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An improved colorimetric method for chlorine dioxide and chlorite ion in drinking water using lissamine green B and horseradish peroxidase

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Abstract

Lissamine Green B (LGB) was carefully selected as a potential candidate for the development of a new U.S. Environmental Protection Agency (EPA) method that is intended for use at water utilities to determine chlorine dioxide (ClO_2) in drinking water. Chlorine dioxide reacts with LGB in aqueous solution to decrease the absorbance of LGB in direct proportion to the ClO_2 concentration. LGB was confirmed to have adequate sensitivity, and to suffer less interference than other dyes reported in the literature. The stoichiometry for the reaction between LGB and ClO_2 was found not to be 1:1 and is dependent on the LGB concentration. This required calibration of each LGB stock solution and prompted the investigation of alternate means of calibration, which utilized a horseradish peroxidase (HRP)-catalyzed conversion of chlorite ion (ClO_2^-) to ClO_2. This approach allowed the simultaneous determination of ClO_2^- concentration, which is also required each day at water plants that use ClO_2. Studies were conducted to characterize and carefully optimize the HRP-conversion of ClO_2^- to ClO_2 in order to yield reaction conditions that could be accomplished in less than 30 min at modest cost, yet meet EPA's sensitivity and robustness requirements for routine monitoring. An assessment of method detection limit, linearity and slope (or sensitivity), precision, and accuracy in finished drinking water matrices indicated that this approach was suitable for publication as EPA Method 327.0.
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Keywords: Chlorine dioxide and chlorite analysis; Drinking water; Lissamine green B; Horseradish peroxidase

1. Introduction

Chlorine dioxide (ClO_2) is used for many purposes during the production of drinking water, such as control of taste and odor problems, removal of iron and manganese, and a disinfection strategy for control of halogenated organic disinfection byproducts. When ClO_2 reacts with constituents in the water, the anions chlorite, chloride, and chlorate are formed [1]. Due to potential adverse health effects associated with short-term exposure to ClO_2 and chlorite ion (ClO_2^-) [2], the U.S. Environmental Protection Agency (EPA) established a maximum residual disinfectant level (MRDL) of 0.8 mg L^{-1} for ClO_2 and a maximum contaminant level (MCL) of 1.0 mg L^{-1} for ClO_2^- in drinking water as part of the Stage 1 Disinfectant/Disinfection Byproducts (D/DBP) Rule [3].

Drinking water plants that use chlorine dioxide are required to test the water entering their distribution system on a daily basis using an EPA-approved method to confirm that their water is below the MRDL and MCL for ClO_2 and ClO_2^-. Standard Method 4500-ClO_2 D [4] involves the reaction between *N,N′*-diethyl-*p*-phenylenediamine (DPD) and ClO_2 to form an oxidized product that is measured at 550 nm. Limitations of this method include the potential interference posed by manganese (4), and the potential interference caused by free available chlorine (FAC) even in the presence of the masking reagent glycine, which is used to suppress this interference [5]. Standard Method 4500-ClO_2 E, involves a successive amperometric

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titration procedure to determine ClO_2 and ClO_2^- concentrations [6]. Method 4500-ClO_2 E is subject to interferences from manganese, copper, and nitrate, which are commonly found in drinking waters.

While the methods approved for daily monitoring under the Stage 1 D/DBP Rule were deemed adequate by EPA at the time of rule promulgation, EPA initiated studies aimed at developing a new method for ClO_2 that might offer improved selectivity and simplicity. A comprehensive search of the literature identified four different dyes that had been investigated as potential alternative methods to determine chlorine dioxide in water—acid chrome violet K (ACVK), amaranth, chlorophenol red (CPR) and lissamine green B (LGB) [7–15]. Each involves a colorimetric technique similar to the DPD method. Amaranth and ACVK were reported to have interferences with permanganate, which is commonly used for drinking water treatment. CPR was reported to exhibit an interference with chlorite, which is a serious limitation since chlorite is formed during disinfection with ClO_2. All methods showed some interference associated with colored waters. LGB was selected for evaluation because it appeared to have the least number of potential interferences and because it offered the most sensitivity.

The reaction of HRP with ClO_2^- was originally investigated over 30 years ago [16]. Horseradish peroxidase was reported to react with ClO_2^- to form another reactive molecule that was capable of chlorinating monochlorodimedone [17]. The intermediate produced, however, was the subject to debate. One group provided evidence that the reactive intermediate is ClO_2 [16,18–20] and proposed the following reaction [18].

$$5HClO_2 \rightarrow 4ClO_2 + Cl^- + 2H_2O + H^+$$

A second group presented data supporting the production of hypochlorite ion rather than ClO_2 by the reaction of HRP and ClO_2^- [17,21].

Because this enzymatic reaction offered the potential to develop a single method to analyze both ClO_2 and ClO_2^-, a number of experiments were designed to demonstrate the feasibility of using LGB to measure the ClO_2 formed by HRP. Data from these studies supported preliminary feasibility, and warranted additional studies to determine if objectives for a viable compliance method could be met. These objectives include: (i) sufficient precision at low concentration to yield a method detection limit for both ClO_2 and ClO_2^- that are at least a factor of five below their MRDL and MCL; (ii) precision and accuracy at concentrations near the MRDL/MCL that would allow the users to routinely achieve quality assurance recovery criteria (which are typically set at 70–130% in EPA methods); (iii) no significant interference from either free available chlorine or chloramines at levels at or above their MRDLs; (iv) a sample analysis time of less than 30 min; and (v) a simple method format that can be readily implemented by water treatment personnel.

This manuscript describes work to develop a single method that utilizes LGB and HRP to determine ClO_2^- and ClO_2 concentrations in drinking water. This included the evaluation of the reaction between LGB and ClO_2, and characterizing and optimizing the HRP-catalyzed conversion of ClO_2^- to ClO_2 so that the reaction would proceed to completion in a reasonable time. This included evaluating the effect of pH, HRP concentration, HRP activity, and temperature on the reaction rate. Finally, this paper describes the work to develop a common calibration procedure that did not require ClO_2 standards, and the evaluation of method sensitivity, accuracy, and precision in finished waters. This work ultimately formed the basis for EPA Method 327.0 [22], which was recently approved for daily monitoring of ClO_2^- and ClO_2 concentrations in drinking water from public water systems using ClO_2 [23].

2. Experimental

2.1. Reagents

All chemicals were ACS grade unless otherwise noted. Types I, II and VI-A horseradish peroxidase were purchased from Sigma (St. Louis, MO) and had activities of 148, 158, and 288 U mg^{-1}, respectively. Specified activity ranges were 15–150, 150–250, and 250–330 U mg^{-1}, respectively. Sigma defines the activity (U) as the amount of enzyme, which oxidizes 1 μM of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) per minute at pH 6.0 and 25 °C. According to Sigma, the Type I HRP is a crude product concentrated from extract of horseradish. The Type II product is further fractionated to remove some of the non-heme protein, and the Type VI-A product is further purified using ion chromatography [24]. Cost per analysis using the small volume technique at the optimized HRP concentration using the final Method 327 procedure is $0.23, $0.39 and $3.02 for the Type I, II and VI-A HRP, respectively.

A chlorite ion standard (1.00 mg L^{-1}) was purchased from Absolute Standards (Hamden, CT). Ammonium hydrogencitrate, trisodium citrate, sodium dihydrogen citrate sesquihydrate, glycine and chloroform were purchased from Sigma (St. Louis, MO, USA). Borate buffer was purchased from Fluka (St. Louis, MO). LGB (technical grade, 60%), sodium chlorite and potassium persulfate were purchased from Aldrich (Milwaukee, WI). Deionized, organic free water, which is designated as reagent water in this manuscript, was obtained by using a Milli-Q Elix 3 reverse osmosis system with a Milli-Q Gradient A10 water purification system (Bedford, MA).

2.2. Chlorine dioxide standard preparation

ClO_2 was generated by combining a solution containing 16 g sodium chlorite in 100 mL of reagent water with a solution of 8 g potassium persulfate in 200 mL of reagent water in a 500-mL gas washing bottle (Ace Glass, Vineland, NJ) [25]. The reaction was allowed to proceed for 30 min while sparging nitrogen through the gas washing bottle at 250 mL min^{-1}. The ClO_2 was collected in a clear, 1-L bottle containing 500 mL of reagent water cooled with ice and protected from room light. The stock ClO_2 solution was stored in headspace-free 6-mL amber vials with Teflon screw top caps at 4 °C. Prior to use, each vial was equilibrated to room temperature and diluted 1:5 with reagent water to prepare the working standard with a concentration near 1.4 g L^{-1}. This working standard was immediately transferred

to a gastight syringe (either a 2.5 or a 5.0-mL Hamilton, Reno, NV) and calibrated as described below.

A KD Scientific Model 100 syringe pump (Holliston, MA) equipped with a Hamilton gastight syringe was used to deliver the ClO_2 working standard. A small Teflon tube (9 in. long) was attached to the syringe needle to allow accurate delivery of small aliquots. Because ClO_2 is very volatile, standard delivery was accomplished by inserting the Teflon tube well below the surface of the sample being fortified. The concentration of the standard in each syringe was determined spectrophotometrically using a headspace-free technique and a 1-cm pathlength quartz cell with a Teflon stopper. The ClO_2 concentrations were calculated assuming a molar absorptivity coefficient for aqueous solutions of ClO_2 at 360 nm of 1225 cm^{-1} M^{-1} [25]. Each syringe was calibrated at the beginning, middle and end of the single-day experiments. Absorbance values for the ClO_2 solutions typically varied less than 2% during an experimental day. Average concentrations were used for all calculations.

2.3. Reagent preparation

For the initial ClO_2 studies, LGB stock solutions were prepared by dissolving 240 mg in 250 mL of reagent water and allowing the solution to stir for 24 h. Working stock solutions of LGB were prepared in a manner such that when diluted (as discussed below), they yielded an initial absorbance near 1.0. All HRP studies were conducted using a concentrated stock of HRP in reagent water that was stored at or below 6 °C. For the LGB/HRP combined reagent studies, a concentrated citric acid/glycine buffer was prepared by mixing 9 g of trisodium citrate, 5 g sodium dihydrogen citrate, and 1 g glycine in 127 mL of reagent water. A buffered HRP solution was prepared by combining 240 mg of the Type II HRP and 12.2 mL of the concentrated citric acid/glycine buffer in a 200-mL volumetric flask and bringing it to volume with reagent water. An 80-mL aliquot of the LGB stock solution was added to 12.2 mL of the concentrated citric acid/glycine buffer and brought to volume with reagent water in a 200-mL volumetric flask to prepare a buffered LGB solution. The two buffered solutions were combined and stored in a 500-mL amber glass bottle with a Teflon screw cap. A 200-μL aliquot of chloroform was added as a biocide. This LGB/HRP combined reagent, which had a final pH of 6.0, was shaken well and allowed to stand for at least 1 h prior to use. The combined LGB/HRP reagent was prepared fresh every two weeks.

2.4. Large bottle ClO_2 procedure

A headspace-free procedure was developed for the initial ClO_2 studies that employed tared, 125-mL amber bottles with Teflon screw caps. Buffer was first added to each bottle (either 63 mg of pH 8 Tris preset crystals or an equivalent amount of the other buffers), and each bottle was filled to volume with reagent water. A calibration curve was prepared by delivering an aliquot of the working ClO_2 standard (60, 180, 300, 420, or 600 μL) deep into each bottle using the syringe pump. The bottles were quickly sealed and thoroughly mixed by inverting. A 10-mL aliquot of the solution was withdrawn from the sample bottle using pipette, and a 10-mL aliquot of the LGB stock was added to yield an initial absorbance near one. Solutions were capped, and mixed again, and inverted to confirm they were free of air bubbles. Any bottle that contained headspace was rejected. A blank was prepared in an identical manner but did not contain ClO_2. Precision and accuracy data were obtained by processing samples (or fortified reagent waters) in an identical manner to standards.

The blank and calibration standards were analyzed sequentially in a 1-cm cell using an Agilent (Model 8453) diode array spectrophotometer. The absorbance value was determined for each standard and blank and a calibration curve was established by plotting the absorbance difference (blank—calibration standard) at 633 nm versus ClO_2 concentration. A liner regression that was not forced through zero was used to fit the data. Sample concentrations were calculated by comparing the absorbance difference (blank—sample) to the calibration curve.

2.5. Micro procedure for ClO_2^- (or ClO_2)

A scaled-down, headspace-free procedure was used for the HRP experiments. A set of about forty 16-mL amber glass vials were hand selected by gravimetric determination of their volumes. Vials were chosen such that all vials in the set had volumes within ±1% of the average volume of the set.

The 16-mL amber glass vials were completely filled with reagent water (or sample matrix) fortified with known ClO_2^- concentrations. A 1-mL aliquot was removed from each vial and replaced with a 1-mL aliquot of the citric acid/glycine buffer. Vials were capped and shaken and a second 1-mL aliquot was removed from each vial and replaced with a 1-mL aliquot of the LGB/HRP combined reagent. Vials were capped, shaken and allowed to react for 20–30 min. A blank was prepared in an identical manner without the addition of ClO_2^-. Absorbance differences between the blank and calibration standards (or samples) were measured at 633 nm in a 1-cm cell.

2.6. Final method evaluation conditions

The final method protocol is described in detail elsewhere [22]. It uses the micro procedure described above, but contains a combined HRP/LGB reagent, which was necessary to yield a single calibration curve as discussed below. A single procedural calibration curve is determined for ClO_2^-, which is used to calculate the concentration of both analytes. Samples are first measured to determine a total concentration of both species, and then a second, sparged sample is measured to determine the ClO_2^- concentration. The ClO_2 concentration is calculated as the difference between the total and ClO_2^- concentrations.

2.7. Temperature studies

The effect of temperature on the reaction of HRP with chlorite ion was investigated by equipping the Agilent spectrophotometer with a Peltier (Model 89090A) thermostated cell holder. Prior to collecting data, an external temperature probe (HP Betather-

mastat) was used to confirm that the contents of the cells reached the desired temperature. Experiments were conducted at 15, 25, 35, 45, and 55 °C, using a Type I HRP concentration of 0.28 mg mL^{-1} and a ClO_2^- concentration of 1 mg L^{-1}. In the experiments, all reagents except the HRP were allowed to equilibrate for 10 min in the thermostated cell holder prior to addition of the HRP, which was added (63 μL to 2.5 mL in the cell) at time zero.

3. Results and discussion

3.1. Preliminary assessment of LGB detection of ClO_2

LGB is a triphenylmethane dye with a high standard redox potential (1.0 V) [7] making it less subject to interferences from combined chlorine or chlorite ion. LGB has a pH-dependent absorption maximum in the red region of the visible spectrum. For example, in Tris buffer the absorbance maxima were 615, 622 and 631 nm at pH 9, 8 and 7, respectively. The original evaluation of LGB was conducted at pH 9.0 using an ammonium chloride/ammonia buffer [7]. The reason for using this pH value is that at pH 9, hypochlorite ion is the predominant FAC species, and it is a less powerful oxidant than hypochlorous acid. In addition, ammonia binds FAC to form chloramines, which are even weaker oxidants than hypochlorite ion [7]. A number of alternate reagents to remove FAC have been studied since this original work, allowing the investigation of alternate pH values. Eliminating the potential FAC interference was a key consideration since many drinking water systems use FAC to maintain a disinfectant residual in the distribution system.

Several buffer systems, including ammonium chloride/ammonia, borate, phosphate buffers at pH 9, and Tris buffer at pH 7, 8 and 9 were examined. Buffered reagent water solutions were prepared containing initial FAC concentrations of 8 mg L^{-1}, which is twice the MRDL allowed under the Stage 1 D/DBP Rule [3]. For the ammonium chloride/ammonia and Tris buffers, which sequester FAC, the predominant chlorine species in solution is combined chlorine. Each solution was fortified with LGB and monitored at their absorbance maximum over a 2-h period. At pH 9, each of the buffer systems exhibited a 4% loss in LGB absorbance in the absence of FAC (or combined chlorine) with the exception of borate buffer, which exhibited a 20% loss over the 2-h period. The pH 9 phosphate buffer fortified with FAC showed a 59% decrease in LGB absorbance over the 2-h period, but was stable (4% loss) when the FAC was sequestered by adding 200 mg L^{-1} of ammonium chloride prior to the addition of LGB. The ammonium chloride/ammonia buffer fortified with combined chlorine, exhibited a 6% loss over the same period (2% more than the blank control which contained LGB at the same concentration in the same buffer without any FAC). The absorbance of LGB in Tris buffer at pH 9 and 7 in the presence of combined chlorine diminished by 4% and 1%, respectively over the same 2-h period, indicating that lower pH values could be considered and may even be preferred as lower pH seems to slow the degradation of LGB.

Precision, accuracy and method detection limit [25] were evaluated using the large bottle technique described above in several buffer systems. Method detection limits were calculated according to the procedure described by Glaser et al. [26]. These data are presented in Table 1. All buffers performed acceptably with the exception of the borate buffer system presumably due to the excessive downward drift in LGB absorbance noted above. A preliminary assessment of method performance in a local surface and groundwater performed similarly.

3.2. Method design considerations

Aqueous chlorine dioxide standards require daily calibration, a syringe pump or alternate headspace-free delivery device with appropriate accuracy, and a high degree of skill in headspace-free manipulation of potentially toxic, volatile standards. These requirements were incompatible with EPA's original method objectives; therefore a calibration approach that is used in the indigo method [27,28] for determining ozone concentrations in water, the sensitivity coefficient adjustment, was considered. This approach would, if feasible, simplify calibration. To use this approach, however, the reaction stoichiometry must be known.

The apparent molar absorptivity coefficients were determined for two commercially available sources of LGB based on triplicate measurements in pH 6.0 citric acid buffer, where the term "apparent" is used to indicate that the calculated molar absorptivities are not corrected for dye purity. The LGB obtained from Acros and Aldrich, which had manufacturer's purity ratings of 51.4% and 74.8%, respectively, yielded apparent molar absorptivities of 44,890 and 71,510 (cm^{-1} M^{-1}) at their absorbance maxima (634 nm). By using the Beer Lam-

Table 1
Evaluation of the ClO_2 procedure in various buffer systems over the pH range of 7–9 using the large bottle technique and gaseous ClO_2 calibration standards

Buffer system	MDL (mg L^{-1})	Low-level[a]		Mid-level[a]		High-level[a]	
		Rec. (%)	R.S.D. (%)	Rec. (%)	R.S.D. (%)	Rec. (%)	R.S.D. (%)
Ammonia/NH_4Cl buffer pH 9	0.043	95	5.9	115	9.5	114	8.0
Borate buffer pH 9	0.14	159	12	126	5.3	105	3.2
Tris buffer pH 9.0	0.12	101	16	105	4.0	96	2.6
Tris buffer pH 8.5	0.075	88	12	113	8.4	98	1.8
Tris buffer pH 8.0	0.080	73	15	105	7.1	101	0.3
Tris buffer pH 7.5	0.081	90	11	113	2.7	104	0.5
Tris buffer pH 7.0	0.077	110	9.2	111	2.1	110	0.6

[a] Fortification levels for the low-, mid- and high-levels were 0.24, 0.7 and 2.3 mg L^{-1}, respectively. R.S.D. (%), percent relative standard deviation. Rec. (%), percent recovery.

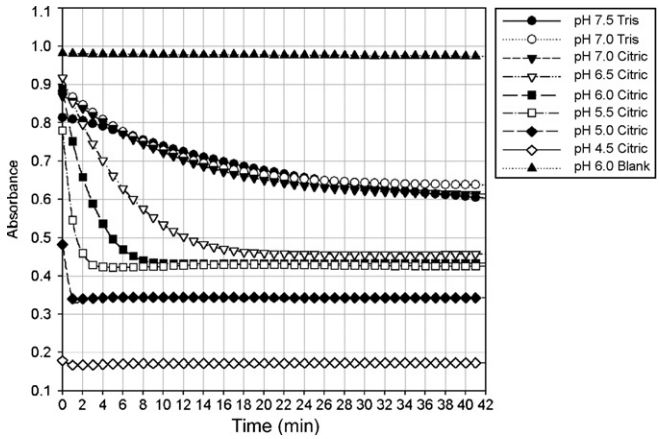

Fig. 1. The pH dependence of the HRP conversion of ClO_2^- to ClO_2 measured by the disappearance of LGB with an initial ClO_2^- concentration of 1.0 mg L^{-1}.

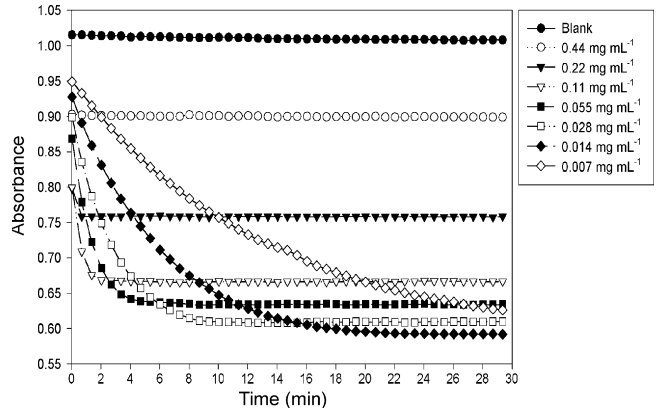

Fig. 2. Reaction rate in pH 6.0 citrate buffer as a function of HRP concentration for a chlorite concentration of 1.0 mg L^{-1}.

bert Law, this predicted an absorbance change of 1.1 for a 1.0 mg L^{-1} concentration of ClO_2 assuming a 1:1 reaction stoichiometry. Because most of the calibration curves yielded slopes near 0.35 (ΔA mg^{-1} L), the calculated reaction stoichiometry is not 1:1. This ruled out using the sensitivity coefficient adjustment approach [27,28] and shifted focus on devising a calibration routine that employed the HRP conversion of ClO_2^- to ClO_2.

3.3. The effect of pH on the Type I HRP reaction rate

Original studies of the reaction rate for HRP-catalyzed conversion of ClO_2^- to ClO_2 reported that the optimal pH was 4.1 [16]. Research conducted since then has indicated that disproportionation of ClO_2^- to form ClO_2 [29,30] might contribute to the reported reaction rates at low pH. Organic acids, like citric acid, have been shown to catalyze this reaction at even higher pH values [31]. This warranted studies to determine what effect, if any, disproportion plays in the conversion of ClO_2^- to ClO_2.

A series of kinetic studies were conducted using Tris and citric acid buffer systems that spanned the pH range from 4.5 to 7.5. Tris has a single pK_a at 8.07 and is not a suitable buffer below pH 7.0. Citric acid, with pK_a values of 3.14, 4.77, and 6.39, is a much better buffer in the pH range of 2–7. In each experiment, the initial ClO_2^- concentration was 1.0 mg L^{-1} (15 μM), the HRP concentration was 0.055 mg mL^{-1} (1.3 μM), and the LGB concentration was 14 μM (using the apparent molar absorptivity coefficient reported above). As indicated in Fig. 1, the reaction rate exhibited a dramatic dependence on pH, as did the apparent stoichiometry of the reaction, which is currently not understood. An experiment conducted similar to the pH 4.5 experiment but without HRP showed no noticeable decrease in LGB absorbance and ruled out any contribution from disproportionation over the pH range studies. The behavior shown in Fig. 1, clearly illustrates the important role that buffer pH plays in method performance. Based on these data, the pH 6 citric acid buffer was chosen for two reasons: the absorbance change with time (or method sensitivity) was not affected by small changes in pH; and the reaction required less than 30 min for completion.

3.4. Optimization of Type I HRP concentration

Hewson and Hager [18] reported that lower concentrations of HRP are more efficient at producing ClO_2, e.g., more moles of ClO_2 are produced per mole of HRP, and for a constant concentration of HRP, higher concentrations of ClO_2^- also formed more ClO_2. This warranted an investigation of reaction rates over the ClO_2^- concentration range of interest, which was initially established as 0.25–2.0 mg L^{-1} (3.7–30 μM). For these studies, Type I HRP obtained from Sigma-Aldrich was utilized. The relevance of enzyme "type" is discussed below. A series of 30-min kinetic experiments were conducted at seven HRP concentrations ranging from 0.44 to 0.0070 mg L^{-1} (10–0.16 μM) for four ClO_2^- concentrations (0.25, 0.50, 1.0 and 2.0 mg L^{-1}). All experiments were conducted at room temperature in pH 6.0 ammonium hydrogencitrate buffer using the small volume technique described above. The enzyme concentrations were specifically chosen to achieve a sufficiently fast reaction at all concentrations ultimately yielding reaction times of 20 min or less. The data from the mid-level ClO_2^- concentration experiments are presented in Fig. 2. The figures obtained at other ClO_2^- concentrations are available as supplemental information (Supplemental information, Figs. SI1–SI3).

The kinetic studies presented in Fig. 2 exhibited inhibition at high HRP concentrations, which was apparent in the 0.22 mg mL^{-1} HRP experiment (5.0 μM HRP with 15 μM ClO_2^-) and increased at the higher HRP concentration. Inhibition was more evident in the lower ClO_2^- concentration experiments. For example, the 0.25 mg L^{-1} (3.7 μM) ClO_2^- solutions began to exhibit this phenomenon at 0.055 mg mL^{-1} (1.2 μM) HRP and total inhibition of ClO_2 production was observed in the $\geq$0.22 mg mL^{-1} (5.0 μM) HRP concentration experiments. Only the highest HRP concentration (0.44 mg mL^{-1} or 10 μM) showed any inhibition in the 2.0 mg L^{-1} (30 μM) ClO_2^- concentration experiments. In all of these experiments, the initial LGB absorbance was near 1, which equates to an initial LGB concentration of about 14 μM.

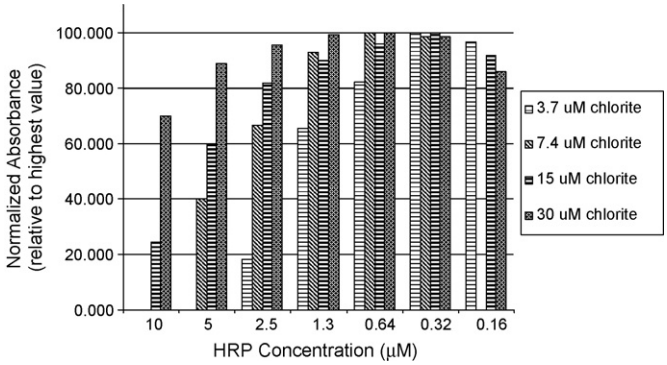

Fig. 3. Absorbance change as a function of HRP concentration normalized at each ClO_2^- concentration.

This inhibition was also investigated as a function of enzyme activity. These data are reported in the section below.

Hewson and Hager [18] suggested that ClO_2 could react with the HRP and at high enough ClO_2 concentrations this reaction could inactivate the enzyme. To investigate this further, an experiment was conducted in triplicate in which ClO_2^- 1.0 mg L^{-1} (15 μM) and HRP 0.028 mg mL^{-1} (0.64 μM) were allowed to react in a headspace-free container for 20 min prior to the addition of LGB. In each case, the absorbance of the solution was not different from the blank indicating that the ClO_2 had been consumed by an alternate or competing reaction.

Although inhibition details remain unclear, the HRP concentration affects the rate and amount of LGB consumed by ClO_2. HRP concentration optimization studies (Fig. 3) were done by normalizing all data relative to the largest absorbance change (blank—sample). With the exception of the data set at the ClO_2^- and HRP concentrations of 0.50 mg L^{-1} (7.4 μM) and 0.0070 mg mL^{-1} (0.16 μM), which was unintentionally omitted in the experiment, all data sets without a histogram bar represent complete inhibition (no change in LGB absorbance). The optimum Type I HRP concentration was 0.028 mg mL^{-1} (0.64 μM). Measurements at this concentration exhibited a relative absorbance near 100% for all concentrations. Furthermore, the reactions at 0.028 mg mL^{-1} were completed faster than those using 0.014 mg mL^{-1} HRP.

3.5. Evaluation of enzyme type

Numerous types of HRP enzyme are available. Experiments described above were conducted exclusively with Type I HRP. As indicated in Section 2.1, the lot of Type I enzyme available for these studies was at the top of the specified activity range (148 U mg^{-1}, range 15–150 U mg^{-1}). This warranted the evaluation of additional types of HRP. This included the evaluation of a Type II material that was near its lower limit of activity and a Type VI-A HRP with average activity. Kinetic studies were conducted at room temperature using Type II and Type VI-A HRP in a manner identical to the experiments described above for the Type I enzyme at a ClO_2^- concentration of 1.0 mg L^{-1} (Supplemental information, Figs. SI4 and SI5). The reactions with the Type VI-A and II HRP were faster than the Type I enzyme. This was most pronounced at low enzyme concentration. In addition, both Type II and VI-A showed less susceptibility towards inhibition at high HRP concentration. The Type I enzyme showed inhibition at the two highest HRP concentrations evaluated, the Type II HRP at only the highest HRP concentration, and the Type VI-A HRP did not exhibit inhibition at any concentration. These data seem to indicate that the competing reaction that results in diminished degradation of LGB is not an intramolecular reaction (with respect to HRP). It is possible that an impurity, removed during HRP refinement, reacts preferentially with ClO_2. Ruling out such an occurrence would not be a trivial task.

The Type VI-A HRP has the highest reaction rate and as a result is least subject to inhibition; however, the increase in cost is significant (see Section 2.1). Since the Type I enzyme evaluated in these studies was very near the upper purity limit of the manufacturer's specification, the Type II enzyme which added only modest cost was the obvious choice for the method. In addition, because the Type I enzyme was essentially at the lower activity limit for the Type II HRP, the concentration optimized in the above section was not altered.

3.6. Effect of temperature on HRP reaction rate

Temperature is widely understood to affect enzyme turnover rates, which could adversely affect method robustness and precision. To address this concern, the effect of temperature on enzyme kinetics was investigated for the Type I HRP. The activity of the Type I enzyme was very near the lower range limit of the Type II HRP. Experiments were conducted at 15, 25, 35, 45, and 55 °C using the optimum HRP concentration of 0.028 mg mL^{-1} and a ClO_2^- concentration of 1.0 mg L^{-1}, respectively (Supplemental information, Fig. SI6). The rate of ClO_2 generation (or LGB consumption) increased with increasing temperature, but the rate increases were smaller than expected. At 55 °C, the reaction was complete in about 10 min, while the 15 °C experiment required 17 min—both acceptable reaction times. Reaction rates at other temperatures fell between these two values. Based on these results, it was determined that the effect of temperature on this reaction was negligible over the range of temperatures that will likely be encountered in the laboratory environment.

3.7. Initial assessment of chlorite ion method performance

An initial assessment was conducted on the ClO_2^- portion of the method using the micro procedure that had been optimized using pH 6 citric acid/glycine buffer. Method detection limits were determined according to Glaser et al. [26] in reagent water, a chlorinated surface water, and ClO_2-treated groundwater. These were 0.063, 0.045 and 0.12 mg L^{-1}, respectively, for each matrix fortified with ClO_2^- at a concentration of 0.50 mg L^{-1}. The groundwater was sparged for 10 min to remove any residual ClO_2 prior to fortification with ClO_2^-. Precision and accuracy in these matrices were also assessed and are reported in Table 2. Method performance was adequate in all matrices.

Table 2
Accuracy and precision for the micro procedure using HRP for the determination of chlorite reagent water and two finished tap waters

Matrix	Low-level			Mid-level			High-level		
	Spike level ($mg\,L^{-1}$)	Mean Rec. (%)	R.S.D.[a] (%)	Spike level ($mg\,L^{-1}$)	Mean Rec. (%)	R.S.D.[a] (%)	Spike level ($mg\,L^{-1}$)	Mean Rec. (%)	R.S.D.[a] (%)
RW[b]	0.50	97.6	4.1	1.0	109	1.9	2.0	98	0.5
SW[c]	0.50	85	6.7	1.0	103	1.4	2.0	97	1.3
GW[d]	0.50	81	9.5	1.0	104	0.8	2.0	97	0.6

[a] R.S.D., percent relative standard deviation.
[b] RW, reagent water.
[c] Finished surface water from a municipality disinfected with chlorine.
[d] Groundwater from a municipality disinfected with chlorine with a hardness >300 $mg\,L^{-1}$ ($CaCO_3$).

3.8. Developing a common calibration technique for ClO_2 and ClO_2^-

The technical challenges associated with the headspace-free manipulation of ClO_2 standards together with its caustic nature ruled out calibration using aqueous ClO_2 standards. The stoichiometry of the reaction between ClO_2 and LGB was not one-to-one, a necessity for sensitivity coefficient adjustment. This meant external calibration was required for ClO_2 and ClO_2^-. However, data collected during the initial evaluation of LGB for the detection of ClO_2 and ClO_2^- seemed to indicate that the slopes for each analyte differed, and that the slope of the calibration curves might be dependent on the concentration of LGB.

The initial concentration of LGB was varied to provide an absorbance range from approximately 1.0–3.5 in a 1-cm cell. Actual absorbance measurements were all made using a 5-mm pathlength cell in an attempt to remain within the linear region of the spectrophotometer. Experiments were conducted at pH 6 using the citric acid/glycine buffer, with a relatively high concentration of both ClO_2 and ClO_2^- (e.g., 1.7 $mg\,L^{-1}$). The ClO_2^- experiments contained Type II HRP at 0.028 $mg\,mL^{-1}$; the ClO_2 experiments did not (Supplemental information, Fig. SI7). Both reactions exhibited a sensitivity (ΔA per $mg\,L^{-1}$ of analyte) that was dependent on LGB concentration, and at all LGB concentrations the sensitivity for ClO_2^- was higher than ClO_2. This is surprising, since the detection of ClO_2^- first requires its HRP-catalyzed conversion to ClO_2, and this reaction was not expected to be 100% efficient.

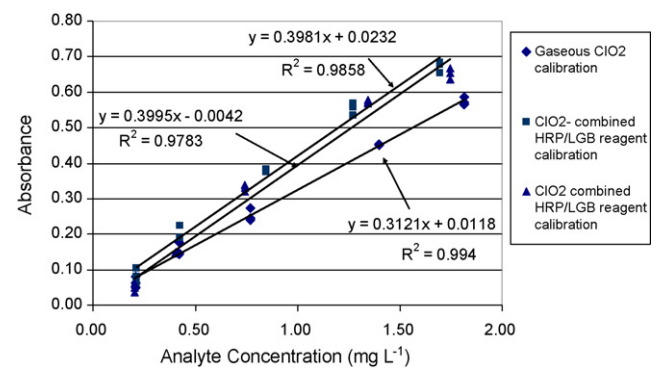

Fig. 4. Slopes of calibration curves recorded for ClO_2 with gaseous ClO_2 calibration, and ClO_2^- and ClO_2 using the combined HRP/LGB reagent.

Perhaps HRP plays a role in increasing the sensitivity by reacting with impurities in the LGB that would otherwise react with chlorine dioxide. To investigate this further, the micro procedure and a combined LGB/HRP reagent were used to generate calibration curves for ClO_2^- and ClO_2 over a concentration range of 0.25 to 1.8 $mg\,L^{-1}$. These curves were compared to a third prepared for ClO_2 with LGB but without the HRP (Fig. 4). The micro technique using the combined reagent yielded slopes for both analytes that were in good agreement.

3.9. Evaluation of final method performance

The combined reagent was further optimized to yield a linear dynamic range of 0.25 to 2.3 $mg\,L^{-1}$ ClO_2^- for evaluation

Table 3
Method detection limits and precision and accuracy determined in fortified reagent water using the combined HRP/LGB reagent and a single chlorite calibration curve according to the final Method 327.0 procedure

Fortification concentration		Chlorite			Chlorine dioxide		
ClO_2^- ($mg\,L^{-1}$)	ClO_2 ($mg\,L^{-1}$)	Recovery (%)	R.S.D. (%)	MDL ($mg\,L^{-1}$)	Recovery (%)	R.S.D. (%)	MDL ($mg\,L^{-1}$)
0.25	SNF	112	12	0.11	SNF	SNF	SNF
0.25	0.95	118	8.5	0.078	116	3.6	NC
SNF	0.26	SNF	SNF	SNF	102	5.1	0.042
1.0	0.26	103	2.9	NC	124	16	0.16
1.0	0.94	98.5	3.2	NC	111	4.8	NC

SNF, sample not fortified. NC, MDLs were not calculated because the fortification concentration was too high. MDL, method detection limit calculated according to [26].

Table 4
Method precision and accuracy in surface water and groundwater using the combined HRP/LGB reagent and a single chlorite calibration curve according to the final Method 327.0 procedure

Fortification level		Chlorite		Chlorine dioxide	
ClO_2^- (mg L^{-1})	ClO_2 (mg L^{-1})	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Surface water[a]					
1.0	SNF	107	4.2	SNF	SNF
SNF	0.82	SNF	SNF	100	1.9
1.0	0.82	109	3.7	91.3	8.9
2.0	SNF	105	1.4	SNF	SNF
SNF	1.94	SNF	SNF	96.9	1.0
Groundwater[b]					
1.0	SNF	110	4.4	SNF	SNF
SNF	0.90	SNF	SNF	92.0	3.3
1.0	0.90	107	1.6	93.8	3.0
2.0	SNF	100	2.7	SNF	SNF
SNF	2.0	SNF	SNF	110	1.7

[a] Finished surface water contained 0.9 mg L^{-1}FAC.
[b] Finished groundwater contained 0.8 mg L^{-1} FAC and had a hardness of 325 mg L^{-1} ($CaCO_3$). SNF, sample not fortified.

in the final method. The resulting method [22] is a method by difference, which first analyzes total ClO_2^- and ClO_2, and then determines the ClO_2^- concentration in a second, sparged sample. ClO_2 concentrations are then calculated by difference. Studies were designed to determine MDLs, accuracy and precision in reagent water in a manner that would pose a reasonable challenge to the method. For example, MDLs were determined for reagent waters containing single analytes and for reagents with one analyte at the lowest calibration concentration and the other at its regulatory limit, the MRDL or the MCL as reported above. These data, reported in Table 3, demonstrated acceptable method performance. Studies were next designed to evaluate method precision and accuracy in a finished groundwater and surface water. These studies were designed to assess single analyte performance near the regulatory limit and near the upper limit of the calibration range and to assess method performance with both analytes near their regulatory limits. These data (Table 4) also met method performance objectives.

4. Conclusions

LGB was selected for the analysis of ClO_2 in finished drinking water because it had fewer interferences and better sensitivity than other approaches. While investigating LGB, several properties were uncovered that posed significant challenges requiring the use of an alternate calibration procedure. The HRP-catalyzed conversion of ClO_2^- into ClO_2 was selected as a potential answer.

Kinetics studies showed that the HRP conversion of ClO_2^- to ClO_2 accelerated as pH was decreased, and that disproportionation did not contribute to the formation of ClO_2 even at the lowest pH studied. The enzyme concentration was optimized to avoid a competing reaction that consumes ClO_2 at high HRP concentrations while achieving a suitable reaction analysis time at a reasonable cost. The competing reaction was less prominent and/or absent for the more purified forms of HRP. The mechanism of this inhibition reaction is currently not understood.

The slope of the calibration curves were larger for the HRP-generated ClO_2 than for ClO_2 added directly into solution. This required the use of a combined HRP/LGB reagent and the use of an identical reaction time for both ClO_2 and ClO_2^-. The latter was required because the oxidation of LGB by ClO_2 in the presence of HRP exhibited both a fast and a slow reaction with a kinetic profile similar to that of the HRP-catalyzed reaction that contributed to sensitivity.

The performance of the final method was evaluated in reagent water and in finished waters fortified with ClO_2 and ClO_2^-. Method detection limit, linearity and sensitivity (or slope), accuracy, precision and robustness met the original project objectives and warranted publication as EPA Method 327.0. Future studies could include investigation of reagent stability, and a full characterization of potential method interferences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2007.06.006.

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Figure SI1: Reaction rate in pH 6.0 citrate buffer as a function of Type I HRP concentration for a chlorite concentration of 0.25 mg L^{-1}.

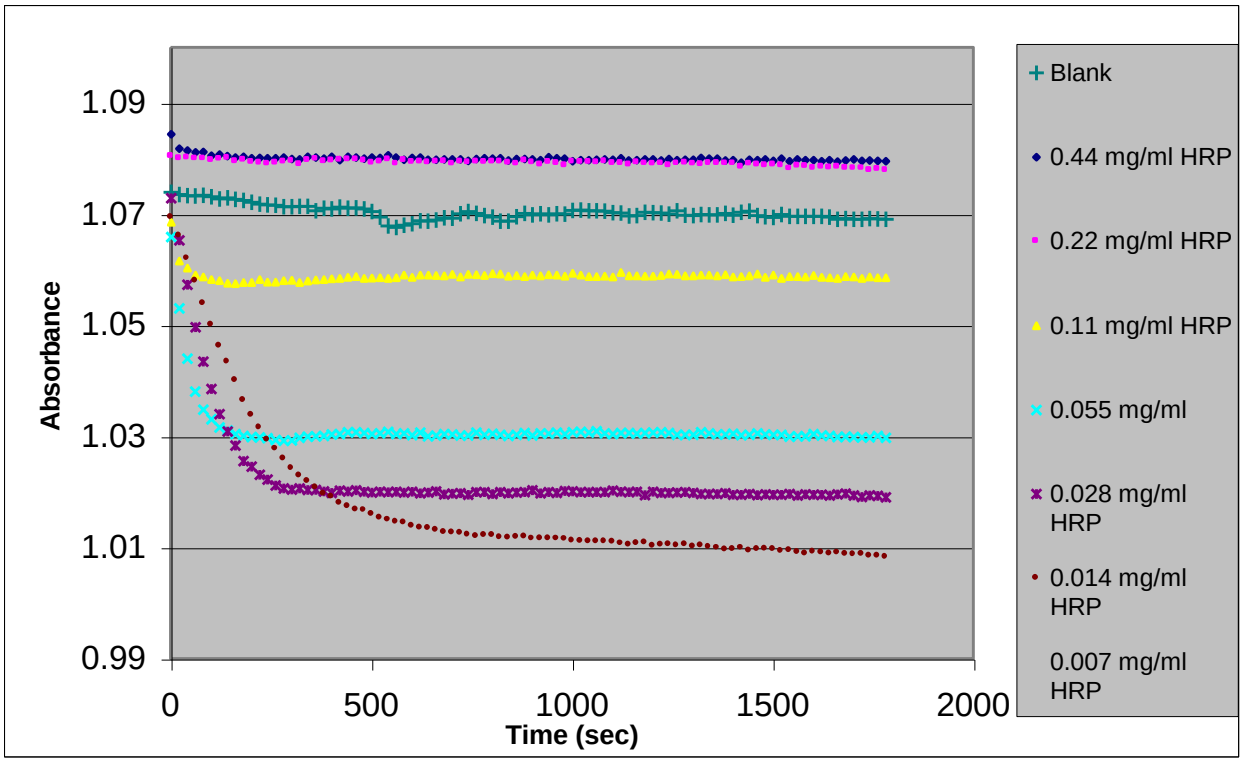

Figure SI2: Reaction rate in pH 6.0 citrate buffer as a function of Type I HRP concentration for a chlorite concentration of 0.50 mg L^{-1}.

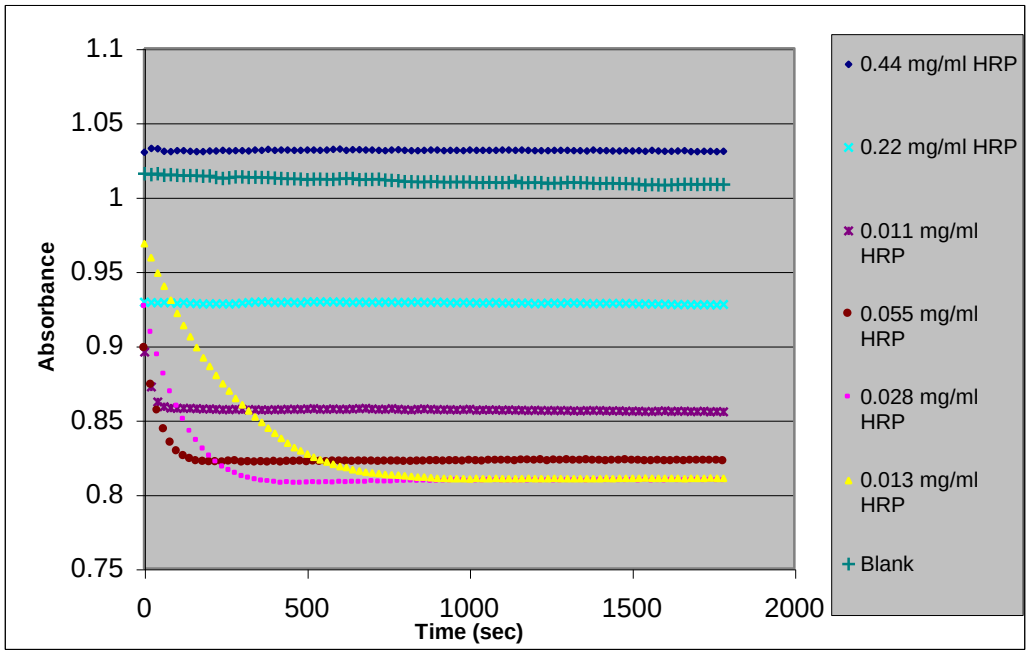

Figure SI3: Reaction rate in pH 6.0 citrate buffer as a function of Type I HRP concentration for a chlorite concentration of 2.0 mg L^{-1}.

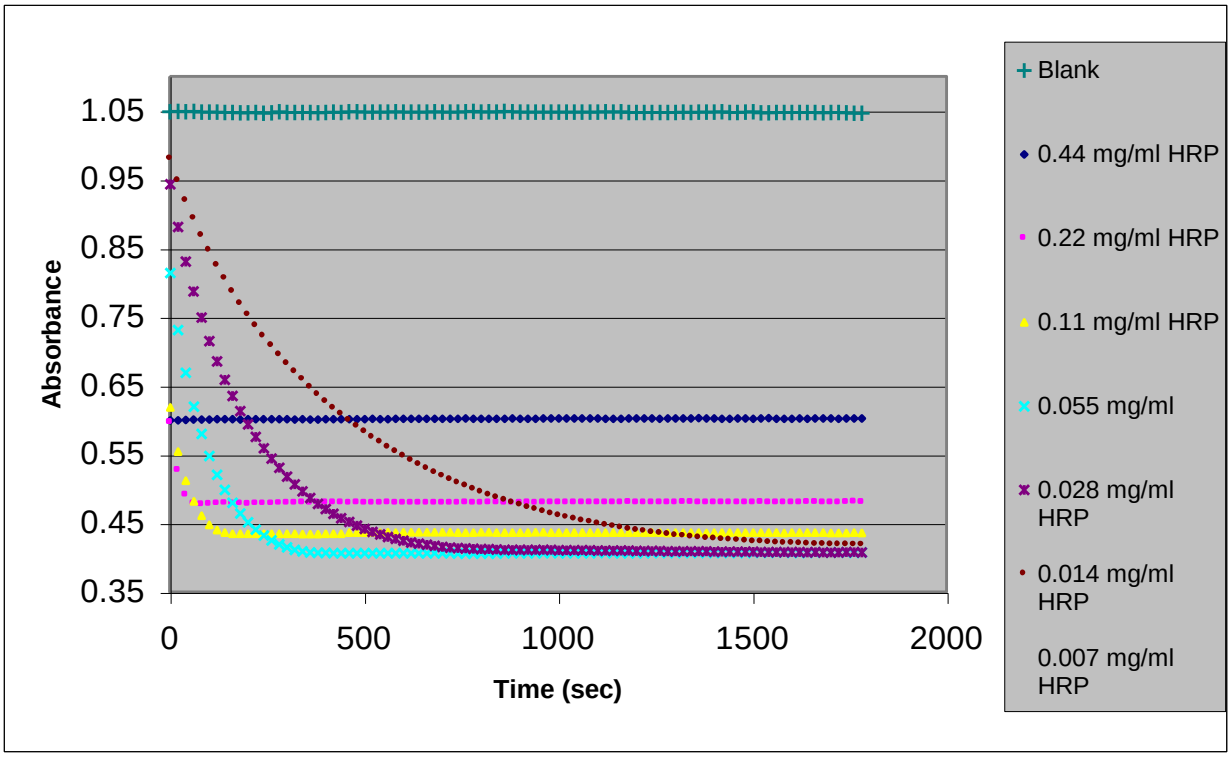

Figure SI4: Reaction rate in pH 6.0 citrate buffer as a function of Type II HRP concentration for a chlorite concentration of 1.0 mg L^{-1}.

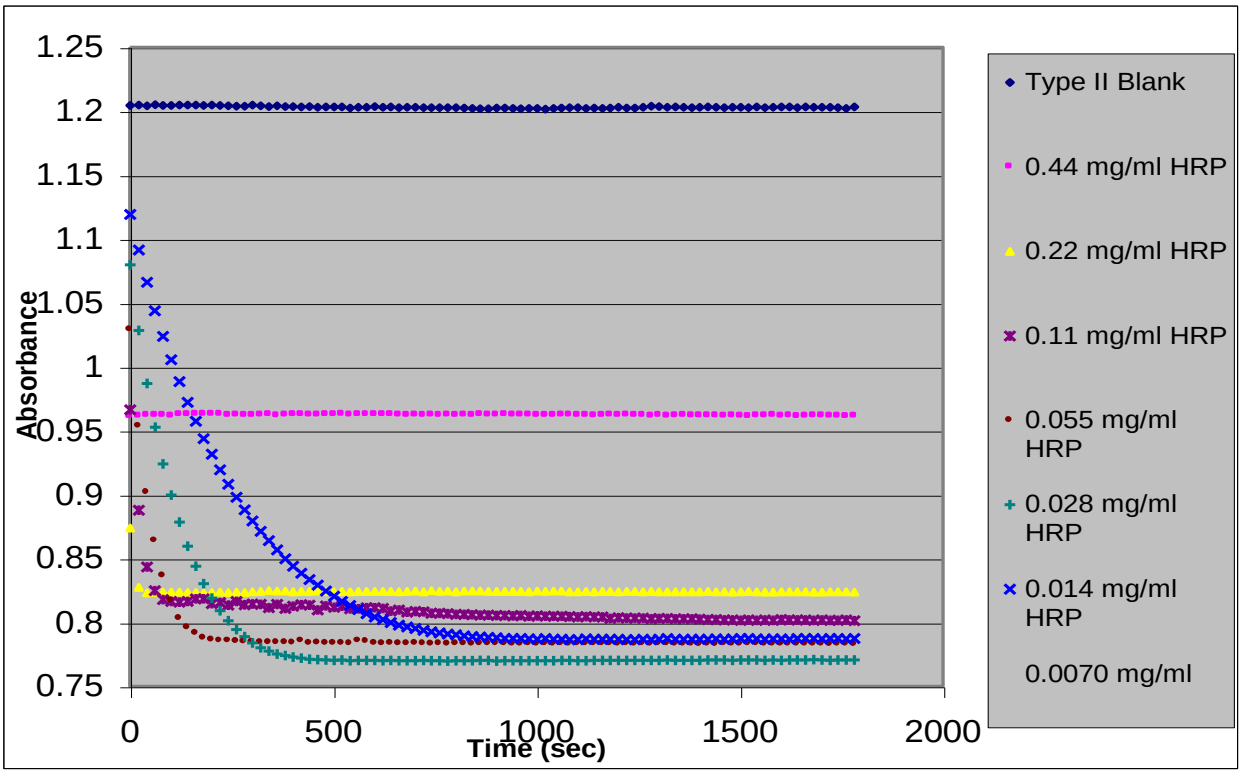

Figure SI5: Reaction rate in pH 6.0 citrate buffer as a function of Type VIA HRP concentration for a chlorite concentration of 1.0 mg L^{-1}.

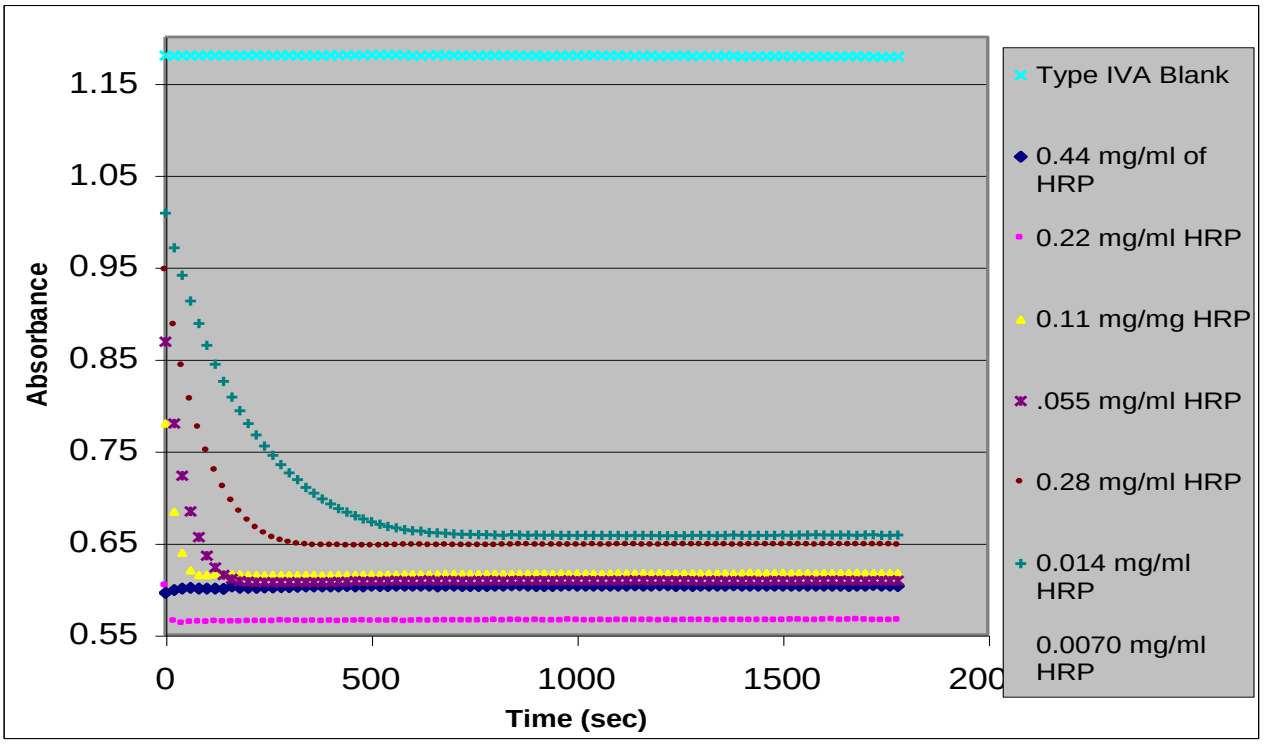

Figure SI6: Type I HRP (0.028 mg mL^{-1}) reaction rate as a function temperature for a chlorite concentration of 1.0 mg mL^{-1}.

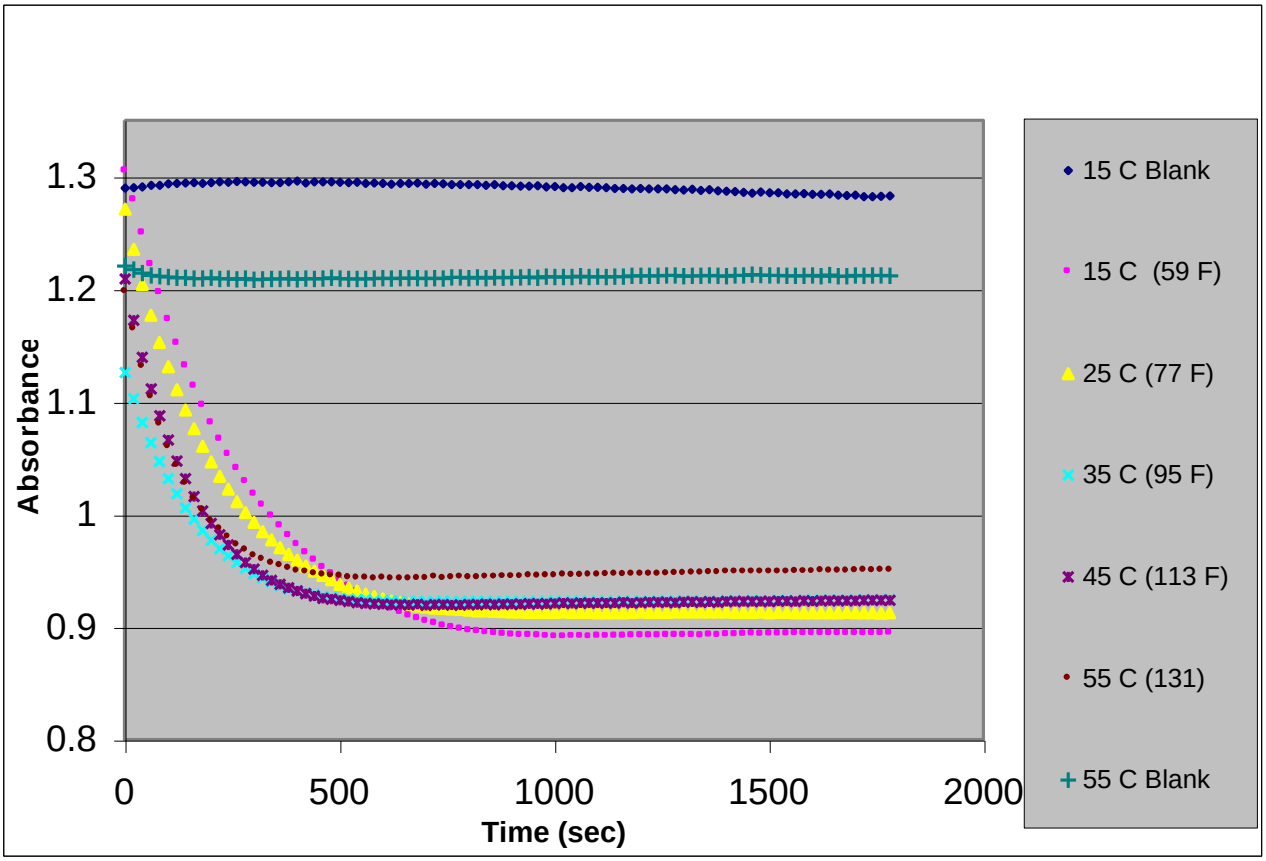

Figure SI7. The dependence of method sensitivity for the chlorite and chlorine dioxide analyses as a function of LGB concentration. All absorbances measured in a 5-mm pathlength cell, but multiplied by two to be consistent with data presented elsewhere. Error bars are ± one standard deviation based on triplicate measurements.