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Determination of 4-Methylimidazole and 2-Acetyl-4(5)-tetrahydroxybutylimidazole in Caramel Color and Processed Foods by LC-MS/MS

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ABSTRACT: In this study, the quick HPLC-MS/MS method for the simultaneous separation of 2-acetyl-4(5)-tetrahydroxybuthylimidazole (THI) and 4-(5)-methylimidazole (4-MI) in alkaline medium was used for caramel color and processed foods in Korea. After a simple sample pretreatment, 51 4-MI-labeled samples were positive for 4-MI and 2 also contained THI. The concentration of 4-MI was 260.5～24,499.3 μg/kg in caramel color, less than LOD～1,712.5 μg/kg in sauce, 1,242.3, 5,972.2 μg/kg in balsamic vinegar, 2,118.3～5,802.4 μg/kg in complex seasoning, 82.7～5,110.6 μg/kg in curry, and 29.9～464.4 μg/kg in soft drinks. The recovery rate of 4-MI was 97.1～111.0% in sauce and 81.9～110.0% in powder and that of THI was 83.6～106.4% in sauce and 61.2～99.4% in powder. Our results concluded a safe amount of 4-MI and THI compared to the limit of Korea additive code but the processed foods do not have a limit of caramel color and 4-MI in Korea. Therefore, research and monitoring of 4-MI and THI is needed for processed foods in Korea.

Keywords: 2-acethyl-4(5)-tetrahydroxybuthyl imidazole, caramel color, LC-MS/MS, 4(5)-methylimidazole

INTRODUCTION

The addition of color in the food industry is necessary to attract the attention of consumers and to improve appetite. Of these, caramel color is widely used as natural additives in general food industry and restaurants because of its color, flavor and stabilization of colloidal system. Caramel color is classified as natural additives in Korea Food Additives Code. Natural additives were obtained by extraction of biological resources such as animals and plants and defined as the insoluble mineral substances and the materials gained by enzyme reaction and fermentation (1).

The caramel color comes in four types (I～IV), and is used just to improve the color rather than to prevent loss of nutrients. Recent interests appeared in caramel color III and Ⅳ with ammonia processing. 4(5)-methylimidazole (4-MI) and 2-acethyl-4(5)-tetrahydroxybuthylimidazole (THI), unwanted byproducts, were reported to be toxic with adverse effects on the immune system. 4-MI was considered a carcinogenic substance in animal experiments (2) and classified with the group 2B substances that are possible carcinogens to human (3). California's Office of Environmental Health hazard Assessment (OEHHA) in USA registered 4-MI as a carcinogen with no significant risk level (NSRL) when ingesting 16 μg/day (4). European Union (EU) and the World Health Organization (WHO) put the legal limit 4-MI 250 mg/kg or less in caramel color III and IV and THI 10 mg/kg or less in caramel color III. On the other hand, the European Food Safety Authority concluded that 4-MI exposure from caramel color was not of concern (5). In Korea, 4-MI set the limit less than 250 mg/kg in caramel color III & IV, and less than 25 mg/kg for THI as of December, 2012 (5). 4-MI was analyzed by gas chromatography with hydrogen-ionization detector after sample purification and THI was proposed liquid chromatography with UV detector through the extraction process of using separation funnel in Korea (1).

Many experiments have studied 4-MI and THI: simultaneous analysis of the THI and 4-MI using High Performance Liquid Chromatography (HPLC)/ESI-MS after solid-phase extraction pretreatment (6), 4-MI, 2-MI, THI LC-MS/MS analysis without pretreatment in coke and caramel color (7), 4-MI and THI HPLC/ESI-MS analysis after supercritical fluid extraction in grained coffee (8), and 4-MI LC-MS/MS analysis after Solid-Phase Extraction in soy sauce and food (9), GC-MS analysis of 4-MI after

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ion-pair extraction in roasted coffee beans (10) and 4-MI analysis in soft drinks and stout beer (11). In addition, much research has been conducted on the toxicity or physiological activity of the THI and 4-MI are accomplished (2,12,13).

Nowadays, consumers are more sensitive to the complex materials than natural ingredients that are added during food processing. In Korea, 4-MI and THI have the limit in caramel color classified in natural additives; on the other hand, the processed food has no restrictions on 4-MI and THI.

Therefore, the present study was designed to validate the method for the simultaneous quantification of both 4-MI and THI after simple pretreatment and to know the use of caramel color in processed food. Finally this study provides the basic data of 4-MI and THI by the different type of processed foods in Korea.

MATERIALS AND METHODS

Samples

A total of 78 samples were analyzed. All samples were purchased from the market in Seoul, Korea. The number of caramel color, sauces, curry, mixed drinks, vinegar, compound seasonings is 3, 47, 9, 20, 3 and 3 respectively. All samples were analyzed by the existence of caramel color labeled in food.

Standard and reagents

The chemical structures of 4(5)-methylimidazole (4-MI) and 2-acethyl-4(5)-tetrahydroxybuthylimidazole (THI) are shown in Fig. 1. The 2 standards and the mobile phase of LC-MS/MS were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Acetonitrile was used HPLC grade (Merck-KGaA, Darmstadt, Germany) in the extraction. 0.2 μm syringe filter (RC membrane, 0.2 μm, 26 mm; Phenomenex, Torrance, CA, USA) was used in filter of solution. Distilled water (18.2 MΩ) was used for the preparation of reagents and standards with a Milli-Q water purification System (Millipore, Billerica, MA, USA).

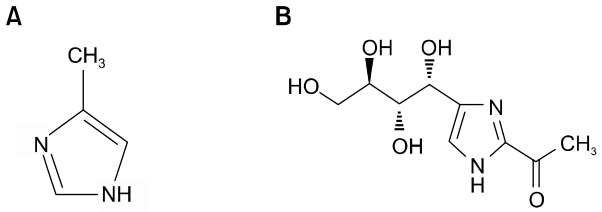

Fig. 1. Chemical structures of 4-MI (A) and THI (B).

Extraction and purification

The sample (about 1.0 g) was placed in a centrifuge tube and 5∼10 mL acetonitrile were added. Samples were centrifuged at 5,000 rpm, 0°C to prevent active ingredients' spoilage.

The separated supernatant was filtered through a 0.2 μm syringe filter and used in the analysis.

Analytical instruments and conditions

Centrifuges used in the sample preparation were MICRO CENTRIFUGE (Tomy Seiko JP/MX-305, Tokyo, Japan). All samples were determined by Rapid Resolution Liquid Chromatography (RRLC) (Agilent, Palo Alto, CA, USA). LC separation was performed on a Luna C18 (2×100 mm, 3 μm; Phenomenex) column-thermostatted at 35°C using mobile phase A (5 mM NH_4HCO_3 in high-purity water; pH 9) and mobile phase B (5 mM NH_4HCO_3 in methanol; pH 9) with a flow of 0.2 mL/min. Mass spectrometer was used with API 3200 Qtrap (Applied Biosystems, Foster, CA, USA) and the multiple reaction monitoring (MRM) mode was monitored for quantitative analysis.

The instrumental conditions for both are shown in Table 1.

Table 1. LC-MS/MS conditions for analysis

Instrument	Parameter	Conditions
LC	Column	Luna C_{18}, 2×100 mm, 3 μm
	Mobile phase	Solvent A (70): 5 mM NH_4HCO_3 (pH 9)
		Solvent B (30): 5 mM NH_4HCO_3 50 mL+950 mL MEOH (pH 9)
	Flow rate	0.2 mL/min
	Injection volume	5 μL
	Column Temp.	30°C
MS/MS	Standard	4-MI/THI
	Polarity	Positive/Negative
	Curtain gas	25 psi
	Collision gas	Medium
	Ionspray voltage	5,500 V
	Temperature	500°C
	Ion source gas 1	40 psi
	Ion source gas 2	50 psi
	Interface heater	On

Method validation

Several experimental parameters such as LOD (limit of detection), LOQ (limit of quantification), linearity, recovery, stability and repeatability were evaluated for validation of the HPLC-MS/MS method. The calibration curves were linear in the concentration ranges 5～1,000 μg/kg for 4-MI and THI. The recovery of the method was verified analyzing sauce and powder samples after addition of three known amounts (10, 50, and 100 μg/kg) of 4-MI and THI standard solution. The LOD was calculated S/N ratio>3 and the LOQ S/N ratio<10 with LC-MS/MS.

RESULTS AND DISCUSSION

HPLC-MS/MS

In the present work, the HPLC-MS/MS system was used to achieve the unambiguous identification of THI and 4-MI. The analytes produced protonated molecules [M+H]- at m/z 230, which were used as a parent ion giving three daughter ions (m/z 150.8, 122.9, 108.7) for THI and [M+H]+ at m/z 83, which had three daughter ions (m/z 56, 41, 42) for 4-MI. The MS/MS was therefore used in order to obtain additional structural information by detecting diagnostic product-ions obtained by in-source CID of the parent ion. MRM transition of m/z from 83 to 56 was used for the quantification of 4-MI and from 228.8 to 150.8 for THI (Table 2). All samples examined gave MS/MS spectra equivalent to the standard solution. MS TlC chromatogram and MS/MS spectra of standard and sample are shown in Fig. 2 and 3. The retention time of 4-MI and THI was 8.0 and 3.7 minutes respectively and reproducible results were obtained for run-to-run repeatability of retention times, peak areas and concentrations of 4-MI and THI. Therefore this method was suitable to analyze 4-MI and THI.

Results of method validation

The method was linear between 5～1,000 μg/kg in the measuring solutions with correlation coefficient r>0.9990 for 4-MI and r>0.9999 for THI. The LODs were found to be 3 μg/kg for 4-MI and 0.5 μg/kg for THI in the measuring solutions. The LOQs for 4-MI and THI were 5 μg/kg and 2 μg/kg respectively. The results are summarized in Table 3. The recovery of the method was verified analyzing curry and powder samples after addi-

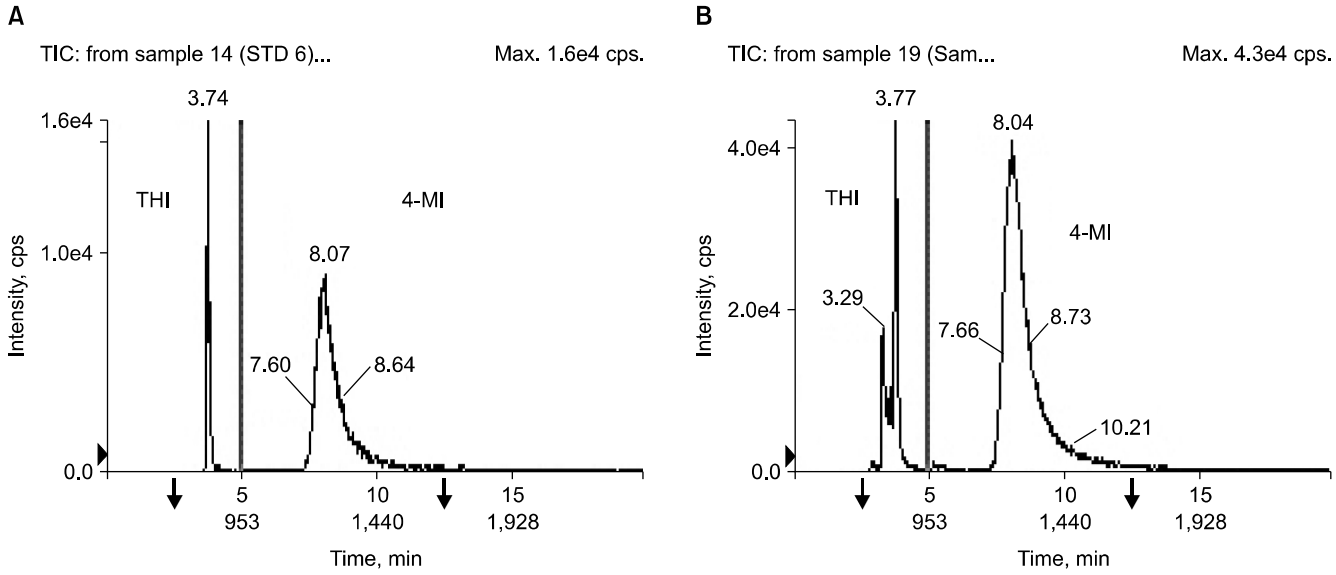

Fig. 2. Total ion chromatograms of 4-MI and THI. (A) Standard solution (500 μg/L), (B) Caramel colors sample.

Table 2. MRM condition of detected compounds in samples

Compound	Exact mass	Q1 Mass (amu)	Q3 Mass (amu)	EP (V)	DP (V)	CE (V)	CXP (V)
4-MI	82.10	83	56 41 42	6.5	46	23 51 27	4
THI	230.0	228.8	150.8 122.9 108.7	-9	-35	-22 -32 -32	0

EP, enterance potential; DP, dedustering potential; CE, collision energy; CXP, collision cell exit potential.

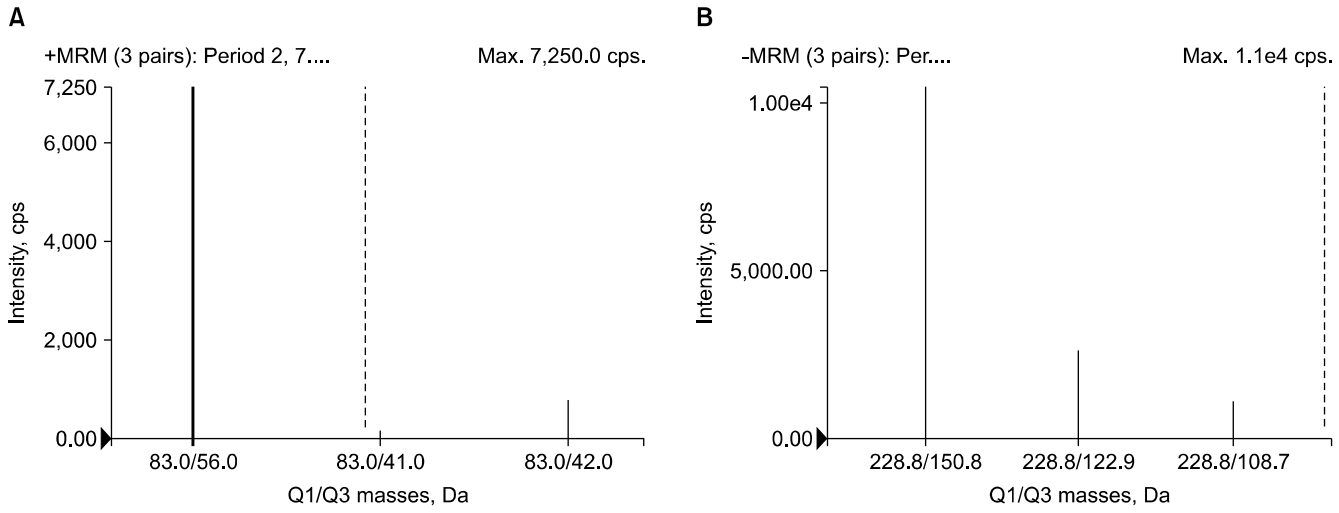

Fig. 3. MS/MS spectra of 4-MI (A) and THI (B) using LC-MS/MS.

Table 3. Linear equations, correlation coefficients, LODs and LOQs of 4-MI and THI[1)]

	Linear equation	Correlation coefficient (r)	LOD (μg/kg)	LOQ (μg/kg)
4-MI	Y=1490.2X	0.9990	3	5
THI	Y=290.41X	0.9999	0.5	2

[1)]Instrument linearity range: 5~1,000 μg/kg.

Table 4. Recovery of 4-MI and THI in different matrix sample (n=5)

Matrix	Spiked level (μg/kg)	Recovery (%)±relative standard deviation (RSD)	
		4-MI	THI
A (sauce)	10	111.0±0.2	83.6±0.9
	50	97.1±3.9	106.4±0.9
	100	98.9±3.1	86.5±1.1
B (curry)	10	110.0±0.2	99.4±0.4
	50	97.2±1.9	68.9±0.7
	100	81.9±0.9	61.2±1.0

tion of three known amounts of 4-MI and THI standard solution with the same pretreatment. The theoretical concentrations of the spiking 4-MI and THI standards were 10, 50, 100 μg/kg (n=5). The recovery rate of 4-MI spiked in sauce and curry was 102.3% and 96.4% (average of three concentrations). The recovery rate of THI spiked in sauce and curry was 92.2% and 76.5%. The mean recovery and RSD for five replicates are given in Table 4.

Survey of 4-MI and THI in food samples

Table 5 shows the amount of 4-MI and THI analyzed in commercial foods. 51 of 57 samples, caramel-color-labeled, were detected in the 4-MI concentrations. 1 caramel color and 1 dressing were detected in THI and 4-MI.

According to caramel color types, 3 caramel colors contained 4-MI 260.5~24,499.3 μg/kg, compared with the limit of 4-MI in caramel color, 250 mg/kg, in Ministry of Food and Drug Safety, which was considered a safe amount. 1 caramel color also detected a high THI concentration, 14,031.4 μg/kg. With these data, we can assume that this caramel color was caramel type III.

4-MI levels in sauces were detected in a wide range from <LOD to 1,712.5 μg/kg. It seemed that the level of 4-MI was originated from the caramel color, soy sauce and sugars of materials. In Yamaguchi and Masuda study (9), they reported that soy sauce samples were detected 0.43~4.8 μg/g with high concentration of 4-MI and the natural soy sauce also were found extremely low 4-MI concentration by the Millard reaction. 4-MI in 2 dark brown balsamic vinegars was detected at 1,242.3, 5,972.2 μg/kg and it also seemed that soy sauce and sugars affected the 4-MI amount. 3 complex seasonings were all Jajang powders and they had high 4-MI detection rates, 2,118.3, 5,496.3, and 5,802.4 μg/kg respectively. It seemed that they were used the caramel color because of their flavor and color. 9 curries all were detected 4-MI, 82.7~605.6 μg/kg, and a dark brown curry showed a

Table 5. Results of the quantitative determination of 4-MI and THI by LC-MS/MS

Type of food	Samples analyzed (n)	Sample detected (n)	Range of levels (μg/kg)	
			4-MI	THI
Caramel colors	3	1	260.5	N.D
		1	574.4	
		1	24,499.3	14,031.4
Sauce	29	6	N.D	N.D
		5	101~200	
		3	201~300	
		7	301~400	
		1	410.2	
		1	582.2	
		1	664.8	
		1	782.1	
		1	1,032.7	
		1	1,137.5	
		1	1,640.4	
		1	1,712.5	
Balsamic vinegar	2	1	1,242.3	N.D
		1	5,972.2	
Complex seasonings	3	1	2,118.3	N.D
		1	5,496.3	
		1	5,802.4	
Curry	9	3	0~100	N.D
		4	101~110	
		1	605.6	
		1	5,110.6	
Soft drinks	10	4	0~100	N.D
		3	101~200	
		2	301~400	
		1	464.4	
Dressing	1	1	617.1	166.7
Total	57			

N.D, not detected.

higher level, 5,110.6 μg/kg.

10 soft drinks, recently issued in Korea, all were found 4-MI, 29.9～464.4 μg/kg. These are similar to previously reported values as follows: cola type soft drinks, ND～560 μg/L (7) and 37～613 μg/kg (11). Also, 1 balsamic dressing showed 4-MI 617 μg/kg and THI 166.7 μg/kg. This could be caused from caramel color itself and sugar contents from material. Brown 21 samples without caramel-color label, all didn't contain the 4-MI and THI.

In our research, 2 samples with THI detection seemed to use the different caramel color type compared with the other samples. Samples with soy sauce and sugars may have the higher 4-MI concentration (10).

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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