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Analytical Methods

Rapid and quantitative detection of 4(5)-methylimidazole in caramel colours: A novel fluorescent-based immunochromatographic assay

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ABSTRACT

A novel fluorescence-based immunochromatographic assay (ICA) for rapid detecting 4(5)-methylimidazole (4-MI) is presented in this study. In our work, the conjugates of fluorescent microspheres (FMs) and 4-MI monoclonal antibody were used as probe for ICA. Under optimal conditions, a standard curve of ICA-based detection of 4-MI was developed, linear detection ranged from 0.50 to 32.0 mg/L. The cross-reactivities were observed less than 3.93% by detecting 6 selected structural analogues of 4-MI. The recoveries of 4-MI in caramels detection were ranged from 82.85% to 102.31%, with the coefficient of variation ($n = 3$) below 9.06%. Quantitative comparison of the established fluorescence-based ICA with high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) and indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) analysis of real caramel colour samples indicated a good correlation among the methods. Therefore, our developed fluorescence-based ICA method shows great potential for simple, rapid, sensitive, and cost-effective quantitative detection of 4-MI in food safety control.

1. Introduction

Caramel colour is one of the most widely used colourings for variety of foods and beverages (Lee, Jang, & Shibamoto, 2013). Based on the reactants used in manufacturing, caramel colours are classified into four classes: class I, plain caramel or caustic caramel; class II, caustic sulphite caramel; class III, ammonia caramel, and class IV, sulphite ammonia caramel (Sengar & Sharma, 2014). Unfortunately, some by-products such as 4-MI are also produced during the manufacturing process of class III or class IV caramel (Hengel & Shibamoto, 2013; Moon & Shibamoto, 2010). 4-MI has been identified as a chemical, which can cause cancer, by the National Toxicology Program of United States in 2007 (National Toxicology Program, 2007). Moreover, 4-MI is expected to be classified as group 2B (possibly carcinogenic to humans) by International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer (IARC), 2012). Moreover, the office of Environmental Health Hazard Assessment (OEHHA) within the California Environmental Protection Agency recommended that the acceptable daily intake (ADI) of 4-MI should be less than

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29 μg/day (OEHHA (Office of Environmental Health Assessment). Notice of intent to list 4-methylimidazole, 2011).

Various methods have been developed for detecting 4-MI in caramel colours, including gas chromatography–mass spectrometry (GC–MS) (Fernandes & Ferreira, 1997), liquid chromatography (LC) (Moretton, Crétier, Nigay, & Rocca, 2011) and LC–MS/MS (Schlee et al., 2013). These methods are essentially very specific and sensitive. However, they require professional training for the operator and very expensive apparatus. Furthermore, the pre-treatment of samples is time-consuming and it is implausible for *in situ* detection.

Compared with the above mentioned methods, immunoassay is simple, cost-effective, together with its high sensitivity, high selectivity, and simple sample extraction processes. Regarding immunoassay, enzyme-linked immunosorbent assay (ELISA) and ICA are two well-known and established examples (Wang et al., 2014). Indirect competitive ELISA (ic-ELISA) method has been developed by Wu et al. to detect 4-MI in caramels (Wu, Yu, & Kang, 2015). Despite its high accuracy and efficiency, the ELISA method showed some disadvantages like time-consuming cleaning process (approximately 90 min), well-trained operator and very specific apparatus for detecting, which make it unsuitable for *in situ* detection. However, ICA is considered to be one of the most preferred real-time analytical tools for its simple, rapid (approximately 15 min), selective and cost-effective characters (Bai et al., 2013; Li et al., 2013). Therefore, ICA has been widely used for qualitative and quantitative detection of both high-throughput screens and individual testing.

Conventionally, gold is used as the tracer in ICAs because the gold-based assays are cheap, fast, and sensitive and capable of screening many substances, including antibiotics, pathogens and drugs. But limits have been observed when ultra high sensitivity is required (Chiao, Shyu, Hu, Chiang, & Tang, 2004; Peng et al., 2007; Valecha, Sharma, & Devi, 1998; Wang et al., 2007). In recent years, fluorescent microspheres, instead of colloidal gold, have been developed and used in ICA format with increased sensitivity and accuracy (Majdinasab et al., 2015; Wang et al., 2014). For example, fluorescence-based ICA has been established for sulfamethazine and enrofloxacin residue detection (Chen et al., 2013; Huang et al., 2013). The fluorescence-based ICA was shown to be able to test low-molecular weight compounds with high sensitivity and high specificity.

Therefore, in the present work, we developed a novel fluorescence-based ICA for fast and accurate detection of 4-MI in caramels. The conditions of the fluorescence-based ICA have been optimised. We strongly believe that our work contributes to the development of fast, efficient and accurate immunoassay of hazardous low molecular compounds in Food.

2. Materials and methods

2.1. Meterials

4-MI, 1-methylimidazole, 5-benzimidazolecarboxylic acid, 2-methylimidazole, 1,2-dimethylimidazole, histamine, polyoxyethylene sorbitan monolaurate (Tween-20), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fluorescent microspheres with a diameter of 100 nm were purchased from Merck (Damstadt, Germany). Rabbit immunoglobulin G (IgG) and goat anti-mouse IgG (GAM IgG) was purchased from Vector (Burlingame, CA, USA). The 4-MI monoclonal antibody (mAb) and the coating antigen were prepared previously (Wu et al., 2015). The sample pad, nitrocellulose (NC) membrane and polystyrene card were obtained from Millipore Corp. (Bedford, MA, USA). The absorbent pad was supplied by Shanghai Kinbio Tech. Co., Ltd (Shanghai, China). Caramels were purchased from local supermarket: brand A and B were class I caramel (E150a), brand C, D and E were class III caramel (E150c), and brand F and G were class IV caramel (E150d).

2.2. Preparation of the FM labelled mAb conjugates and FM labelled GAM IgG conjugates

The FM labelled mAb (FM-mAb) conjugates and FM labelled GAM IgG (FM-GAM IgG) conjugates were prepared as follows: FMs (100 μL) were dispersed in 900 μL MES buffer (0.1 M, pH 6.0), then NHS and EDC were added and stirred at room temperature in the dark for 20 min. The mixture was centrifuged at 18 °C at 11,000 rpm for 20 min and washed by 1 mL PBS (0.1 M, pH 7.4). The washing steps repeated twice, the collected precipitate was redispersed in 1 mL PBS and 50 μL of mAbs or GAM IgGs (10 mg/mL) were added dropwise. The mixture was maintained on a shaker for 2 h at room temperature, and then centrifuged at 18 °C at 11,000 rpm for 20 min. The FM-mAb conjugates and FM-GAM IgG conjugates were obtained after centrifugation at 11,000 rpm for 20 min at 18 °C. The pellets were resuspended in 1 mL PBS (0.1 M) containing 0.5% (w/v) OVA and 0.05% Tween 20. The suspension was blended by sonicated for 15 min and stored at 4 °C in the dark for further experiment.

2.3. Fluorescence-based ICA

The immunochromatographic strip consists of four sections, including a NC membrane, a sample pad, an absorbent pad and a backing card (Fig. 1). A band of coating antigen (4 mg/mL) was drawn on the NC membrane as the test line (T-line) and a band of rabbit IgG (1 mg/mL) was drawn as the control line (C-line) by using the ZX1000 Dispensing Platform (Irvine, CA, USA). The coated membranes were dried at 37 °C for 12 h. The sample pad was soaked in PBS containing 1% OVA and 0.05% Tween 20 and completely dried at 37 °C for 24 h. Then the NC membrane, the sample pad, and the absorbent pad were fixed on the polystyrene support plates illustrated in Fig. 1. The plate was cut into 4 mm wide strips, and placed inside a plastic cartridge designed to fit the holder of optical reader.

The subsequent ICA experiments were performed as follows: 50 μL aliquot of series of concentration of 4-MI (or samples) solution was mixed with 25 μL FM-mAb conjugates (diluted 2400-fold by PBS) and 25 μL FM-GAM IgG conjugates (diluted 1200-fold by PBS), and blended on a shaker for 30 s. The sample (70 μL) was then added into the window of the sample pad. After 15 min incubation, the strip was scanned with an optical reader, which was designed by Guangzhou Wondfo Biotech Co., Ltd, (Guangzhou, China). The fluorescent intensity of test lines (F_T) and control lines (F_C) were recorded, the ratio of F_T/F_C was then calculated. The 4-MI concentration was determined using the built-in linear regression equation of the 4-MI calibration curve. The standard 4-MI solutions were prepared by spiking stock 4-MI solution (1 g/L) in PBS, with a final concentration of 0 (as negative control), 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, and 64.0 mg/L. The standard curve was established by plotting the $B_x/B_0 \times 100\%$ against the logarithm of the 4-MI concentration, where B_0 and B_x represented F_T/F_C ratio between the negative control and positive samples, respectively. 20 blank samples (only PBS solution) were analysed and recorded by the optical reader for the calculation of the limit of detection (LOD) and the limit of quantification (LOQ) (Majdinasab et al., 2015; Wang et al., 2014). The LOD and LOQ were calculated as three times and ten times of the standard deviation of the negative samples, respectively.

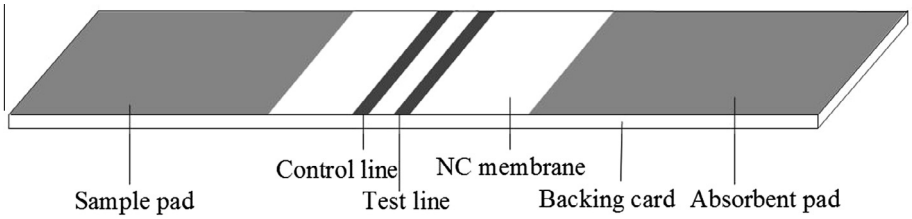


Fig. 1. A schematic diagram of the ICA strip. Coating antigen was immobilised at the test line, and rabbit IgG was immobilised at the control line of the detection zone.

2.4. Specificity Assay

Structural analogues of 4-MI were chosen for cross-reactivity (CR) study to determine the specificity of the FM-mAb conjugates. The CR of each compound was quantitated from the individual standard curve by using the following formula:

$$\text{CR } (\%) = (\text{IC}_{50} \text{ of } 4-\text{MI}/\text{IC}_{50} \text{ of other analogues}) \times 100$$

where IC_{50} was the concentration of analyte that produces 50% of the maximum normalised response (Fang et al., 2011; Guan, Wu, & Meng, 2013).

2.5. Recovery test

The recovery of spiked samples was used to confirm the reproducibility of ICA. Caramel samples (10 mg) were diluted 50-fold (w/v) using PBS, and spiked with 4-MI standards (1, 2, 4, 6 and 8 mg/L). Then, the spiked samples were analysed by ICA to estimate the recoveries.

2.6. HPLC–MS/MS and indirect competitive immunosorbent assay (ic-ELISA) analysis

The real caramel colour samples were determined by HPLC–MS/MS. The pretreatment of the samples was according to the method described by Moon (Lee et al., 2013). The HPLC–MS/MS system consisted of a Waters (MA, USA) HPLC system (600 pump and Rheodyne 7725i Manual Injector) coupled with a Thermo Scientific (San Jose, USA) LTQ mass spectrometer. HPLC separation was performed on a Waters XBridge BEH Shield RP18 Column (3 μm, 3.0 mm × 150 mm) using mobile phase A (10 mM of ammonia in high-purity water) and mobile phase B (methanol) in a gradient program with a flow of 0.3 mL/min: 0–10 min: 5% B; 11–15 min: 5% B to 100% B; 15–25 min: 100% B; 25–35 min, 100% B to 5% B. In the mass spectrometer, electrospray ionisation (ESI) in positive ion mode was used at a capillary temperature of 275 °C with nitrogen gas (35 psi). The collision energy was set at 33 eV. The mass spectrometer was operated in selected reaction monitoring (SRM) mode to observe the transition of m/z 83–56 for 4-MI. The real caramel colour samples were also determined by ic-ELISA method, as described by the published literature (Wu et al., 2015).

2.7. Statistical analyses

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

3. Results and discussion

3.1. Fluorescence-based ICA development

In the subsequent ICA experiments, the 4-MI detection experiments were carried out using the FM-mAb and FM-GAM IgG conjugates. The sample containing 4-MI was first mixed with the FM-mAb and FM-GAM IgG conjugates, and then introduced to the sample pad. The mixture added in the sample pad then moved forward, migrated smoothly and accumulated on the C-line and T-line areas. At C-line area, FM-GAM IgG conjugates bound with rabbit IgG and the fluorescent intensity was recorded on the reader. The concentrations of rabbit IgG and FM-GAM IgG conjugates were fixed, thus the fluorescent intensity of the C-line (Fc) was also constant and independent of 4-MI concentration. The fluorescent intensity of C-line was used as a reference to reduce the error between different strips. In the meantime, coating antigen in the T-line and the free 4-MI in the standards or samples bound with FM-mAb conjugates competitively. As a result, only free FM-mAb conjugates could migrate smoothly and finally accumulate on the T-line area, suggesting that the fluorescent intensity of the test line (F_T) decreases as the concentration of 4-MI in sample increases. According to the principle described above, the ratio (F_T/F_C) would be inversely proportional to 4-MI concentrations in the sample, which could be used for quantitation of 4-MI in samples.

As the competition reaction mainly occurred at T-line area, the parameters of T-line should be optimised. The concentration of FM-mAb conjugates and the amount of coating antigen were considered as the most important factors which have been revealed to affect the fluorescent intensity of the T-line (Li et al., 2013). To achieve the best sensitivity of the test strip, a series of concentrations of coating antigen (1.0, 2.0, 4.0, 8.0 mg/mL) and different FM-mAb conjugates dilutions (1/600, 1200, 1/2400, 1/4800) on the T-line were evaluated to obtain the optimal assay performance. The main parameters used to evaluate immunoassay property were IC_{50} and the ratio of maximum F_T/F_C (F_{max}) to IC_{50} (i.e. F_{max}/IC_{50}). Similar to ELISA, higher F_{max}/IC_{50} ratio always means higher sensitivity of ICA (Hao et al., 2009). As shown in Table 1, coating concentration of 4 mg/mL and FM-mAb conjugates dilution

Table 1
Effects of the coating antigen and FM-mAb conjugates on the ICA sensitivity.

Item	Parameter	IC_{50} (mg/L)	F_{max}/IC_{50}
Coating antigen concentration (mg/mL)	1.0	8.61	0.24
	2.0	5.14	0.53
	4.0	4.50	0.79
	8.0	5.48	0.72
FM-mAb conjugate dilutions	1/600	4.81	0.53
	1/1200	5.15	0.68
	1/2400	4.51	0.79
	1/4800	5.48	0.70

of 2400-fold were selected as the optimised conditions for the ICA of 4-MI detection. Based on the optimised conditions, a standard inhibition curve for the detection of 4-MI was obtained and shown in Fig. 2. The percentage ratio of B_x/B_0% was plotted against the logarithm of concentration of 4-MI (mg/L), a liner correlation was observed: $y = -42.596x + 79.024$ ($R^2 = 0.991$, $n = 3$). The linear detection range was 0.50–32.0 mg/L. The LOD was calculated as three times the standard deviation of the negative samples, and LOQ was measured as ten times the standard deviation, which were 0.18 mg/L and 0.6 mg/L, respectively.

3.2. CR analysis

CR was widely used to evaluate the specificity of an established ICA (Ren et al., 2014). In this work, CR of 6 structural analogues (1-methylimidazole, 2-methylimidazole, 1,2-dimethylimidazole, histamine, histidine and 5-benzimidazolecarboxylic acid) of 4-MI were tested to evaluate the specificity of ICA. As showed in Table 2, CR between 4-MI and its analogues was less than 3.93%. The results suggested that specificity of ICA-based detection of 4-MI established in this study was very high.

3.3. Matrix effects

Detection of 4-MI in caramel colour samples by immunoassay often requires previous dilution to avoid matrix interferences. Class I caramel (brand A) was used to evaluate the matrix effect on the ICA efficiency, because no 4-MI presenting in this kind of caramels. The caramel samples were diluted (1:2, 1:5, 1:10, 1:20, and 1:50) with PBS and a 4-MI standard curve was prepared with each sample dilution. It was observed that complete inhibition curves without interference were obtained when 1:20 or even higher dilution of the caramel sample was used (data not shown). The results indicated that low dilution ($\leqslant$10-fold) of sample matrix significantly affected the results. However, the matrix effects could be eliminated by diluting the matrix at least to 20-fold with PBS. In addition, Class III caramel (brand C) and Class IV caramel (brand F) were diluted (1:5, 1:10, 1:20, and 1:50) with PBS, and then spiked with 4-MI standards (5, 10 and 20 mg/L). The recoveries and coefficient of variation (CV) values were detected to evaluate the matrix effect. The recoveries of Class III caramel were calculated as 66.59–78.12% for 1:5 dilution, 74.33–85.76% for 1:10 dilution, 88.34–108.22% for 1:20 dilution and 90.56–110.01% for 1:50 dilution, respectively. In all the cases, CV value was lower than 10%. The recoveries and CVs of the 20-fold and 50-fold dilutions were suitable for the detection of 4-MI. Similar results were shown for Class IV caramel: the recoveries were calculated as 71.33–128.24% for 1:5 dilution, 81.25–123.16% for 1:10 dilution, 90.11–106.94% for 1:20 dilution and 92.97–101.48% for 1:50 dilution, with the CV value lower than 12%. Thus, in order to eliminate the matrix interference, samples should be diluted at least 20-fold with PBS prior to the assay.

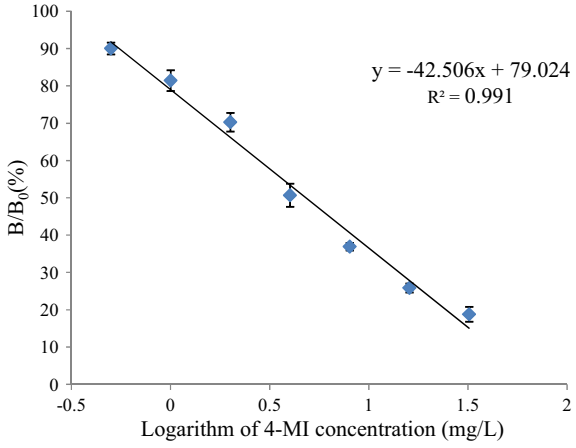
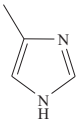
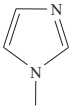
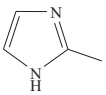
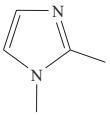
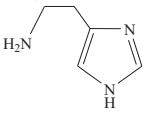
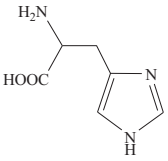
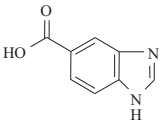


Fig. 2. Standard inhibition curve of ICA for 4-MI.

Table 2
CRs of the Analogues of 4-MI.

Analogue	Structure	FM-mAb conjugates	
		IC_{50} (mg/L, mg/kg)	CR%
4-MI		5.140437	100.0
1-Methylimidazole		130.6502	3.93
2-Methylimidazole		134.5698	3.82
1,2-Dimethylimidazole		500.7998	1.03
Histamine		>2000	<0.4%
Histidine		>2000	<0.4%
5-Benzimidazolecarboxylic acid		812.1975	0.63

3.4. Validation of the assay

To test the reliability of practical application, optimised ICA was applied to the determination of 4-MI in spiked caramel colour samples (brand A and B). The samples were diluted 50-fold (w/v) with PBS and spiked with 4-MI at 1, 2, 4, 6 and 8 mg/L, then directly

Table 3
Recovery of 4-MI in spiked samples.

Caramels	Spiked concentration (mg/L)	Recovery (%)	CV (%)
Brand 1	1	96.70	4.35
	2	82.85	6.89
	4	100.675	4.52
	6	95.90	5.66
	8	101.34	9.06
Brand 2	1	102.31	3.53
	2	98.73	5.64
	4	84.66	6.89
	6	84.93	4.75
	8	98.92	6.69

Table 4
Concentrations of 4-MI found in real caramel colour samples.

Spiked concentration	4-MI concentration (mg/kg)		
	HPLC–MS/MS (n = 3)	ICA (n = 3)	ic-ELISA (n = 3)
Brand A (class I)	–	–	–
Brand B (class I)	–	–	–
Brand C (class III)	85.24 ± 1.31	87.16 ± 2.07	90.52 ± 1.65
Brand D (class III)	179.90 ± 1.43	186.78 ± 1.99	168.61 ± 2.76
Brand E (class III)	91.86 ± 0.89	95.06 ± 2.50	88.18 ± 2.39
Brand F (class IV)	41.21 ± 1.05	44.08 ± 1.36	43.22 ± 1.11
Brand G (class IV)	126.50 ± 1.18	123.97 ± 2.73	120.07 ± 0.92

analysed by the ICA without any treatment. As seen in Table 3, the recoveries and coefficient of variation (CV) values of ICA for 4-MI in spiked samples were in the range of 82.85–102.31% and 3.53–9.06%, respectively. The recoveries and CVs of ICA are suitable for screening purposes.

To further demonstrate the reliability and practicability of the proposed ICA in real samples, 7 caramel colour samples were analysed by ICA, ic-ELISA and HPLC–MS/MS. The results in Table 4 indicated that the results of ICA are closed to the results of the other two methods. The established ICA method did not show any false-negative or false-positive result. In addition, a good correlation was obtained between the ICA method and HPLC–MS/MS method with linear regression equation of $y = 1.0219x + 0.25645$ ($R^2 = 0.9985$), and it's also a good correlation between the ICA method and ic-ELISA method with linear regression equation of $y = 0.858x + 10.468$ ($R^2 = 0.9924$). Thus, it can be concluded safely that the established ICA can be applicable for detecting 4-MI in caramels.

4. Conclusions

An accurate and specific fluorescence-based ICA method for 4-MI detection within 15 min has been established in this study. The linear range was observed in the range of 0.5–32 mg/L. The recoveries and CVs for 4-MI detection in commercial caramel samples were 82.85–102.31% and 3.53–9.06%, respectively. The assay was simple, rapid, and sensitive, providing not only qualitative results for rapid assessment but also quantitative results for the accurate determination of 4-MI in caramel colour samples.

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