactured by heating carbohydrates with sulfite-containing compounds. Class III compounds are created by heating the carbohydrates with ammonium compounds, and class IV compounds are produced by heating carbohydrates with both sulfite-containing and ammonium-containing compounds. Classes I and II are usually used for alcoholic beverages. Class III is often used in confectionery, beer, and soy sauce. Class IV is widely used in soft drinks.

Table 2 shows the amounts of 4(5)-MI reported in commercial caramel colors and beverages. In this table, unit amounts were harmonized to parts per million because the units used in the different studies were varied (e.g., ppm, mg/kg, and μg/g). However, some units, such as μg/L and ng/mL, which could not be changed to ppm, are shown as they were originally reported. Reports on 4(5)-MI in caramel colors began to appear around 1990. Amounts of 4(5)-MI reported in caramel color classes III and IV ranged from 0.025 to 463 ppm and from 0 to 1276 ppm, respectively.

Initially, analysis of 4(5)-MI had not been a main focus of research until the NTP's report in 2007 revealed 4(5)-MI carcinogenicity in caramel colors.[5] Studies on the characterization of caramel colors I, II, III, and IV for various physical data reported that 4(5)-MI was not detected in colors I and II but was found at <10−34 and 157−983 mg/kg in colors III and IV, respectively.[51,59] When the results of genotoxicity studies on caramel colors were reported, the amount of 4(5)-MI was shown as one item among various physical data, including color intensity, solid percent, nitrogen percent, sulfur percent, THI concentration, ammoniacal nitrogen concentration, and sulfur dioxide concentration, obtained from the caramel sources.[52,53] The first reports on 4(5)-MI in various beverages appeared in the early 1980s.[63] In this study, thin layer chromatography (TLC) and gas chromatography with a nitrogen−phosphorus detector (GC-NPD) as well as densitometry after samples prepared using an acid−base extraction were used for qualitative determination of 4(5)-MI in sauces and beverages. The results showed 0.17−0.70 ppm 4(5)-MI in cola drinks. In another paper, 6.6−351 ppm of 4(5)-MI was determined via HPLC-UV in caramel color after ion-pair extraction or ion-pair chromatography.[60]

Some derivatives have been used to analyze 4(5)-MI by GC. For example, 4(5)-MI in caramel color was derivatized into 1-acetyl-4(5)-MI with acetic anhydride and then analyzed by GC with FID.[54] Later, this derivative was more accurately analyzed by GC-MS.[8] The same study developed an automated heart-cutting two-dimensional liquid chromatography technique (LC-LC), which determined free 4(5)-MI directly in <30 min. The other commonly used reagent to prepare 4(5)-MI derivatives for GC analysis is isobutylchloroformate.[55] 4(5)-MI forms two derivatives corresponding to 4- or 5-methylimidazole with isobutylchloroformate as shown in Figure 5. These two compounds were separated satisfactorily by a DB-5 GC column, and their identification was confirmed by MS spectra.[55] Later, this method was used for GC-MS analysis of 4(5)-MI in brand cola (188−416 μg/L)[64] and in roasted coffee (0.307−1.241 ppm).[66] The determination of 4(5)-MI in ammonia caramel colors using this derivative required an ion-pair extraction with bis(2-ethylhexylphosphate) in chloroform.[55,64]

The use of HPLC with a UV detector became the mainstream method for 4(5)-MI analysis from the early 1990s to the late 1990s. However, after HPLC-MS became available in the late 1990s, direct analysis of highly water-soluble organic compounds, which do not have appropriate UV absorption, in an aqueous solution became possible. For example, carcinogenic water-soluble acrylamide was reported in heated foodstuffs using HPLC-MS for the first time in 2002.[68,69] Even though acrylamide has been formed in Maillard reaction systems,[70] many studies on the formation of cooked flavor chemicals associated with the Maillard reaction did not report the presence of acrylamide because it was not extracted with organic solvents.[71] Although an aqueous solution can be applied directly to HPLC, some sample preparation steps are still required to obtain satisfactory quantitative analysis of 4(5)-MI by HPLC-MS.

As shown in Table 2, use of solid phase extraction (SPE) is the major method of sample preparation for 4(5)-MI analysis today. Because the $pK_a$ of 4(5)-MI is 7.6,[60] silica modified with $C_{18}$ is the most widely used SPE. A typical cleanup procedure for 4(5)-MI analysis in a soft drink using SPE is as follows:[25]

**Table 2. 4(5)-MI Reported in Caramel Colors, Sauces, and Beverages**

| sample | analytical method | preparation method | amount (ppm) | year reported | ref |
|---|---|---|---|---|---|
| caramel color | | | | | |
| class III | HPLC-UV or RI | Soxhlet extraction, column fractionation | <10−43 | 1992 | 51 |
| class III | HPLC-UV (610 nm) | not reported | 34−463 | 1992 | 52 |
| class III | HPLC-UV (610 nm) | not reported | 12−436 | 1992 | 53 |
| class III | GC | acetyl derivative | 25−303 | 1975 | 54 |
| class III | GC-MS | isobutylchloroformate derivative | 7.5−212.0 | 1997 | 55 |
| class III | HPLC-UV (215 nm) | sonicated | 24−50 | 1998 | 56 |
| class III | GC | ion pair extraction | 0.025−0.3 | 2005 | 57 |
| class III | HPLC-MS | SPE (Waters Oasis MCX 3 $cm^3$/60 mg; Applied Separations Spe-ed; benzenesulfonic SCX 500 mg/3 mL; Ansys SPEC SCX Disc 15 mg/3 mL | 73.3−187.8 | 2006 | 58 |
| class IV | HPLC-UV or RI | Soxhlet extraction, column fractionation | 157−983 | 1992 | 51 |
| class IV | HPLC-UV or RI | Soxhlet extraction, column fractionation | 112−1276 | 1992 | 59 |
| class IV | HPLC-UV (610 nm) | not reported | 0−387 | 1992 | 52 |
| class IV | HPLC-UV (610 nm) | not reported | 146−215 | 1992 | 53 |
| class IV | HPLC | none | 6.6−351 | 1981 | 60 |
| class IV | HPLC-UV | sonicated | 130−480 | 1998 | 56 |
| classes III and IV | GC-MS, LC-LC | acetyl derivative | 20−120 | 2011 | 8 |
| classes III and IV | HPLC-MS/MS | SPE (Sep-Pak Vac 12 cc, 2 g, C18) | 121 | 2011 | 61 |
| not specified | capillary isotachophoresis | none | 122−406 | 1989 | 62 |
| sauces | | | | | |
| Worcestershire sauce | TLC, densitometry, GC | acid−base extraction | 1.0−3.4 | 1981 | 63 |
| Worcestershire sauce | HPLC-MS/MS | SPE (Sep-Pak Vac 12 cc, 2 g, C18) | 0.027 | 2011 | 61 |
| soy sauce | TLC, densitometry, GC | acid−base extraction | 0.35−0.55 | 1981 | 63 |
| soy sauce | HPLC-MS/MS | SPE (Sep-Pak Vac 12 cc, 2 g, C18) | 0.002−4.8 | 2011 | 61 |
| beverages | | | | | |
| cola | TLC, densitometry, GC | acid−base extraction | 0.17 − 0.70 | 1981 | 63 |
| brand cola | GC-MS | isobutylchloroformate derivative | 188−416 μg/L | 2011 | 64 |
| brand cola | HPLC-MS | SPE (Ansys SPECSCX Disc 15 mg/3 mL) | 0.30−0.36 | 2011 | 25 |
| white label cola | GC-MS | isobutychloroformate derivative | 219−613 | 2011 | 64 |
| carbonated soft drinks | HPLC-MS/MS | SPE (Sep-Pak Vac 12 cc, 2 g, C18) | 0.67 | 2011 | 61 |
| carbonated guarana | GC-MS | isobutylchloroformate derivative | 438 | 2011 | 64 |
| carbonated beverages | HPLC-MS/MS | 4-methyl-$d_3$-imidazole | 0−692 ng/mL | 2012 | 65 |
| roasted coffee | GC-MS | isobutylchloroformate derivative | 0.307−1.241 | 2002 | 66 |
| roasted coffee | HPLC-MS | SPE (Waters Oasis MCX 3 $cm^3$/60 mg; Applied Separations Spe-ed; benzenesulfonic SCX 500 mg/3 mL | 0.39−2.05 | 2006 | 58 |
| roasted coffee powder | HPLC-ESI-MS | supercritical fluid extraction | 0.77−1.45 | 2006 | 67 |
| canned coffee | HPLC-MS/MS | SPE (Sep-Pak Vac 12 cc, 2 g, C18) | 0.048 | 2011 | 61 |
| dark beer | GC-MS | isobutylchloroformate derivative | 0−424 mg/L | 2011 | 64 |
| dark beer | HPLC-MS | SPE (Waters Oasis MCX 3 $cm^3$/60 mg; Applied Separations Spe-ed; benzenesulfonic SCX 500 mg/3 mL | 1.58−28.03 | 2006 | 58 |
| dark beer | HPLC-MS/MS | SPE (Sep-Pak Vac 12 cc, 2 g, C18) | 0.017 | 2011 | 61 |
| whiskey | TLC, densitometry, GC | acid−base extraction | 0.12−0.14 | 1981 | 63 |
| energy drink | GC-MS | isobutylchloroformate derivative | 0−37 | 2011 | 64 |
| no carbonated flavor | GC-MS | isobutylchloroformate derivative | 0−3 | 2011 | 64 |


dx.doi.org/10.1021/jf304855u | J. Agric. Food Chem. 2013, 61, 780−789

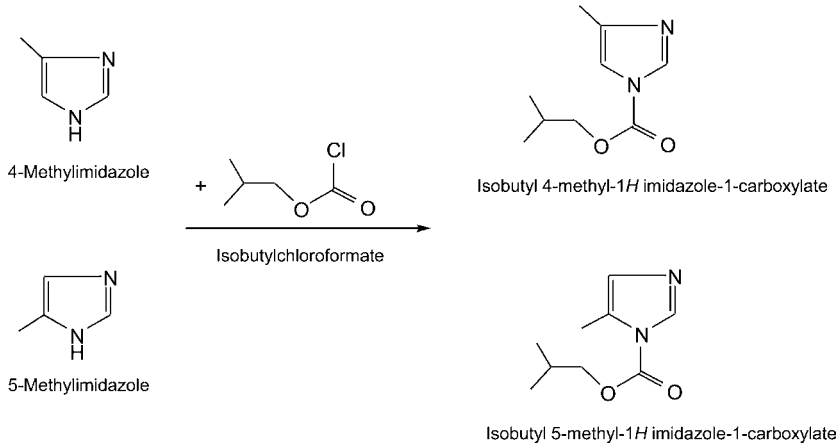


**Figure 5.** Reaction formula of 4(5)-MI with isobutylchloroformate to form derivatives.

**Table 3. Additional in Vivo and in Vitro Studies of 4(5)-MI**

| exptl animal | results of observation | dose | year reported | ref |
|---|---|---|---|---|
| cow | acute clinical signs: trembling, excessive salvation, and incoordination by oral dose | >1.5 g | 1986 | 75 |
| young calves | acute clinical signs: hypersalvation, mouth chomping, diarrhea, muscle fasciculations, tremors, hyperexcitability, convulsions, coma, and death (within 3 h) by oral dose | 400 mg/kg | 1987 | 76 |
| sheep | no apparent ill effect at body weight by oral dose | 20 mg/kg | 1990 | 77 |
| goats and heifers | 4(5)-MI and its metabolites were excreted mainly in urine but also in milk and feces by oral dose | 20 mg/kg | 1993 | 78 |
| mice | no morphological changes in the olfactory mucosa by ip injection | 38 mg/kg | 1995 | 79 |
| F344 rat | formation of metabolite and renal clearance of 4(5)-MI appeared to be a saturable process by gavage administration | 50 mg/kg | 1995 | 80 |
| cow | became febrile and hyperexcitable, and displayed an abnormal circling behavior by oral dose | 50−300 mg/kg | 1997 | 81 |
| rat | suppress testosterone secretion by injection | 10−300 mg/kg or 122−4407 μg/kg | 1998 | 82 |
| rat liver microsomes | inhibited *p*-nitrophenol hydroxylase (in vitro) | 0−10 mM | 1994 | 83 |
| rat and mice | induced tremors and ataxia by oral dose for 14 weeks | 10000 ppm in feed | 2006 | 36 |
| mouse brain tissue | inhibited glutamate decarboxylase activity (in vitro) | 2 mM | 2009 | 84 |

After a commercial cola (5 mL) with the addition of 30 μL of 0.1 N HCl is passed through an Anasys SPEC SCX Disc 15 mg/3 mL cartridge, the cartridge is washed with 1 mL of methanol. The 4(5)-MI trapped is removed with 2 mL of acidified (5 M HCl) methanol. After removal of methanol, the condensed residue is dissolved into 500 μL of Milli-Q water for LC-MS analysis.

Currently, the use of LC-MS is at the forefront of 4(5)-MI analysis because most samples are aqueous, which can be injected into LC directly. One drawback of LC-MS is that the instrument is still rather expensive. Less expensive GC-MS or GC-NPD methods have been also used, but these methods require somewhat tedious sample preparations compared to the LC-MS method. However, the sensitivity of GC is comparable of that of LC-MS as long as satisfactory sample preparations are performed.

Amounts of 4(5)-MI in sauces were rather low compared with those in either caramel or beverage samples, ranging from 0.027 to 3.4 ppm in Worcestershire sauce and from 0.002 to 4.8 ppm in soy sauce.[61,63] In the case of sauces, the amounts used for cooking or seasoning are relatively low. Therefore, amounts ingested by humans through soy sauce may not be significant enough to cause concern about possible toxicological effects. On the other hand, beverages, such as cola (0.17−613 ppm),[25,63,64] coffee (0.048−2.05 ppm),[58,61,66,67] and beer (0.017−28.03),[58,61] which are consumed in rather large amounts, all contain levels sufficiently high as to receive attention by regulatory agencies.

## BIOLOGICAL ACTIVITIES

Among the biological activities of 4(5)-MI, toxicity has received the most attention over the past decade. There are several comprehensive reviews focused on the toxicities of 4(5)-MI written by the National Toxicology Program.[5] Since the National Cancer Institute nominated 2- and 4(5)-MI as candidates for toxicity and carcinogenicity studies, many toxicology studies have been performed using mice and rats.[72] For example, induction of thyroid lesions in Fischer 344/N rats and B6C3F1 mice was observed in 14 week toxicity studies of 4(5)-MI.[73] Later, a more comprehensive report on the toxicity and carcinogenicity of 4(5)-MI using the same experimental animals was published.[36] In this report, when male and female F344/N rats and B6C3F1 mice were exposed to 4(5)-MI for two years, there was clear evidence of the carcinogenic activity of 4(5)-MI in male and female B6C3F1 mice based on increased incidences of alveolar/bronchiolar neoplasms.[36] On the basis of this report, the State of California's Proposition 65 determined cancer potency and the "no significant risk level (NSRL) of 4(5)-MI as 0.045 mg/kg·day and 16 μg/day, respectively.[74] This report describes the


dx.doi.org/10.1021/jf304855u | J. Agric. Food Chem. 2013, 61, 780−789

**Table 4. Toxicity and Related Studies on Caramel Colors**

| color type | study objectives | dose | year reported | ref |
|---|---|---|---|---|
| ammonia processed in a pan | long-term toxicity in rats by oral dose | 1, 3, or 6% | 1977 | 89 |
| III | effects on mammalian vitamin $B_6$ metabolism | 0.6% (in vitro) | 1982 | 90 |
| IV | pattern of absorption, distribution, and excretion using [U-$^{14}$C] glucose in rats by oral gavage | 2.5 g/kg | 1992 | 91 |
| III | effects on the immune system with human volunteers | 20 mg/kg | 1992 | 92 |
| III | effects on the immune system of rats by oral dose | 4% in diet | 1992 | 93 |
| III | comparative study between rat and human studies on immunotoxicity by oral dose | 200 mg/kg | 1992 | 94 |
| IV | sub- and chronic toxicity (carcinogenicity) in F344 rats and B6C3F1 mice by oral dose | 2−10 g/kg | 1992 | 95 |
| II | subchronic toxicity in F344 rats by oral dose | 4−16 g/kg | 1992 | 96 |
| III | toxicity studies in F344 rats by oral dose | 10−20 g/kg | 1992 | 97 |
| III and IV | genotoxiciy hazard assessment by in vitro study | 1−50 mg/plate | 1992 | 52 |
| I | assessment of the genotoxicity potential in cytogenetics and lymphoma in vitro assays | 0.1−10 mg/plate | 1992 | 98 |
| I, II, III, and IV | absence of mutagenic and clastogenic activities in CHO cells in in vitro assay | 2.5−20 mg/plate | 1992 | 53 |
| I, III | immunotoxic effects in rats by oral dose | 4% in drinking water | 1993 | 99 |
| III | effects of subcronic exposure on the immune system in mice by oral dose | 2 and 4% in drinking water | 1994 | 100 |

details of how to obtain the values of cancer potency and NSRL using the results of the animal studies. For example, the NSRL is the intake associated with a lifetime cancer risk of $10^{-5}$. The cancer potency was estimated on the basis of the combined incidence of alveolar/bronchiola adenoma or carcinoma in male mice. NSRL was calculated using the cancer potency value and other factors including a lifetime cancer risk. Additionally, some typical in vivo and in vitro studies of 4(5)-MI are summarized in Table 3.

In the 1980s, the presence and toxicity of 4(5)-MI were also recognized in ammoniated forage[85] as a result of animal feeds treated with anhydrous ammonia to improve nutritive value.[86] Thus, studies were conducted on the biological activities of 4(5)-MI associated with ammoniated forage feeds in various animals, such as cows,[75] young Holstein calves,[76] sheep,[77] goats and heifers,[78] and young lambs.[87] Most information on 4(5)-MI toxicity can be found in the reviews prepared by NTP.[5]

In vivo studies on 4(5)-MI began to be performed in the mid 1980s. When relatively high doses (400 mg/kg body weight) of 4(5)-MI were orally administered to Holstein bull calves, various clinical signs were observed, such as hypersalivation, diarrhea, tremors, and convulsions, followed by coma and death within 3 h.[76] Adult cows also developed trembling and excessive salivation after oral administration of ≥1.5 g of 4(5)-MI.[75] In vitro studies reported inhibition of *p*-nitrophenol hydroxylase and glutamate decarboxylase in rat liver microsomes[83] and mouse brain tissue,[84] respectively. These reports indicate that 4(5)-MI causes some clinical disorders at high levels of ingestion. On the other hand, the most recent study reported that 4(5)-MI exhibited tumor preventive activity in the rat.[88]

Table 4 summarizes the toxicity studies on caramel colors. One of the early studies using experimental rats indicated that caramel colors prepared in a pan in the laboratory caused a reduced rate of body weight gain but no evidence of a carcinogenic effect when included at levels of 1−6% in the diet for 52 weeks.[89] In the early 1990s, intensive toxicity studies were performed on various types of caramel colors by a research group in The Netherlands.[92−94,99] One typical study performed by this group demonstrated that 0.4 or 4% caramel color III in the diet of rats for 28 days influenced various immune function parameters, including suppressing thymus-dependent immunity, decreasing natural cell-mediated cytotoxicity in the spleen, and enhancing clearance of *Listeria monocytogenes*.[99] A unique study using elderly male volunteers with a marginal deficit in vitamin $B_6$ reported that ingestion of 200 mg/kg body weight/day (acceptable daily intake) of commercial caramel color III for 7 days did not affect any immune-related factors.[94] Prior to this study, the acceleration of vitamin $B_6$ release from a rabbit brain by caramel color III had been reported.[90] In addition to these studies on caramel color III, studies of caramel colors I, II, and IV have been conducted, but they are generally considered not to be toxic or carcinogenic on the basis of the results of animal studies.[52,53,95,96,98]

There have been many reports on heat-induced toxic chemicals, particularly on Maillard reaction products (MRPs), in foods since the mid 20th century. Humans have been consuming Maillard reaction products continuously and consistently since cooking food was discovered. Consequently, it is proposed that many toxic chemicals, including 4(5)-MI, produced via the Maillard reaction, must be ingested by humans today as they have been for centuries. In addition to 4(5)-MI, some other chemicals studied include polycyclic aromatic hydrocarbons (PAHs) such as benz[*a*]pyrene, aromatic amines such as Glu-P-1, acrylamide, and diacetyl. The amounts of these chemicals formed are generally found only in trace levels (around the level of μg/kg of food or ppb), but for most of these compounds, detailed information about possible human toxicity at these levels is not yet well established. Continued investigation of these chemicals to determine the levels that cause adverse effects to human is in order.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATION USED

4(5)-MI, 4(5)-methylimidazole; NTP, National Toxicology Program; WHO, World Health Organisation; THI, 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole; CAS, Chemical Abstracts Service; GC, gas chromatography; GC-MS, gas chromatography–mass spectrometry; NPD, nitrogen–phosphorus detector; HPLC, high-performance liquid chromatography; UV, ultraviolet detector; FID, flame ionization detector; LC, liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; SPE, solid phase extraction; NSRL, no significant risk level; PAHs, polycyclic aromatic hydrocarbons; IR, infrared spectrometry; RI, refractive index; TLC, thin layer chromatography; ip, intraperitoneal

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