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Indirect competitive ELISA based on the prepared polyclonal antibody for highly sensitive detecting illegal additive 4-chloro-oxyphenisatin diacetate in weight loss food products

Qingqing Li ^a, Jinyue Wei ^a, Chenming Cai ^a, Peiying Hao ^a, Feng Jiang ^b, Xinyuan Wang ^c, Xiaoping Yu ^a, Biao Zhang ^{a*}

^a Key Laboratory of Microbiological Metrology, Measurement & Bio-product Quality Security, State Administration for Market Regulation, College of Life Sciences, China Jiliang University, Hangzhou 310018, China

^b Hubei Provincial Institute for Food Supervision and Test, Wuhan 430075, China

^c Hangzhou Institute of Food and Drug Control, Hangzhou 310022, China

ABSTRACT: 4-chloro-oxyphenisatin diacetate (Cl-Bisatin-DA), frequently added illegally to weight loss foods, has been proven to exert severe health hazards, highlighting the crucial importance of developing a rapid and sensitive analytical method. In this work, the hapten, antigens, and polyclonal antibody of Cl-Bisatin-DA were prepared, and the highly sensitive indirect competitive ELISA (ic-ELISA) using the prepared polyclonal antibody was established for the detection of Cl-Bisatin-DA in weight loss food products. Under optimal conditions, the detection limit (IC₁₅) and sensitivity (IC₅₀) of the established ic-ELISA were 1.07 ng/mL and 27.67 ng/mL, respectively. The cross-reactivity values of ten non-target analogs were below 0.2%. The application evaluation results showed that the spiked recovery rates of Cl-Bisatin-DA in zero-calorie jelly, solid beverage, enzyme beverage, pressed candy, and fruit vinegar beverage ranged from 76.1% to 106.6%, which were consistent with the LC-MS analysis results. The developed ic-ELISA provides a novel potential tool for the rapid and highly sensitive detection of Cl-Bisatin-DA in weight loss food products.

Keywords: 4-Chloro-oxyphenisatin diacetate, Polyclonal antibody, ic-ELISA, Illegal addition, Weight loss food products

1. Introduction

Obesity is recognized as one of the most severe public health challenges of the 21st century [1], with its prevalence increasing "explosively" globally over the past few decades [2]. With the growing obese population, the market demand for weight-loss products has surged, leading to a proliferation of various products [3]. However, lured by high profits, some illegal manufacturers disregard laws and regulations [4], illegally adding 4-chloro-bisacetophenone (Cl-Bisatin-DA) and other cathartic substances in weight loss foods to exaggerate and misleading consumers with exaggerated claims [5], endangering human health severely [6]. 4-Chloro-oxyphenisatin diacetate (Cl-Bisatin-DA), a structural analog of oxyphenisatin acetate,

*Corresponding author
Biao Zhang, email: zhangbiao9129@163.com

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is a commonly illegally added laxative in weight-loss products [7]. It stimulates intestinal peristalsis and causes diarrhea to achieve short-term weight loss effects [8], but long-term intake can induce intestinal dysfunction, electrolyte imbalance, and even irreversible liver damage [9]. Although numerous national and regional regulations explicitly ban the use of oxyphenisatin acetate and its analogues in health foods [10], producers continue to evade supervision by introducing structural analogs or novel derivatives, making detection increasingly challenging [11,12].

Currently, the existing detection methods of Cl-Bisatin-DA and its analogues mainly include high-performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS) [13], mass spectrometry [14], and gas chromatography-mass spectrometry (GC-MS) [15,16], etc. And there is still a lack of research on the detection method of Cl-Bisatin-DA based on ELISA. Nevertheless, most of these methods rely on complex sample pretreatment procedures and often suffer from issues such as insufficient sensitivity [17], long separation times [18], and severe interference from matrix components [19]. While demonstrating high sensitivity, LC-MS/MS and GC-MS are hampered by high cost, stringent operational requirements [20], and often lengthy derivatization [21]. Therefore, rapid and highly sensitive detection methods are urgently needed to be developed for on-site rapid detection of Cl-Bisatin-DA.

Enzyme-linked immunosorbent assay (ELISA) technology relies on the high specificity and affinity of antibodies for target recognition [22], coupled with enzyme-labeled conjugates that catalyze substrate reactions to generate detectable signals for quantitative or qualitative analysis [23]. It offers advantages such as high sensitivity, good specificity [24], high throughput, simple operation, low cost, and suitability for on-site application, which makes it widely used in food safety [25], environmental monitoring [26], and clinical diagnosis [27]. In recent years, ELISA has been applied to detect illegal drugs in weight-loss products, providing a basis for standard establishment.

In this study, a highly sensitive, accurate and specific ic-ELISA using immunometry based on standard samples was established and applied to the detection of Cl-Bisatin-DA in weight loss food products. A novel hapten of Cl-Bisatin-DA was designed and synthesized to produce high-affinity polyclonal antibody. After systematic optimization of key assay parameters, including coating antigen concentration, blocking buffer, and incubation conditions, the based highly sensitive and stable ic-ELISA for Cl-Bisatin-DA was established. The ic-ELISA was rigorously evaluated for specificity, accuracy, and precision in real samples, including zero-calorie jelly, solid beverages, enzyme drinks, pressed candy, and fruit vinegar drinks, and validated against LC-MS. This ic-ELISA provides a robust technical approach for rapid screening and regulation of illegally added substances in weight-loss foods, while also offering a solid scientific foundation for future development of standardized methodologies and implementation of regulatory measures.

2. Materials and methods

2.1 Materials

The 4-Chloro-oxyphenisatin diacetate (Cl-Bisatin-DA) was obtained from Hubei Provincial Institute For Food Supervision And Test. The anti-Cl-Bisatin-DA antibody (Ab₁) was obtained in the laboratory. Ethyl

chloroacetate, methanol, K_2CO_3 , NaOH, concentrated hydrochloric acid, dimethyl sulfoxide (DMSO) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Bovine serum protein (BSA) was purchased from Solarbio (Beijing, China). Ovalbumin (OVA) was purchased from Sigma-Aldrich (Shanghai, China). HRP labeled Goat anti-rabbit IgG ($Ab_2@HRP$) was purchased from Jackson ImmunoResearch Laboratories (West Grove, USA). oxyphenisatine, phenolphthalein, thymolphthalein, difluorenylidene, orlistat, sennoside B, and α -naphtholphthalein were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Oxyphenisatine diacetate, bisphenol TMC were obtained from Shanghai Macklin Biochemical Technology Co., Ltd. Bisoxatin acetate was purchased from Bidepharm (Shanghai, China). Ultrapure water was obtained from a Millipore Milli-Q system and used for the preparation of all solutions.

2.2 Instruments

A MILLI-Q ultrapure water system was purchased from Millipore (Billerica, USA). A Varioskan LUX multimode microplate reader (VLBL00D2) was purchased from ThermoFisher Scientific (USA). 96-well EIA/RIA plates were purchased from Corning Incorporated (USA). The Agilent 1100-Thermos TSQ Quantum Ultra was purchased from Thermo Fisher Scientific (USA). The ultraviolet spectrophotometer was purchased from MAPADA instruments (Shanghai, China). A Venusil MP C18 column was purchased from Agela Technologies (Tianjin, China).

2.3 Synthesis of hapten, immunogen and coating antigen

The synthesis route for the hapten is shown in Figure 1A. Cl-Bisatin-DA (0.1 mmol, 43.58 mg) was dissolved in 5.0 mL of methanol. Ethyl chloroacetate (0.2 mmol) and K_2CO_3 (0.6 mmol) were added, and the mixture was stirred at 300 rpm room temperature (25 °C) for 12 h. Subsequently, 5.0 mL of a 2 mol/L NaOH solution was added to the reaction mixture, and stirring was continued for 3 h. After the reaction was complete, the pH was adjusted to about 6.5 by dropwise addition of HCl, and the mixture was allowed to stand for phase separation. The upper organic phase was collected and washed three times with ultrapure water (10 mL each time), then purified by silica gel column chromatography (200 mesh silica gel, eluent: dichloromethane/methanol = 95:5, v/v); the eluent containing the hapten was collected and concentrated to dryness under reduced pressure (40 °C, 0.08 MPa) to obtain the hapten as a white solid. The synthesis processes for the immunogen and coating antigen were illustrated in Figure 1B and 1C, respectively. The hapten was completely dissolved in methanol, and BSA and OVA were added respectively. The molar ratio of hapten to BSA (or OVA) was 1.5:1. The mixture was stirred at 25 °C for 18 h and then incubated overnight at 4 °C. The solution was collected and dialyzed in 0.01 mol/L phosphate buffer for 72 h to obtain immunogen (hapten-BSA) and coating antigen (hapten-OVA), which were stored at 4 °C for use.

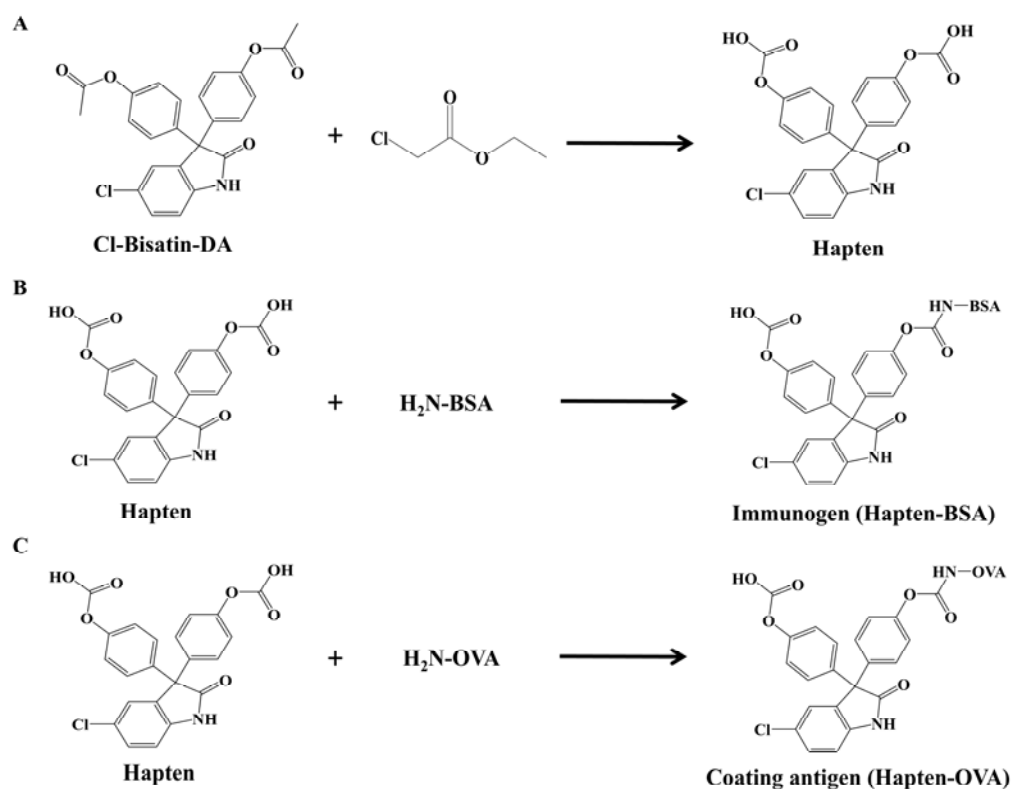


Figure 1. The synthesis route for the hapten (A), immunogen (B), and coating antigen (C).

2.4 Preparation and purification of anti-Cl-Bisatin-DA antibody

New Zealand White rabbits were selected for the production of polyclonal antibodies against Cl-Bisatin-DA [28]. The specific experimental steps are shown in the supporting materials. In simple terms, the initial immunization was performed by multi-point subcutaneous injection (back and inguinal lymph node region) 1.0 mg/mL immunogen (Hapten-BSA) emulsified with Freund's complete adjuvant. Subsequent booster immunizations used Freund's incomplete adjuvant and were administered every two weeks for a total of six immunizations. Antiserum was collected after bleeding and separated by centrifugation. Polyclonal antibodies were purified by specific adsorption of IgG antibodies using Protein A-Sepharose 4B. Animal experiments were conducted in accordance with the "Regulations for the Administration of Laboratory Animals" issued by the Ministry of Science and Technology of the People's Republic of China.

2.5 Establishment of ic-ELISA

The coating antigen solution (100.0 $\mu\text{L}/\text{well}$) was added to a 96-well plate and incubated at 4 $^{\circ}\text{C}$ overnight. After washing three times with PBST (10 mmol/L phosphate buffer containing 0.05% Tween-20), blocking buffer (200.0 $\mu\text{L}/\text{well}$) was added, and the plate was incubated at 37 $^{\circ}\text{C}$ for 1 h. Following washing, 50.0 μL of the Cl-Bisatin-DA standard solution, 50.0 μL of the metrological calibration solution, and 50.0 μL of the antibody solution were added to the corresponding microwells, respectively. The metrological calibration solution was prepared with PBST (10 mmol/L PBS containing 0.05% Tween-20,

pH 7.4) as the solvent, and the concentration gradient was set as 0, 10.0, 50.0, 100.0, 200.0 ng/mL Cl-Bisatin-DA; the calibration solution was stored at 4 °C and freshly prepared within 72 h for use.

The microplate was incubated at 37 °C for 1 h. After washing again, 100.0 µL of the Ab₂@HRP was added to each well and incubated at 37 °C for 30 mins. After washing, 100.0 µL of substrate solution was added and incubated at 37 °C for 15 mins for color development. The reaction was terminated by adding 50.0 µL of 1.25 mol/L H₂SO₄, and the absorbance value at 450 nm was measured by the multimode microplate reader. The inhibition rate was calculated as follows:

$$\text{Inhibition rate (\%)} = 100 \times (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})$$

Where OD_{control} is the absorbance value without the standard (sample replaced by PBS/buffer); OD_{blank} is the absorbance value with only PBS (no Ab₁, or other appropriate blank). Using Cl-Bisatin-DA concentration as the abscissa and the inhibition rate (%) as the ordinate, a sigmoidal standard inhibition curve was fitted using Origin software. The sensitivity (expressed as the 50% inhibitory concentration, IC₅₀) and the limit of detection (LOD, calculated as IC₁₅ based on the curve fitting) were determined from the fitted equation.

2.6 Optimization of ic-ELISA parameters

To improve the performance of the ic-ELISA, key parameters were optimized based on the detection limit and coefficient of variation, including the concentration of the coating antigen, the coating conditions, the blocking buffer, the incubation time of the Ab₁, the dilution ratio of the Ab₂@HRP, and the color reaction time.

2.7 Specificity evaluation

The structural analog and the functional analog of Cl-Bisatin-DA may affect the specificity of the construction ic-ELISA. Therefore, Cl-Bisatin-DA and its ten analogs, including Oxyphenisatine, phenolphthalein, thymolphthalein, difluorenylidene, orlistat, sennoside B, α-naphtholphthalein, oxyphenisatine diacetate, bisphenol TMC, and bisoxatin acetate, were selected for the specificity evaluation of the ic-ELISA. The cross-reactivity (CR) rate as the evaluation index was calculated as follows:

$$\text{Cross-reactivity (\%)} = 100 \times \text{IC}_{50}(\text{Cl-Bisatin-DA}) / \text{IC}_{50}(\text{analog})$$

Where IC₅₀(Cl-Bisatin-DA) is the concentration of Cl-Bisatin-DA when the inhibition rate is 50.0%, and IC₅₀(analog) is the concentration when the inhibition rate of one analog is 50.0%.

2.8 Application of the ic-ELISA in real samples

Real samples were selected from five common types of commercially available weight-loss products: zero-calorie jelly, solid beverage, enzyme beverage, pressed candy, and fruit vinegar beverage. As complex sample matrix components might interfere with ELISA results, these five types of real samples were pretreated, which integrated homogenization, extraction, centrifugation, and filtration as core operations. In addition, the sample matrices were diluted to different extents to reduce interference from complex components.

Accurately weigh 20.0 g of solid beverage and pressed candy. The solid beverage is directly ground by a high-speed grinder (10,000 r/min, 30 s). The pressed candy is ground into coarse powder with a mortar and further homogenized with a grinder to obtain a uniform and delicate powder. For zero-calorie jelly, 20.0 g of samples were uniformly mixed using a homogenizer (8,000 r/min, 2 min). For liquid samples such as enzyme beverage and fruit vinegar beverage, 20 mL of each sample was placed in a plugged beaker and magnetically stirred at a speed of 300 rpm for 5 mins to ensure complete dispersion. All processed samples were fully mixed before further analysis. Subsequently, each homogenized sample was transferred to a 50.0 mL sealed centrifuge tube, 40.0 mL of methanol was added, and ultrasonic extraction was performed for 15 mins. After extraction, it was centrifuged at 4000 rpm for 5 min at room temperature (25°C). The supernatant was taken to a 50.0 mL volumetric flask, and the inner wall of the centrifuge tube was washed three times with methanol, 5.0 mL each time, and all the flushing fluid was combined into the same volumetric flask, brought to volume and mixed thoroughly. The appropriate amount of supernatant was filtered through a microporous filter membrane, and the methanol-water mixture (1:1, volume ratio) was appropriately diluted according to the actual concentration for subsequent determination.

2.9 LC-MS/MS analysis

The mass spectrometry reference conditions are listed in Tables S1. The content of Cl-Bisatin-DA in the samples was analyzed by LC-MS/MS with detailed parameters described below (modified from the reported method). The Venusil MP C18 column (2.1 mm×150 mm, 3 µm) was employed for the chromatographic separation at a column temperature of 30 °C and a flow rate of 0.4 mL/min. The injection volume was 5 µL. The mobile phase A was 0.1% formic acid aqueous solution and the mobile phase B was acetonitrile. The linear gradient conditions for LC-MS/MS analysis are listed in table S2.

3. Results

3.1 Principle of the immunometrological ic-ELISA

The highly sensitive immunometrological ic-ELISA was successfully constructed based on the prepared anti-Cl-Bisatin-DA antibody (Ab₁). As shown in Figure 2, the immunometrological ic-ELISA detection process is based on the form of antigen-antibody indirect competitive binding reaction, achieving precise quantitative analysis of the target substance. The coating antigen of hapten-OVA was modified in the 96-microplate well through physical adsorption. The coating antigen competed with the added targets for binding to the added Ab₁. As the concentration of Cl-Bisatin-DA increases, the probability of coating antigen-capturing Ab₁ decreases. Subsequently, the Ab₂@HRP was added in the above 96-microplate well. The amount of the complex "hapten-OVA-Ab₁-Ab₂@HRP" decreased with increasing Cl-Bisatin-DA concentrations. Meanwhile, the extent to which the added substrate TMB was catalyzed by HRP to oxidized TMB was weakened, and the absorbance value (OD₄₅₀) corresponding to oxidized TMB was reduced. Furthermore, the inhibition rate showed an upward trend with the increase of Cl-Bisatin-DA concentrations. To reduce the interference of the external environment and the operational errors among personnel, the

metrological calibration solution module was introduced in this ic-ELISA. In other words, the positive corrected microwells and the negative sample microwells were operated simultaneously while the standard curve was established by adding different Cl-Bisatin-DA concentrations. Based on the proportional relationship between the detected value of the positive sample concentration and the actual set value (10.0 ng/mL and 50.0 ng/mL), the detected Cl-Bisatin-DA concentration for constructing the standard curve was corrected, and then the standard curve was constructed to improve to enhance the precision and accuracy of detecting Cl-Bisatin-DA.

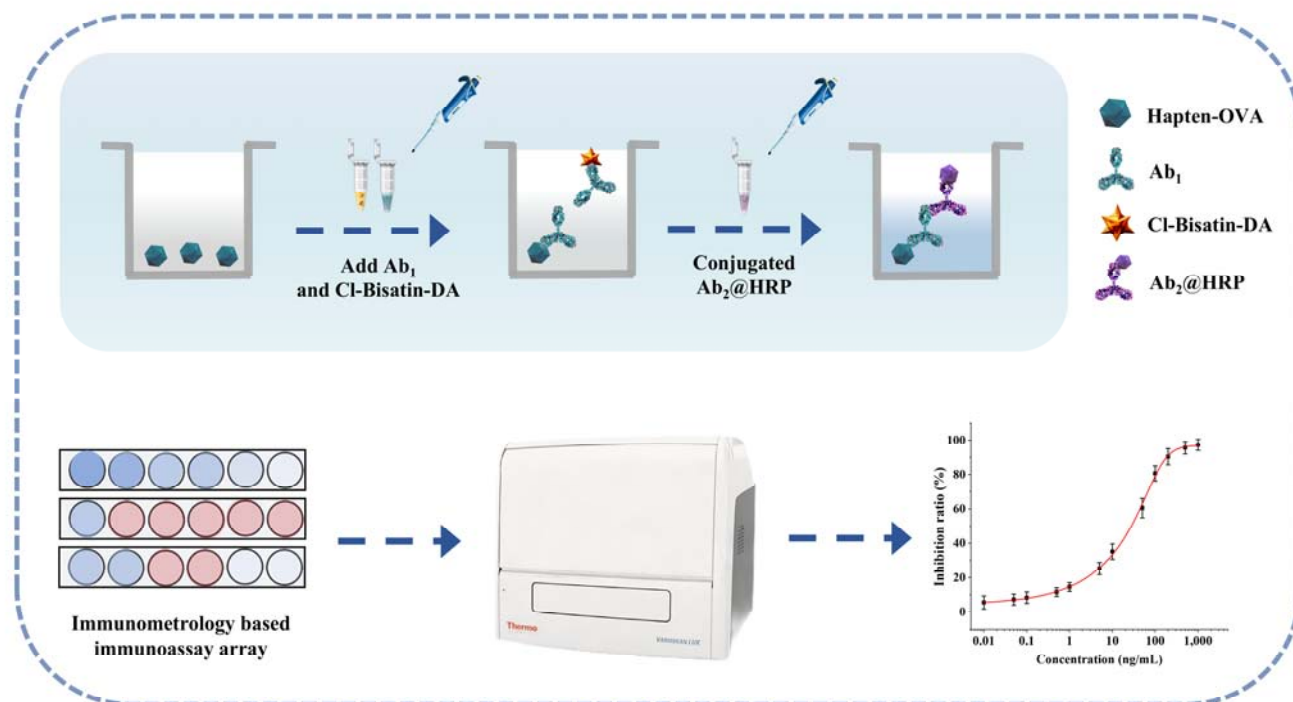


Figure 2. Scheme of the immunometrological ic-ELISA detection process for Cl-Bisatin-DA.

3.2 Characterization of the hapten, immunogen and coating antigen

The successful preparation of hapten and immunogens was crucial for the production of Ab_1 . Mass spectrometry was used to analyze the Cl-Bisatin-DA hapten. The theoretical molecular weight of the Cl-Bisatin-DA hapten was 441.07 Da (dalton). The mass spectrometric analysis revealed that the mass-to-charge (m/z) ratio of the prepared Cl-Bisatin-DA hapten was 442.85 Da (Figure S1), indicating a slight discrepancy from the theoretical molecular weight. The main cause of this phenomenon was the presence of two chlorine isotopes with atomic masses of 34.97 Da and 36.97 Da respectively, which were manifested as two molecular ion peaks at the mass-to-nucleus ratios of 155.05 Da and 157.05 Da in the mass spectrum, respectively. Therefore, it can be demonstrated that Cl-Bisatin-DA hapten has been successfully prepared. Subsequently, the peak changes in the absorption spectra of BSA, Cl-Bisatin-DA, and hapten-BSA obtained by the ultraviolet spectrophotometer can also prove the successful preparation of immunogen (Figure S2). The subsequent experimental results also confirmed the successful preparation of the hapten and immunogen of Cl-Bisatin-DA.

3.3 Parameters optimization of ic-ELISA

The accuracy, sensitivity and repeatability of the enzyme-linked immunosorbent assay (ELISA) results are highly dependent on the precise control of experimental conditions. Therefore, in this study, the concentrations of the coating antigen, the coating conditions, the blocking buffer, the incubation time of Ab₁, the dilution ratio of Ab₂@HRP, and the time of color reaction were optimized, with the results presented in Figure 3.

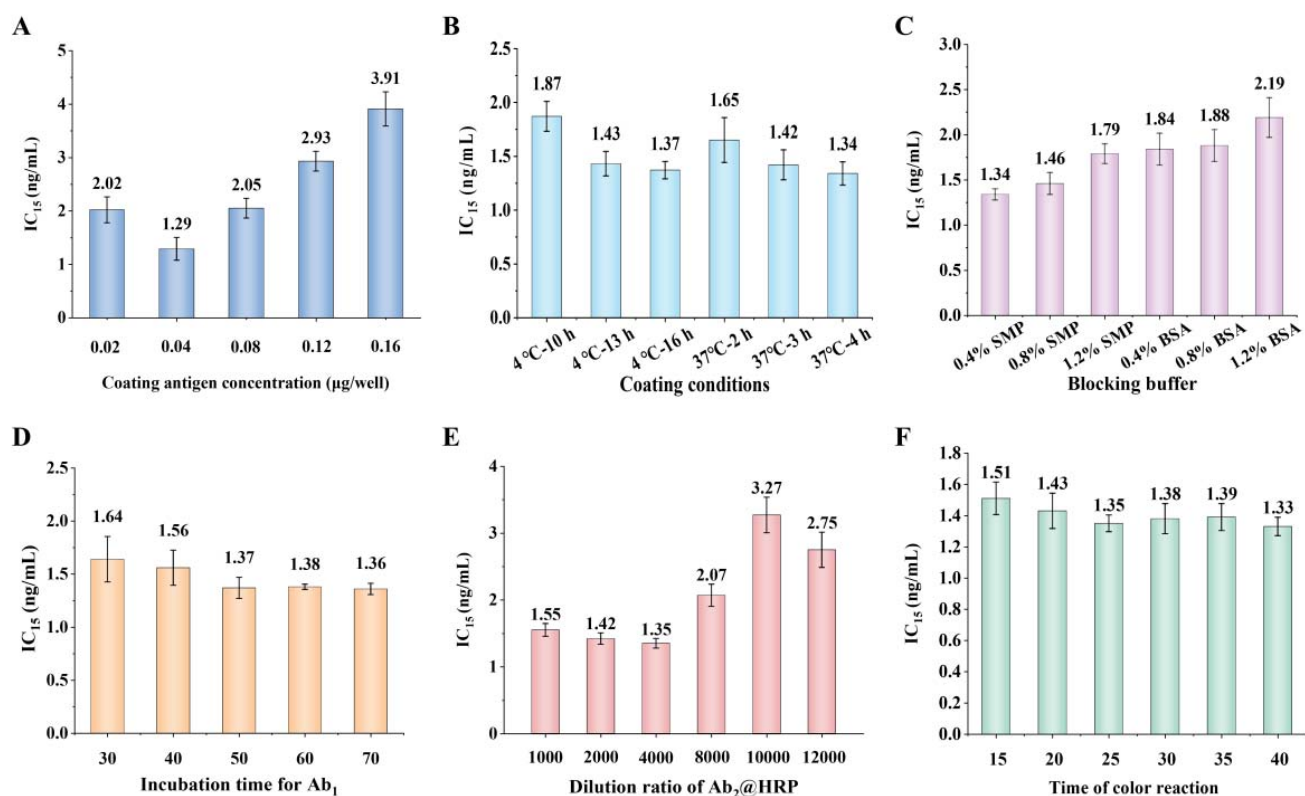


Figure 3. Optimization of working conditions of ic-ELISA. Optimization of coating antigen concentration (A), coating conditions (B), the blocking buffer (C), the incubation time for Ab₁ (D), the dilution ratio of Ab₂@HRP (E), and time of color reaction (F).

3.3.1 Coating antigen concentration

Coating antigen concentration directly affects the binding amount of the solid-phase carrier and antibody. A lower concentration of the coating solution results in less binding of antibody, leading to a weaker signal and lower sensitivity. As shown in Figure 3A, we first optimized the concentration of the coating antigen (0.02 µg/well, 0.04 µg/well, 0.08 µg/well, 0.12 µg/well, 0.16 µg/well). When the concentration of coating antigen was greater than 0.04 µg/well, the IC₁₅ increased with the increase of the concentration of coating antigen. When the concentration of the coating antigen was 0.04 µg/well, the detection limit (IC₁₅) reached the minimum value of 1.29 ng/mL. Considering all these factors, the optimal concentration of coating antigen was determined to be 0.04 µg/well.

3.3.2 Different coating conditions

The coating process is a physical adsorption procedure. The temperature and time determine whether the coated molecules can be uniformly and stably fixed on the carrier surface and maintain the "active conformation" that can bind to the subsequent antibodies. Low temperature and short time will lead to insufficient binding of coating molecules to the carrier, while too high temperature and too long time may lead to denaturation of coating molecules. It can be seen from Figure 3B that when the coating condition was 4 °C 16 h, this ic-ELISA has the lowest detection limit, which was 1.37 ng/mL. Accordingly, the coating conditions of 4 °C for 16 h was selected for subsequent experiments.

3.3.3 Blocking buffer

After being coated, there are still "empty sites" on the surface of the carrier that have not been occupied by the coated molecules. The function of the sealing solution is to fill these empty sites to prevent non-specific adsorption of subsequent antibodies. If the blocking buffer is not chosen properly or is not applied adequately, it will result in a low signal-to-noise ratio. Excessive blocking reduced the reaction signal by competing for antibody-binding sites, while insufficient blocking (e.g., 0.1% skim milk powder) increased non-specific adsorption. As shown in Figure 3C, the detection limit of the ic-ELISA using SMP as the blocking buffer was lower than that of the experimental group using BSA as the blocking buffer. When 0.4% skim milk powder was used as the blocking buffer, the detection limit and coefficient of variation of ic-ELISA were the lowest, which were 1.34 ng/mL and 4.60%, respectively. Therefore, 0.4% skim milk powder was selected as the best blocking buffer.

3.3.4 Incubation time of Ab_1

Since Ab_1 can specifically bind to the coating antigen, we also need to optimize the incubation time of Ab_1 . If the incubation time of Ab_1 is too short, the binding between it and the antigen is insufficient, resulting in a decrease in the sensitivity of the detection. If the incubation time is too long, it will increase the non-specific binding of Ab_1 . Within 50 mins, the detection limit decreased significantly with the increase of time, and there was no significant change in the detection limit after 50 mins ($P > 0.05$). Therefore, the incubation time of the Ab_1 was finally determined to be 50 mins, (Figure 3D).

3.3.5 Dilution ratio of $Ab_2@HRP$

Excessive concentration of $Ab_2@HRP$ will result in a large amount of unbound $Ab_2@HRP$ remaining, which further leads to an increase in false positive reactions. Insufficient concentration of $Ab_2@HRP$ will result in a low binding amount and insufficient sensitivity. Therefore, the dilution ratio of the $Ab_2@HRP$ needs to be further optimized. As shown in Figure 3E, when the dilution factor of the $Ab_2@HRP$ was 1:4000, the detection limit and coefficient of variation of the ic-ELISA were the lowest, which were 1.35 ng/mL and 5.27%, respectively. At this point, the performance of the ic-ELISA was the best, consequently, the dilution ratio of the $Ab_2@HRP$ was finally determined to be 4000.

3.3.6 Time of color reaction

The color reaction time will affect the accuracy and repeatability of the results. If the incubation time of the color reaction is insufficient and the reaction between the enzyme and the substrate is not sufficient, the output signal will be weak. If the color reaction time is too long, it may lead to the increase of background signal and false positive reaction. Therefore, this experiment optimizes the color reaction time. As shown in Figure 3F, the results show that when the time of color reaction was 25 mins, this ic-ELISA has the highest sensitivity and the best measurement effect.

3.4. Detection performance evaluation

Under the optimal conditions of 0.04 µg/well in the coating solution, 4 °C for 16 hours for coating, 0.4% skim milk powder as the blocking buffer, incubation time of the Ab₁ for 50 mins, dilution ratio of the Ab₂@HRP at 4000 times, and incubation time of color reaction for 25 mins, this study developed an ic-ELISA to detect different concentrations of Cl-Bisatin-DA. The standard inhibition curve of Cl-Bisatin-DA was plotted (Figure 4A). As shown in the figure, the curve is a typical S-shaped curve. The detection limit (IC₁₅) and sensitivity (IC₅₀) were 1.07 ng/mL and 27.67 ng/mL, respectively, indicating that the ic-ELISA has high sensitivity.

The core objective of the ELISA stability test is to verify the reliability, repeatability and long-term effectiveness of the detection results, providing data support for the standardized application of the ELISA (such as quantitative analysis in scientific research and development of kits). Therefore, this experiment evaluated the stability of the developed ELISA, and the experimental results are shown in Figure 4B, and the specific data are shown in Table S3. Standard samples of different concentrations were randomly tested six times within a day or over several days. The results indicated that there was no significant difference in the intra-day and inter-day stability test results of the standard samples at the same concentration, suggesting that the proposed ELISA has good stability (Figure 4C). As shown in Figure 4D, the intra-day spiked recovery rate was 90.2%-102.2%, with a coefficient of variation (CV) of less than 4.12%. The inter-day spiked recovery rate was 83.0%-100.5%, with a CV less than 6.72%. Therefore, the stability and repeatability of this ELISA are acceptable.

To reduce the interference from non-target components, this experiment employed the dilution method to eliminate the interference from the matrix. The PBS solution group was used as the control system. By comparing the signal differences between the PBS group and the sample group, it was determined whether the interfering substances in the matrix had been effectively removed, ensuring the specificity of the detection. The results are shown in Figure 4D. When the dilution factor was greater than ten times, the signal value of the sample group was close to that of the PBS group, indicating that the interference from the matrix components had been successfully eliminated and the subsequent detection could be continued.

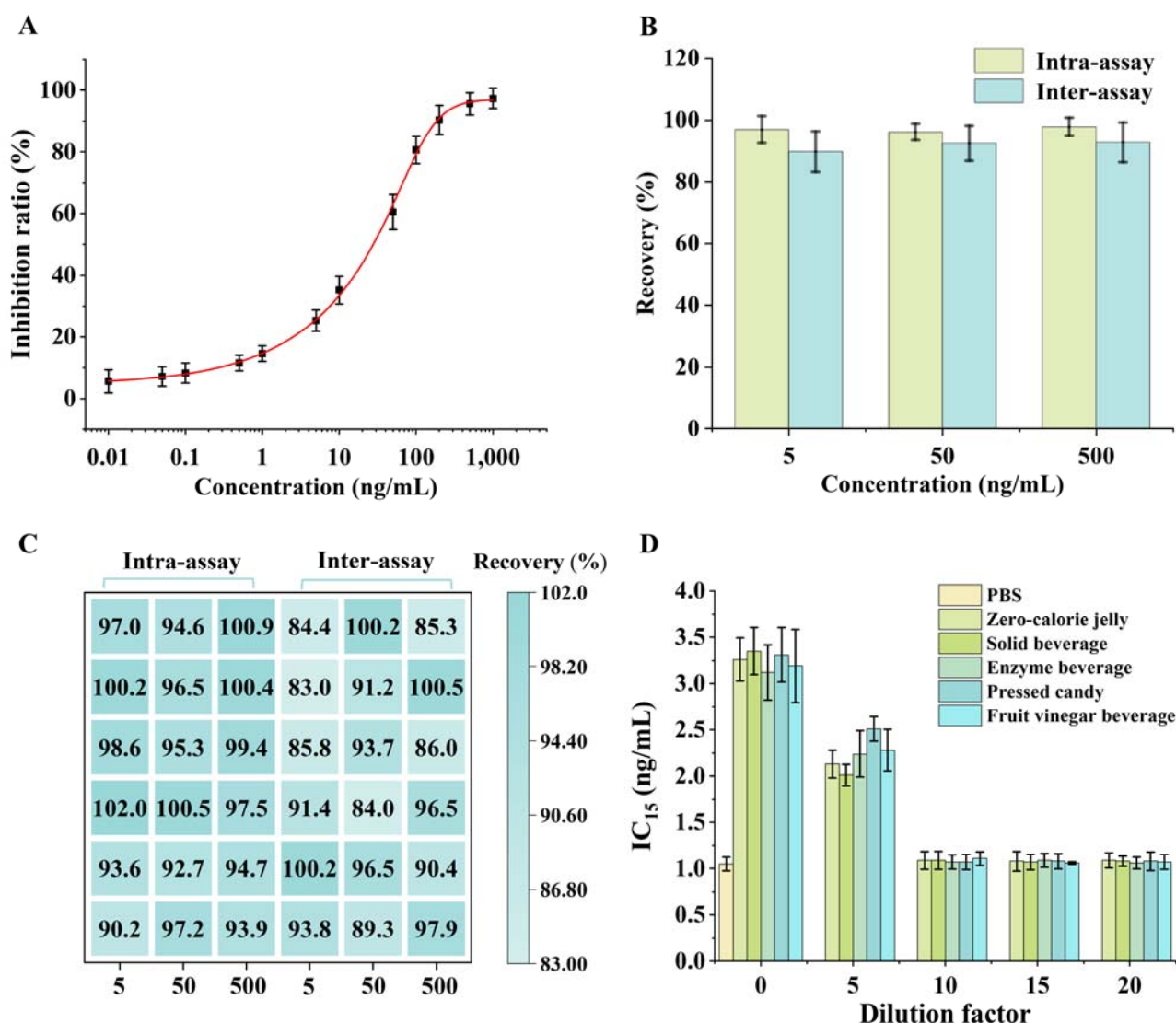
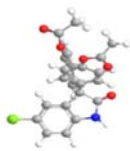
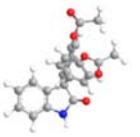
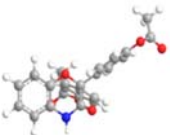
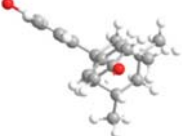
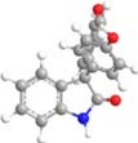
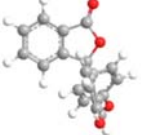
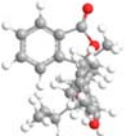
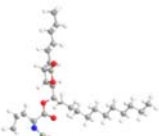
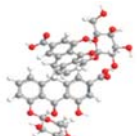


Figure 4. Performance evaluation of ic-ELISA for Cl-Bisatin-DA detection. (A) Standard inhibition curve; (B) Intra-day and inter-day inhibition rate results; (C) Intra-day and inter-day spiked recovery rates; (D) Matrix interference elimination by dilution.

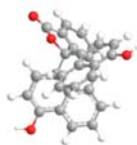
3.5. Determination of cross-reactivity

To evaluate the specificity of the developed ic-ELISA for Cl-Bisatin-DA detection, substances structurally or functionally similar to Cl-Bisatin-DA (i.e., sharing weight-loss effects) were selected for cross-reactivity testing, the specific results are listed in Table 1. As shown in Table 1, the structures of oxyphenisatine, oxyphenisatine diacetate, bisoxatin acetate, and phenolphthalein are all similar to Cl-Bisatin-DA. Additionally, the specific structural diagrams of six other functionally similar substances (thymolphthalein, difluorenylidene, bisphenol TMC, orlistat, sennoside B, and α -naphtholphthalein) were provided. The results indicate that the IC₅₀ of Cl-Bisatin-DA is 27.67 μ g/L. Moreover, the cross-reactivity rates for oxyphenisatine diacetate, oxyphenisatine, phenolphthalein, bisoxatin acetate, thymolphthalein, difluorenylidene, bisphenol TMC, orlistat, sennoside B, α -naphtholphthalein were all less than 0.2%, indicating that the prepared antibody possesses high specificity for Cl-Bisatin-DA and this ic-ELISA can be used for the specific detection of Cl-Bisatin-DA.

Table 1. Results of cross-reactivity test (n=3)

Number	Variety	Structural formula	IC ₅₀ (ng/mL)	Cross reaction rate (%)
0	4-Chloro-oxyphenisatin diacetate		27.67	100
1	Oxyphenisatine		>20000	<0.2
2	Bisoxatin acetate		>20000	<0.2
3	Bisphenol TMC		>20000	<0.2
4	Oxyphenisatine diacetate		>20000	<0.2
5	Phenolphthalein		>20000	<0.2
6	Thymolphthalein		>20000	<0.2
7	Difluorenylidene		>20000	<0.2
8	Orlistat		>20000	<0.2
9	Sennoside B		>20000	<0.2

10

 α -naphtholphthalein

>20000

<0.2

3.6. Application evaluation in real samples

This developed ic-ELISA was applied to the detection of target substance in five types of weight loss foods (zero-calorie jelly, solid beverage, enzyme beverage, pressed candy, and fruit vinegar beverage) for application evaluation. The sample pretreatment process is shown in Figure 5A. The pretreatment of the five samples was carried out in accordance with the steps in Section 2.8. No target was detected in the actual samples purchased. Spiking experiments were performed at different levels on zero-calorie jelly, solid beverage, enzyme beverage, pressed candy, and fruit vinegar beverage. The final spiked concentrations were 20.0 ng/mL, 100.0 ng/mL and 500.0 ng/mL. The concentrations of the different levels added to the zero-calorie jelly were detected as 17.5 ng/mL, 95.4 ng/mL, and 448.8 ng/mL, and the concentrations detected by the liquid chromatography-mass spectrometry method were 18.4 ng/mL, 94.1 ng/mL, and 499.4 ng/mL (Table S4). Based on the detected results of this ic-ELISA and LC-MS method, it can be found that the recovery rates of zero-calorie jelly range from 81.8% to 105.2% with a coefficient of variation of 6.76%, and from 85.9% to 100.6% with a coefficient of variation of 5.64%, respectively (Figure 5B). As shown in Figures 5C-F, based on the detection results of this developed ic-ELISA (Table S4), the recovery rates in zero-calorie jelly, solid beverage, enzyme beverage, pressed candy, fruit vinegar beverage ranged from 78.1% to 106.6%, and the coefficient of variation was 7.49% to 9.06%. Based on the detected results of LC-MS, the recovery rates in the above-mentioned samples ranged from 83.8% to 105.9% (Figures 5C to 5F), with a coefficient of variation was 5.79% to 6.15%. The detection results of this developed ic-ELISA and LC-MS were linearly fitted, with the correlation equation being $y=1.0906x-1.4079$, and the corrected R^2 was 0.9984 (Figure S3). The above detected results indicate that the constructed ic-ELISA has acceptable reliability, accuracy and repeatability. It can be used as a detection tool for the rapid detection of Cl-Bisatin-DA substances in weight-loss foods products.

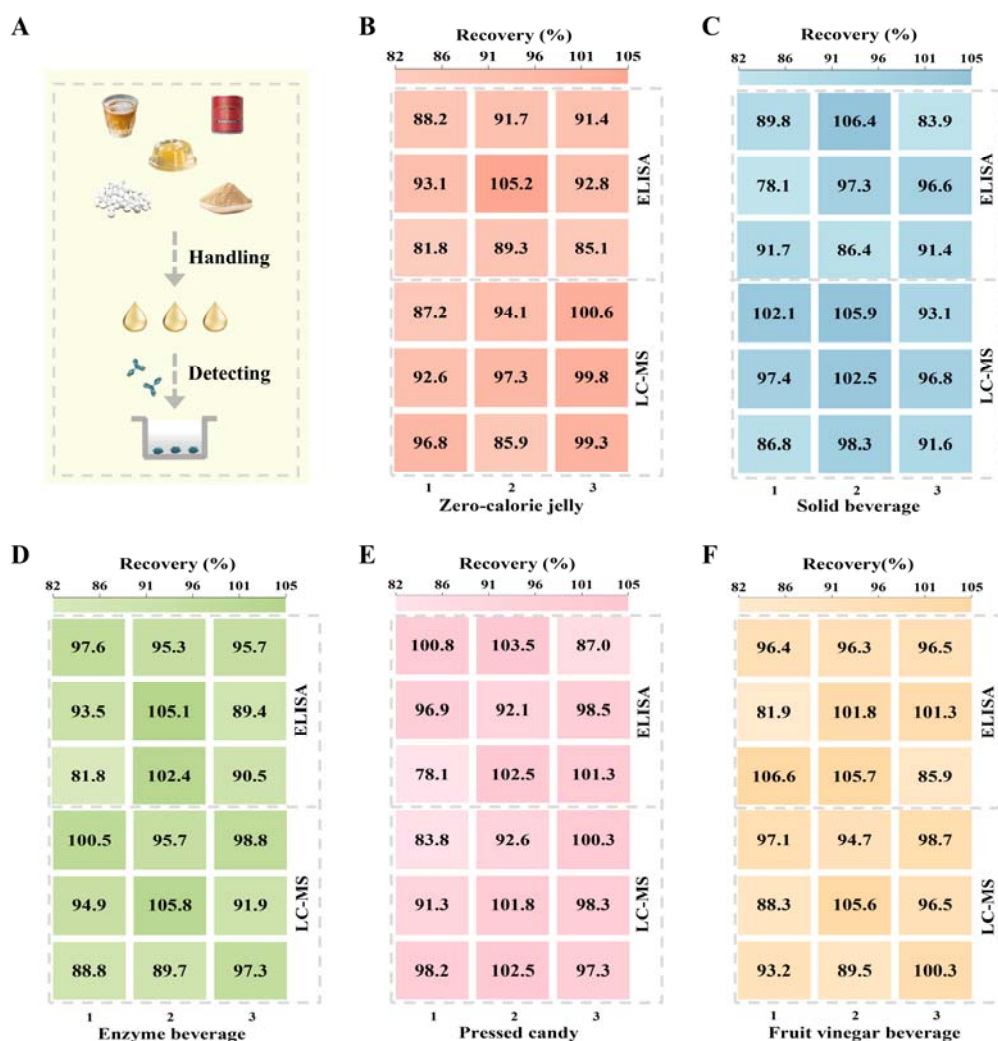


Figure 5. Pretreatment process of samples (A), recoveries of Cl-Bisatin-DA in zero-calorie jelly (B), solid beverage (C), enzyme beverage (D), pressed candy (E), fruit vinegar beverage (F) performed using the ic-ELISA (The numbers 1, 2, and 3 respectively represent the concentrations of Cl-Bisatin-DA at 20.0 ng/mL, 100.0 ng/mL, and 500.0 ng/mL).

4. Discussion

An efficient ic-ELISA based on prepared antibody has been developed to meet the detection requirements of illegal additive Cl-Bisatin-DA in weight loss foods products. This developed ic-ELISA demonstrates significant advantages, with a detection limit as low as 1.07 ng/mL and a detection range of 2.3-1000 ng/mL, providing a reliable tool for trace analysis in complex matrices. A multi-dimensional performance comparison (Table S5) was performed with the reported detection methods of Cl-Bisatin-DA and its analogues (HPLC, LC-HRMS/MS, SERS), and the core advantages of the developed ic-ELISA in the on-site rapid screening scenario were clarified. Compared with chromatography / mass spectrometry, ic-ELISA has the advantages of simple operation, low instrument cost, wide linear range and strong field applicability. Through the evaluation of stability and repeatability, the CV value of the intra-day and inter-day of this ic-ELISA was less than 4.12% and 6.72%, respectively, confirming the stable performance of the developed ic-ELISA during long-term storage and repeated use, and ensuring the consistency of results among laboratories. The specific test further verified the precise recognition ability of the developed ic-ELISA for Cl-Bisatin-DA. The cross-reaction rate (CR) of this developed ic-ELISA for ten structural or functional

analogs was less than 0.2%, which effectively excluded common interfering substances (e.g., phenolphthalein, oxyphenisatine) and significantly reduced the risk of false positivity. In practical applications, the constructed ic-ELISA was successfully applied to the detection of Cl-Bisatin-DA in five spiked weight loss foods samples, with spiked recovery rates ranging from 76.1% to 106.6%, which were highly consistent with the LC-MS detection results ($R^2=0.9984$), efficiently screening out Cl-Bisatin-DA and highlighting its practicality and promotion potential in food safety supervision.

In recent years, detection strategies based on novel functional materials, including chiral nanomaterials, rare metals, metal-organic frameworks (MOFs), and related organic-inorganic hybrid frameworks [29-31], have undergone rapid development in the monitoring of illegal food additives. These methods based on novel materials have the advantages of ultra-high sensitivity and fast response, and can be used as complementary technologies for the developed ic-ELISA in the future. The combination of the developed antibody with these novel nanosensor technologies will be the focus of future research, which can further expand the application scope of Cl-Bisatin-DA detection methods. Despite the excellent performance of ic-ELISA, the combination of multiple techniques or expanded sample validation still needs to be explored in future studies to enhance the universality of the constructed ic-ELISA. For example, the developed ic-ELISA can be further validated in more types of weight-loss foods (e.g., meal replacement shakes, weight-loss tea) and actual contaminated samples; the preparation of monoclonal antibodies against Cl-Bisatin-DA can further improve the specificity and stability of the antibody; the development of a portable ic-ELISA kit can realize the on-site rapid screening of Cl-Bisatin-DA by food supervision personnel without professional laboratory conditions. Overall, this study provides an innovative solution for the monitoring of illegal additive Cl-Bisatin-DA in weight-loss foods, and has profound significance for promoting industry standardization and public food safety.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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