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LITERATURE DISCREPANCY: WHAT IS THE MOLAR ABSORPTIVITY OF ABTS?

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Abstract

In 1975 Childs and Bardsley first described the now widely-used redox chromophore ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate)], reporting that the molar absorptivity of the prominent visible region peak at 414 nm for the oxidized $ABTS^{\cdot+}$ radical was 36 $mM^{-1}cm^{-1}$. Since then, many authors have reported ε_{414} values in the 31 – 37 $mM^{-1}cm^{-1}$ range, but many others have reported ε_{414} = 26 – 28 $mM^{-1}cm^{-1}$. Here we carefully characterize the kinetics of ABTS: oxidation, first to $ABTS^{\cdot+}$, and then to $ABTS^{2+}$. After accounting for the second oxidation step, we find ε_{414} of $ABTS^{\cdot+}$ to be 26.0 ± 0.4 $mM^{-1}cm^{-1}$ in phosphate buffer at pH 6. We discuss possible reasons for the discrepancy between the two molar absorptivity values.

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Introduction

The redox chromophore ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate)] is widely used in the spectrophotometric analysis of oxidants[1], especially free radicals,[2-4] peroxides,[5-15] transition metal complexes,[16-20] and halogens[16,21]. Lately, ABTS has also been used to measure antioxidant activity in natural products and biological samples.[22-28]

Reduced ABTS:, which is colorless (ε_{340} = 36 $mM^{-1}cm^{-1}$, ref.5), can lose an electron to form the amine radical $ABTS^{\cdot+}$ (Figure S1, Appendix). This blue-green compound has three visible absorbance peaks, with the most prominent at ≈ 415 nm. The peak around 730 nm is less intense, but it is sometimes used because interfering absorbances from other compounds at this wavelength are rare.

In their initial paper introducing the use of ABTS to detect H_2O_2, Childs and Bardsley reported a molar absorptivity of 36 $mM^{-1}cm^{-1}$ for both the reduced ABTS: (ε_{340}) and the oxidized $ABTS^{\cdot+}$ radical (ε_{414}).[5] Their spectrophotometric redox titration curve (Figure *1*) supported the former value: ε_{340}(ABTS:) = 1.8 (A_{340})/0.050 mM/1.00 cm = 36 $mM^{-1}cm^{-1}$; however, the molar absorptivity of the oxidized $ABTS^{\cdot+}$ was clearly significantly lower. From their spectra, one can calculate $\varepsilon_{414}(ABTS^{\cdot+})$ = 1.125 (A_{414})/0.050 mM/1.00 cm = 22.5 $mM^{-1}cm^{-1}$. Finally, correcting for the 12% of ABTS: that remained unoxidized after the last addition of H_2O_2, as judged by the remaining small peak at 340 nm, we get ε_{414}(ABTS:) = 25.6 $mM^{-1}cm^{-1}$. In agreement with this, Maruthamuthu et al. reported $\varepsilon_{417}(ABTS^{\cdot+})$ = 27 $mM^{-1}cm^{-1}$.[16] However, Scott et al. criticized this latter value and reported $\varepsilon_{417}(ABTS^{\cdot+})$ = 34.7 $mM^{-1}cm^{-1}$ at pH 0, in agreement with Childs and Bardsley's value.[17] On the other hand, the spectrophotometric redox titration of ABTS: with Ce^{4+} published by Scott et al. clearly showed the final $ABTS^{\cdot+}$ peak at 417 nm to be 20.5% lower than the initial ABTS: peak at 340 nm; thus, their measured value of $\varepsilon_{417}(ABTS^{\cdot+})$ should actually be 27.6 $mM^{-1}cm^{-1}$. This latter value agrees with the value of Maruthamuthu et al. (criticized by Scott et al.) and the value of Childs & Bardsley calculated from their own spectrophotometric redox titration (Figure *1*).

Over the years, the molar absorptivities for $ABTS^{\cdot+}$ reported by Childs & Bardsley and Scott et al. (36 and 34.7 $mM^{-1}cm^{-1}$, respectively) were cited and employed by many researchers.[2,3,4,9,12,24] Interestingly, however, researchers who independently determined the molar absorptivity of $ABTS^{\cdot+}$ reported values that clustered in the two distinct regions reported by Childs/Scott vs. Marutahmuthu et al. (Table *1*).

Of the 17 reported $ABTS^{\cdot+}$ molar absorptivities in Table *1*, 12 were "high" as reported by Childs/Scott: average = 33.7 ± 1.7 $mM^{-1}cm^{-1}$ (range: 31 – 37), while 5 were "low" (cf. Marutahmuthu et al [16]): average = 27.1 ± 1.0 $mM^{-1}cm^{-1}$ (range: 26 – 28). Note that the difference between these two sets of values is greater than two standard deviations. The "high" values were measured mostly at pH -0.5 to 4.4, however, three were measured at pH 6 – 8. The "low" values were measured mostly at pH 4.5 – 6.5, but one was measured at pH 0. At first glance, one might be tempted to conclude, as did Pinkernell et al[10,21] and Wang and Reckhow [1], that "higher pH results in lower apparent values of ε." However, this seems unlikely for two reasons. First, 3 of the 12 "high" values (25%) were measured at high pH, while 1 of the 5 "low" values

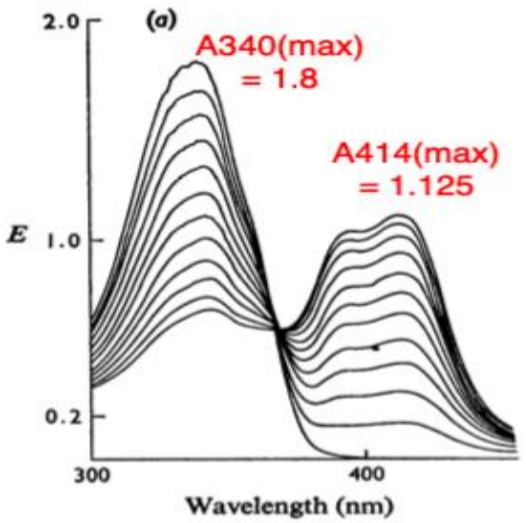

Figure 1. Spectrophotometric redox titration of ABTS with H_2O_2 in phosphate buffer, pH 6.0. Adapted from ref.*5*.

(20%) was measured at low pH. Based on the entire sample set of all literature values, any dependence on pH seems to be statistically insignificant: Averaging all molar absorptivities obtained at pH < 5, we get 32 ± 3 $mM^{-1}cm^{-1}$, and for those at pH ≥ 6, 31 ± 5 $mM^{-1}cm^{-1}$. The difference of only 1 $mM^{-1}cm^{-1}$ is clearly not statistically significant, with $P = 0.5$. Alternatively, fitting all of the reported values in Table *1* to the pH-dependence expected for an acid-base titration (Fig. S2, Appendix), the R^2 value for the resulting fit is low (0.1), the Chi-square value is high (174), and the fitted pK_a (3.6 ± 3.2) has an uncertainty nearly as large as its value.

Furthermore, ABTS: has only a single titratable group (the azino -NH^+= group)[7] whose pK_a has been found[17] to be 2.1, while the oxidized $ABTS^{\cdot+}$ radical has no titratable group at all (pK_a < 0).[17] For pH > 3, ABTS: and $ABTS^{\cdot+}$ will both be deprotonated, and their physical characteristics should not change with pH. This in fact matches results reported for the pH range 3 to 10.5.[7,29] Thus, the contention that the $ABTS^{\cdot+}$ molar absorptivity is pH-dependent is not supported by experimental evidence.

Noting that in the narrow range 6.0 < pH < 7.5, $ABTS^{\cdot+}$ molar absorptivities of both 27 and 35 $mM^{-1}cm^{-1}$ were reported, we set out to carefully measure the value at pH 6.0. ABTS: can be oxidized by strong oxidizing agents (e.g., permanganate, persulfate, Cr(VI)), but in the absence of catalysts, the reaction can take hours.[24] If peroxide is the oxidant, then the enzyme peroxidase effectively catalyzes ABTS: oxidation. We have characterized the double-exponential kinetics of peroxidase-catalyzed ABTS: oxidation to $ABTS^{\cdot+}$, and its subsequent oxidation to $ABTS^{2+}$. By separating these two processes, we have created careful standard curves showing that ε_{414} for $ABTS^{\cdot+}$ = 26.0 ± 0.4 $mM^{-1}cm^{-1}$.

Experimental Methods

All reagents were purchased at maximum purity from Sigma-Aldrich; stock solutions were kept on ice. Spectrophotometric measurements were made with a Varian Cary-3 UV-Vis spectrophotometer at room temperature, unless otherwise specified. In order to make the ABTS: calibration curve for absorbances at 340 nm, sample solutions were placed in quartz cuvettes containing a final volume of 3.0 mL; sample cuvettes were blanked with a reference cuvette containing deionized water. For all ABTS: oxidation reactions, semi-micro plastic cuvettes contained a final volume of 1.0 mL.The sample cuvette contained final concentrations of 0.5 µM horseradish peroxidase, 0.1 M phosphate buffer (pH 6.0), 0 – 100 µM ABTS:, and $[H_2O_2] = 4\cdot[ABTS:]$; the reference cuvette contained everything except the enzyme. Nonlinear regression of absorbance kinetic traces was performed with Kaleidagraph software.

Table 1. Reported literature values of the molar absorptivity of ABTS.

pH	λ_{max} (nm)	$\varepsilon_{\lambda max}$ ($mM^{-1}cm^{-1}$)	Reference	comments
-0.5	415	36.9 ± 0.1	Fan [19]	
0	417	34.7	Scott [17]	
0	417	27.6	Scott [17]	Calculated*
2	405	31.6 ± 0.2	Pinkernell [10]	
2	415	33.0 ± 0.2	Pinkernell [10]	From Fig. 1
2	415	33.6 ± 0.5	Wang [1]	
2.1	415	33.4 ± 0.4	Wang [1]	
4.2	415	33.0 ± 0.1	Lee [18]	
4.3	414	31.1 ± 0.2	Arnao [8]	
4.5	414	26.7	Arnao [22]	From Fig. 1
4.4	412	32.4	Shindler [6]	
6.0	414	36	Childs [5]	
6.0	414	25.6	Childs [5]	Calculated*
6.0	417	27	Maruthamuthu [16]	
6.5	405	28.5 ± 1.0	Pinkernell [21]	
7.5	414	33	Cano [23]	From Fig. 1
8	420	35.7	Van Hellemond [12]	?**

* calculated from the A_{417}/A_{340} peak height ratio

** value reported at pH 8, 420 nm, but cited Childs & Bardsley (at pH 6, 414 nm; ref.[5])

Results

We determined the molar absorptivity of the reduced ABTS: chromophore to be ε_{340} = 39.4 ± 0.3 $mM^{-1}cm^{-1}$ (Figure S3, Appendix), in reasonably good agreement with the values determined by Childs and Bardsley and Scott *et al*. (36 and 36.6 $mM^{-1}cm^{-1}$, respectively).[5,17]

For the peroxidase-catalyzed oxidation of ABTS: by H_2O_2, we were able to fit the fast rise in A_{414} due to $ABTS^{\cdot+}$ formation, followed by the slow fall in A_{414} due to further oxidation of $ABTS^{\cdot+}$ to $ABTS^{2+}$, to double-exponential kinetics (Equation *1*):

Equation 1: $$A_t = A_{final} + \Delta A_{fast} \cdot e^{-k_{fast} \cdot t} + \Delta A_{slow} \cdot e^{-k_{slow} \cdot t}$$

where k_i is a first-order rate constant (in s^{-1}), ΔA_i is the amplitude of the first-order process (negative for rising absorbance, and positive for falling abosorbance), and A_{final} is the asymptotic absorbance as $t \rightarrow$ infinity. It can be shown that the initial absorbance, $A_0 = A_{final} + \Delta A_{fast} + \Delta A_{slow}$; also, the absorbance due to complete oxidation of the initial ABTS: to $ABTS^{\cdot+}$, without any subsequent loss of $ABTS^{\cdot+}$ from further oxidation = $A_{max} = A_0 + |\Delta A_{fast}| = A_{final} + \Delta A_{slow}$. Data for the oxidation of 25 µM ABTS: in the presence of 1 mM H_2O_2 are shown in Figure *2*. It is clear that the fit of Equation 1 (red curve) to the measured absorbances (blue points) is excellent. The residuals from this fit are plotted in Figure *S4* in the Appendix.

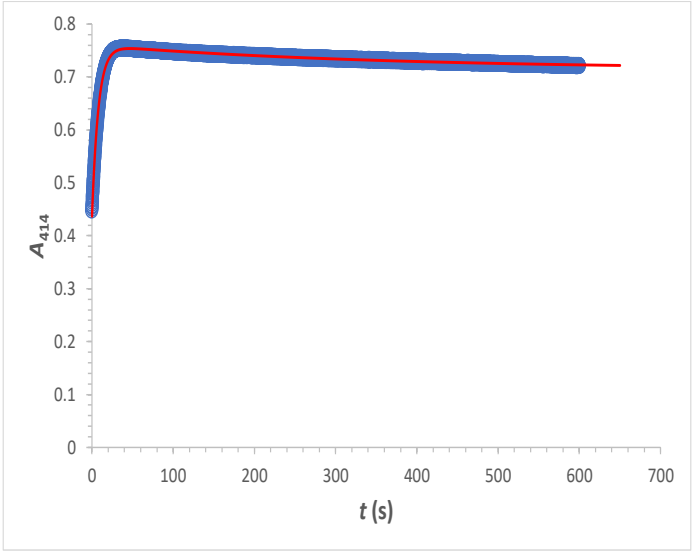

Figure 2. Peroxidase-catalyzed oxidation of 25 µM ABTS by 1 mM H_2O_2 (40-fold excess). Data (blue points) are fit to Equation 1 (red curve). The solution contained 0.5 µM horseradish peroxidase, and 0.1 M phosphate buffer, pH 6.0. Fitted parameters from nonlinear regression: A_{final} = 0.71460 ± 0.00017; ΔA_{fast} = -0.32562 ± 0.00027; k_{fast} = 7.579 ± 0.011 min^{-1}; ΔA_{slow} = 0.04561 ± 0.00013; k_{slow} = 0.1716 ± 0.0015 min^{-1}; R^2 = 0.9976.

The average first order rate constant for ABTS: oxidation to $ABTS^{\cdot+}$ was 7.4 ± 2.1 min^{-1} (range: 4 – 11 min^{-1}). Included in this average value were reactions run with [ABTS:]=10-100μM, and $[H_2O_2]$ = 0.01-500 mM. As expected, A_0, ΔA_{fast}, A_{final}, and A_{max} (= A_{final} + ΔA_{slow}) were all linearly proportional to the initial concentration of ABTS:. Because A_{max} represents the absorbance due to complete oxidation of the initial ABTS: to $ABTS^{\cdot+}$ without any further oxidation, plotting A_{max} vs. initial [ABTS:] yielded an accurate calibration curve and molar absorptivity for the radical $ABTS^{\cdot+}$.

In addition, because k_{slow} was generally well over 10-fold slower than k_{fast} (range: 9-fold to 230-fold), absorbances at t < 40 s represented almost exclusively the first oxidation step and could be successfully fit to a single exponential (Equation *2*) indicative of the rise in $[ABTS^{\cdot+}]$:

Equation 2: $$A_t = A_0 + \Delta A_{fast} \cdot (1 - e^{-k_{fast} \cdot t})$$

The asymptotic maximum absorbance as $t \rightarrow$ infinity = $A_{max} = A_0 + \Delta A_{fast}$, and this is linearly proportional to the maximum $[ABTS^{\cdot+}]$, which in turn equals the initial concentration of reduced ABTS:. Thus the slope of the calibration curve plotting $A_{414,max}$ vs. the initial [ABTS:] would also give $\varepsilon_{414}(ABTS^{\cdot+})$, the molar absorptivity of the $ABTS^{\cdot+}$ radical. From both types of calibration curves, we determined $\varepsilon_{414}(ABTS^{\cdot+})$ = 26.0 ± 0.4 $mM^{-1}cm^{-1}$ (*Figure 3*).

Discussion

$ABTS^{\cdot+}$ molar absorptivities reported in the literature have fallen into two ranges: low (26 – 28 $mM^{-1}cm^{-1}$) and high (31 – 37 $mM^{-1}cm^{-1}$). A few possible explanations can be discounted: Our low value could not be due to contaminants in the stock ABTS:, because our molar absorptivity of the reduced ABTS: matched that reported by Childs and Bardsley and by Scott et al.. Alternatively, Pinkernell et al.[10,21] and Wang and Reckhow[1] suggested that the differences were due to pH, but as we discussed in the Introduction, these pH-related differences were not statistically significant.

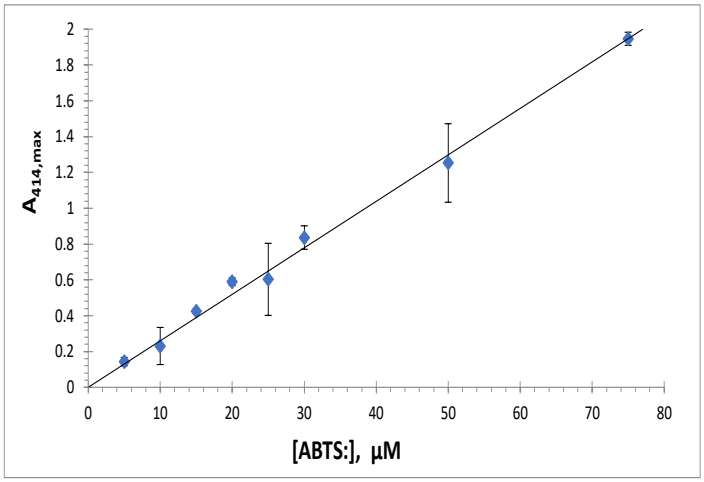

Figure 3 $ABTS^{\cdot+}$ standard curve. Solution contained 0.5 μM horseradish peroxidase, 0.1 M phosphate buffer, pH 6.0, and $[H_2O_2]$ = 4·[ABTS:]. Triplicate samples were run at each [ABTS:]; error bars = 1 standard deviation. The *y*-intercept was statistically insignificant (0.02 ± 0.03, P = 0.5), so the fit was forced through the origin: slope = 26.0 ± 0.4 mM^{-1}, R^2 = 0.998.

The explanations that remain are calculation error and matrix effects. We believe that both of these have cropped up in the literature. The fact that both Childs & Bardsley[5] and Scott et al.[17] reported high values (36 and 34.7 $mM^{-1}cm^{-1}$, respectively), but calculations using their spectrophotometric redox titration curves yielded low values (25.6 and 27.6 $mM^{-1}cm^{-1}$, respectively), suggests a calculation error. Similarly, Arnao et al[8] reported a high value (31.1 $mM^{-1}cm^{-1}$), but a calculation using their absorbance spectra (Fig. 1 in ref. [22]) yielded a low value (26.7 $mM^{-1}cm^{-1}$); both of these Arnao et al. experiments were done in the same buffer at the same pH.

On the other hand, Pinkernell et al.[10] reported a high value (33 $mM^{-1}cm^{-1}$) at pH 2 in tap water acidified with acetic acid, and a low value (28.5 $mM^{-1}cm^{-1}$) at pH 6.5 in phosphate buffer.[21] It seems possible that in this case, and perhaps others, matrix effects in different buffers could account for the discrepancy.[1] This certainly bears further exploration. What we can say now with good confidence is that the molar absorptivity of $ABTS^{\cdot+}$ in phosphate buffer at pH 6 lies in the low range, between 26 and 28 $mM^{-1}cm^{-1}$. Furthermore, we recommend that researchers who plan to measure oxidant concentration using the molar absorptivity of $ABTS^{\cdot+}$, carefully determine its value in the appropriate solution matrix rather than rely on a particular literature value.

Acknowledgments

All of the data in this paper (and some of the text) were adapted from Tanor Morinaka's senior thesis [2013, Chemistry Department, Willamette University], done in partial fulfillment of his requirements for graduation.

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[1]Note that Childs and Bardsley[5] used essentially the same phosphate buffer (pH 6) as Pinkernell et al.. Although Childs and Bardsley reported a high value for $\varepsilon_{414}(ABTS^{\cdot+})$, as recounted above, we calculated a low value from their spectra that matches the low value reported by Pinkernell et al.[21]

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Appendix

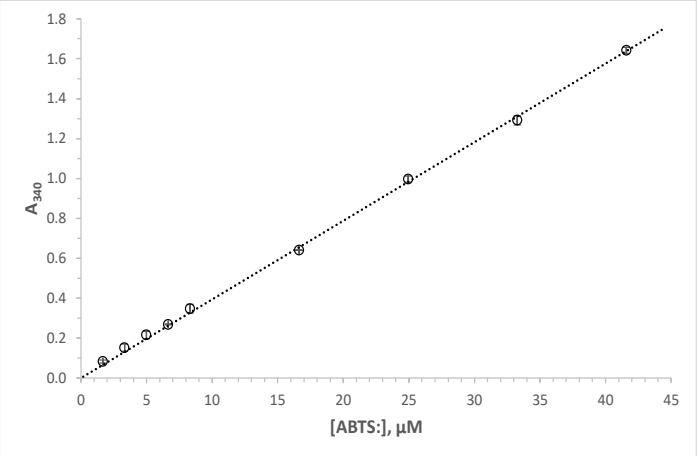

Figure S3. ABTS: calibration curve. Error bars (one standard deviation from triplicate measurements) are smaller than point symbols. Slope = 39.4 ± 0.3 mM^{-1}; R^2 = 0.9996.

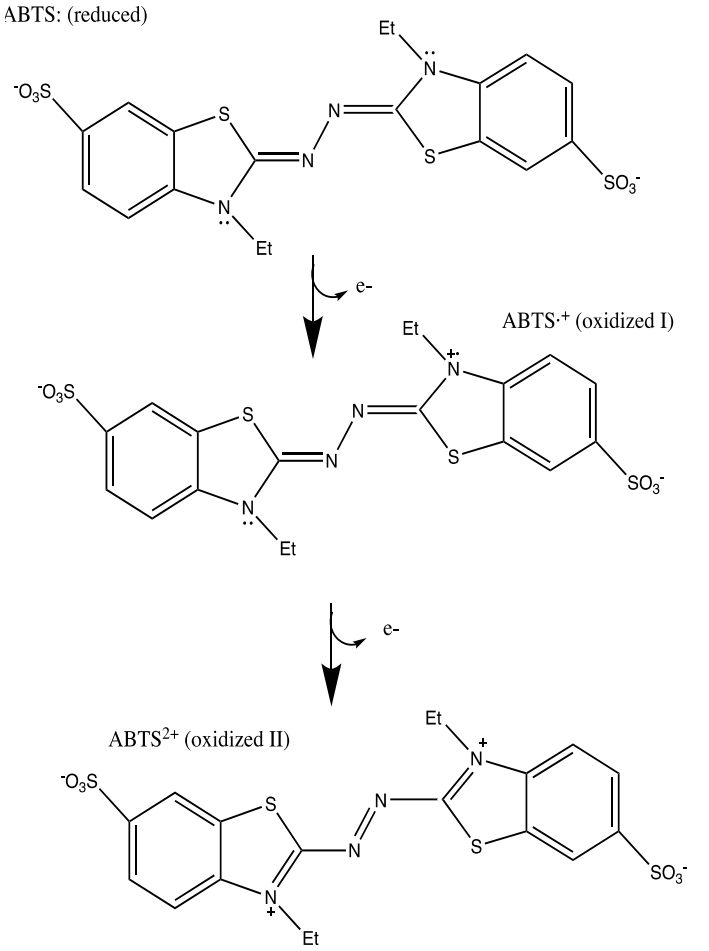

Figure S1. Oxidation of ABTS

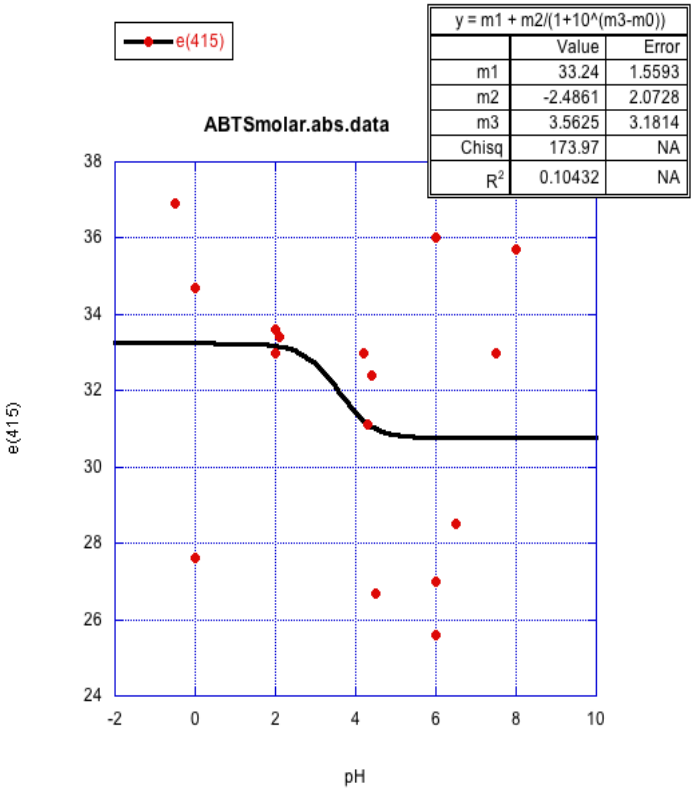

Figure S2. ABTS·+ molar absorptivity dependence on pH is statistically insignificant. Data from Table 1.

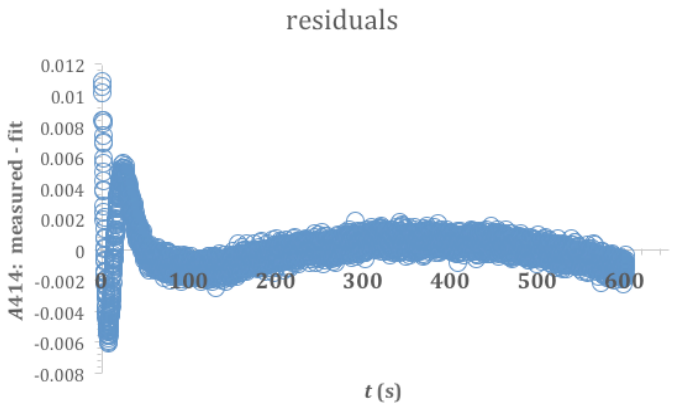

Figure S4: Residuals ($A_{414,obs} – A_{414,fit}$) for the data from Figure 2. Essentially all of the residuals (99.9%) lie between -0.006 and +0.006, which is less than the uncertainty in the absorbance measurement.