Formation of carcinogenic 4(5)-methylimidazole in caramel model systems: A role of sulphite

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ABSTRACT

Aqueous caramel model systems consisted the D-glucose/NH_3/sulphite were heated at 100 °C for 2 h and amounts of carcinogenic 4(5)-methylimidazole (4-MI) formed were determined. The amount formed ranged from 7.0 to 155.0 ppm. A system with 0.1 M sulphite yielded the greatest amount of 4-MI, which was 54% more than that yielded from a system without sulphite. When the amount of sulphite increased over 0.1 M, the amount of 4-MI reduced. The greatest reduction was achieved with 0.2 M sulphite by 68% compared to 0 M sulphite, suggesting that sulphite plays an important role in the formation of carcinogenic 4-MI in caramel colour. Also, a system with 0.1 M sulphite yielded the most intense caramel colour but the other levels of sulphite did not change the colour intensity significantly. Sulphite contributed slightly to the level of flavour chemicals evaluated using pyrazine formation. The results suggest that sulphite addition at appropriate amount reduces 4-MI formation in caramel colour without sacrificing flavour and colour formation.

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1. Introduction

Caramel colour is one of the oldest and most widely used colourings for variety of foods and beverages, including batters, baked goods, jams, pickles, ice creams and frozen desserts, gravies, sauces, fillings and toppings, prepared meats, spirits, wines, beers, cola beverages, root beers, ginger ales, and confectionary (Chappel & Howell, 1992). Even though, caramel colour was commercially produced in the US in 1963, toxicity evaluation and regulatory set up were rather slow. Comprehensive report on regulatory history of caramel colour was published in 1992 (Chappel & Howell, 1992).

There are four classes of caramel colour (Class I, II, III, and IV) based on the application and the reactants used in their manufacture (Chappel & Howell, 1992; Yamaguchi & Masuda, 2011). Class I is prepared by heating carbohydrates alkali or acid. Class II is manufactured by heating carbohydrates with sulphite-containing compounds. Class III is created by heating the carbohydrates with ammonium compounds. Class IV is produced by heating carbohydrates both with sulphite-containing and ammonium-containing compounds. Class I and II are usually used for alcoholic beverages (Chappel & Howell, 1992; Miller, Vandome, & McBtewster, 2010).

Class III is often used in confectionery, beer, and soy sauce. Class IV is widely used in soft drinks.

Since ammonia-treated molasses feed was demonstrated to show some neurological impairment in cattle (Bartlett & Broster, 1957; Morgan & Edwards, 1986), many studies on the toxicity of caramel colour have been conducted. For example, a commercial Class III caramel colour was mutagenic against Salmonella typhimurium TA 100 without metabolic activation (Jensen, Willumsen, & Knudsen, 1983). Mutagenicity of Class III was also observed by the micronuclei test (Dong, Xiong, & Huang, 1984). On the other hand, no evidence of genotoxicity was found in caramel colours tested by the S. typhimurium assay (Brusick, Jagannath, Galloway, & Nestmann, 1992). Also, the studies using experimental mice demonstrated that Class IV at 10 g/kg body weight (the highest dose level tested in long-term studies) was not toxic or carcinogenic (MacKenzie et al., 1992). Class III may have toxicity because ammonium treatment produced some toxic chemicals (Bartlett & Broster, 1957). From the view point of possible toxicity, ammonia-free process for manufacturing caramel is therefore recommended (Dong et al., 1984).

It is well known that the major reaction that occurs in a caramel model system is Maillard reaction. Carbohydrate produces a tremendous amount of heterocyclic compounds when it is heated with an amine compound, such as ammonia and amino acid upon this reaction (Shibamoto, 1983). Many studies, which revealed the toxic principles of caramel colours (Class III and IV) using Maillard reaction systems, consisted of a sugar and ammonia. For example,

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2-acetyl-4(5)-(1,2,3,4-tetrahydroxy-butyl)-imidazole, which one of the heterocyclic compounds forms in Maillard reaction, was found to be responsible for the immunotoxicity of Class III (Houben & Penninks, 1994).

Among heterocyclic compounds formed in Maillard reaction systems, 4(5)-methylimidazole (4-MI) has begun to receive much attention both in regulatory agencies and consumers because the National Toxicology Program (NTP) has identified 4-methylimidazole as causing cancer (National Toxicology Program, 2007) and the Office of Environmental Health Hazard Assessment (OEHHA) within the California Environmental Protection Agency plans to list 4-methylimidazole as known to the state of California to cause cancer (OEHHA (Office of Environmental Health Assessment). Notice of intent to list 4-methylimidazole., 2011). In Europe, the European Food Safety Authority published recommendations and regulations of caramel such as admissible daily intake (European Food Safety Authority (EFSA), 2011).

4-MI was first reported in L-rhamnose/ammonia Maillard reaction system (Shibamoto & Bernhard, 1978). Later, 4-MI was found in commercial roasted coffee samples ranging from 0.307 to 1.231 mg/kg but not in green coffee samples (Casal, Fernandes, Oliveira, & Ferreira, 2002). Recently, formation of 4-MI in Maillard reaction model systems in significant amounts and its presence in commercial cola soft-drinks (ranging from 0.30 to 0.36 mg/ml) were reported (Moon & Shibamoto, 2011). Also, recent reports indicated that Class III and IV contained 4-MI ranging from 20 to 500 ppm (Moretton, Crétier, Nigay, & Rocca, 2011). Moreover, 4-MI levels found in naturally brewed soy sauce were extremely low (ranging from <0.002 to <0.023 µg/g), whereas those in soy sauces containing caramel colour were relatively high (ranging from 0.43 to 4.8 µg/g), suggesting that a principle source of 4-MI is caramel colour (Yamaguchi & Masuda, 2011).

So far, most studies on 4-MI are focused on levels or toxicity but not in the process. The formation mechanisms of carcinogenic 4-MI in foods must be investigated to prevent or reduce its formation in foods. The role of ammonium compounds has been studied in the reports shown above but there is virtually no report on a role of sulphite compounds in its formation (Bartlett & Broster, 1957; Dong et al., 1984). Therefore, the role of sulphite compounds in 4-MI formation in Maillard reaction systems and its correlations between the formation of 4-MI and pyrazines with typical roasted or toasted flavours were investigated.

2. Materials and methods

2.1. Chemicals

D-Glucose, ammonium hydroxide solution (29%), sodium sulphite, and 4-MI were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Standard stock solution of 4-MI was prepared in HPLC grade water at a concentration of 10 mg/l. The solution was stored in the dark at 4 °C until used. Pyrazines used in caramel model study were bought from TAKATA Koryo Co., Ltd. (Osaka, Japan).

2.2. Sample preparations of caramel model systems

A 30 ml aqueous solution containing three levels of D-glucose and ammonium hydroxide—1.0 M each (Group I), 0.5 M each (Group II), and 0.1 M each (Group III)—and sodium sulphite (0 M, 0.1 M, 0.2 M, 0.5 M, 0.7 M, or 1.0 M) was prepard in a swing top bottle and heated at 100 °C for 2 h in an oven. After the samples were cooled to room temperature, the reaction samples were diluted with a deionized water 1–10-fold. Each sample was stored at 4 °C until used.

2.3. Analysis of 4-MI

4-MI analysis was conducted using a Hewlett Packard 1100 liquid chromatograph interfaced to an Applied Biosystems API 2000 MS/MS via an electrospray ionisation (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 (Varian, Walnut Creek, CA, USA) with a 3 µm particle size. The mobile phase was water (15 mmol ammonium hydroxide, solvent A) and acetonitrile (15 mmol ammonium hydroxide, solvent B). A linear gradient that follows at 0–3 min, A/B = 98/2; at 10–13 min, 60/40; and at 15–25 min, 98/2 was used with flow rate at 0.4 ml/min. Under these conditions, 4-MI was eluted at 9.1 min.

The mass spectrometric data were acquired in multiple reactions monitoring (MRM) mode. The 4-MI peak area from the transition of m/z 83 to m/z 56 was used for the conformation and quantification.

A standard calibration curve was prepared in 0.25, 0.5, 1.0, 5.0, and 10.0 µg/ml concentrations by diluting the standard solution (10 mg/l) with HPLC grade water and each solution (20 µl) was injected to LC-MS. Recovery efficiency of 4-MI was examined using 10 µg/ml aqueous solutions.

2.4. Analysis of pyrazines in Maillard reaction products

Pyrazines were identified by comparison with the Kovats gas chromatographic retention index I and by the mass spectral fragmentation pattern of each component compared with those of authentic compounds. The identification of the GC components was also conducted with the NIST AMDIS version 2.1 software.

Each sample (3 ml) was placed in a 20 ml head space vial closed by silicone-PTFE septa and chemicals in a head-space and was trapped on a SPME device with 50/30 mm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco Inc., Bellefonte, PA, USA) at 50 °C for 30 min under magnetic stirring. SPME fibre was conditioned into the injection port of the gas chromatography (GC) at 250 °C for 1 h before used. The SPME fibre was immediately injected into GC by exposing the fibre at 260 °C for 5 min. The fibre was baked at 250 °C for 30 min following the each extraction. An HP Model 6890 GC interfaced to a 5971A mass selective detector (GC–MS) was used for mass spectral identification of the GC components at an MS ionisation voltage of 70 eV. A 60 m × 0.25 mm i.d. (df = 0.25 µm) DB-Wax bonded-phase fused-silica capillary column (J&W Scientific Inc., Folsom, CA, USA) was used. The helium carrier gas flow rate was 1.0 ml/min at a splitless mode (20/1). The injector and detector temperatures were 260 °C and 250 °C, respectively. The oven temperature was programmed from 50 °C to 220 °C at 3 °C/min, and was maintained for 10 min.

2.5. Colour measurement of samples from caramel model systems

A reaction mixture (100 µl) was diluted with 10 ml purified water and the absorbance of the resulting solution was measured at λ = 420 nm with a Hewlett Packard 8452A Diode Array Spectrophotometer running UV–Visible Chemstation software (Agilent Technologies, 1995–2000). Water was used as a blank. All experiments were repeated three times.

3. Results and discussion

3.1. Analytical results of 4-MI in samples from caramel model systems

As discussed earlier, four classes of caramel colour (Class I, II, III, and IV) are based on the manufacture application and the reactants

used to heat the carbohydrates (Chappel & Howell, 1992; Miller et al., 2010). Among them Class IV is widely used in soft drinks such as soda drink. Therefore, Class IV system was used to investigate 4-MI formation in caramel colour in the present study.

A satisfactory standard curve was obtained ($y = 176,000x + 5820$, $R^2 = 0.997$) for 4-MI analysis in the present study. Recovery efficiency of 4-MI was $101.2 \pm 1.8\%$ from an aqueous solution. Fig. 1 shows the results of 4-MI analysis in the samples from caramel model systems. When D-glucose and ammonium hydroxide was heated without sulphite, amount of 4-MI formed was 100.22 ± 15.84 µg/ml from Group I (D-glucose/ammonium hydroxide = 1 M/1 M), 69.78 ± 3.79 µg/ml from Group II (D-glucose/ammonium hydroxide = 0.5 M/0.5 M), and 7.18 ± 1.36 µg/ml from Group III (D-glucose/ammonium hydroxide = 0.1 M/0.1 M); suggesting that 4-MI formation was a dose response to the reactants. However, dose response was not observed when sulphite was added. On the other hand, it is obvious that the addition of sulphite increased formation of 4-MI.

In the case of Group I, the formation of 4-MI ranged from 154.52 ± 24.60 µg/ml (with 0.1 M sulphite) to 26.15 ± 10.43 µg/ml (with 0.5 M sulphite). There was a significant increase of 4-MI formation in a sample with 0.1 M sulphite (54% increase), and then it was decreased sharply in a sample with 0.2 M sulphite (68% decrease). It was further decreased in a sample with 0.5 M sulphite by 47% and the increased slightly to the samples with 0.7 and 1.0 M sulphite by 5% each. In the case of Group II, 4-MI also increased significantly from a sample without sulphite to a sample with 0.1 M sulphite by 64%, and then decreased to the sample with 1 M sulphite steadily by ranging from 10 to 20%. In the case of Group III, formation of 4-MI in the samples was relatively low because the amount of reactants used was 1/10-fold that of Group I and 1/5-fold that of Group II. However, the pattern of curve in the graph was similar to those of Groups I and II. The highest level was obtained from the sample with 0.1 M sulphite.

Addition of sulphite more than 0.2 M reduced the formation of 4-MI in Group I. In particular, addition of 0.5 M sulphite decreased its formation by nearly 75%. However, it is difficult to rationalise this phenomenon because when sulphite was added to Group II, less 4-MI formation was observed, but the amount of 4-MI was not less than that of the sample without sulphite. Also, when sulphite was added more than 0.2 M in Group II, amounts of 4-MI formed was less than those formed in Group I. For example, when
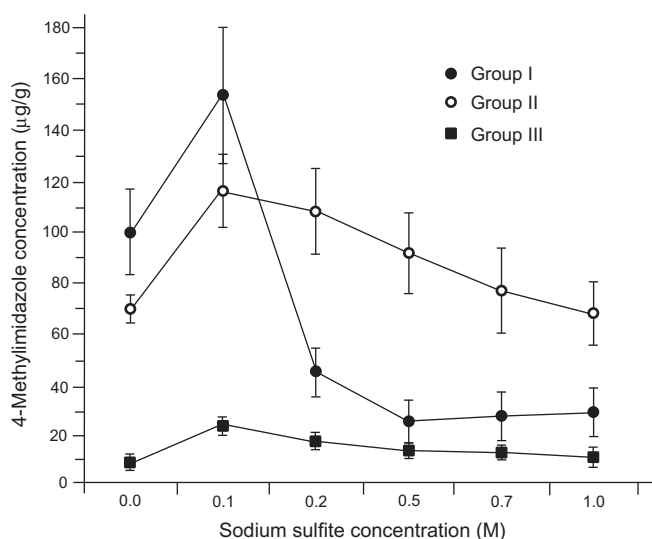
0.2 M sulphite was added, Group II produced 107.24 ± 18.04 µg/ml of 4-MI whereas Group I produced 48.87 ± 10.78 µg/ml of 4-MI which resulted in 54% reduction.

3.2. Colour absorbance of the samples

Fig. 2 shows UV absorbance of the samples prepared by caramel model systems. The wavelength used in the present study ($\lambda = 420$ nm) has been widely used for assessing the colour intensity of Maillard reaction mixtures (Ajandouz & Puigserver, 1999). The intensity of absorbance (420 nm) has shown a similar curve tendency as 4-MI concentration in Group I. Intensity increased from system with 0.0 M sulphite to system with 0.1 M sulphite by 26.7% and then decreased in the system with 0.2 M by 17.0%. The intensity changed only slightly when amount of sulphite increase from 0.2 M. However, an absorbance curve of Group II samples has shown a gradual increase from a system with 0.0 M sulphite to a system with 0.5 M sulphite (62.0%), followed by a slight decrease from there to a system with 1.0 M sulphite (4%). An absorbance curve from Group III exhibited only slight increase from a system with 0.0 M sulphite to a system with 1.0 M sulphite (10%).

3.3. Pyrazines formed in Caramel Model Systems

According to the significance of flavour formation in Maillard reaction systems (Shibamoto, 1983), 9 kinds of pyrazines were selected from over 30 heterocyclic flavour compounds identified in the present study to investigate a role of sulphite in caramel-flavour formation. Table 1 shows the analytical results of 9 pyrazines. 2-Methylpyrazine is formed in the greatest amount ranged from 65.10 µg/ml (Group I with 0.5 M sulphite) to 52.33 µg/ml (Group III without sulphite), followed by 2,6-dimethylpyrazine from 21.23 µg/ml (Group II with 1.0 M sulphite) to 5.18 µg/ml (Group III without sulphite), and 2,5-dimethylpyrazine from 12.30 µg/ml (Group III with 0.5 sulphite) to 1.91 µg/ml (Group III without sulphite). The lower reactant levels the lower pyrazine formation obtained are a general phenomenon. It is interesting that the lower sulphite level the higher the pyrazine formation, which is generally consistent with 4-MI formation, was observed. The composition of pyrazines formed was consistent with the results of previous studies on the sugar/amine Maillard reaction systems (Shibamoto & Bernhard, 1978). It is, however, difficult to rationalise the role of sulphite for each pyrazine formation.
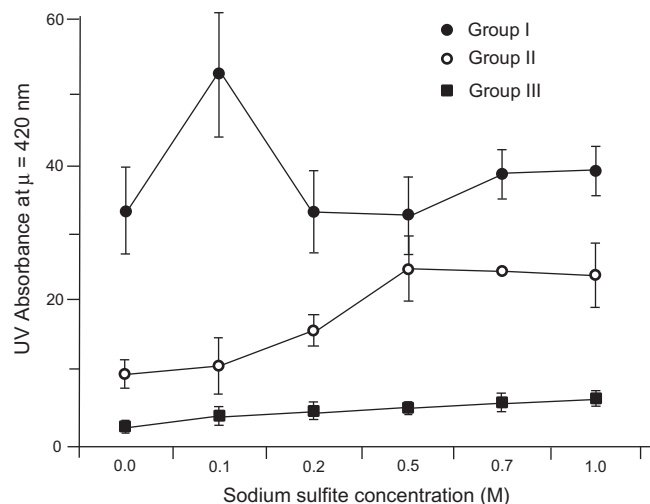

Fig. 1. Concentrations of 4-MI found in the samples from caramel model systems. Values are mean ± SD ($n = 3$).

Fig. 2. UV absorbance of the samples from caramel model systems. Values are mean ± SD ($n = 3$).

Table 1
Amounts of pyrazines formed in caramel model systems (μg/ml): Group I, II, and III.

Pyrazine	Sulphite concentration (M)								
	0			0.5			1.0		
	I	II	III	I	II	III	I	II	III
Unsubstituted-	2.30	2.39	3.68	1.60	1.63	–	–	–	–
2-Methyl-	62.05	64.91	52.33	65.10	62.48	57.21	55.80	56.56	52.55
2,5-Dimethyl-	3.67	4.89	1.91	6.76	8.10	12.30	8.05	11.66	11.94
2,6-Dimethyl-	10.91	11.63	5.18	16.97	18.41	14.71	16.76	21.23	19.32
2-Ethyl-	0.73	0.92	–	–	–	–	–	–	–
2,3-Dimethyl-	1.47	1.51	0.96	2.40	1.98	–	1.98	1.99	1.13
2-Ethyl-6-methyl-	0.57	0.74	–	–	–	–	–	–	–
Trimethyl-	1.34	1.26	–	1.36	1.60	–	1.60	2.21	1.91
Ethenyl-	0.85	0.73	–	–	–	–	–	–	–

In conclusion, use of caramel colour prepared using sugar/ammonia/sulphite reaction systems is expected to continue regardless the formation of carcinogenic 4-MI in the food industry. Therefore, it is important to investigate formation mechanisms of 4-MI in the caramel preparations in order to reduce its formation without sacrificing factors for palatability, including colour, taste, and flavour. The results in the present study suggest that sulphite plays an important role in 4-MI formation in caramel colour and sulphite addition at appropriate amount reduces its formation without sacrificing flavour and colour formation. In future study more accurate addition level of sulphite would be investigated according to the optimization method. The impact of sulphite on the colour stability in caramel or foods will be also studied.

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