Electronic Supplementary Information

Highly Sensitive Flow Injection Chemiluminescence Method for Myoglobin Detection in Beef Samples Based on Zinc Ion-Induced "Dragging" of Heme Iron

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Experimental Section

Instruments

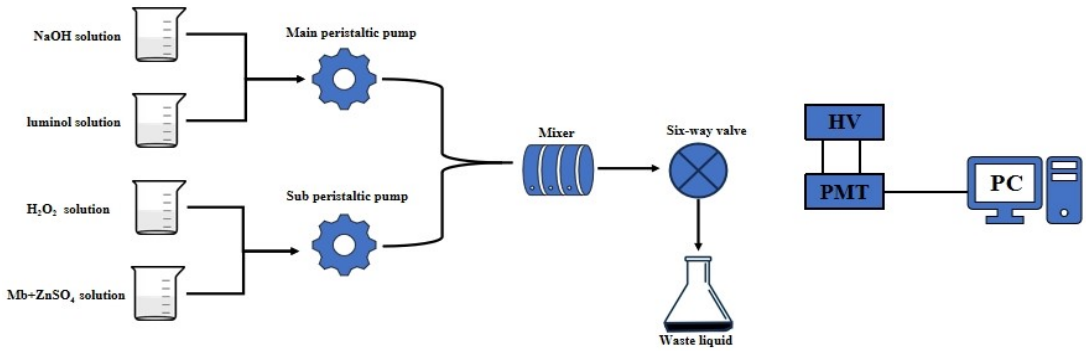
A flow injection chemiluminescence analyzer (IFFM-E, Xi'an Ruimai Analytical Instrument Co., Ltd.), a double-beam UV-vis spectrophotometer (Shanghai Yuanxi Instrument Co., Ltd.), an automatic triple-distilled water system (SZ-97A, Shanghai Yarong Biochemical Instrument Factory), a high-speed refrigerated centrifuge (TGL-16, Changsha Xiangyi Centrifuge Instrument Co., Ltd.), and a vortex mixer (QL-901, Haimen Kylin-Bell Lab Instruments Co., Ltd.) were used.

Reagents

Sodium hydroxide (NaOH, 1.0 $mol \cdot L^{-1}$) and zinc sulfate ($ZnSO_4$, 0.1 $mol \cdot L^{-1}$) stock solutions were prepared in ultrapure water; hydrogen peroxide (H_2O_2, 30% w/w) was used as received. Luminol stock solution (5×10^{-2} $mol \cdot L^{-1}$) was prepared in 0.1 $mol \cdot L^{-1}$ NaOH and stored at 4 °C in the dark. Tris-EDTA (TE) buffer solution (10 $mmol \cdot L^{-1}$ Tris-HCl, 1 $mmol \cdot L^{-1}$ EDTA, pH 8.0) was prepared for dilutions. Myoglobin (Mb) stock solution and buffer solution (0.2 $mol \cdot L^{-1}$ NaH_2PO_4-Na_2HPO_4, pH 7.0) were used. The myoglobin aptamer (Mb aptamer, 5′-CCC TCC TTT CCT TCG ACG TAG ATC TGC TGC GTT GTT CCG A-3′) was purchased from Sangon Biotech (Shanghai) Co., Ltd. The aptamer was centrifuged, serially diluted with TE buffer to appropriate concentrations, and then stored at −20 °C. All other solutions were freshly prepared by diluting stock solutions as required.

Procedure of Flow Injection Chemiluminescence (FICL)

The four solutions participating in the chemiluminescence (CL) reaction—luminol, NaOH, H_2O_2, and a mixture of the sample Mb (or blank solution) with $ZnSO_4$ solution—are introduced into the four channels of a FICL analyzer. NaOH and luminol are first mixed, followed by mixing with H_2O_2 and the sample/blank-$ZnSO_4$ mixture via a six-way valve and passed into the detector for CL signal measurement. Excess waste liquid is expelled from the opposite end of the six-way valve before detection. The schematic diagram is provided in Scheme S1.



Scheme S1 Schematic diagram of the FICL system for Mb detection

Optimization of CL Reaction Conditions

The concentrations of luminol, NaOH, H_2O_2, and $ZnSO_4$ were systematically optimized using the ratio of the Mb sample CL signal to the blank serving as the evaluation metric (denoted as "*Times*").

Optimization of Luminol Concentration

In the study of the effect of luminol concentration on the CL intensity, all other reagent concentrations were kept constant, including H_2O_2 at a mass fraction of 0.03%, NaOH at 0.01 mol·L^{-1}, Zn^{2+} at 5 × 10^{-5} mol·L^{-1}, Mb at 6.25 × 10^{-10} mol·L^{-1}, and the buffer solution at 5.0 × 10^{-4} mol·L^{-1}. The luminol concentration varying from 1.25 × 10^{-5} to 8.75 × 10^{-5} mol·L^{-1} on CL intensity is illustrated in Fig. S1. The maximum "*Times*" value was achieved at a luminol concentration of 5.0 × 10^{-5} mol·L^{-1}, which was selected as the optimal concentration for further experiments.

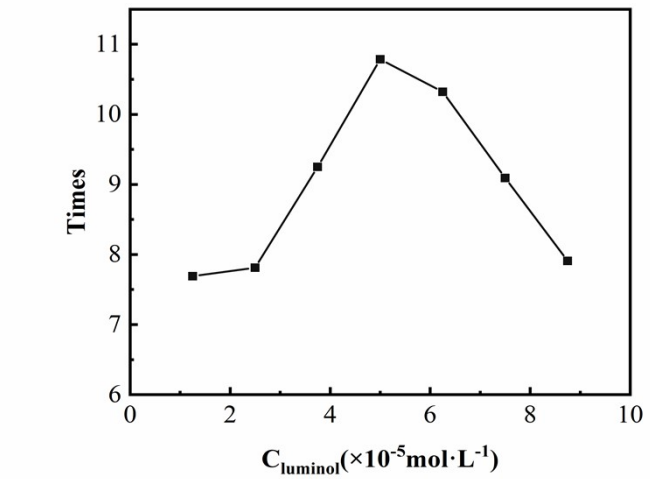


Fig. S1 Effect of luminol concentration on the CL intensity.

Optimization of NaOH Concentration

In the study of the effect of NaOH concentration on the CL intensity, all other reagent concentrations were kept constant, including H_2O_2 at a mass fraction of 0.03%, luminol at 5.0 × 10^{-5} mol·L^{-1}, Zn^{2+} at 5 × 10^{-5} mol·L^{-1}, Mb at 6.25 × 10^{-10} mol·L^{-1}, and the buffer solution at 5.0 × 10^{-4} mol·L^{-1}. The impact of NaOH concentration varying from 0.005 to 0.025 mol·L^{-1} on CL intensity is shown in Fig. S2. The maximum "*Times"* value was achieved at a NaOH concentration of 0.01 mol·L^{-1}, which was selected as the optimal NaOH concentration.

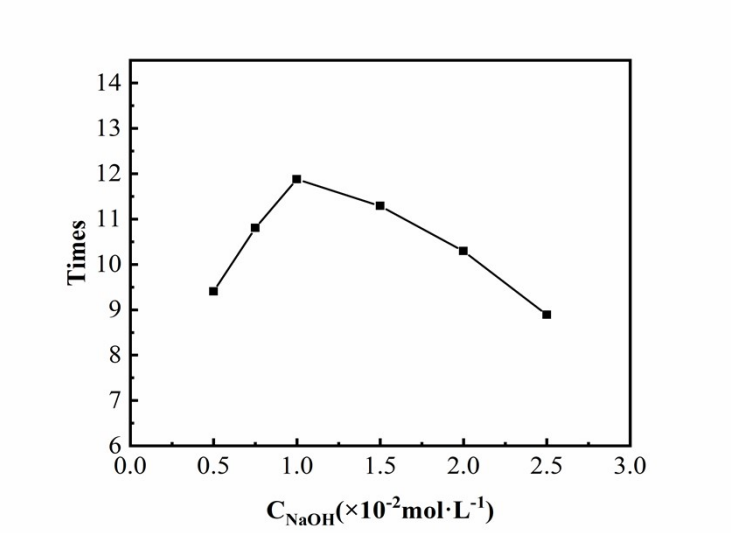


Fig. S2 Effect of NaOH concentration on the CL intensity.

Optimization of H_2O_2 Concentration

In the study of the effect of H_2O_2 concentration on the CL intensity, all other reagent concentrations were kept constant, including luminol at 5.0 × 10^{-5} mol·L^{-1}, NaOH at 0.01 mol·L^{-1}, Zn^{2+} at 5 × 10^{-5} mol·L^{-1}, Mb at 6.25 × 10^{-10} mol·L^{-1}, and the buffer solution at 5.0 × 10^{-4} mol·L^{-1}. The influence of H_2O_2 mass fraction varying from 0.015% to 0.045% on CL intensity is presented in Fig. S3. The highest "*Times"* value was achieved at 0.03% H_2O_2, establishing this as the optimal mass fraction.

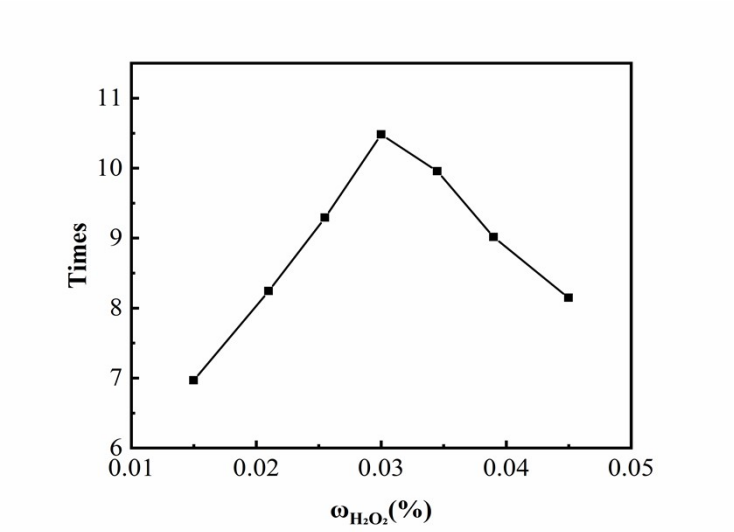


Fig. S3 Effect of H_2O_2 concentration on the CL intensity.

Optimization of $ZnSO_4$ Concentration

In the study of the effect of $ZnSO_4$ concentration on the CL intensity, all other reagent concentrations were kept constant, including H_2O_2 at a mass fraction of 0.03%, luminol at 5.0×10^{-5} mol·L^{-1}, NaOH at 0.01 mol·L^{-1}, Mb at 6.25×10^{-10} mol·L^{-1}, and the buffer solution at 5.0×10^{-4} mol·L^{-1}. The effect of $ZnSO_4$ concentration ranging from 2.0×10^{-5} to 8.0×10^{-5} mol·L^{-1} on CL intensity is depicted in Fig. S4. The maximum "*Times*" was achieved at a $ZnSO_4$ concentration of 5.0×10^{-5} mol·L^{-1}, confirming its suitability for subsequent experiments.

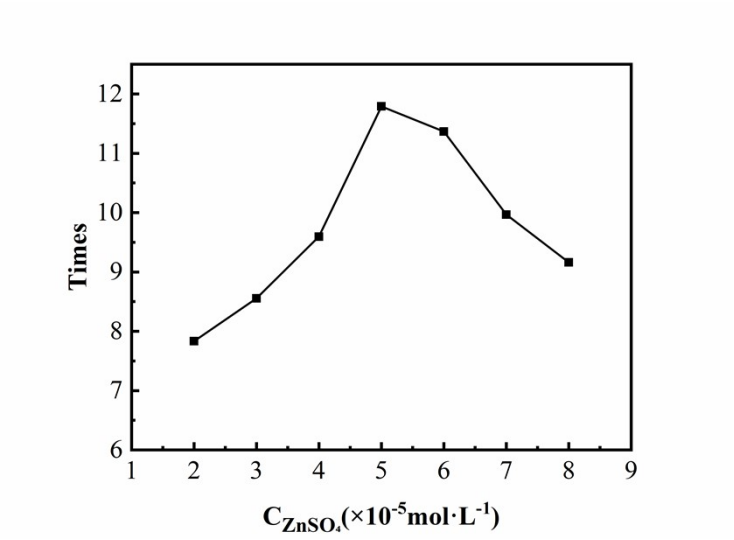


Fig. S4 Effect of $ZnSO_4$ concentration on the CL intensity.

Linear Relationship of Mb Under Optimized Conditions

The CL intensity has a good linear relationship with the concentration of Mb from 3.125×10^{-11} to 2.5×10^{-9} mol·L^{-1} with a correlation coefficient (R^2) of 0.9908 (Fig. S5). The linear equation is $I = 1.85987C - 0.130617$ (I is the CL intensity; C is the concentration of Mb).

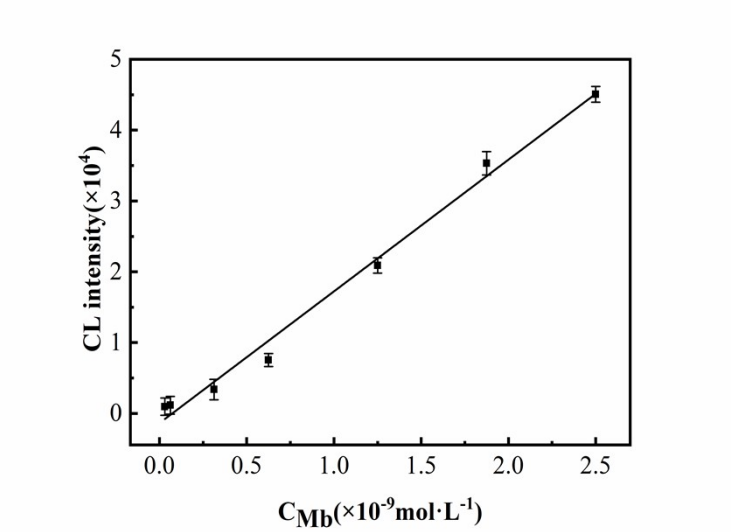


Fig. S5 Calibration curve of the CL intensity versus the concentration of Mb.

Sample Preparation

Pretreatment of Meat Sample

Fat and connective tissues were removed from the meat sample, and residual blood was rinsed with normal saline. The processed meat samples were finely chopped, and an accurate weight of 2.000 g was transferred to a 10 mL centrifuge tube, followed by adding 5 mL of TE buffer solution. The mixture was vortexed at high speed on a mini vortex mixer and then centrifuged at 10,000 rpm for 20 min at 0 °C. The supernatant was collected; the precipitate was added with 3 mL of buffer solution, vortexed again, and centrifuged under the same conditions for 10 min. The supernatants from both centrifugation steps were combined. The pH was adjusted to 7.0 using 1 mol·L^{-1} and 0.1 mol·L^{-1} HCl solutions and the final volume was diluted to 10 mL with ultrapure water. The resulting solution was filtered through a 0.40 μm hydrophilic cellulose membrane and used as the original sample.

Sample Preparation for Total Hemoprotein Determination

The original sample was diluted 5-fold; 10 μL of the diluted solution was added to 490

μL of buffer solution, followed by 500 μL of $ZnSO_4$ solution. After thorough mixing, 500 μL of the mixture was diluted to a final volume of 100 mL to obtain a sample for detecting total hemoprotein concentration.

Sample Preparation for Mb Determination

The original sample was also diluted 5-fold; 10 μL of the diluted sample was mixed with 460 μL of buffer solution and 30 μL of an aptamer solution (0.1 $\mu mol \cdot L^{-1}$). After mixing, 500 μL of $ZnSO_4$ solution was added. The mixture was then thoroughly mixed, and 500 μL of it was diluted to a final volume of 100 mL to obtain a sample for detecting Mb concentration.

Method Validation

Table S1 Recovery and relative standard deviation of total hemoprotein in samples

No.	Amount detected ($\times 10^{-10}/mol \cdot L^{-1}$)	Amount Added ($\times 10^{-10}/mol \cdot L^{-1}$)	Measured amount ($\times 10^{-9}/mol \cdot L^{-1}$)	Recovery %	Mean Recovery %	RSD%
1			1.038	98.10		
2	4.249	6.250	1.036	97.78	98.42	0.86
3			1.046	99.38		
4			1.321	95.58		
5	4.249	9.375	1.325	96.01	95.69	0.29
6			1.320	95.48		
7			1.697	101.8		
8	4.249	12.50	1.702	102.2	102.3	0.55
9			1.711	102.9		