Short communication: Study on the formation of 2-methylimidazole and 4-methylimidazole in the Maillard reaction

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ABSTRACT

2-Methylimidazole (2-MI) and 4-methylimidazole (4-MI) can be formed via the Maillard reaction during dairy thermal treatment. In this study, different reactions between α -dicarbonyl compounds (methylglyoxal, glyoxal) and aldehydes (formaldehyde, acetaldehyde) in the presence of ammonium sulfate were performed to investigate the formation of 2-MI and 4-MI. Two formation pathways of 2-MI and 4-MI were proposed. One pathway is that α -dicarbonyl compound reacts with equivalent ammonia to form an intermediate, while aldehyde reacts with equivalent ammonia to form another intermediate, then the 2 intermediates react together to generate 2-MI or 4-MI. Alternatively, α -dicarbonyl compound can react with double ammonia to form an intermediate, and subsequently reacts with aldehyde to form 2-MI or 4-MI. Additionally, possible mechanisms were also proposed to explain the phenomenon that the 2-MI content was much lower than 4-MI in Maillard reaction.

Key words: 2-methylimidazole, 4-methylimidazole, methylglyoxal, glyoxal

Short Communication

2-Methylimidazole (2-MI) and 4-methylimidazole (4-MI) are undesired byproducts generated in Maillard reaction where carbohydrates (glucose, lactose, and so on) react with amino groups or ammonia (Casal et al., 2002; Moretton et al., 2011; Wang and Schnute, 2012). These 2 compounds have been identified in ammoniated hay forage for livestock animals and milk from cows fed ammoniated forage (Chan et al., 2006). 2-Methylimidazole and 4-MI have also been determined in many other kinds of food, such as soy sauce and bever-

ages originated from additional caramel colors (Klejdus et al., 2003, 2006; Petrucu et al., 2013).

In 2007, the National Toxicology Program identified 2-MI and 4-MI as chemicals that cause cancer (National Toxicology Program, 2004, 2007). Additionally, many researchers also reported that 2-MI and 4-MI were carcinogenic based on animal model studies (Chan et al., 2006, 2008a,b). For example, Chan et al. (2006) reported that 2-MI can induce thyroid hyperplasia and hypertrophy, and both 2MI and 4MI induce anemia in rats and mice. Thus, the International Agency of Research on Cancer listed both the methylimidazoles as group 2B compounds for the reason that they are possibly carcinogenic to humans in 2011 (Grosse et al., 2011; Lim and Shin, 2013; Schlee et al., 2013). In the same year, the Office of Environmental Health Hazard Assessment within the California Environmental Protection Agency recommended that the acceptable daily intake of 4-MI was less than 29 $\mu\text{g}/\text{d}$ (Office of Environmental Health Hazard Assessment, 2011). Recently, many researchers have focused on the levels of 4-MI in food products, especially beverages and coffees (Casal et al., 2002; Schlee et al., 2013; Gosciniy et al., 2014; Xu et al., 2015). For instance, 4-MI was detected in commercial roasted coffee ranging from 0.307 to 1.241 mg/kg (Casal et al., 2002). So humans are easily exposed to 4-MI in common diets and might exceed the acceptable daily intake level in some cases.

In dairy thermal processes, imidazoles might be produced via Maillard reaction as the presence of reducing sugars (lactose and glucose) and amino compounds (including AA and proteins). Therefore, it is important to investigate the formation mechanism of 2-MI and 4-MI in food processing. Recently, many studies focus on toxicity and levels of 2-MI and 4-MI, but few studies focus on the formation mechanism of the 2 compounds. Moon and Shibamoto (2011) reported a formation pathway of 4-MI in which methylglyoxal (MGO) interacted with ammonia (NH_4OH). Jang et al. (2013) reported that α -dicarbonyl compounds were the precursors of 4-MI. However, details of the formation mechanisms of

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2-MI and 4-MI have not been well established yet. It has been reported that some imidazole derivatives can be formed in methylglyoxal/amines systems or glyoxal/amines systems (Galloway et al., 2009; De Haan et al., 2011; Kua et al., 2011). For instance, De Haan et al. (2011) found that methylimidazole and dimethylimidazole were produced in reactions of methylglyoxal with AA. The proposed pathway of the imidazole derivatives is important to our understanding of 2-MI and 4-MI formation.

In Maillard reaction, 2 common α -dicarbonyl compounds [MGO and glyoxal (GO)] are always degraded from reducing sugars (Thornalley et al., 1999). Previous studies indicated that these 2 α -dicarbonyl compounds might be the important precursors of 2-MI and 4-MI (Yaylayan and Haffenden, 2003; Moon and Shibamoto, 2011). In the present study, MGO, GO, and ammonium sulfate (used to supply ammonia) were reacted to investigate the formation of 2-MI and 4-MI in model Maillard reaction. Moreover, different reactions between α -dicarbonyl compounds (MGO, GO) and aldehydes (formaldehyde, acetaldehyde) in the presence of ammonium sulfate were also performed to assess the role of MGO and GO in the formation process of 2-MI and 4-MI.

The 2-MI, 4-MI, GO (40%), MGO (40%), formaldehyde (40%), acetaldehyde (40%), and ammonium sulfate (AS) were purchased from Sigma-Aldrich (St. Louis, MO). The HPLC-grade methanol was provided by Merck (Darmstadt, Germany). Deionized water was obtained from a Millipore water station.

The GO (0.2 M), MGO (0.2 M), and ammonium sulfate (0.2 M; reaction I); MGO and ammonium sulfate (0.2 M) with or without formaldehyde (0.2 M; reactions II and III, respectively); GO and ammonium sulfate (0.2 M) with or without acetaldehyde (0.2 M; reactions IV and V, respectively); MGO (0.2 M), acetaldehyde (0.2 M), and ammonium sulfate (0.2 M; reaction VI); and GO (0.2 M), formaldehyde (0.2 M), and ammonium sulfate (0.2 M; reaction VII) were dissolved in 2 mL of phosphate buffer (pH 7.4, 0.2 M). Then the reactions were carried out in screw-cap tubes using a polyethylene glycol (MW600) bath at 120°C for 30 and 60 min, respectively. The samples were cooled to room temperature by ice water immediately after the reaction and stored at 4°C.

Analyses of 2-MI and 4-MI were referred the literature with a slight modification (Wang and Schnute, 2012; Schlee et al., 2013). The HPLC-MS/MS system used to detect 2-MI and 4-MI consisted of a Waters HPLC system (Waters Co., Milford, MA), coupled with a Thermo Scientific (San Jose, CA) LTQ mass spectrometer. Chromatographic separation was accomplished with a Waters XBridge BEH Shield RP18

Column (3.5 μ m, 3.0 mm $\times$ 150 mm) using mobile phase A (10 mM ammonia in high-purity water) and mobile phase B (methanol) in a gradient program with a flow of 0.3 mL/min: 0 to 10 min: 5% B; 11 to 15 min: 5% B to 100% B; 15 to 20 min: 100% B. The mass spectrometer was performed via an electrospray ionization (ESI) source operated in the positive ion mode at 275°C with nitrogen gas (35 psi; $\sim$ 241 kPa), and the collision energy was set at 33 V. The mass spectrometer was operated in selected reaction monitoring mode. Both 2-MI and 4-MI exhibited a $[M+H]^+$ ion at m/z 83, which was used as a parent ion to obtain fragment ions. The fragment ions were used for 2-MI and 4-MI quantification. The retention time of 2-MI and 4-MI was 7.92 and 9.93 min, respectively.

All experiments were carried out at least triplicate. Means and standard errors of data were calculated for each treatment. An ANOVA was carried out using SAS 9.3 software (SAS Institute Inc., Cary, NC) to determine any significant differences between treatments ($P < 0.05$).

The 2-MI and 4-MI were tested using a HPLC-MS/MS system. Figure 1 shows the ESI-MS/MS data of 2-MI and 4-MI. Obviously, the fragmentation with the highest intensity for 2-MI and 4-MI was 42 and 56, respectively. Therefore, an m/z of 42 was chosen for 2-MI quantification, and an m/z of 56 was used for 4-MI quantification. The limits of detection for 2-MI and 4-MI were 20 and 6 μ g/L, respectively.

Table 1 shows the results of reaction I (MGO/GO/ammonia model system). In the present study, the MGO/GO/ammonia model system was used because previous studies proved that MGO and GO were the primary precursors of imidazoles (Yaylayan and Haffenden, 2003; Jang et al., 2013). In reaction I, both 2-MI and 4-MI were found. However, the content of 4-MI formed in this system was significantly higher than the content of 2-MI. To explain the significant difference of the content of 2-MI and 4-MI and propose the mechanism for 2-MI and 4-MI formation, more experiments should be carried out, as shown in Table 1 (reaction II–VII).

In the Maillard reaction, sugars (glucose, fructose, lactose, and so on) often degrade into aldehydes, forming important precursors of imidazoles. These aldehydes can also form from AA via Strecker degradation (Yaylayan and Haffenden, 2003). Thus, different model reactions (reactions II, III, IV, V, VI, and VII) were designed to confirm the formation pathway of 2-MI and 4-MI. The results of the 6 supplementary reactions are shown in Table 1. It can be seen that 2-MI was found only in reaction IV (GO/acetaldehyde/ammonia), which means that both GO and acetaldehyde were the precursors of 2-MI. Moreover, no 2-MI was detected in reaction VII,

Figure 1. Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) information for 2-methylimidazole (2-MI; A) and 4-methylimidazole (4-MI; B). The data are from fragmenting the $m/z = 83$ ion. The concentrations of 2-MI and 4-MI used to get ESI-MS/MS data are 1 $\mu\text{g/mL}$. RT = retention time; AV = average; NL = normalized level; T = type; ITMS = ion trap mass spectrometry.

indicating that formaldehyde did not participate in the formation of 2-MI. Based on the results, the formation mechanism of 2-MI was proposed and shown in Figure 2. The 2-MI can be formed by the reaction of GO, acetaldehyde, and ammonia through 2 pathways. For one thing, GO can react with equivalent ammonia to form intermediate 1. Meanwhile, acetaldehyde reacts

with equivalent ammonia to form intermediate 2. Then, the 2 intermediates interact with each other to generate 2-MI. Alternatively, GO can react with double ammonia to form intermediate 3. Subsequently, it reacts with acetaldehyde to produce 2-MI.

In addition, Table 1 shows that 3 of the supplementary reactions (reactions II, III, IV, V, VI, and VII)

Figure 2. Proposed formation pathways of 2-methylimidazole (2-MI) and 4-methylimidazole (4-MI). For 2-MI: $R_1 = \text{H}$, $R_2 = \text{CH}_3$; and for 4-MI: $R_1 = \text{CH}_3$, $R_2 = \text{H}$.

could produce 4-MI. They were reaction II (MGO/formaldehyde/ammonia), reaction III (MGO/ammonia), and reaction VI (MGO/acetaldehyde/ammonia). It was observed that all the reactions generated 4-MI containing MGO. Thus, MGO should be the primary precursor in 4-MI formation. Comparing reaction II with III, more 4-MI was formed in the presence of formaldehyde. Therefore, formaldehyde may also play an important role in the generation of 4-MI. The synthesis method of 4-MI presented by Debus (1858) and Radziszewski (1882) also demonstrated that formaldehyde was a precursor in 4-MI formation. Similar to the generation of 2-MI, 4-MI can be formed through the same 2 pathways and was shown in Figure 2. Based on the pathways proposed in Figure 2, it can be inferred that reactions VI and VII mainly produce 2,4-dimethylimidazole and imidazole, respectively. To verify this

deduction, ESI-MS with positive ion mode was used to detect the samples of reactions VI and VII. The m/z of 97 ($[\text{M}+\text{H}]^+$) and 69 ($[\text{M}+\text{H}]^+$) was primarily found in reactions VI and VII, which corresponded to the molecular weight (**MW**) of 2,4-dimethylimidazole (MW 96) and imidazole (MW 68) (as shown in Figure 3). This result was in accordance with Yu et al. (2011), who found that imidazole was formed in the glyoxal/AS system.

Additionally, the mechanism for reactions III and IV forming 4-MI needs to be confirmed. According to the pathway proposed in Figure 2, formaldehyde needs to be formed in reactions III and IV. A possible way to form formaldehyde is likely the hydrolysis of MGO (shown in Figure 4), as proposed in the literature that α -dicarbonyl compound may hydrolyze into aldehyde and carboxylic acid (Brands and van Boekel, 2001).

Table 1. Content of 2-methylimidazole (2-MI) and 4-methylimidazole (4-MI) formed in different model reactions

Reactions (reactants) ¹	Content of 2-MI/4-MI ² ($\times 10^6 \mu\text{g/L}$)			
	2-MI		4-MI	
	30 min	60 min	30 min	60 min
I (MGO/GO/AS)	0.12 $\pm$ 0.02	0.13 $\pm$ 0.02	2.59 $\pm$ 0.05	3.32 $\pm$ 0.02
II (MGO/formaldehyde/AS)	—	—	4.88 $\pm$ 0.05	5.34 $\pm$ 0.02
III (MGO/AS)	—	—	1.62 $\pm$ 0.04	1.77 $\pm$ 0.06
IV (GO/acetaldehyde/AS)	1.61 $\pm$ 0.06	1.71 $\pm$ 0.03	—	—
V (GO/AS)	—	—	—	—
VI (MGO/acetaldehyde/AS)	—	—	0.79 $\pm$ 0.04	0.83 $\pm$ 0.03
VII (GO/formaldehyde/AS)	—	—	—	—

¹MGO = methylglyoxal; GO = glyoxal; AS = ammonium sulfate.

²— = the content of 2-MI or 4-MI was less than the limit of detection of the corresponding compound. The limits of detection for 2-MI and 4-MI were 20 and 6 $\mu\text{g/L}$, respectively.

Figure 3. Electrospray ionization-mass spectrometry (ESI-MS) information for reaction VI (A) and reaction VII (B). RT = retention time; AV = average; NL = normalized level; T = type; ITMS = ion trap mass spectrometry.

However, some subsequent literature found that no aldehyde was found in α -dicarbonyl degradation (Davidek et al., 2006; Smuda and Glomb, 2013). Thus, the formation of 4-MI in reactions III and IV may occur in other ways. Yu et al. (2011) proposed a mechanism for imidazole that formed in the glyoxal/AS system. The formation of 4-MI in reactions III and IV may occur through a similar pathway and is shown in Figure 5 (pathway A). Furthermore, the lowest content of 4-MI was detected in reaction VII, possibly caused by the addition of acetaldehyde reacting with MGO to produce other compounds and inhibiting 4-MI formation.

Looking back to reaction I, the mechanism by which it generated both 2-MI and 4-MI is shown in Figure 5

Figure 4. Possible pathways for aldehyde formation via methylglyoxal degradation.

Figure 5. Proposed mechanisms for 2-methylimidazole (2-MI) and 4-methylimidazole (4-MI) formation in the methylglyoxal/glyoxal/ammonia system.

(pathway A, B, C), which was similar to the literature (Yu et al., 2011). It can be seen that imidazoles (2-MI, 4-MI) can be formed by the interaction of 2 mol α -dicarbonyl compounds (methylglyoxal, glyoxal) and 2 mol ammonia. And, paying attention to the phenomenon that the amount of 4-MI was much more than the amount of 2-MI in reaction I, a possible explanation might be that 4-MI can be formed in 2 ways (Figure 5, pathway A, B), whereas 2-MI can only be produced in one way (Figure 5, pathway C). Most importantly, the CH_3 of C2 (the bold one in Figure 5, pathway C) impeded the N1 to attack C2 to form C-N bond, resulting in less 2-MI being formed. In this case, 4-MI was found to be more easily formed than 2-MI.

It is well known that MGO and GO are formed through sugar degradation (Thornalley et al., 1999). Thus, 2-MI and 4-MI can be found in food thermal processing that undergoes Maillard reaction. Because the carcinogenic risk of the 2 imidazoles has been a concern of regulatory agencies and consumers, many researchers have tried to simultaneously detect 2-MI and 4-MI in food. Interestingly, almost no 2-MI was detected compared with 4-MI, which was detected in cola, caramel, and so on (Schlee et al., 2013; Goscinny et al., 2014; Xu et al., 2015), which might be explained by the proposed mechanism in this study.

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