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Analytical Methods

Development of a monoclonal antibody-based indirect competitive immunosorbent assay for 4(5)-Methylimidazole detection in caramels

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ABSTRACT

In this study, an indirect competitive enzyme-linked immunoassay (ic-ELISA) based on monoclonal antibody for 4(5)-Methylimidazole (4-MI) detection was described. The artificial antigens were prepared by conjugating bovine serum albumin (BSA) or ovalbumin (OVA) with the hapten of 4-MI. And monoclonal antibody, evaluated by ic-ELISA, was obtained by immunizing BABL/c mice. After optimizing, a standard curve for ic-ELISA detection on 4-MI was obtained with the linear detection range of 0.64–20.48 mg/L. The cross-reactivity (CR) of all the structural analogues of 4-MI was less than 5.62%. The recoveries of 4-MI in caramels detection were ranged from 88.69% to 114.09%, with relative standard deviation ($n = 3$) below 8.07%. The results suggested that the established ic-ELISA is promising for 4-MI commercial detection in caramels.

1. Introduction

The 4(5)-Methylimidazole (4-MI) is a nitrogen heterocyclic compound formed in the Maillard reaction (Casal, Fernandes, Oliveira, & Ferreira, 2002; Fernandes & Ferreira, 1997). Previous researchers have tested 4-MI in commercial food. For example, 4-MI was detected in commercial roasted coffee ranging from 0.307 to 1.231 mg/kg (Casal et al., 2002). Early studies showed that 4-MI was a potential mutagen and carcinogen (Chan, Hills, Kissling, & Nyska, 2008; Chan, Mahler, Travlos, Nyska, & Wenk, 2006). Thus, 4-MI was always concerned by regulatory agencies and consumers. About 7 years ago, i.e. 2007, the National Toxicology Program (NTP) of United States has identified 4-MI as a chemical to cause cancer (Moon & Shibamoto, 2010; National Toxicology Program, 2007). Subsequently, the Office of Environmental Health Hazard Assessment (OEHHA) within the California Environmental Protection Agency recommended that the acceptable daily intake (ADI) of 4-MI was less than 29 μg/day (Office of Environmental Health Assessment, 2011). Moreover, The European Food Safety Authority (EFSA) suggested the maximum limit content of 300 mg/kg for ammonia process caramels (European Food Safety Authority, 2012). Therefore, developing an effective means for 4-MI detection is necessary.

4-MI content has always been detected using gas chromatography–mass spectrometry (GC–MS), high performance liquid chromatography (HPLC) and high performance liquid chromatography–tandem mass spectrometry (HPLC–MS) (Cunha, Barrado, Faria, & Fernandes, 2011; Klejdus, Moravcová, & Kubáň, 2003; Klejdus, Moravcová, Lojková, Vacek, & Kubáň, 2006; Yamaguchi & Masuda, 2011). Although these methods stated above were

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sensitive, expensive measurement cost and complicated cleanup procedures always limited their wide use. Moreover, all of these analytical methods require a long time for sample preparation. Compared with instrumental methods, immunoassays are portable and cost-effective, with adequate sensitivity, high selectivity, and simple extraction processes. Hence, it is significant to develop an immunoassay method for 4-MI detection. Several immunoassays based on the enzyme-linked immunosorbent assay (ELISA) have been developed for the analyses of pesticides and veterinary drugs in food and the environment. This indicates ELISA has been one of the best means to test low-molecular weight compounds with higher specificity (Chin, Wong, Pont, & Karu, 2002; Li & Li, 2000; Mickova et al., 2003; Wanatabe et al., 2001; Yang et al., 2008). Moreover, still no recent literature about 4-MI detection by (ic-)ELISA was reported.

The present study will contribute to enriching the information on the application of ic-ELISA in low-molecule weight compound detection. The objective of this study was to establish an accurate ic-ELISA means based on monoclonal antibody to detect 4-MI in caramels.

2. Materials and methods

2.1. Materials

4(5)-Methylimidazole, 1-methylimidazole, histamine, 2-methylimidazole, 1,2-dimethylimidazole, 5-benzimidazolecarboxylic acid, 4-nitrobenzyl bromide, glutaraldehyde (25%), BSA, OVA, potassium carbonate (K_2CO_3) and tetrabutylammonium iodide, reduced iron powder, polyoxyethylene sorbitan monolaurate (Tween-20), Freund's complete and incomplete adjuvants, hypoxanthine-aminopterin-thymidine (HAT) and hypoxanthine-thymidine (HT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The RPMI Media 1640 was purchased from AppliChem GmbH (Darmstadt, Germany). Polyethylene glycol (PEG 1500) was purchased from Boehringer Mannheim (Mannheim, Germany). The horseradish peroxidase (HRP)-labeled goat anti-mouse IgG was purchased from Vector (Burlingame, CA, USA). Color reagents A (0.004% urea hydrogen peroxide acetate solution, pH 5.4–5.6) and B (3,3′,5,5′-Tetramethylbenzidine, TMB, 0.2 mg/mL ethanol–water solution) were purchased from Tai Tian He Biological limited company (Jinan, Shandong, China). HPLC-grade acetonitrile was provided by Merck (Damstadt, Germany). 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). BALB/c mice were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China). All animal studies were performed in accordance with institutional guidelines.

2.2. Hapten preparation and identification

The 4-MI hapten was synthesized and shown in Fig. 1. Briefly, 4-MI (3 mmol) was dissolved in 5 mL acetonitrile, then 4-nitrobenzyl bromide (4.5 mmol), K_2CO_3 (5 mmol) and tetrabutylammonium iodide (0.2 mmol) were added. The mixture was stirred at 600 rpm, 70 °C on a C-MAG HS10 magnetic stirrer (IKA, Germany) for 24 h. Subsequently, the reacted mixture was dissolved in 50 mL water, and extracted using ethyl acetate (90 mL, 3 times). The ethyl acetate phase was concentrated in vacuum. After the residue was dissolved in 8 mL ethanol–water solution (ethanol:water, 5:3, v/v), excess HCl solution (1 N, 20 mL) and reduced iron powder (600 mg) were added, and stirred at room temperature for 48 h. The reaction mixture was filtered, and adjusted to pH 8.0 using 2 N NaOH, then extracted by dichloromethane (90 mL) subsequently. The dichloromethane phase was concentrated in vacuum and stored at 4 °C until used.

For further isolation, the concentrated dichloromethane phase was separated by a preparative liquid chromatography using a C_{18} (10 μm) column (25 × 600 mm, inside diameter × length, Lisui II, Suzhou Lisui Technology Co., Ltd, Suzhou, China) with an ultraviolet detector (Lisui, Suzhou Lisui Technology Co., Ltd, Suzhou, China). The mobile phase of the system was 100% water for the period of 0–30 min, then a linear gradient from 100% water to 100% methanol from 30 to 120 min, and finally, eluted by 100% methanol, from 120 to 150 min. The flow rate was 12 mL/min, and monitor wavelength was 225 nm. All the peaks monitored by ultraviolet detector were collected and characterized by electro spray ionization-mass spectra (ESI-MS). ESI-MS was carried out in a LCQ-Fleet mass spectrometer (ThermoFisher, USA), with an electro spray ionization source using a positive ion mode (m/z 50–800). The target compound was concentrated and dried in vacuum. A yellow solid was obtained and analyzed by nuclear magnetic resonance (NMR), which was operated at 400 MHz for ^{1}H NMR in a Bruker Avance 400 instrument (Bruker Co., Switzerland). Hapten structure data: ESI-MS m/z 169.00 $[M+H]^+$, 336.78 $[2M+H]^+$; ^{1}H NMR data (400 MHz, $CDCl_3$): δ: 2.14(d, 3H, CH_3); δ: 3.58(s, 2H, NH_2); δ: 4.90 (d, 2H, CH_2); δ: 6.57–6.79 (m, 3H, CH=CCH, NCH=C); δ: 6.85–6.99 (m, 2H, CHC=CH); δ: 7.35–7.45 (d, 1H, NCH=N).

2.3. Preparation of hapten–protein conjugates

Hapten was conjugated with BSA and OVA for immunogen (hapten–BSA) and coating antigen (hapten–OVA) preparation, respectively, using the glutaraldehyde method (Shen et al., 2011). Briefly, 6 mg hapten was dissolved in 200 μL methanol, then 5 mL of carbonate buffer (0.5 N, pH 9.6) containing 65 mg BSA or 300 mg OVA was added. Subsequently, 50 μL glutaraldehyde (25%) diluted in 1 mL carbonate buffer was added to the above solution with stirring at 600 rpm at room temperature (25 °C) for 4 h, and then dialyzed in 0.015 mol/L PBS (pH 7.4) at 4 °C for 2 days. The conjugates were scanned within a wavelength range of 190–400 nm on a DU-800 spectrophotometer (Puxi, Beijing, China).

2.4. Immunization and monoclonal antibody preparation

The immunogen emulsions were injected into 7-week-old BALB/c female mice subcutaneously. The dosage of immunogen

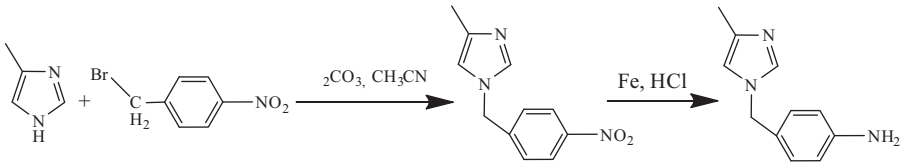


Fig. 1. Synthetic route for hapten.

for each mouse was 100 μg, which was dissolved in 100 μL physiological saline and emulsified with an equal volume of Freund's complete adjuvant. Three additional injections with the same dosage of immunogen emulsified in Freund's incomplete adjuvant were given after two-week interval. Antiserum was obtained from the tail vein of mouse for one week after the fourth immunization. The antibody titers and recognition properties with 4-MI were tested by ic-ELISA. Ten days after the third booster injection, the mouse that showed higher titer and significant competition was selected for the cell fusion according to the literature (Guan, Wu, & Meng, 2013). Briefly, the splenocytes of mouse were fused with SP2/0 myeloma cell at a ratio of 5:1 (37 °C). PRMI Media 1640 (20 mL) was added to end the cell fusion at the time of 4 min. The fused cells were distributed in 96-well culture plates supplemented with HAT medium containing 15% FBS with peritoneal macrophages. When the hybridoma cells were grown to approximately 30–40% confluent in the well, culture supernatants were collected and screened using ic-ELISA. Selected hybridoma cells were cloned by limiting dilution, and stable antibody-producing clones were injected into the enterocoelia of F1 mouse. Ascites were purified using ammonium sulphate precipitation.

2.5. ic-ELISA

The development of ic-ELISA was performed according to the method reported by Fang et al. (2011) with a slight modification. The ic-ELISA was carried out on 96-well polystyrene microplates. All incubations were performed at 37 °C except for the coating antigen. Microplate wells were coated with 100 μL of hapten–OVA (1 μg/mL in 0.05 mol/L carbonate buffer at pH 9.6, 100 μL/well) at 4 °C overnight. The plate was washed 5 times with 0.015 mol/L PBST solution (1000 mL phosphate buffer with 0.5 mL Tween-20) and blocked by adding 1% casein in PBST solution (200 μL/well). After incubation for 2 h, the plate was washed 5 times with PBST solution. Subsequently, analyte (4-MI or its analogues) with different concentration (50 μL/well) and the diluted antibody (50 μL/well) were added into the wells. After incubation for 1 h, the plate was washed and further incubated with the goat anti-mouse IgG-HRP (1:20,000 in PBST with 1% casein, 100 μL/well) for 30 min. After washing 5 times, equal volume of color reagents A and B were added (total volume is 100 μL/well). The reaction was stopped by adding sulfuric acid solution (2 mol/L, 50 μL/well) after 10 min of incubation at 37 °C. The absorbance was measured at 450 nm with a Multiskan MK3 microplate reader (Thermo Scientific, Waltham, USA).

A series of parameters, including coating antigen concentrations (0.25, 0.5, 1.0, 2.0 μg/ml), reaction buffer ionic strength (15, 30, 60, and 120 mmol/L PBST), buffer pH (5.4, 6.4, 7.4, 8.4) and secondary antibody dilutions (1/1250, 2500, 1/5000, 1/10,000, 1/20,000) were evaluated to obtain the optimal assay performance. The main parameters used to evaluate immunoassay property were IC_{50} and the ratio of maximum absorbance (A_{max}) to IC_{50} (i.e. A_{max}/IC_{50}). Higher A_{max}/IC_{50} ratio always means higher sensitivity of ic-ELISA assay (Hao et al., 2009; Lee, Ahn, Park, Kang, & Hammock, 2001). A standard curve was obtained under the optimum condition by plotting relative absorbance of B/B_0% against the logarithm of 4-MI concentration. B/B_0% was calculated using the Eq. (1), according to the report of Fang et al. (2011) and Guan et al. (2013).

$$B/B_0\% = [(A_x - A_{min})/(A_{max} - A_{min})] \times 100 \quad (1)$$

where A_x is the absorbance in the presence of 4-MI, A_{max} is the absorbance without 4-MI, and A_{min} is the absorbance of the background.

2.6. Cross-reactivity (CR)

CR was tested to evaluate the specificity of the antibody. The CR between 4-MI and the analogues of 4-MI were tested using ic-ELISA. The CR ratio was calculated according to Eq. (2).

$$CR\ (\%) = (IC_{50}\ \text{of 4-MI}/IC_{50}\ \text{of other analogues}) \times 100 \quad (2)$$

where IC_{50} was the concentration of analyte that produces a 50% of the maximum normalized response.

2.7. Recovery test

The recovery of spiked samples was used to confirm the reproducibility of ic-ELISA. Caramel samples (1.0 g) were diluted 50-fold

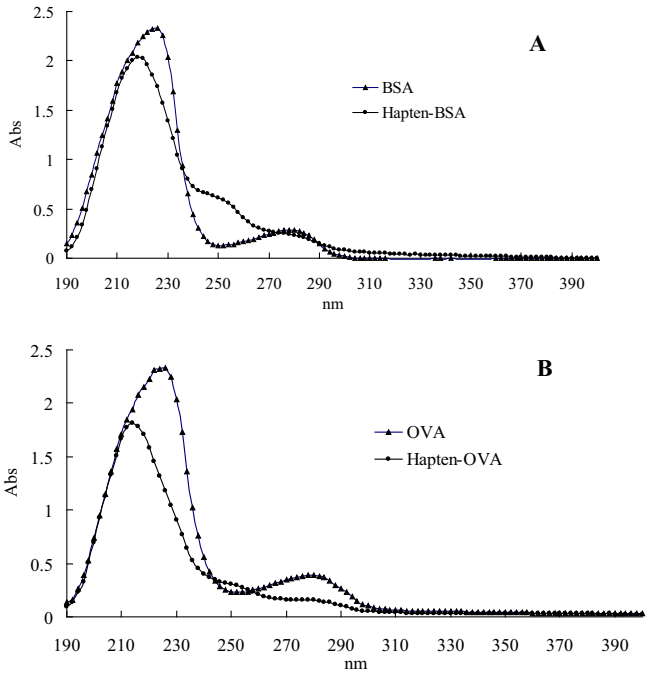


Fig. 2. Analysis of BSA, hapten–BSA conjugates (A) and OVA, hapten–OVA conjugates (B) by UV–Vis spectrophotometry.

Table 1
Effects of various factors on the ELISA sensitivity.

Item	Parameter	IC_{50} (mg/L)	A_{max}/IC_{50}
Coating antigen concentration (μg/mL)	0.25	9.04	0.09
	0.5	5.89	0.15
	1.0	2.28	0.34
	2.0	2.51	0.09
pH of PBST	5.4	11.38	0.03
	6.4	3.56	0.17
	7.4	2.59	0.27
	8.4	3.19	0.13
Ionic strength of PBST (mmol/L)	15	3.50	0.22
	30	2.30	0.28
	60	1.85	0.34
	120	2.56	0.27
Secondary antibody dilutions	1/1250	4.63	0.64
	1/2500	2.88	0.84
	1/5000	2.04	0.81
	1/10,000	1.74	0.68
	1/20,000	1.58	0.44

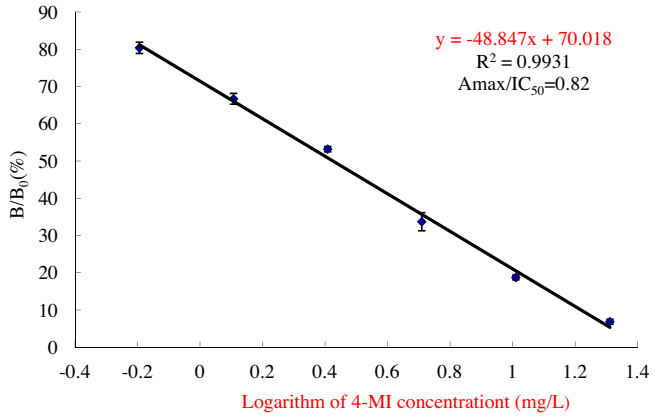


Fig. 3. Standard inhibition curve of ic-ELISA for 4-MI.

using PBST, and spiked with 4-MI standards (2.5, 5 and 10 mg/L). Then, the spiked samples were analyzed by ic-ELISA to estimate the recoveries.

2.8. Statistical analysis

All experiments were carried out in triplicates. Analysis of variance (ANOVA) was carried out to determine any significant differences ($P < 0.05$) among the applied treatments by the SPSS package.

3. Results and discussions

3.1. Identification of conjugations

The Ultraviolet–Visible (UV–Vis) spectra were often used to examine the qualitative difference between the carrier protein and conjugates. Therefore, it can be used to confirm whether the hapten was conjugated to the carrier protein or not. The spectra of BSA, hapten–BSA, OVA, hapten–OVA (1 mg/ml) were recorded from 190 to 400 nm (Fig. 2). Clearly, significant difference between the conjugate spectra and that of the corresponding carrier protein was observed (mainly at 215–280 nm), which indicated that the haptens had been coupled to carrier proteins.

Table 2
CRs of the analogues of 4-MI.

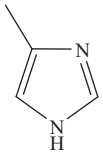
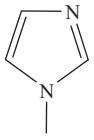
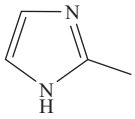
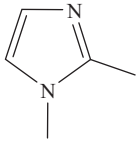
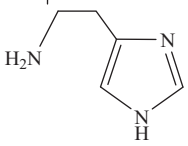
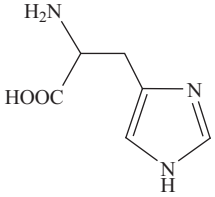
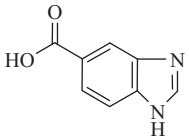
Analogue	Structure	Mouse monoclonal antibody	
		IC_{50} (mg/L, mg/kg)	CR%
4-MI		2.57	100.0
1-Methylimidazole		390.84	0.66
2-Methylimidazole		53.70	4.78
1,2-Dimethylimidazole		45.71	5.62
Histamine		>2000	<0.2
Histidine		>2000	<0.2
5-Benzimidazolecarboxylic acid		>2000	<0.2

Table 3
Recovery of 4-MI in spiked samples.

Caramels[a]	Spiked concentration (mg/L)	Recovery ± SD[b] (%)	RSD (%)
Brand C	2.5	114.09 ± 5.53	4.85
	5	90.78 ± 2.30	2.54
	10	97.48 ± 2.83	2.90
Brand D	2.5	94.93 ± 7.66	8.07
	5	104.08 ± 5.09	4.89
	10	112.22 ± 2.50	2.23
Brand E	2.5	92.71 ± 1.98	2.14
	5	101.05 ± 5.87	5.81
	10	97.88 ± 2.47	2.52
Brand F	2.5	104.56 ± 4.30	4.11
	5	102.39 ± 7.59	7.41
	10	104.92 ± 2.36	2.25
Brand G	2.5	98.50 ± 3.92	3.98
	5	88.69 ± 3.29	3.71
	10	105.12 ± 6.08	5.78

[a] The initial concentration of the 5 brands caramels were 1.74 ± 0.12, 3.70 ± 0.09, 1.86 ± 0.05, 0.74 ± 0.09, 2.53 ± 0.08 mg/L, which were detected by ic-ELISA with 50-fold dilution in PBST, respectively.
[b] SD, standard deviation.

3.2. Optimization of the ic-ELISA

The buffer ionic strength, buffer pH and secondary antibody dilutions were investigated for ELISA reaction optimization (Table 1). It was observed that all the experimental parameters detailed in this experiment showed significant effects on the sensitivity of ic-ELISA ($P < 0.05$). Based on the data in the present study, a coating concentration of 1 μg/mL, pH value of PBST solution of 7.4, ionic strength of PBST at 60 mmol/L, and secondary antibody dilution of 2500-fold were selected as the optimized conditions for the ic-ELISA of 4-MI detection. And a standard inhibition curve for the detection of 4-MI was obtained and shown at Fig. 3 under the optimal condition. The curve was plotted with B/B_0% as the longitudinal coordinates (y) and the logarithm of concentration of 4-MI (mg/L) as the lateral coordinates (x): $y = -0.4885x + 0.7002$ ($R^2 = 0.9931$, $n = 3$). The linear detection range was 0.64–20.48 mg/L.

3.3. CR analysis

It is an inherent problem for ELISA or ic-ELISA detection that antibodies may recognize the structural analogues of target analyte (Watanabe et al., 2004). In this experiment, the 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 obtained monoclonal antibody. As showed in Table 2, the CR between 4-MI and its analogues was less than 5.62%. The results suggested that the ic-ELISA method established in this study showed high specificity for 4-MI detection.

3.4. Matrix effects

Two commercial Class I caramel (brand 1 and 2, without 4-MI) were used to evaluate the matrix effects on the ic-ELISA performance for its negative test. The caramel samples were diluted with PBST (1:2, 1:5, 1:10, 1:20, 1:50, v/v) and then used to prepare inhibition curves, which were compared with that prepared in the absence of cameral samples. Overlapping curves were obtained when the samples were diluted at least 10-fold (data not shown). It was indicated that the sample with low dilution (⩽5-fold) significantly affected the ic-ELISA curves. However, the matrix effects could be eliminated by diluting the matrix at least to 10-fold with PBST. Thus, samples need to be diluted at least 10-fold with PBST prior in this assay.

3.5. Analysis of spiked samples

The reproducibility of established ic-ELISA was evaluated using 5 brands of caramels samples (brand 3, 4, 5, 6 and 7), which were diluted in PBST prior to ic-ELISA. The recoveries and the relative standard deviations (RSDs) of the tested samples were shown in Table 3. Acceptable recoveries of 88.69–114.09% and RSDs of 2.14–8.07% were obtained in the established ic-ELISA method, which suggested the ic-ELISA was accurate in 4-MI detection. Additionally, it is reported that the content of 4-MI in caramels was in range of 20–120 mg/L or mg/kg (Moretton, Crétier, Nigay, & Rocca, 2011). Thus, it can be seen that the established ic-ELISA can be applied for the detection of 4-MI in caramels.

This study provided a simple and accurate method to test 4-MI in caramels without any pretreatment comparing with recent HPLC, HPLC–MS or GC–MS methods (Cunha et al., 2011; Klejdus et al., 2003, 2006; Yamaguchi & Masuda, 2011). Moreover, 96 samples can be tested simultaneously within 4 h by the 96-well plates, meaning it will take less than 2.5 min to test per sample, corresponding at least 20 min for HPLC or HPLC–MS methods (Klejdus et al., 2003, 2006; Yamaguchi & Masuda, 2011). Hence, established ic-ELISA can be used to detect 4-MI with a lower cost and shorter time.

4. Conclusions

The established ic-ELISA method was successfully used to detect 4-MI. The monoclonal antibody was obtained using the immunogen which was prepared by conjugating the synthesized hapten with BSA. Based on the monoclonal antibody, the established ic-ELISA showed a linear detection range of 0.64–20.48 mg/L, with negligible cross-reactivity (<5.62%) to structural analogues. The recovery and RSD in this assay for 4-MI detection in commercial caramel samples were 88.69–114.09% and 2.14–8.07%, respectively. Thus, it was suggested that the established ic-ELISA method was sensitive and specific for 4-MI detection in caramels.

Acknowledgements

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