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Analytical Methods

Fluorometric determination of hydrogen peroxide in milk by using a Fenton reaction system

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ABSTRACT

A simple and highly sensitive fluorometric method is proposed for the determination of H_2O_2 in milk samples. In this method, non-fluorescent coumarin is oxidised to highly fluorescent 7-hydroxycoumarin by hydroxyl radicals (·OH) generated in a Fenton reaction, and the oxidation product has strong fluorescence with a maximum intensity at 456 nm and can be used as a fluorescence probe for H_2O_2. Under the optimal conditions (2.5×10^{-4} mol L^{-1} iron(II) ions, 4.0×10^{-4} mol L^{-1} coumarin, solution pH 3.0, reaction time 9 min, and excitation at 346 nm), the proposed method presents wide linear responses between the fluorescence intensity and H_2O_2 concentration in a wide range from 2.0×10^{-8} to 2.0×10^{-5} mol L^{-1}, with a detection limit ($S/N = 3$) of 5.0×10^{-9} mol L^{-1}. After possible interferences are evaluated for a series of chemical substances, the present method has been applied to the determination of hydrogen peroxide in milk with satisfactory results.

1. Introduction

Hydrogen peroxide (H_2O_2) is widely used in the fields of foods, pharmaceuticals, dental products, textiles, environmental protection, and it is also involved in advanced oxidation processes (AOPs) and various biochemical processes (Demirkol, Mehmetoglu, Qiang, Ercal, & Adams, 2008; Luo, Abbas, Zhu, Deng, & Tang, 2008; Nogueira, Oliveira, & Paterlini, 2005; Zhang, Mao, & Cai, 2000). In many countries, H_2O_2 has been accepted as a food additive of controlling the growth of microorganisms, bleaching (Toyoda, Ito, Iwaida, & Fujii, 1982), removing glucose from dried eggs and controlling microbial growth in stored milk before cheese-making (EU Risk Assessment Report, 2003).

Although H_2O_2 is the primary chemical for sterilization of plastic packaging material used in aseptic systems, the FDA regulations specify that a maximum concentration of 35% (w/w) H_2O_2 may be used for sterilizing food contact surface because the use of H_2O_2 at high concentrations is unfavourable to the lowering of the residual H_2O_2. In a properly designed aseptic packaging system a good microbicidal effect using H_2O_2 can be achieved and the level of residue can also be effectively controlled (Ansari & Datta, 2003; Hanway, Hansen, Anderson, Lyman, & Rushing, 2005). H_2O_2 is approved in the USA for treating milk and its weight cannot exceed 0.05% of the milk weight. Because an excess of residual H_2O_2 is harmful to humans, the level of residue is required to be effectively controlled within permissible limits. For example, the Ministry of Health and Welfare of Japan forces that H_2O_2 has to be either decomposed or removed from the final products (Toyoda et al., 1982). The FDA regulation limits residual H_2O_2 to 0.5 mg L^{-1} in finished food packages (Özkan, Kırca, & Cemeroğlu, 2004). In a national survey made in the USA, zero residues were reported in most foods after treatment with H_2O_2 (EU Risk Assessment Report, 2003).

It is known that H_2O_2 creates serious problems (Chen, Yu, Zhou, & Wang, 2007). H_2O_2 is extremely toxic to cells at high concentrations (Wei & Guo, 2007), and it can cause cancer in the duodenum of mouse after it is administered in the drinking water at 0.1% (w/w) and 0.4% (w/w) (Toyoda et al., 1982). In short-term genotoxicity tests, H_2O_2 also gives predominantly positive results (Desesso, Lavin, Hsia, & Mavis, 2000). Therefore, a monitoring of low levels of H_2O_2 in foods is of great importance for health effects are anticipated, and even the monitoring of H_2O_2 in vapour phase is also an important industrial health issue (Bohrer et al., 2008).

Numerous methods have been developed for the determination of H_2O_2, such as spectrophotometry (Nogueira et al., 2005; Tanner & Wong, 1998; Wei & Wang, 2008), fluorometry (Chen et al., 2007; Sakuragawa, Taniai, & Okutani, 1998), electrochemistry (Zheng & Guo, 2000), and chemiluminescence (Hu, Zhang, & Yang, 2007). Several methods have also been proposed for the determination of H_2O_2 in milk, such as oxygen electrode method (Toyoda et al., 1982), mediator-free amperometric biosensor method (Liang & Mu, 2008), flow injection analysis method (Cerdán, Tortajada,

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Puchades, & Maquieira, 1992) and FT-IR method (Şansal & Somer, 1999). It was noticed that H_2O_2 involved in Fenton reaction, which is very important in both laboratories and industries (Perkowski, Jóźwiak, Kos, & Stajszcyk, 2006). In our laboratory, therefore, a spectrophotometric method for the H_2O_2 detection has been proposed on the basis of decolorization of methyl orange in Fenton reaction system (Luo et al., 2008). Because of the characteristics of Fenton reaction, this method has merits of being rapid and simple in the operation. Although this method can be satisfactorily used for practical samples containing H_2O_2 at concentrations ranging from 5.0×10^{-7} to 1.0×10^{-4} mol L^{-1}, the development of more sensitive method is a challenge for a faster determination of H_2O_2 at lower concentrations.

Determination of H_2O_2 in gas-phase using aromatic hydroxylation has been reported. Lee et al. investigated a fluorescence method for determination of gas-phase peroxides by using Fenton reaction and benzoic acid, which was based on the hydroxylation of benzoic acid by ·OH to form the fluorescent product, hydroxybenzoic acid (Lee & Tang, 1994). Similarly, sodium salicylate can also be oxidised by ·OH to produce dihydroxybenzoic acid. Liu et al. developed a high performance liquid chromatography method for determination of gas-phase hydrogen peroxide in ambient air with a linear range from 2.6×10^{-6} to 4.4×10^{-5} mol L^{-1} (Liu, Steinberg, & Johnson, 2003). However, this method is limited when it was used for the determination of low H_2O_2 concentration. Recently, in our laboratory, coumarin fluorescence probing technique has been used for the detection of ·OH in aqueous systems (Guan, Zhu, Zhou, & Tang, 2008; Luo et al., 2009). Because of the strong oxidising ability of the Fenton reaction, it was intended to be used to oxidise non-fluorescent coumarin to highly fluorescent 7-hydroxycoumarin, leading to a sensitive fluorometric method for the determination of H_2O_2 in milk. As anticipated, it was confirmed that the newly established method was able to be used for the determination of low H_2O_2 concentration in a linear range from 2.0×10^{-8} to 2.0×10^{-5} mol L^{-1} with detection limit as low as 5.0×10^{-9} mol L^{-1}.

2. Materials and methods

2.1. Materials

All reagents, such as coumarin ($C_9H_6O_2$), H_2O_2 (30%), ferrous sulphate ($FeSO_4{\cdot}7H_2O$) and trichloroacetic acid (TCA) (CCl_3CO_2H), were of analytical-reagent grade. Double distiled water was used exclusively. Diluted solutions of NaOH and H_2SO_4 were used to adjusted pH. A iron(II) ions stock solution (0.01 mol L^{-1}) was obtained by dissolving 0.278 g $FeSO_4{\cdot}7H_2O$ in 100 mL of 0.5 mmol L^{-1} H_2SO_4, and a H_2O_2 stock solution (0.01 mol L^{-1}) was prepared from 30% (w/w) H_2O_2 solution and standardised by titration with $KMnO_4$ solution (2.3×10^{-2} mol L^{-1}).

2.2. Apparatus

Spectrofluorometric measurements were performed on a FP-6200 fluorescence spectrophotometer (Jasco, Japan). Each measurement was repeated three times to ensure the reproducibility, and the data were averaged. The excitation wavelength was set at 346 nm, and the emission wavelength was set at 456 nm.

2.3. Sample preparation and analysis procedure

Four different milk samples (A, B, C and D) were commercially obtained from a local supermarket in Wuhan, China. Prior to the determination, 20 mL of 20% (w/w) TCA was added to 20 mL of the milk, followed by stirring for 40 min. Then, the mixture was filtered through a 0.2 μm filter twice, and the obtained solution was used for the analysis of H_2O_2. After the pH was adjusted, 2 mL of the above-prepared solution was added to 3 mL of 6.7×10^{-4} mol L^{-1} coumarin, followed by the addition of 0.1 mL of 1.25×10^{-2} mol L^{-1} iron(II) ions (the final pH was adjusted to 3). Finally, the mixture was reacted for 9 min and the fluorescence intensity was monitored at 456 nm.

3. Results and discussion

3.1. Related reactions in the Fenton system

The Fenton reagent (iron(II)/H_2O_2) makes major contribution in the Fenton reaction, in which iron(II) ions catalyse the reduction of H_2O_2. In more details, highly reactive hydroxyl radicals (·OH) are formed during the reaction of H_2O_2 with iron(II) ions, and the generated hydroxyl radical (·OH) can destroy organic compounds due to its high oxidation potential (Perkowski et al., 2006). The mechanism for the Fenton reaction mainly involves the following steps (Georgi et al., 2007):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {\cdot}OH + OH^- \quad (1)$$

$$H_2O_2 + Fe^{3+} \rightarrow Fe^{2+} + {\cdot}HO_2 + H^+ \quad (2)$$

$${\cdot}OH + H_2O_2 \rightarrow {\cdot}HO_2 + H_2O \quad (3)$$

$${\cdot}OH + Fe^{2+} \rightarrow Fe^{3+} + OH^- \quad (4)$$

$$Fe^{3+} + {\cdot}HO_2 \rightarrow Fe^{2+} + O_2 + H^+ \quad (5)$$

$$Fe^{2+} + {\cdot}HO_2 + H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (6)$$

$${\cdot}HO_2 + {\cdot}HO_2 \rightarrow H_2O_2 + O_2 \quad (7)$$

When coumarin is added into the reaction solution, it can be oxidised to 7-hydroxylcoumarin by the generated hydroxyl radical via Eq. (8) (Guan et al., 2008; Ishibashi, Fujishima, Watanabe, & Hashimoto, 2000).

$$\text{Coumarin} + {\cdot}OH \rightarrow \text{7-hydroxylcoumarin} \quad (8)$$

Because coumarin is non-fluorescent and the product 7-hydroxylcoumarin is highly fluorescent, the fluorescence intensity of the reaction solution will be increased in the time course of reaction. Thus, it is possible to correlate the fluorescence intensity of the reaction solution with the concentration of the oxidant H_2O_2. By monitoring the fluorescence intensity of the reaction solution, we have observed that the fluorescence intensity of 7-hydroxylcoumarin at emission wavelength of 456 nm is proportional to the concentration of H_2O_2, leading to the establishment of a new fluorometric method for the determination of H_2O_2.

3.2. Effects of operation parameters on the determination of H_2O_2

The major operational parameters were further investigated to establish a new fluorescent method for the determination of H_2O_2, and the major parameters were reaction time, pH of reaction solution, initial concentrations of iron(II) ions and coumarin.

3.2.1. Effect of reaction time

The reaction time was investigated at given conditions (pH 3.0, 2.5×10^{-4} mol L^{-1} iron(II) ions, 4.0×10^{-4} mol L^{-1} coumarin, 2.0×10^{-6} or 2.0×10^{-5} mol L^{-1} H_2O_2). Fig. 1 shows the profiles of the fluorescence intensity of the solution during the reaction. At H_2O_2 concentrations of both and 2.0×10^{-6} and 2.0×10^{-5} mol L^{-1}, the fluorescence intensity is increased with the increasing

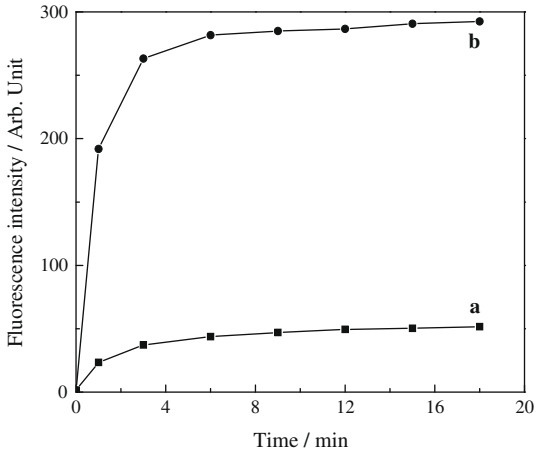

Fig. 1. Effect of reaction time on the fluorescence intensity of the reaction solutions containing H_2O_2 at initial concentrations of (a) 2.0×10^{-6} and (b) 2.0×10^{-5} mol L^{-1}. The initial concentrations of iron(II) ions and coumarin were 2.5×10^{-4} and 4.0×10^{-4} mol L^{-1}, respectively, and the solution pH was pH 3.0.

of reaction time initially, and then becomes almost constant beyond 6 min. The "saturation" of the fluorescence intensity beyond 6 min indicates that the added H_2O_2 is completely consumed by the oxidation of the substrate coumarin. Therefore, the reaction time is selected at 9 min as an optimal value.

3.2.2. Effect of pH

The solution pH is very important to the Fenton reaction, which generally requires low solution pH values ranging from 2 to 4 (Chang, Chen, & Chern, 2008), because at higher pH values ferric ions will precipitate as hydroxide (Georgi et al., 2007). Fig. 2 illustrates the dependence of fluorescence intensity on the solution pH. Under the given conditions, the best solution pH is observed at pH 3.0 in Fig. 2. Hence, the solution pH is optimised at pH 3.0 to establish a fluorometric method for the determination of H_2O_2 in the present work.

3.2.3. Effect of iron(II) ions concentration

The concentration of iron(II) ions in Fenton reaction systems is required to be low in order to avoid the formation of large amounts of iron sludge (Georgi et al., 2007). Moreover, the excess of iron(II) ions will consume the generated hydroxyl radical (·OH) according to Eq. (4), which is harmful to the efficiency of the oxidation of the organic substrate and then is unfavourable to the sensitivity of the method for the determination of H_2O_2. However, there was almost no reaction in the absence of iron(II) ions (Fig. 3A). Therefore, it is important to search a suitable iron(II) ions concentration. Fig. 3A shows that the best initial concentration of iron(II) ions is 2.5×10^{-4} mol L^{-1}, which is considered as the optimal iron(II) ions concentration for the determination of H_2O_2 in the present work.

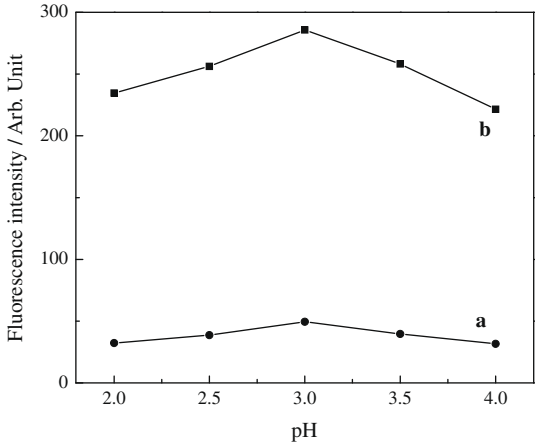

Fig. 2. Effect of pH on the fluorescence intensity of the reaction solutions containing H_2O_2 at initial concentrations of (a) 2.0×10^{-6} and (b) 2.0×10^{-5} mol L^{-1}. The initial iron(II) ions and coumarin concentration were 2.5×10^{-4} and 4.0×10^{-4} mol L^{-1}, respectively, and the reaction time was 9 min.

3.2.4. Effect of coumarin concentration

In the present work, coumarin is used as the organic substrate, which is ready for its oxidation to 7-hydroxycoumarin by reacting with the hydroxyl radicals generated from the reduction of H_2O_2 catalysed by iron(II) ions. The reactant coumarin is non-fluorescent, but the product 7-hydroxycoumarin is highly fluorescent. Thus the excess of coumarin will not influence the measurement of the fluorescence intensity of the reaction solution. A higher coumarin concentration allows the generation of more 7-hydroxycoumarin if there is an enough amount of H_2O_2 in the reaction solution. This indicates that high concentrations of coumarin are favourable to the increase of the upper limit in the possible linear

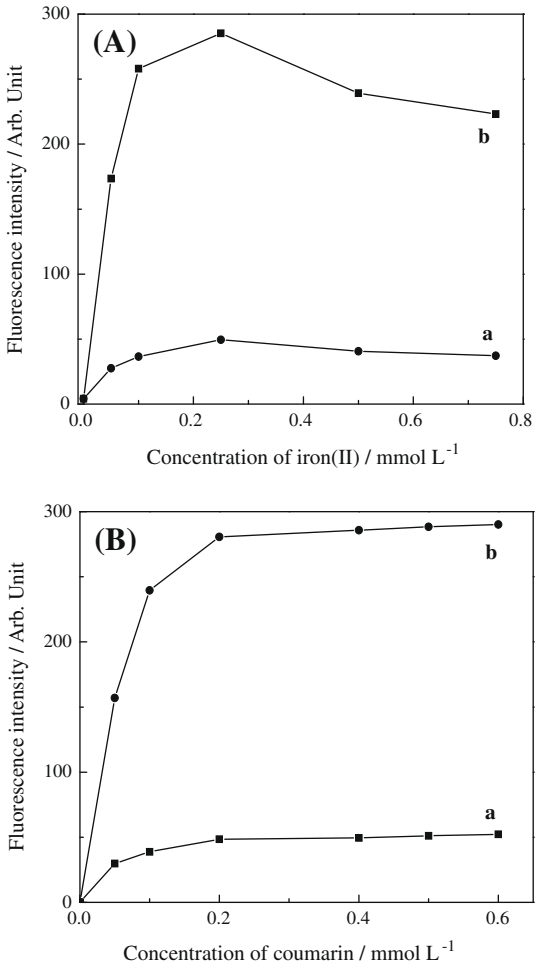

Fig. 3. Effects of (A) iron(II) ions concentration and (B) coumarin concentration on the fluorescence intensity of the reaction solutions (pH 3.0) containing H_2O_2 at initial concentrations of (a) 2.0×10^{-6} and (b) 2.0×10^{-5} mol L^{-1}. Other reaction conditions: the reaction time was 9 min; the initial coumarin concentration 4.0×10^{-4} mol L^{-1} in (A) and the initial iron(II) ions concentration 2.5×10^{-4} mol L^{-1} in (B).

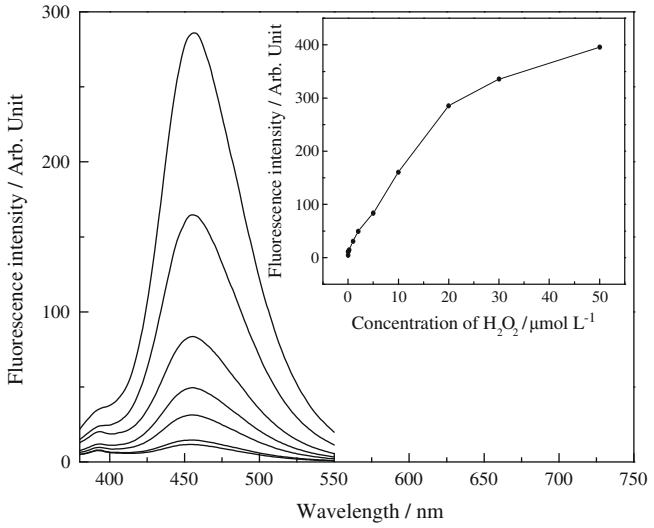

Fig. 4. Influences of H_2O_2 concentration on the emission spectra of the reaction solutions after the reaction under the optimal conditions (initial coumarin concentration 4.0×10^{-4}, initial iron(II) ions concentration 2.5×10^{-4} mol L^{-1}, pH 3.0, reaction time 9 min). The inset gives the calibration curve for H_2O_2 determination under the optimised reaction conditions.

range of the method for the determination of H_2O_2. Fig. 3B illustrates the influence of coumarin concentration on the fluorescence intensity at different concentrations of H_2O_2. It is clearly seen that the fluorescence intensity is increased initially with the increase of coumarin concentration, and then keeps almost constant beyond a given coumarin concentration. In order to expand the linear range of the method for the determination of H_2O_2, the coumarin concentration is selected at 4.0×10^{-4} mol L^{-1}.

3.3. Calibration curve

Under the optimal conditions (4.0×10^{-4} mol L^{-1} coumarin, 2.5×10^{-4} mol L^{-1} iron(II) ions, reaction time of 9 min, and solution pH 3.0), the fluorescent intensity was measured at a series of H_2O_2 concentrations, and the calibration graph for the determination of H_2O_2 was obtained as shown in Fig. 4. It is found in Fig. 4 that the emission envelope at 456 nm is increased in intensity as the H_2O_2 concentration is increased. The inset in Fig. 4 shows that the fluorescence intensity at 456 nm is well linearly correlated with the H_2O_2 concentration ranging from 2.0×10^{-8} to 2.0×10^{-5} mol L^{-1}. The detection limit ($S/N = 3$) is evaluated as 5.0×10^{-9} mol L^{-1}.

To clarify the merits of the fluorometric Fenton method, we have compared with the methods reported for the determination of H_2O_2 in Table 1. In comparison with the available methods reported in the literature, the proposed method has the almost widest linear range and lowest detection limit. Such a wide linear range and a low detection limit demonstrate that the established method may be an excellent method for the determination of H_2O_2 in foodstuffs. Moreover, the reagents used in this method are simple, and the operation procedures for the analysis are very easy.

3.4. Interferences

To investigate the selectivity of the new method, various possible interfering substances were examined under the selected optimal conditions in the presence of 5.0×10^{-6} mol L^{-1} H_2O_2. Because the induced relative error was less than 5% by the addition of the possible interfering compounds, the presence of the following compounds will not interfere with the determination of H_2O_2 from $CaCl_2$ (0–5.0 mmol L^{-1}), $NaNO_3$ (0–5.0 mmol L^{-1}), NaCl (0–5.0 mmol L^{-1}), $ZnSO_4$ (0–1.0 mmol L^{-1}), KH_2PO_4 (0–1.0 mmol L^{-1}), $MnCl_2$ (0–0.5 mmol L^{-1}), $Al_2(SO_4)_3$ (0–0.5 mmol L^{-1}), $SrCl_2$ (0–0.5 mmol L^{-1}), $C_6H_{12}O_6$ (0–0.5 mmol L^{-1}), C_6H_5COONa (0–0.1 mmol L^{-1}), $FeSO_4$ (0–0.02 mmol L^{-1}), $FeCl_3$ (0–0.05 mmol L^{-1}), KI (0–0.05 mmol L^{-1}), vitamin C (0–0.05 mmol L^{-1}) and $CuSO_4$ (0–0.05 mmol L^{-1}). Accordingly, we can see that simple salts give no interference even at high concentrations. However, the tolerant interference of reducing agents (KI, $C_6H_{12}O_6$ and vitamin C) are poorer because they can also be oxidised by H_2O_2 or ·OH. Moreover, the transition metal ions (Fe(II), Fe(III), Mn(II) and Cu(II)) which can initiate Fenton (or Fenton-like) reaction in the presence of H_2O_2. Therefore, the tolerant interference concentration of these metal ions are lower than simple salts.

3.5. Determination of H_2O_2 in milk

The newly proposed method has been used to determine H_2O_2 in four milk samples, and the results are listed in Table 2. In four

Table 2
Results of the determination of H_2O_2 in four milk samples ($n = 5$).

Samples	Added (μmol L^{-1})	Found (μmol L^{-1})	R.S.D (%)	Recovery (%)
Sample A	0	0.431	2.15	
	0.050	0.502	4.85	104.3
	0.500	0.905	3.97	97.2
	5.00	5.115	6.05	94.2
Sample B	0	0.440	2.87	
	0.050	0.518	3.58	105.7
	0.500	0.920	4.11	97.9
	5.00	5.258	5.62	96.6
Sample C	0	0.400	4.58	
	0.050	0.423	5.76	94.0
	0.500	0.926	5.21	102.8
	5.00	5.740	4.65	106.3
Sample D	0	0.385	3.84	
	0.050	0.408	6.23	93.8
	0.500	0.925	4.52	104.5
	5.00	5.458	3.68	101.4

Table 1
Comparison of analytical performances of various methods.

Methods	Major reagents	Linear range (mol L^{-1})	Detection limit (mol L^{-1})	Reference
Potentiometry	MnO_2	3.0×10^{-7}–3.6×10^{-4}	1.2×10^{-7}	Zheng and Guo (2000)
Amperometry	Hb-DNA, ZrO_2/Au	1.1×10^{-6}–2.3×10^{-3}	0.5×10^{-6}	Liang and Mu (2008)
Spectrophotometry	OPDV	1.5×10^{-6}–1.5×10^{-3}	2.9×10^{-7}	Tanner and Wong (1998)
Spectrophotometry	Fe_3O_4, ABTS	5.0×10^{-6}–1.0×10^{-4}	3.0×10^{-6}	Wei and Wang (2008)
Spectrophotometry	OPDA, Hb	5.0×10^{-8}–3.5×10^{-6}	9.2×10^{-9}	Zhang et al. (2000)
Spectrophotometry	MO, $FeSO_4$	5.0×10^{-7}–1.0×10^{-4}	2.0×10^{-7}	Luo et al. (2008)
Fluorometry	Hb	0.0–8×10^{-5}	2.6×10^{-8}	Xu and Zhang (2001)
Fluorometry	[a]	5.0×10^{-7}–9.0×10^{-4}	–	Chen et al. (2007)
Fluorometry	Coumarin, $FeSO_4$	2.0×10^{-8}–2.0×10^{-5}	5.0×10^{-9}	The present work

[a] 3,3-Diethylo-xadicarbocy-anine iodide.

milk samples, a trace of H_2O_2 (4.30, 4.40, 4.00 and 3.85×10^{-7} mol L^{-1}) has been detected. When H_2O_2 at three different concentrations (0.50, 5.00 and 50.0×10^{-7} mol L^{-1}) is added into the milks, the recoveries of H_2O_2 are ranged from 93.8% and 106.3% for all samples. The relative standard deviation (RSD) is less than 6.23%. These results indicate that the new method is satisfactorily good for its practical application to the determination of H_2O_2.

4. Conclusions

We have successfully developed a new fluorometric method for the determination of H_2O_2 in foods such as milk. The new method lays its foundation on the Fenton reaction system, in which the non-fluorescent organic substrate coumarin is oxidised to highly fluorescent 7-hydroxycoumarin by the hydroxyl radicals (·OH) generated from the reduction of H_2O_2 catalysed by iron(II) ions. Under the selected conditions, the fluorescence intensity of the reaction solution is found to be linearly proportional to the H_2O_2 concentration ranging from 2.0×10^{-8} to 2.0×10^{-5} mol L^{-1} with a detection limit of 5.0×10^{-9} mol L^{-1}. This method has merits of cheap reagents, simple operation, rapid analysis, wide linear range and high sensitivity. Therefore, when it is used for practical milk analysis, this new method gives satisfactory results.

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