

Article

# The Scopoletin-HRP Fluorimetric Determination of H<sub>2</sub>O<sub>2</sub> in Seawaters—A Plea for the Two-Stage Protocol

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**Abstract:** A single solution protocol has been widely used for the fluorimetric determination of H<sub>2</sub>O<sub>2</sub> in natural waters by its bleaching of the fluorescing scopoletin in the presence of the enzyme horseradish peroxidase (HRP). In this protocol, the reaction between scopoletin and H<sub>2</sub>O<sub>2</sub> in the sample and the subsequent internal additions, and the measurements of the fluorescence are all carried out at a single pH in a fluorometer cell. It is found that this protocol is prone to four sources of possible error. The variability in the reaction stoichiometry between scopoletin and H<sub>2</sub>O<sub>2</sub> in the presence of varying amounts of excess scopoletin, the effect of pH on the rate of reaction between scopoletin and H<sub>2</sub>O<sub>2</sub>, the photobleaching of scopoletin, and the de-activation of HRP. These possible sources of error can be circumvented in a two-stage protocol in which the reaction between H<sub>2</sub>O<sub>2</sub> and scopoletin is carried out immediately upon sampling at a pH of 7, and the measurement of the fluorescence is carried out later on at a pH of 9. It should be the protocol of choice. Furthermore, in the two-stage protocol, after the initial reaction between H<sub>2</sub>O<sub>2</sub> and scopoletin, the sample may be stored at room temperature for six days and at 4 °C for at least a month before its fluorescence is measured. This option can significantly reduce the logistics in the field.

**Keywords:** hydrogen peroxide; fluorimetric determination; scopoletin; horseradish peroxidase

## 1. Introduction

Hydrogen peroxide is found rather ubiquitously in seawater at concentrations ranging from undetectable (sub-nM) to 10<sup>2</sup> nM [1–7]. A method that has been used extensively for its determination is the scopoletin-horseradish peroxidase (HRP) fluorimetric method [1,7–14]. In this method, a fluorescing agent, scopoletin, reacts with H<sub>2</sub>O<sub>2</sub> in the presence of HRP to form a nonfluorescing compound [15]. The reduction in fluorescence is related to the concentration of H<sub>2</sub>O<sub>2</sub>, which can be quantified by internal additions. Holm et al. [8] described a single-solution protocol for the determination of H<sub>2</sub>O<sub>2</sub> in groundwater samples in which the sample is analyzed immediately upon collection and the fluorometer cell is used as the reaction vessel. The reaction between H<sub>2</sub>O<sub>2</sub> and scopoletin, including the internal additions, is carried out at a fixed pH. Subsequently, Zhang and Wong [1] reported a modified two-stage protocol in which the analytical scheme of Holm et al. [8] is separated into two stages. In the first stage, the reaction between scopoletin and H<sub>2</sub>O<sub>2</sub> in the sample with and without an internal addition is carried out in the field

in separate reaction vessels at the natural pH immediately upon sample collection. The sample may then be stored for up to four days. Then, in the second stage in the laboratory or in the field, the pH of the sample is adjusted to the optimal value of 9.2 and the fluorescence of the solution is measured. The purposes of this two-stage protocol are twofold: to make sample storage possible and to improve the sensitivity of the method. However, we have found that these two protocols frequently yield different results, especially when they are applied to seawater samples. Further examination indicates that there are other factors that favor the two-stage protocol. These factors include: the difference in the optimal pH for the reaction between scopoletin and  $\text{H}_2\text{O}_2$  and for maximizing the fluorescence of scopoletin, the photobleaching of scopoletin in the fluorometer cell, the dependence of the reaction stoichiometry between  $\text{H}_2\text{O}_2$  and scopoletin on the amount of excess scopoletin added, and the deactivation of the enzyme HRP during the analysis. Here, the effects of these processes are discussed and their implications on these two analytical protocols are assessed.

## 2. Materials and Methods

### 2.1. Apparatus

Two fluorometers with excitation light sources of different intensities were used for measuring the fluorescence of scopoletin. The Turner Model 10-AU-005-CE (Turner Designs, San Jose, CA, USA) is a filter fluorometer for both field and laboratory use. Its excitation light source is a low power 4W mercury lamp. The Hitachi Model F-7000 (Hitachi High-Technologies Corporation, Tokyo, Japan) is a high-performance research-grade scanning fluorescence spectrophotometer in which a high intensity 150 W xenon lamp is used as the excitation light source.

### 2.2. Reagents

All chemicals used are of ACS (American Chemical Society) reagent-grade. “ $\text{H}_2\text{O}_2$ -free” Milli-Q reagent grade water is prepared by the method of Zhang and Wong [16] and is used for the preparation of the reagents.

Standard 1 and 5  $\mu\text{M}$   $\text{H}_2\text{O}_2$  solutions: a 1 ml portion of a 30% (*w/w*) solution of  $\text{H}_2\text{O}_2$  was diluted to 1000 mL to make an approximately 0.01 M stock solution. This stock solution was standardized iodometrically by using iodate as the primary standard [17]. Standard 1 and 5  $\mu\text{M}$  solutions were prepared from this standardized 0.01 M solution by serial dilutions.

Scopoletin (10 and 100  $\mu\text{M}$ ): a 15 mg portion of scopoletin (Sigma, Saint Louis, MO, USA); 7-hydroxy-6-methoxy-2H-1-benzopyran-2-one,  $\text{C}_{10}\text{H}_8\text{O}_4$ , molecular weight ( $M_w$ ) = 192.2 g/mol) was dissolved in 500 mL of water to make a 150  $\mu\text{M}$  stock solution. This stock solution was stored at 4 °C in the refrigerator until use. Ten and 100  $\mu\text{M}$  solutions of scopoletin were prepared from the stock solution by serial dilutions immediately before use. These diluted solutions were stored at room temperature.

Horseshoe peroxidase (HRP) (600–1000 purpurogallin units (p.u.)  $\text{mL}^{-1}$ ): a 4 mg portion of HRP (Sigma, Type II, 150–250 p.u.  $\text{mg}^{-1}$ ) was dissolved in 1 mL of water. This solution was stored at 4 °C in a refrigerator until use. The catalytic effect of this reagent on the reaction between  $\text{H}_2\text{O}_2$  and scopoletin could be maintained for at least two months.

Saturated solution of  $\text{Na}_2\text{B}_4\text{O}_7$ : a sufficient amount of  $\text{Na}_2\text{B}_4\text{O}_7$  was added to 100 mL of water so that a small amount of solid remained undissolved.

Phosphate buffer solution (pH 7.0): a 0.27 g portion of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and a 0.43 g portion of  $\text{Na}_2\text{HPO}_4$  were dissolved in 100 mL of water to make a 0.05 M phosphate solution.

### 2.3. Procedures

#### 2.3.1. Single-Solution Protocol

The single-solution protocol for the determination of  $\text{H}_2\text{O}_2$  follows the method of Holm et al. [8].

### 2.3.2. Modified Single-Solution Protocol

Several minor modifications are applied to the method of Holm et al. [8]: the amount of scopoletin added is specified, an additional dose of HRP is added together with each internal addition of  $\text{H}_2\text{O}_2$ , and the reaction time between scopoletin and  $\text{H}_2\text{O}_2$  is fixed at 3 min. Briefly, 5 mL of a sample, 0.05 mL of a 0.05  $\mu\text{M}$  pH 7.0 phosphate buffer solution and 0.05 mL of a 100  $\mu\text{M}$  solution of scopoletin are transferred to a fluorometer cell and the fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 460 nm is measured in a Turner fluorometer (Turner Designs). Then, 0.01 mL of a 600–1000 p.u.  $\text{mL}^{-1}$  HRP solution is added to the cell and the fluorescence is read again after 3 min of reaction time. This is followed by three sequential additions of 0.1 mL of a 5  $\mu\text{M}$  standard  $\text{H}_2\text{O}_2$  solution and 0.01 mL of HRP solution to the cell and the fluorescence is measured 3 min after the addition of both solutions. The concentration of  $\text{H}_2\text{O}_2$  in the sample is calculated as described by Holm et al. [8].

### 2.3.3. Two-Stage Protocol

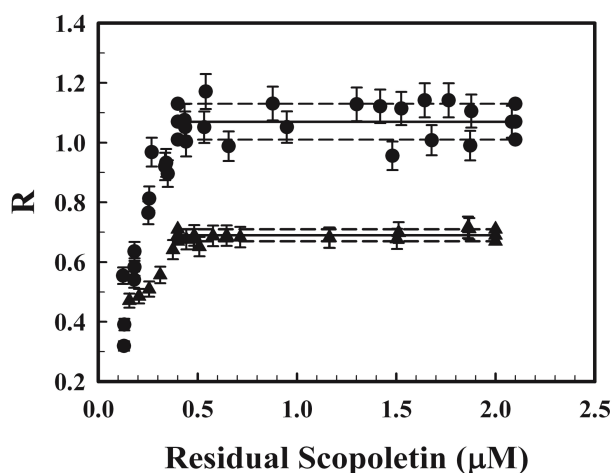
The two-stage protocol generally follows the method of Zhang and Wong [1]. A 5-mL aliquot of the sample and 0.5 mL of 10  $\mu\text{M}$  scopoletin are transferred to each of three 15 mL light tight brown high density polyethylene reaction bottles. Then, 0.5 mL of water is added to two of the bottles while 0.5 mL of a 1  $\mu\text{M}$  standard  $\text{H}_2\text{O}_2$  solution is added to the third. Finally, 0.01 mL of 600–1000 p.u.  $\text{mL}^{-1}$  HRP solution is added to the last two bottles. These samples can be stored in the dark at room temperature for up to 6 days or refrigerated at 4 °C for up to 30 days prior to their analyses. When they are analyzed, 0.2 mL of a saturated  $\text{Na}_2\text{B}_4\text{O}_7$  buffer solution is added to each of the bottles to bring the pH of the solution to 9.2. Then, the fluorescence of each solution is read at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The concentration of  $\text{H}_2\text{O}_2$  in the sample is calculated as described by Zhang and Wong [1].

## 3. Results and Discussion

### 3.1. The Stoichiometry in the Reaction between Hydrogen Peroxide and Scopoletin

The fluorescence of a 2.5 and a 0.6  $\mu\text{M}$  scopoletin solution in water and natural seawater in the presence of 1.2–2 p.u.  $\text{mL}^{-1}$  of HRP and various amounts of  $\text{H}_2\text{O}_2$  was measured. The amount of scopoletin that was destroyed by its reaction with  $\text{H}_2\text{O}_2$  was estimated from the decrease in fluorescence. The mole ratio of the scopoletin lost to  $\text{H}_2\text{O}_2$  reacted, and the amount of residual scopoletin left in the solution was calculated and the relationship between the two is shown in Figure 1. In both water and seawater, the mole ratio increased with increasing concentration of residual scopoletin and reached an approximately constant value of  $1.07 \pm 0.06$  in water and  $0.69 \pm 0.02$  in seawater when the residual scopoletin exceeded 0.4  $\mu\text{M}$ . In both the single solution and the two-stage protocol for the determination of  $\text{H}_2\text{O}_2$ , a linear relationship between the decrease in fluorescence and the amount of  $\text{H}_2\text{O}_2$  reacted is assumed. This assumption requires a constant mole ratio of scopoletin to  $\text{H}_2\text{O}_2$  reacted, and thus an excess in scopoletin of at least 0.4  $\mu\text{M}$  must be maintained. In the two-stage protocol, the amount of scopoletin added results in a 0.83  $\mu\text{M}$  solution in the reaction mixture. The internally added  $\text{H}_2\text{O}_2$  amounts to 0.08  $\mu\text{M}$ . Thus, in order to maintain a constant molar ratio, the concentration of  $\text{H}_2\text{O}_2$  in the sample must not exceed about 0.35  $\mu\text{M}$  at a mole ratio of scopoletin to  $\text{H}_2\text{O}_2$  reacted of 1 in water and 0.54  $\mu\text{M}$  at a mole ratio of scopoletin to  $\text{H}_2\text{O}_2$  reacted of 0.69 in seawater. This would cover the concentrations found in most seawater samples. The presence of an adequate amount of excess scopoletin can be safely assumed if, after each measurement, the fluorescence still exceeds 50% of the value without the addition of HRP. If samples with elevated concentrations of  $\text{H}_2\text{O}_2$  are expected, the amount of scopoletin added needs to be adjusted upward accordingly. Nevertheless, there is an upper limit on the amount of excess scopoletin that should be added. Since the concentration of  $\text{H}_2\text{O}_2$  in the sample is estimated from the decrease in fluorescence resulting from the reaction between  $\text{H}_2\text{O}_2$  and scopoletin relative to

the fluorescence of the added scopoletin, an excessively large amount of added scopoletin will lead to a small fractional decrease in fluorescence and a large uncertainty.



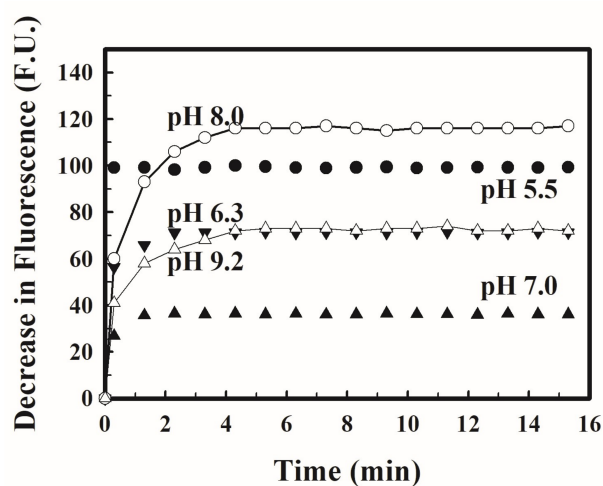
**Figure 1.** The effect of the concentration of residual scopoletin on the stoichiometry in the reaction between  $\text{H}_2\text{O}_2$  and scopoletin in reagent-grade water (●) and in seawater (▲). R is the molar ratio of scopoletin lost to  $\text{H}_2\text{O}_2$  reacted. Duplicate samples were at least analyzed at each level of residual scopoletin. —: average R at residual scopoletin exceeding 0.4  $\mu\text{M}$ ; - - -: one standard deviation from the mean.

In the single-solution protocol, the amount of scopoletin added was not clearly specified. It is given as “80% excess over the amount quenched by the natural  $\text{H}_2\text{O}_2$ ” and the optimum volume of the internally added  $\text{H}_2\text{O}_2$  quenched “approximately half of the fluorescence” [8]. If the concentration of  $\text{H}_2\text{O}_2$  in the sample is 0.2  $\mu\text{M}$ , a typical concentration in surface natural water, at a mole ratio between scopoletin and  $\text{H}_2\text{O}_2$  reacted of 1, the amount of scopoletin added would be  $180\% \times 0.2$  or 0.36  $\mu\text{M}$  and the residual scopoletin left after it that has reacted with the  $\text{H}_2\text{O}_2$  in the sample would be 0.16  $\mu\text{M}$ . This residual concentration is already below the threshold concentration of 0.4  $\mu\text{M}$ , and the internally added  $\text{H}_2\text{O}_2$  will further reduce it. Thus, in the single-solution protocol, the concentration of residual scopoletin is likely to be in the range where the molar ratio of scopoletin lost to  $\text{H}_2\text{O}_2$  reacted is not at a constant value. Indeed, Holm et al. [1] reported variable molar ratios as low as 0.1. If a decreasing molar ratio, as a result of the decreasing amount of residual scopoletin at the different steps in the protocol, does occur, it is a potentially significant source of error and may lead to overestimations and degraded precision in the measurement. It is interesting to note that the mole ratio of scopoletin lost to  $\text{H}_2\text{O}_2$  reacted was close to 1 in pure water when a sufficient amount of excess scopoletin was present as previously reported [15]. However, in seawater, the ratio only reached 0.7, suggesting that there may also be a matrix effect on the stoichiometric ratio of the reaction.

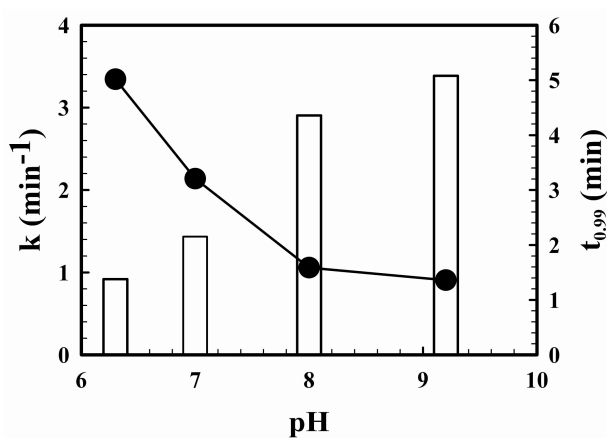
### 3.2. The Effect of pH on the Reaction between Hydrogen Peroxide and Scopoletin

The pH of a solution containing 0.1  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  and 2  $\mu\text{M}$  of scopoletin was adjusted to pH 5.5, 7, 8 and 9.2 by the addition of appropriate amounts of a 0.1 M NaOH and/or 0.1 M HCl solution. The natural pH of the  $\text{H}_2\text{O}_2$ -scopoletin solution without addition of any acid or alkali was 6.3. Then, 0.01 mL of a 600–1000 p.u.  $\text{mL}^{-1}$  HRP solution was added to each of these mixtures. The fluorescence of these solutions at the optimal excitation and emission wavelength at each of these pH values was then followed with time and the resulting time courses of reduction in fluorescence are shown in Figure 2. In all cases, the decrease in fluorescence increased with time and eventually reached a constant maximum value. At pH 5.5, the maximum reduction in fluorescence, indicating the completion of the reaction, was reached approximately instantaneously. At the other pH values, the changes in fluorescence could be approximated as a first order reaction. The resulting first order rate constants decreased with increasing pH (Figure 3). The corresponding reaction times for

the reaction to reach 99% of completion were 1.4, 2.1, 4.4 and 5.1 min at pH 6.3, 7.0, 8.0 and 9.2. Thus, the reaction time decreased with decreasing pH. The reaction time was shortened at a lower pH, but the sensitivity of the method was increased at a higher pH [1], so a minimum reaction time and a maximum sensitivity cannot be achieved simultaneously when a single pH is used in the analysis as in the single-solution protocol. In the two-stage protocol, this difficulty is circumvented as the completion of the reaction is assured, since it is allowed to proceed at the low natural pH of scopoletin for up to days while the sensitivity of the analysis is maximized since the fluorescence is measured after the pH of the reaction mixture has been adjusted to the optimal value. For this reason alone, the two-stage protocol should be the protocol of choice. However, another potentially more significant issue in the single-solution protocol [8] is the possibility of incomplete reactions. At the pH of 7.0 used in the single-solution protocol [8], a reaction time in excess of 2 min is needed to ensure completion of the reaction. However, the reaction time for the reaction between scopoletin and the  $\text{H}_2\text{O}_2$  in the sample and the  $\text{H}_2\text{O}_2$  in each standard internal addition has not been clearly specified [8,12]. If it is not stringently controlled, a variable and unpredictable error may appear.



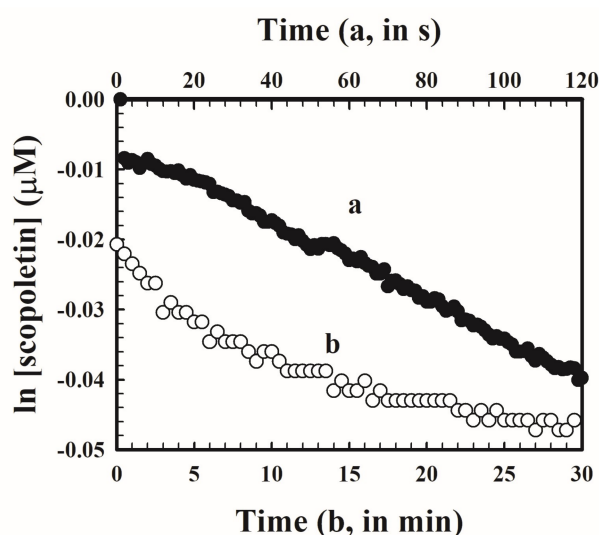
**Figure 2.** The time course of the decrease in the fluorescence, in fluorescence unit (f.u.) of the reaction mixture of  $\text{H}_2\text{O}_2$  and scopoletin at different pH. pH was adjusted by the addition of HCl and/or NaOH. (●: pH 5.5; ▲: pH 6.3; ▼: pH 7.0; ○: pH 8.0; △: pH 9.2). Fluorescence at the different pH were not recorded contemporaneously using the same setting and thus could not be compared with each other.



**Figure 3.** The effect of pH on the rate of the reaction between hydrogen peroxide and scopoletin. ●: first order rate constant (left axis); bars: time needed for the reaction to reach 99% completion.  $k$ : first order rate constant;  $t_{0.99}$ : time needed for the reaction to reach 99% completion.

### 3.3. Photobleaching of Scopoletin

The decomposition of scopoletin by photobleaching was studied by following the time courses of change in fluorescence of scopoletin solutions, at initial concentrations ranging between 0.5 and 2  $\mu\text{M}$ , at the excitation and emission wavelengths for the determination of  $\text{H}_2\text{O}_2$  in a fluorometer cell that is exposed to the light source in a Turner Model 10-AU filter fluorometer or a Hitachi Model F-7000 fluorescence spectrophotometer (Hitachi High-Technologies Corporation). The fluorescence intensity was converted to the corresponding concentrations of scopoletin and the results at one initial concentration of scopoletin are shown in Figure 4. The observed changes in concentration with time may be represented by first order kinetics well. The resulting first order reaction rate constants are listed in Table 1. In the Hitachi fluorescence spectrophotometer where a high intensity 150 W xenon lamp was used as the light source, there was a conspicuous decrease in concentration with time. The first order rate constant was  $3 \times 10^{-4} \text{ s}^{-1}$  at all the initial concentrations. In the Turner Model 10-AU filter fluorometer, a low-power 4 W mercury lamp was used as the light source and the first order rate constant was only  $1.3 \times 10^{-5} \text{ s}^{-1}$ , or about thirty times smaller than that in the Hitachi fluorescence spectrophotometer. In the single-solution protocol, the exposure of the reaction mixture to the light source is prolonged. Any photobleaching of scopoletin while the fluorescence of the sample is measured will appear as an apparent additional amount of  $\text{H}_2\text{O}_2$  and lead to an overestimation in the concentration of  $\text{H}_2\text{O}_2$  in the sample. On the other hand, photobleaching of scopoletin that occurs during the internal additions of  $\text{H}_2\text{O}_2$  will increase the specific fluorescence and lead to an underestimation. The net result is uncertain and likely will be variable. In fluorimetric analysis, a light source with the highest light intensity is usually preferred, as it will provide the highest sensitivity. However, in this case, if the single-solution protocol is used, a fluorometer with a weaker light source should be preferred in order to minimize the effect of photobleaching. At the slow rate of photobleaching, the effect should be negligible in the Turner fluorometer at an exposure time of several minutes. In the two-stage protocol, the exposure time of the reaction mixture to the light source is sufficiently short such that the effect of photo-bleaching will be minimal regardless of which spectrophotometer is used, and the higher sensitivity provided by the more intense light source may be utilized.



**Figure 4.** The time course of change in the natural logarithm of scopoletin concentration (in  $\mu\text{M}$ ) by photobleaching in two fluorometers. (a) ●: Hitachi Model F-7000 fluorescence spectrophotometer, upper scale (in seconds); (b) ○: Turner Model 10-AU-005-CE filter fluorometer, lower scale (in minutes)

**Table 1.** The first order reaction rate constants in the photobleaching of scopoletin in the two fluorometers.

Fluorometer	C <sub>0</sub> (μM)	k (10 <sup>-4</sup> s <sup>-1</sup> )	r
Hitachi Model F7000	2.003	3	0.9987
	1.487	3	0.9895
	0.994	3	0.9960
	0.493	3	0.9835
Turner Model10-AU	0.980	0.13	0.9375
	0.494	0.13	0.9465

C<sub>0</sub>: initial concentration of scopoletin; k: the first order rate constant; r: the correlation coefficient of the natural logarithm of concentration against time.

### 3.4. Deactivation of Horseradish Peroxidase

The stability of the enzymatic activity of HRP for catalyzing the reaction between scopoletin and H<sub>2</sub>O<sub>2</sub> during the prolonged exposure of the reaction mixture to heat and light in the Turner fluorometer in the single-solution protocol was examined by estimating the specific fluorescence in samples of reagent grade water and seawater with and without the addition of another dose of HRP upon the internal additions of H<sub>2</sub>O<sub>2</sub>. The results are shown in Table 2. Invariably, the specific fluorescence was higher when HRP was added together with each internal addition of H<sub>2</sub>O<sub>2</sub> before the fluorescence was read. Furthermore, when additional HRP was added, the specific fluorescence was similar in the same type of water, at 369 ± 8 fluorescence units f.u. μM<sup>-1</sup> in reagent grade water and 587 ± 17 f.u. μM<sup>-1</sup> in seawater, regardless of the concentration of H<sub>2</sub>O<sub>2</sub> in the sample. Without adding another dose of HRP, the specific fluorescence was lower and more variable, at 348 ± 18 f.u. μM<sup>-1</sup> in reagent grade water and 372 ± 77 f.u. μM<sup>-1</sup> in seawater. These behaviors are consistent with the deactivation of the HRP during the analysis so that the HRP added initially can no longer effectively and reproducibly catalyze the reaction between scopoletin and the H<sub>2</sub>O<sub>2</sub> added in the internal additions in the later stage of the analysis. The deactivation of HRP has been noted by the supplier of the chemical [18] and its effect on the fluorimetric determination of H<sub>2</sub>O<sub>2</sub> in the stored sample has been noticed previously [1]. However, its effect on the single solution protocol has not been evaluated. On the other hand, the deactivation of HRP is irrelevant to the two-stage protocol since HRP is used to catalyze the reaction only once when it is added to the sample immediately after sample collection.

**Table 2.** Effect of the addition of horseradish peroxidase (HRP) upon the internal additions of H<sub>2</sub>O<sub>2</sub> on the specific fluorescence of scopoletin.

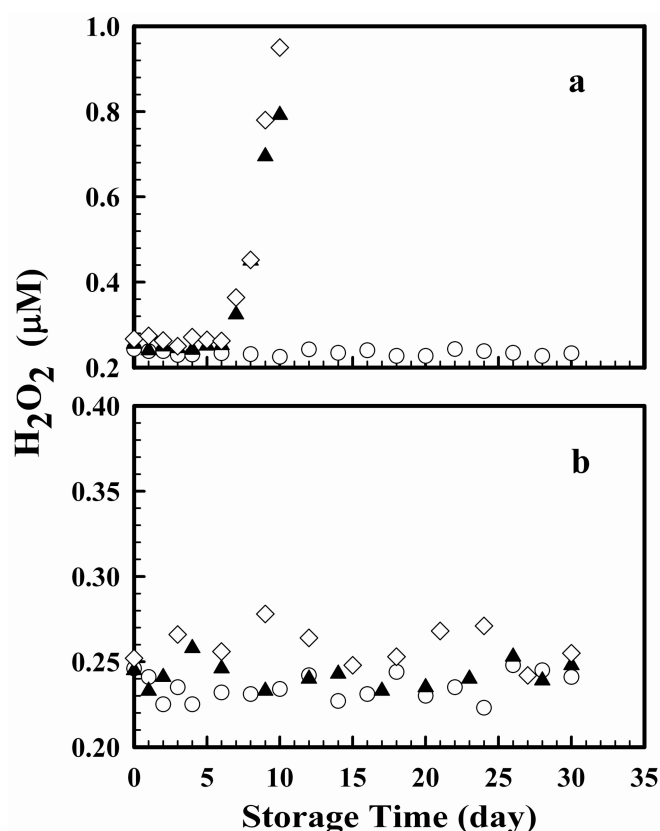
Sample	Specific Fluorescence (f.u. μM <sup>-1</sup> )	
	Without Additional HRP	With Additional HRP
RW	357, 369	370, 379
RW + 0.1 μM H <sub>2</sub> O <sub>2</sub>	334, 333	367, 361
Average	348 ± 18	369 ± 8
SW	438, 438	582, 609
SW + 0.1 μM H <sub>2</sub> O <sub>2</sub>	306, 305	568, 590
Average	372 ± 77	587 ± 17

RW: reagent-grade water; SW: surface seawater; Specific fluorescence: decrease in fluorescence per μM of internally added H<sub>2</sub>O<sub>2</sub>.

### 3.5. Sample Storage

Known amounts of H<sub>2</sub>O<sub>2</sub> were added to samples of “H<sub>2</sub>O<sub>2</sub>-free” reagent grade water, aged estuarine water and aged surface seawater collected from the South China Sea. Scopoletin and HRP were then added to subsamples of these samples as specified in the two-stage protocol. One of the samples was analyzed immediately for the concentration of H<sub>2</sub>O<sub>2</sub>. The other subsamples were stored either at

room temperature (24 °C) or in a refrigerator at 4 °C. The concentrations of H<sub>2</sub>O<sub>2</sub> in these subsamples were quantified after various times of storage. The results are shown in Figure 5. In a previous study [1], the concentration upon storage at room temperature was followed to a maximum storage time of four days and no significant change in the concentration was observed. In this study, the maximum storage time at room temperature was extended to 10 days. In the first six days, the results of natural water samples were similar to those reported previously as the concentration of H<sub>2</sub>O<sub>2</sub> stayed constant at an average concentration of  $0.248 \pm 0.006 \mu\text{M}$  in the seawater sample and  $0.265 \pm 0.006 \mu\text{M}$  in the estuarine water sample. The standard deviations were within the analytical uncertainty of  $\pm 5\%$ . However, at storage times longer than six days, the concentration of H<sub>2</sub>O<sub>2</sub> increased rapidly with time, probably as a result of the decomposition of the scopoletin. The concentration after 10 days of storage was three times those in the first six days of storage. Thus, sample storage should be limited to less than six days at room temperature. In contrast, in Milli-Q reagent-grade water, the concentration of H<sub>2</sub>O<sub>2</sub> stayed constant at  $0.234 \pm 0.006 \mu\text{M}$  for at least 30 days. At a storage temperature of 4 °C, there was no significant change in the concentration of H<sub>2</sub>O<sub>2</sub> up to the maximum storage time of 30 days used in the experiment. The average concentrations of H<sub>2</sub>O<sub>2</sub> over those 30 days of storage time were  $0.242 \pm 0.008 \mu\text{M}$  in the seawater sample,  $0.259 \pm 0.011 \mu\text{M}$  in the estuarine water sample, and  $0.235 \pm 0.008 \mu\text{M}$  in the Milli-Q reagent-grade water. The variations were within the analytical uncertainty of the method and the concentrations found were indistinguishable from those measured within the first six days of storage at room temperature. Thus, in the two-stage protocol, under refrigeration, the storage time of the natural water samples may be extended to at least a month.



**Figure 5.** Variations in the concentration of H<sub>2</sub>O<sub>2</sub> in reagent grade water (○), seawater (▲) and estuarine water (◇) at (a) 24 °C and (b) 4 °C upon storage.



### 3.6. Comparison of Results Obtained from the Single-Solution Protocols and the Two-Stage Protocol

The concentrations of H<sub>2</sub>O<sub>2</sub> in samples of reagent grade water and aged surface seawater from the South China Sea with and without the addition of a known amount of H<sub>2</sub>O<sub>2</sub> were determined with the two-stage protocol. The samples with added H<sub>2</sub>O<sub>2</sub> were also analyzed by using both single-solution protocols, and the results are shown in Table 3. The two-stage protocol yielded highly precise results. The average deviation from the mean was about  $\pm 0.003$   $\mu\text{M}$  at a concentration level of around 0.1  $\mu\text{M}$  in duplicate samples and a quantitative recovery of the added H<sub>2</sub>O<sub>2</sub>, averaging  $102 \pm 4\%$ , was found. When the modified single-solution protocol was used, comparable results, averaging about 104% of the concentrations by the two-stage protocol, were found in reagent-grade water. However, in seawater, noticeably higher concentrations, averaging about 126% of those by the two-stage method, were found. Even higher concentrations were found when the unmodified single-solution method was used. The deviations were larger in seawater than in reagent-grade water. These results indicate that significant overestimations in the concentration of H<sub>2</sub>O<sub>2</sub> may occur when the single-solution protocol is used and the discrepancies may be larger in the analyses of seawater. Even with the added precautions, the modified single solution protocol cannot eliminate the overestimations totally in seawater. The two-stage protocol should be the protocol of choice. It is free from the potential sources of error discussed previously and it allows the samples to be stored for an extended period of time after relatively simple pretreatments.

**Table 3.** Determination of H<sub>2</sub>O<sub>2</sub> in reagent grade water and seawater by three protocols.

Sample	H <sub>2</sub> O <sub>2</sub> Added $\mu\text{M}$	Protocol A Found $\mu\text{M}$	Protocol B Found $\mu\text{M}$	B/A %	Protocol C Found $\mu\text{M}$	C/A %
RW	0	0.013, 0.011				
Average		$0.012 \pm 0.001$				
Average	0.10	0.116, 0.115	0.120, 0.121		0.158, 0.152	
Recovery		$0.116 \pm 0.001$	$0.120 \pm 0.001$	103	$0.155 \pm 0.005$	134
% Recovery		$104 \pm 2$				
RW	0	0.021, 0.020				
Average		$0.021 \pm 0.001$				
Average	0.10	0.120, 0.118	0.128, 0.122		0.156, 0.162	
Recovery		$0.119 \pm 0.001$	$0.125 \pm 0.004$	105	$0.159 \pm 0.005$	134
% Recovery		$98 \pm 2$				
SW	0	0.024, 0.021				
Average		$0.023 \pm 0.002$				
Average	0.10	0.130, 0.124	0.158, 0.161		0.291, 0.271	
Recovery		$0.127 \pm 0.003$	$0.159 \pm 0.002$	125	$0.281 \pm 0.014$	221
% Recovery		$104 \pm 5$				
SW	0	0.022, 0.016				
Average		$0.019 \pm 0.004$				
Average	0.10	0.121, 0.127	0.146, 0.154		0.285, 0.290	
Recovery		$0.124 \pm 0.004$	$0.150 \pm 0.005$	121	$0.288 \pm 0.004$	232
% Recovery		$105 \pm 8$				
SW	0	0.015, 0.012				
Average		$0.014 \pm 0.002$				
Average	0.10	0.114, 0.117	0.140, 0.150		0.219, 0.252	
Recovery		$0.111 \pm 0.005$	$0.145 \pm 0.007$	130	$0.235 \pm 0.023$	212
% Recovery		$97 \pm 7$				

RW: reagent-grade water; SW: surface seawater; protocol A: two-stage protocol; protocol B: modified single-solution protocol; protocol C: single-solution protocol; B/A: Average concentration by protocol B/Average concentration by protocol A; C/A: Average concentration by protocol C/Average concentration by protocol A.

#### 4. Conclusions

When the scopoletin-HRP fluorimetric method is used to determine H<sub>2</sub>O<sub>2</sub> in seawater, the traditional single solution protocol described by Holm et al. [8] is prone to four sources of possible errors. First, as the amount of scopoletin decreases during the internal additions of H<sub>2</sub>O<sub>2</sub>, the stoichiometry of scopoletin to H<sub>2</sub>O<sub>2</sub> reacted does not stay constant if the amount of excess scopoletin drops below 0.4 μM. Secondly, while the fluorescence of scopoletin is higher at a more basic pH, the rate of the reaction between scopoletin and H<sub>2</sub>O<sub>2</sub> is slower. In the single solution protocol where a single neutral to basic pH is used, incomplete reaction may occur if the reaction time is not carefully controlled. Thirdly, photobleaching of scopoletin may occur during the prolonged exposure of the reaction mixture to the light source while it is kept in the fluorescence spectrophotometer. The lost scopoletin may appear as an apparent H<sub>2</sub>O<sub>2</sub> in the sample. This effect is especially acute in high performance research grade fluorescence spectrophotometers fitted with high intensity light sources. Fourthly, HRP in the reaction mixture may undergo deactivation as the reaction mixture is kept at or above room temperature in the fluorescence spectrophotometer. The modified two-stage protocol reported here can not only circumvent these possible sources of possible error in the single solution protocol, but also extend the storage time of samples for the determination of H<sub>2</sub>O<sub>2</sub> to up to at least a month. At concentrations of around 0.02 and 0.1 μM, the precision of the modified method were about ±15% and ±3% at concentrations of 0.02 and 0.1 μM, respectively. The virtually quantitative recovery of added H<sub>2</sub>O<sub>2</sub> indicates that the method is highly accurate.

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**Conflicts of Interest:** The results of the report have conflicts of interest for Thomas R. Holm, Gregory K. George and Michael J. Barcelona.

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