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Fluorometric Determination of Hydrogen Peroxide in Groundwater

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The fluorometric scopoletin–horseradish peroxidase method has been modified for field determinations of hydrogen peroxide concentrations in groundwaters. Standard additions calibration compensates for background fluorescence and inconsistent stoichiometry of the fluorescence quenching reaction due to interferences by the matrix. The detection limit, defined as the blank plus three standard deviations, ranged from 3.6 to 44.6 nM. However, this limit was more an indication of the difficulty of preparing peroxide-free water than the actual limit imposed by the sensitivity of the method for the peroxide contamination introduced with the reagents. For 111 field determinations the weighted average (uncorrected) hydrogen peroxide concentration was 20.2 nM and the pooled standard deviation was 7.7 nM. The average of 45 field blanks was 7.8 nM with a pooled standard deviation of 5.2 nM. At nanomolar concentration levels, it is essential that samples are analyzed for H_2O_2 in the field. Storage periods exceeding 1 h caused serious errors and irreproducible results.

Oxidation and reduction processes play important roles in the solution chemistry and geochemistry of natural waters. Dominant redox couples, such as O_2/H_2O or Fe(II)/Fe(III), influence the speciation of other chemical constituents by establishing a redox condition (*1*). The redox condition of a surface water/sediment or groundwater/aquifer system can control equilibria between dissolved and solid phases, influencing the solubility, mobility, and reactivity of metal ions or nutrient species as well as potential chemical pollutants (*2, 3*). Similarly, oxidizing conditions favor the oxidation or aerobic biotransformation of organic compounds, while reducing conditions may result in the preservation of organic matter (*4*). It is therefore important to identify redox-active species and controlling redox reactions because of their direct influence on the stability and transport of chemical substances which may impact the quality of our water resources.

The redox condition of groundwater systems is of great concern to environmental scientists involved in investigations of the fate of dissolved chemical contaminants as well as efforts to clean up contaminated aquifers. In the past, geochemists have considered groundwater to be in a reducing condition due to the depletion of dissolved oxygen in recharge waters during the oxidation of organic matter (*5*). A number of investigators have demonstrated that the predicted thermodynamic sequence of electron acceptors (i.e., O_2, NO_3^-, Mn(IV), Fe(III), and SO_4^{2-}) may be evidenced by redox zonation within groundwater flow systems (*6–8*). However, the interpretive power of either redox potential measurements or calculated equilibrium potentials alone is only qualitative in many situations (*9–12*).

In order to effectively manipulate subsurface conditions to chemically oxidize chemical contaminants or stimulate microbial transformation of organic compounds, it is necessary to have more reliable indicators of redox condition than calculated or measured oxidation–reduction potentials. Because oxic (oxygen-containing) waters have been postulated to have an effective redox potential of the couple O_2/H_2O_2 (*13*), it was felt that H_2O_2 is an important intermediate in O_2 reduction and may be found in oxic groundwaters. The reliable determination of H_2O_2 in groundwater was the primary goal of this study.

The fluorometric horseradish peroxidase–scopoletin method was chosen for H_2O_2 determinations because it is sufficiently sensitive to determine nanomolar concentrations and the procedure can be performed in the field (*14*). The basis of the method is fluorescence quenching of scopoletin in the reaction with H_2O_2 catalyzed by horseradish peroxidase. The method has been shown to be applicable to H_2O_2 determinations at micromolar to nanomolar concentrations in natural waters (*15, 16*).

EXPERIMENTAL SECTION

Site Description. The groundwater samples which were collected for chemical analysis were taken from PTFE cells (5 cm o.d., Fluorocarbon Co., Anaheim, CA) finished at depths of 11, 15, 21, and 32 m between November 1984 and January 1986. The wells were constructed by hollow-stem auger techniques without the use of drilling aids or foreign additives. The study area is located in the Sand Ridge State Forest near Manito, IL. The geology and hydrology of the site have been described previously (*17–19*). Sampling was done with positive-displacement bladder pumps (Well Wizard, Q.E.D. Environmental Systems, Ann Arbor, MI) constructed of PTFE with PTFE gas and water transfer lines which were dedicated to each well.

Water Sampling. Sampling was conducted according to a published procedure (*20*) to assure that each well was purged of stagnant water prior to sample collection. Field measurements of pH, Eh (platinum electrode potential), temperature, and specific conductance were made at the surface with an in-line flow-cell device that was designed in our laboratories (*21*). Separate aliquots of each sample were taken for the determination of major cations, anions, trace metals, organic carbon, dissolved oxygen, and hydrogen peroxide. The H_2O_2 samples were collected in a glass stoppered, aluminum foil covered, glass bottles. The sampling tubing from the wellhead was covered with commercial pipe insulation to minimize temperature changes and exposure to light.

Apparatus. A Turner Model 111 filter fluorometer, powered by a portable generator, was used for fluorescence measurements. Filters for the analysis were selected by choosing the combination which gave the highest sensitivity to scopoletin in distilled H_2O. The excitation filter was a Corning 7-60 (365-nm primary wavelength); the emission filter was a Wratten 65 (495-nm primary wavelength, transmittance falling to less than 1% below 430 and above 570 nm) coupled with a Wratten 2A (sharp cut off below 415 nm). The light source was a general purpose mercury ultraviolet lamp with its highest efficiency at 360 nm (range 310–390 nm).

Pyrex serum vials (7 cm × 0.75 cm i.d.) were used as cuvettes in the instrument. Vials were selected after measurement of the fluorescence of distilled water in the most sensitive range of the instrument. Vials which differed significantly from the mean reading were rejected.

Reagents. Water used for reagents and blanks was deionized and doubly distilled, with the final distillation within 24 h of use. Reagent water was stored in precombusted, dark glass bottles capped with Teflon-linked caps. All glassware used for fluores-

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cence measurements was cleaned with laboratory detergent free of fluorescent dyes, soaked in 2 M HCl, and thoroughly rinsed with reagent water. A hydrogen peroxide standard was prepared fresh in the field, and renewed on evidence of deterioration, by dilution of a 30% w/w stock solution (Superoxol, Baker $Na_2Sn-O_3{\cdot}3H_2O$ preservative). The concentration of the stock solution was determined periodically in the laboratory by titration with standard $KMnO_4$ (*22*, *23*). Scopoletin (7-hydroxy-6-methoxy-2*H*-1-benzopyran-2-one) stock solution (1×10^{-5} M) was prepared fresh within 24 h of use by dissolving a weighed quantity of scopoletin crystals (Sigma) in double-distilled water. The stock solution was diluted 1:10 immediately before use in the field. The diluted reagent was stable in the dark for 24 h without refrigeration. A 0.05 M phosphate buffer (pH 6.86) was prepared from distilled water and commercial buffer salts (Beckman). Horseradish peroxidase (HRP) (Sigma P8125, type II, 150–200 purpogallin units/p mg of solid) reagent was prepared within 6 h of use. Twenty milligrams of solid HRP and 3 mg of NaN_3 were dissolved in 5 mL of pH 6.86 buffer. Phenol (*24*, *25*) is less effective than NaN_3 at limiting the development of microbes, as evidenced by a strong odor and increasing fluorescence which developed in refrigerated, sealed, phenol-preserved enzyme preparations. When NaN_3 was used, the enzyme was found to be useful for up to 8 h without refrigeration. Storage on ice prolonged its useful life. The crystalline enzyme develops increasing fluorescence after a period of months at −10 °C, and must be periodically replaced.

Procedure. All groundwater samples were analyzed within 10 min of collection. The fluorometer was zeroed by using an opaque rod in place of a cuvette. Five milliliters of groundwater was pipetted into a serum vial, and the background fluorescence was measured and recorded. Next, scopoletin reagent was added by using an adjustable micropipet, the sample was mixed for 5 s with a vortex mixer, and the fluorescence was measured and recorded. Next, 10 μL of HRP reagent was added, the sample was mixed, and the fluorescence was measured and recorded. A decrease in fluorescence after HRP addition indicated the presence of H_2O_2 in the sample. Three spikes of H_2O_2 were added with mixing followed by measurement of the fluorescence after each addition. The H_2O_2 concentration was calculated by the method of standard additions (*26*). Blanks were treated identically to samples except that 50 μL of phosphate buffer was added before the scopoletin.

Preliminary H_2O_2 determinations were necessary to optimize the amount of scopoletin added, the volume of the H_2O_2 spikes, and the fluorometer sensitivity. The amount of scopoletin reagent was calculated to provide up to an 80% excess over the amount quenched by the natural H_2O_2 with the constraint that fluorescence readings, i.e., background plus scopoletin, must remain on-scale. The optimum volume of the H_2O_2 spikes was calculated to quench approximately half of the scopoletin fluorescence. One or two preliminary determinations on each sample were usually sufficient to optimize reagent volumes and instrument settings.

For a subset of groundwater samples, the stoichiometry of the fluorescence quenching reaction was determined. This was done by adding spikes of scopoletin to a fresh aliquot of groundwater and measuring the fluorescence. Scopoletin concentrations were adjusted to bracket the concentration range covered by the H_2O_2 standard additions. The reaction stoichiometry, in moles of scopoletin per mole of H_2O_2, was calculated as the ratio of the slopes of the standard additions curve and the scopoletin calibration curve.

RESULTS AND DISCUSSION

Buffering. Buffering of the pH of water samples for H_2O_2 determinations is recommended (*24*, *25*, *27*). However, groundwaters commonly exhibit pH values close to the specified values and alkalinities in the range of 2–5 mequiv L^{-1} (*28*), which is much greater than the specified buffer concentrations. Added buffer would contribute very little to the pH buffering of the groundwater/reagent mixtures. The buffer was therefore only used for blank determinations. Buffering would be necessary for H_2O_2 determinations in acidic or basic groundwaters having pH values far removed from the optimum for HRP or in the range in which scopoletin

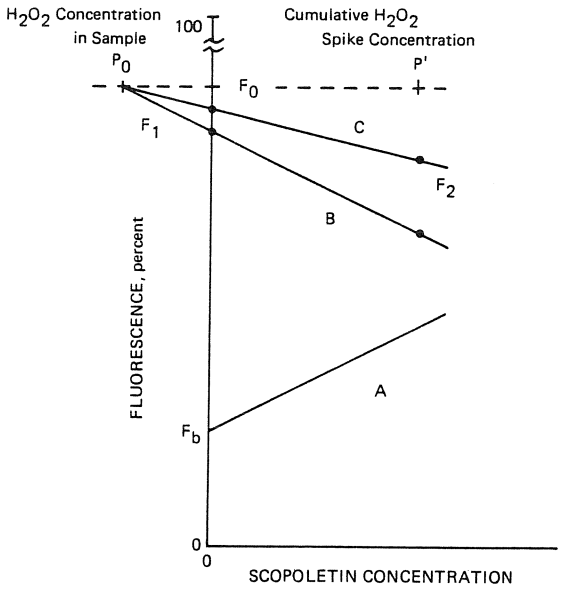

Figure 1. Fluorescence quenching and standard additions: (A) scopoletin in groundwater (F_b is background fluorescence); (B) standard additions curve with ideal stoichiometry, i.e., 1 mol of H_2O_2/mol of scopoletin; (C) standard additions curve with nonideal stoichiometry. Concentration scales for scopoletin and H_2O_2 are arbitrary but identical.

fluorescence is weak.

Calibration Problems and Nonstoichiometry. The stoichiometry of the fluorescence quenching reaction in distilled water has been shown to be 1 mol of scopoletin/mol of H_2O_2 in distilled water (*27*). However, in the present study, the reaction stoichiometry in groundwater analyses, calculated as the ratio of the slope of the standard additions curve to the slope of the distilled water calibration curve, was observed to be as low as 0.1. For a groundwater sample in which 0.1 mol of scopoletin is oxidized for every mole of H_2O_2, comparison with a distilled water calibration curve (*15*, *16*) would underestimate H_2O_2 by a factor of 10. This potentially serious source of error has not been previously recognized in the literature.

There are many reactions that can compete with the fluorescence quenching reaction. Reducing substances such as ascorbic acid, glutathione, and manganous ion have been shown to interfere with the HRP catalyzed oxidation of scopoletin by H_2O_2. Nitrite and ethanol can be oxidized (*14*). Certain amino acids, phenols, thiols, diamines, and iodide also react (*29*). Maloney et al. (*30*) have shown that HRP can catalyze the oxidation of chlorinated phenols by H_2O_2. Perdue (*31*) has shown that phenolic functional groups account for a significant fraction of ionizable protons in aquatic humic substances. Thus, the reduced stoichiometry of the scopoletin–H_2O_2 reaction in groundwaters may have been caused by oxidative coupling of natural organic matter in the presence of HRP and H_2O_2.

The method of standard additions can be shown to correct for nonideal stoichiometry. The derivation of this follows the schematic diagram provided in Figure 1. The fluorescence of a solution containing scopoletin may be given by $F = aS + F_b$, where F is fluorescence, a is the slope of the calibration curve, S is scopoletin concentration, and F_b is background fluorescence. This scopoletin calibration curve is shown as line A in Figure 1. We define the stoichiometry of the fluorescence quenching reaction in a groundwater sample as f mol of scopoletin for every mole of H_2O_2 ($0 < f \leq 1$). The apparent concentration of H_2O_2 by external calibration is then given by $C = (F_0 - F_1)/a$, where C is the calculated H_2O_2 concentration and F_0 and F_1 are the fluorescence levels measured before and after addition of HRP, respectively. If

Table I. Field and Laboratory Performance of the Method

Blanks

date	location[a]	n[b]	H_2O_2 concn, nM	detection limit, nM[c]	precision (% RSD)[d]
7/23/85	f	5	8.0	15.4	31
11/18/85	f	2	4.0	13.9	105
6/2/86	f	3	13.1	30.3	44
5/16/86	l	7	2.3	4.6	33

Spike Recoveries

date	depth	N	initial H_2O_2 concn, nM	H_2O_2 added, nM	H_2O_2 found, nM	recovery,[g] %	precision (% RSD)
7/23/85	11	2	0.0[f]	20.0	21.5	108	12
11/18/85	21	1	2.0	40.0	39.5	94	
6/2/86	21	3	0.0[f]	40.0	37.1	93	23
5/16/86	L[e]	6	2.3	20.0	13.0	53	47

[a] f, field; l, laboratory. [b] Number of replicates. [c] Mean blank + three standard deviations. Standard deviation for 11/18/85 estimated from range (*37*). [d] RSD is relative standard deviation. Standard deviation could not be calculated for the 11/18/85 21-m sample. [e] Distilled water spiked in the laboratory. [f] No quenching on HRP addition. [g] All initial concentrations were below detection limit, but recoveries were calculated by using these initial concentrations.

P_0 is the actual concentration of H_2O_2 in the sample, then the calculated cumulative concentration is fP_0. If the stoichiometry is 1:1, then $f = 1$ and the calibration method yields the correct result. Otherwise, the H_2O_2 concentration may be seriously underestimated. The formula for calculating the H_2O_2 concentration from a single standard addition is $C = (F_0 - F_1)P'/(F_1 - F_2)$, where P' is the concentration of the H_2O_2 spike and F_2 is the fluorescence after addition of the spike. In Figure 1, line B is the standard additions curve for ideal stoichiometry. Its slope is equal in magnitude and opposite in sign to the slope of the scopoletin calibration curve, line A. Line C is the standard additions curve for nonideal stoichiometry. For multiple standard additions (P', F_2) is a point on the regression line. By straightforward algebra, this formula yields the correct H_2O_2 concentration regardless of reaction stoichiometry.

H_2O_2 Background and Blank Determinations. The detection limits (DL), calculated as the blank plus three standard deviations (*32*), were greater than calculated H_2O_2 concentrations at all depths in three of the nine times when DL was estimated and at least at one depth in eight of the nine times, even though there was measurable quenching. We suspected that the distilled water used for blank determinations contained H_2O_2 and tested this hypothesis by varying the concentrations of scopoletin and peroxidase and by adding the peroxidase before the scopoletin in groundwater samples. Varying the reagent concentrations had no effect on the blanks. The contribution of H_2O_2 to the blank fluorescence was eliminated by addition of the enzyme prior to the scopoletin. Therefore, the measured quenching in the reagent blanks was probably due to H_2O_2 present at the 5–25 nM range in the distilled water.

We further investigated the origin of the H_2O_2 contamination in the distilled water. Storage did not produce increases in the levels of H_2O_2 in the water over those in freshly distilled water. Also, sparging the distilled water with oxygen-free nitrogen for several hours did not reduce the levels of H_2O_2. Distillation and collection under nitrogen reduced the level of apparent H_2O_2 in the distilled water by approximately 50%. The most likely sources of the H_2O_2 contaminant are production in the distillation process or carry-over from the original amount in the deionized water.

Blanks for this method have not been addressed in detail in the literature. A number of authors have suggested that H_2O_2 can be produced in deionized distilled water by microbial activity (*33*), by photochemical reactions (*34*), by sparging with air (*16*), or even by free-radical reactions during storage under He (*29*). However, few of the previous workers reported any problems with background H_2O_2 concentrations at the levels of interest in their experiments. Zika and Saltzman (*25*) distilled reagent water from a $KMnO_4$ solution. Perschke and Broda (*27*) suggested successive distillation of blank and reagent water from $KMnO_4$, from $AgNO_3$, and finally from a quartz still to achieve sufficient water purity. Perschke and Broda intended to establish the stoichiometry of the reaction. They did not report the background contribution of H_2O_2 in the distilled water. Kok et al. (*35*) used catalase to prepare H_2O_2-free water. However, this may reduce the stoichiometry of the fluorescence quenching reaction through competition with the HRP. Also, the fluorescence of their blank was 8–12% of the fluorescence of a 1.2 μM H_2O_2 solution. Thus, the blank reduction may not be adequate for H_2O_2 determinations in the nanomolar concentration range. (While this manuscript was in review, a method was published (*36*) for reducing H_2O_2 blanks in distilled water. The method involves pumping the distilled water through a MnO_2 column. Subsequent measurements in our laboratories confirm that the method effectively reduces blank levels of H_2O_2 such that there is no detectable fluorescence quenching on addition of HRP. The method is recommended for field applications of the modified HRP–scopoletin method.)

It is likely that hydrogen peroxide is a normal trace component of distilled water, produced from the water itself and varying with inputs of radiation and dissolved oxygen. Because the fluorescence quenching method is so sensitive and because the H_2O_2 concentrations in groundwaters are so low, distilled water blanks tested the ability to prepare H_2O_2-free water more than the validity of apparent H_2O_2 signals in the analytical method.

Laboratory Analytical Performance. Table I contains averaged analytical performance measures for the modified HRP–scopoletin method determined on several occasions over a 12-month period. These data were collected on aliquots of H_2O_2-spiked groundwater or double-distilled water. The data show that the method is quite sensitive and precise over the concentration range of interest. In most cases recovery of the H_2O_2 spike from groundwater exceeded 95%. The recoveries from spiked distilled water were erratic and averaged ap-

Table II. Field Determinations of Hydrogen Peroxide in Groundwaters[d]

	blank				11 m			15 m			20 m		
date	av[a]	n[b]	SD[c]	MDL[e]	av[a]	n[b]	SD[c]	av[a]	n[b]	SD[c]	av[a]	n[b]	SD[c]
12/13/84	8.16	6	0.64	10.1	60.6	6	13.2	27.4	2	12.8	66.3	2	2.69
1/17/85	20.4	7	8.07	44.6	61.2	3	6.2	–		–	39.6	2	2.38*
2/19/85	7.58	1	(6.07)	(25.8)	32.4	5	11.7	2.94	2	0.36*	7.59	2	1.58*
3/19/85	2.36	6	1.74	7.58	1.20	1	–*	7.89	1	–	8.97	5	0.37
4/23/85	6.37	6	2.20	13.0	11.2	3	0.78	9.59	3	2.22*	17.7	4	9.25
5/15/85	7.38	7	7.02	28.4	9.72	2	5.23*	22.5	2	10.3*	19.3	3	2.91*
7/23/85	8.0	5	2.4	15.4	−1.75	3	–*	11.0	2	13.1*	0.72	2	0.82*
9/19/85	1.07	3	0.85	3.61	14.3	3	17.7	−4.70	2	–*	−1.21	1	–*
10/17/85	0.65	1	(6.07)	(18.9)	20.8	6	10.4				25.2	2	4.54
11/18/85	4.0	2	3.32	13.9	7.63	3	6.41*				2.54	3	2.98*
12/9/85	16.7	2	1.42	20.9	2.21	3	2.02*				3.73	3	2.30*
1/28/86	(4.67)		(6.07)	(22.9)	18.1	5	2.48*				65.0	6	7.23
2/17/86	3.54	2	4.20	16.1	1.06	3	1.67*				8.20	4	4.95*
3/10/86	0.50	2	9.10	27.8	7.41	3	7.84*				15.0	5	10.6*
4/6/86	−1.43	1	(6.07)	(16.8)	2.19	4	1.77*				9.55	3	2.23*

[a] av, average (mean) concentration (nM). [b] *n*, number of replicates. [c] SD, standard deviation (nM). [d] –, quantity could not be calculated. (x), estimated quantity. *, result was below detection limit established by lab blank. Blank space, analysis not performed. [e] MDL, method detection limit (MDL = blank + 3 SD) (nM).

proximately 50%. Also, one should recognize that the H_2O_2 blank problem practically limits the precision and usefulness of an external calibration method. It should be noted that the potential errors involved in external calibration methods due to nonstoichiometric oxidative coupling reactions are far more serious at the nanomolar level than those which may arise from blanking problems. Thus, the minor methodological modifications and use of the standard additions methods permitted the routine quantitation of H_2O_2 in the nanomolar concentration range in groundwater samples.

Field Results. In well-mixed natural surface waters it is generally accepted that dissolved oxygen controls the system redox potential (*1*). Similarly, the redox conditions of the shallow, unconfined sand and gravel aquifer were dominated apparently by the distribution of dissolved oxygen. The samples from the shallow well at 11 m consistently showed dissolved oxygen levels greater than 80% saturated at the in situ temperatures. The concentration gradient with depth from the 11-m well was approximately −0.3 $mg \cdot L^{-1} \cdot m^{-1}$ O_2 to a depth of 32 m where the groundwater exhibited oxygen levels at or near the detection limit for both the azide-modified Winkler titration and Clark polarographic electrode methods. There was a correspondence between dissolved oxygen levels and measurements of platinum electrode potential. The gradient in O_2 noted above closely matched the corresponding gradient in Eh which was approximately −20 $mV \cdot m^{-1}$ with depth in the aquifer. Oxygen was the most concentrated oxidant measured in the system, far exceeding the dissolved levels of other potential electron acceptors, such as Fe(III), NO_3^-, or SO_4^{2-} ions. Therefore, in this shallow sand and gravel aquifer we have observed strong vertical gradients in redox conditions which are bounded by the dissolved oxygen saturation near the water table and levels below the detection limit of practical field methods at a depth of 32 m below land surface. Reports of relatively high levels of dissolved oxygen in unconfined aquifers at depths exceeding 200 m suggest that subsurface oxygen removal processes may be quite slow in certain hydrogeologic settings (*37*). While we have not explored the kinetics of oxygen uptake in our study situation, there is evidence that the activity of the O_2/H_2O electrochemical couple is mediated by the persistence of H_2O_2 in shallow groundwater. This would have the effect of substantially reducing the equilibrium oxidizing intensity of the O_2/H_2O redox couple (*1*).

The results of the H_2O_2 field analyses are presented in Table II. When five or more replicates of a sample were run, the extreme high and low values were ignored in calculating averages and standard deviations. When two replicates were run, the standard deviation was estimated from the range of concentrations (*38*). One replicate blank was run on 2/19/85, 10/17/85, and 4/6/85, and no blank was run on 1/28/86. The square root of the sum of squares of the blank variances of the other dates was used to estimate the standard deviation for these dates. The weighted average of the blanks was used to estimate the standard deviation for these dates. For 1/28/86, the weighted average of the blanks from the other dates was used to estimate the blank. Because of the difficulty of preparing H_2O_2-free water mentioned above, measured H_2O_2 concentrations were frequently below the method detection limit (MDL). However, on 8 of the 15 sampling runs, observed H_2O_2 concentrations for at least one depth were greater than the MDL or estimated MDL.

There was some indication that peroxide maxima were related to maxima in dissolved oxygen, particularly at a depth of 11 m. Although this shallow well consistently showed near-saturation oxygen levels and most frequent detectable peroxide concentrations, the acutal concentrations were not correlated over time. Also, the increased reducing gradient with depth noted above for O_2 and Eh was not observable in the H_2O_2 data.

If H_2O_2 is to be applied to aquifer rehabilitation efforts, we must better understand the reactions that control oxidant or reductant stabilities. The scopoletin–horseradish peroxidase method with the modifications described in this paper permits reliable H_2O_2 determinations in groundwaters and may be useful for the study of H_2O_2 geochemistry.

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