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James Kearns

University of Massachusetts Amherst

Julian Tyson

University of Massachusetts Amherst

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Improving the accuracy and precision of an arsenic field test kit: increased reaction time and digital image analysis

James Kearns and Julian Tyson*

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Two procedures to improve the performance of the Hach EZ test kit for quantifying inorganic arsenic concentrations in drinking water have been investigated. In the first, a digital image of the colored spot formed on the test strip, obtained with a flat-bed scanner was analyzed, by the computer program *Colors*, for the R, G, and B values. Calibrations were constructed by plotting the B values as a function of concentration. Agreement between the experimentally determined B-values and those of the printed chart was only obtained by either increasing the reaction time (to 40 min) or increasing the reaction temperature. The precision as a function of concentration was quantified. A comparison with previously estimated values for visual comparison of the colours, showed that the improved precision of the digital analysis would produce fewer false positive and fewer false negative results at the important threshold values of 10 and 50 $\mu\text{g L}^{-1}$. By running the test for 24 h, improved performance at the low concentration (around 10 $\mu\text{g L}^{-1}$) end of the response scale was obtained.

Introduction

Arsenic compounds, which are widely distributed in the environment as a consequence of natural processes and anthropogenic activities, are implicated in the adverse health of millions of people around the world.¹ The majority of those exposed are drinking contaminated water.² Chronic consumption of arsenic-contaminated water causes skin lesions, neurological disorders and cancers, including cancer of the kidneys and lungs.³ Probably the greatest suffering is in Bangladesh and West Bengal, India, whose rural communities are currently battling “the largest mass poisoning of a population in history.”⁴ The World Health Organization currently suggests a limit of 10 $\mu\text{g L}^{-1}$ for arsenic in water, which is also the “maximum contaminant level” set by the US Environmental Protection Agency; however, 50 $\mu\text{g L}^{-1}$ is currently the threshold value in Bangladesh and India.⁵

In the rural regions of southeast Asia, and elsewhere, affected populations often obtain their water from tube wells sunk into shallow arsenic-contaminated aquifers. Such locations are generally remote from laboratory-based facilities, making laboratory analysis of the very large numbers of local well waters impractical.⁶ In 2005, Melamed reviewed technologies with field measurement potential for monitoring arsenic in the environment.⁷ He concluded: “Accurate, fast measurement of arsenic in the field remains a technical challenge... the central goal of developing field assays that reliably and reproducibly quantify arsenic has not been achieved.” Bangladesh alone has more than ten million tube wells, and field test-kits are the only realistic

means of measuring the arsenic content of the water abstracted.⁶ The analysis is based on the Gutzeit modification of the Marsh reaction, in which arsine gas (AsH_3), formed by reaction of inorganic arsenate or arsenite with zinc in acid solution, reacts with mercuric bromide, impregnated into a paper strip exposed to the head-space of the reaction vessel, to produce a yellow-brown product. The colour is related to the concentration of arsenic in solution, which is found by comparing the colour of the strip with colours on a printed chart provided by the manufacturer. There has been adverse criticism voiced over the performance of earlier versions of these field test kits. Hossain⁸ alludes to the Bangladesh water crisis and the practice of painting a Bangladeshi tube well green if the water contains less than 50 $\mu\text{g L}^{-1}$ and red if it contains more than 50 $\mu\text{g L}^{-1}$, when he writes (in 2006) “field kits used to measure As in the region’s groundwater are unreliable” and “many wells in Bangladesh have been labeled incorrectly.” In an earlier study, published in 2002, Rahman *et al.*⁹ conclude, after evaluating results from the kits made by Merck, the National Institute of Preventional and Social Medicine (NIPSOM) in India, the Asia Arsenic Network (AAN), the All India Institute of Hygiene and Public Health (AII&PH), and Hach, that “millions of dollars are being spent without scientific validation of the field kit method.”

However, the performances of more recent versions of the kits are much improved. Van Geen *et al.* compared¹⁰ results obtained with the Hach EZ kit with those based on laboratory measurements by HG-AAS for the analysis of the water from 799 Bangladeshi tube wells. They found that provided the reaction time was doubled to 40 min, the field kit results were accurate (with respect to the 50 $\mu\text{g L}^{-1}$ value) for 88% of the samples. Steinmaus *et al.* evaluated¹¹ the Hach EZ kit (and the Quick Arsenic kit) in

Department of Chemistry, University of Massachusetts Amherst, MA 01003, USA. E-mail: tyson@chem.umass.edu

the context of the $10 \mu\text{g L}^{-1}$ standard by the analysis of 136 water samples from western Nevada, USA. The laboratory reference method involved HG-AFS. They increased the reaction time to 40 min for the Hach kit as suggested by van Geen *et al.* and found that for the 109 samples that contained more than $15 \mu\text{g L}^{-1}$, the EZ kit correctly identified the concentration as being above $10 \mu\text{g L}^{-1}$. For the 27 samples that contained less than $10 \mu\text{g L}^{-1}$, the Hach EZ kit registered 2 false positives. The status of test kits based on the Gutzeit reaction has been reviewed by Tyson.¹²

The US Environmental Protection Agency's Environmental Technology Verification (ETV) Program¹³ has, for several years, been evaluating arsenic test kits according to a rigorous and extensive set of standard protocols. The ETV program has, to date, evaluated eight test kits based on the Gutzeit reaction submitted by the manufacturers. On testing these kits for accuracy when measuring $10 \mu\text{g L}^{-1}$ As, the percentage of false positives was as high as 18 and the percentage of false negatives was as high as 62.

The issue of the occurrence of false positives and false negatives was examined in detail by Kinniburgh and Kosmus.⁶ They pointed out that the frequency of these was related to the precision of the measurement, which would be a function of the analyte concentration. To calculate this, they adopted the model of Thompson and Howarth,¹⁴ $s_c = s_0 + kC$ where s_c is the standard deviation of replicate analyses at concentration C , s_0 is the standard deviation at zero concentration, and k is a constant. For the Hach EZ kit, they calculated, based on data supplied by the Hach company, s_0 to be $7 \mu\text{g L}^{-1}$ and k to be 0.3. They plotted the percentage chance of an inaccurate result as a function of analyte concentration for a $50 \mu\text{g L}^{-1}$ decision value. This plot showed, for example, that for a field test-kit, the probability of a false negative for a sample of $100 \mu\text{g L}^{-1}$ would still be about 5%, and the probability of a false positive at $25 \mu\text{g L}^{-1}$ would be about 2%. They applied their treatment to 3208 real samples. In a simulation of 1000 analyses of each, they deduced that the Hach EZ kit would misclassify 12% of the wells, whereas a laboratory-based instrumental method (for which $s_0 = 0.3 \mu\text{g L}^{-1}$ and $k = 0.088$) would misclassify just under 1% of the wells. Clearly if the precision of the test kit could be improved, the reliability of the results would be improved (*i.e.* numbers of wells misclassified would be decreased).

We propose that one possible way of improving the precision of colorimetric determinations is to work with digital images obtained with a flat-bed scanner, which was first described in the 1993 paper by Durst and co-workers.^{15,16} They pointed out that the grayscale value was independent of the absorption spectrum of the dye used. The grayscale approach was also used by Bannur *et al.*,¹⁷ and by Johnson.¹⁸ Abrazhev *et al.*¹⁹ determined arsenic by a modification of the Gutzeit method in which a function analogous to absorbance was calculated. The first report of the use of the separated red, green, and blue (RGB) colour intensities was in 2002 by Kompany-Zareh *et al.*,²⁰ who derived a similar absorbance function. Paciornik *et al.*²¹ compared the performance of the RGB; cyan, magenta, yellow (CMY); and hue, lightness, saturation (HLS) colour space models and concluded that the best parameter to use was the hue, H. More recently, Sharma *et al.*²² analyzed the images produced by reaction of arsenic with sulfanilic acid and *N*-(1-naphthyl)ethylene diamine dihydrochloride solution. The detection limit of $60 \mu\text{g L}^{-1}$ is,

unfortunately, not low enough to be of any use for the monitoring of arsenic in groundwater. Mathews *et al.*²³ analyzed JPEG images by a program, *Colors*, that is available *via* the supplemental material on the journal website.²⁴ While the current manuscript was under review, Salman *et al.* described a method for arsenic based on the Gutzeit method in which the colored spots were scanned and a colour density value computed by adding the R, G, and B values together.²⁵

In this paper, we present results of studies to improve measurement precision by the analysis of the digital images, obtained with a flat-bed scanner, of the exposed test-strips from the Hach EZ test kit for the determination of arsenic in solution. We also present results of our studies of the effects of temperature and time. We discuss the implications of these findings for the analytical performance of such tests.

Experimental

Test kit vessels

Reaction vessels and lids were obtained from the Hach Company (Loveland, CO) in the EZ Arsenic Test Kit (cat. 2822800).

Reagents and standards

High purity water ($18 \text{ M}\Omega \text{ cm}$) was obtained from a Barnstead/Thermodyne (Dubuque, IA) E-pure unit. The reagents used for arsine gas generation were those provided with the EZ Arsenic Test Kit, namely sulfamic acid (cat. 28229-99) and zinc (cat. 28230-99). Standard solutions were prepared from laboratory grade sodium arsenite, NaAsO_2 , (cat. 2251) from Fisher Scientific (Pittsburgh, PA). Standard solutions of arsenic were prepared in deionized water with the concentrations ranging from 0, 10, 25, 50, 100, 250, and $500 \mu\text{g L}^{-1}$.

Measurement procedure

All experiments were carried out according to the procedure described by the manufacturer of the test kit. Reaction vessels were filled with 50 mL of the given solution. Sulfamic acid was added and dissolved. The second reagent, zinc, was added, and the vessel was capped with a cap into which a test strip had been inserted and which contained a pea-sized piece of cotton wool in the holder on the inside face. The cotton wool was not moistened with the lead acetate solution provided (the procedure for the removal of any interference by sulfide) as only standard solutions were involved. During the measurement, a significant amount of hydrogen gas is evolved. When bubbles burst at the surface, aerosol droplets can be ejected that reach the mercuric bromide sensing surface of the strip, giving rise to uneven coloration. Except where indicated, five replicate measurements were made.

Digital image analysis

The strips were scanned with an Epson 2480 Perfection Photo flat-bed scanner operating at 600 dpi and a 24-bit colour scale. The resulting images were cropped to display only the colored reaction product. Red, blue, and green intensity values were determined by *Colors*, run on a Windows XP platform, downloaded from the *Journal of Chemical Education*.²⁴ The R, G, and

B values for an image were each assigned a value from 0 to 255. The colour intensity values were recorded in Microsoft *Excel*, which was used for calculations and curve-fitting.

The colored reference chart printed on the side of the strip container was scanned and the average red, green and blue pixel intensities for each spot determined. The chart, shown in Fig. 1, was reconstructed with Adobe Illustrator so that the spots have uniform colour.

Method development

Reaction time. The manufacturer currently recommends removing the strips after 20 min. To investigate the effect of increasing the reaction time, a series of experiments was performed for each concentration in which the test strips were removed at 20, 30, 40 min, and at 24 h.

Temperature. The manufacturer does not specify a recommended operating temperature, and most reactions were run at room temperature (20 °C). To investigate the effect of operating temperatures typical of, say, Bangladesh, measurements were made at 35 °C for a reaction time of 20 min. The reaction vessels were weighted and submerged so that the liquid levels in the vessels were below the water level in a thermostatically controlled water bath.

Data analysis. Plots of average blue pixel intensity, measured by the *Colors* program, as a function of arsenic concentration were constructed. To estimate the standard deviation in the concentration domain, s_c , the quadratic equation corresponding to each successive group of three points, calculated in *Excel*, was solved for the concentration values that corresponded to the limits of \pm one standard deviation in the response domain (the blue intensity value). To examine the validity of the obtained Thompson–Howarth model and to obtain estimates of k and s_0 , plots of s_c as a function of C were created and examined for the agreement with the straight line relationship described above.

Results and discussion

Analysis of image of calibration chart

The reference chart for the Hach EZ kit, in which the colours expected after 20 min reaction time for arsenic concentrations ranging from 0 to 500 $\mu\text{g L}^{-1}$, is shown in Fig. 1. The difficulties of interpolating between adjacent colours can be seen, as can the difficulties of distinguishing between the responses of concentrations just below and above the 10 $\mu\text{g L}^{-1}$ and 50 $\mu\text{g L}^{-1}$ values.

The mean R, G and B intensity values are shown in Table 1, from which it can be seen that the B values are most responsive to the changes in the colours of the reaction spots on the test strips.

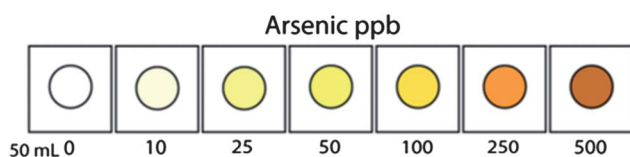


Fig. 1 Scanned image of the color chart corresponding to solutions containing 0, 10, 25, 50, 70, 300, and 500 $\mu\text{g L}^{-1}$ of arsenic.

Table 1 Red, green and blue values from a scan of the Hach kit colour chart

As(III) concentration $\mu\text{g L}^{-1}$	Red	Green	Blue
0	255	255	255
10	254	254	223
25	254	254	137
50	254	254	101
100	252	222	65
250	251	157	53
500	201	113	44

The primary trend is that the intensity of the reflected “blue” light (*i.e.* as defined by the blue filters in the scanner) decreased as concentration of arsenic increased.

Effect of reaction time

The mean blue intensity values for reaction times of 20, 30, 40 min, and 24 h and for the Hach kit colour chart are shown in Table 2; the plots corresponding plots of blue intensity value as a function of concentration are shown in Fig. 2. It can be seen that curves have an approximately exponential shape, and that even for a 40 minute reaction time, the blue values are not as intense as the values in the printed colour chart. As the values for the 24 hour reaction time are more intense than those of the printed chart, it may be concluded that the best match to the chart for reaction at room temperature would be obtained for reaction times longer than 40 minutes but shorter than 24 hours. We have preliminary results that indicate that this might be as long as 4 h; however, the situation is complicated by the fact we also have evidence that the colour fades on prolonged exposure to the headspace vapours. Clearly reaction times of hours are not compatible with the activities of a single technician tasked with visiting as many sites as possible in the working day, but are less problematic if the tests are being performed by multiple individuals. We therefore endorse the suggestion by van Geen *et al.*¹⁰ that when operating at 20 °C, the reaction time be increased to 40 min. A 24 h reaction time can work well with the scheduling of classes in schools, where students can set up the test in one laboratory class period and “read” the strip during the class the following day. In addition, it can be seen that the slope of the calibration for the low concentrations (between 0 and about 10 $\mu\text{g L}^{-1}$) is greatest for the 24 h version of the test, and thus more reliable results for the values near the WHO’s critical value will be obtained by increasing the reaction time.

Effect of temperature

The blue intensities obtained after 20 min at 35 °C are also shown in Table 2 and the corresponding plots of blue intensity as a function of concentration are shown in Fig. 2. It may be seen that raising the reaction temperature has a marked effect on the outcome of the test and that the values obtained at 35 °C are closer to the values for the printed chart than the values obtained at 20 °C. Thus, it would seem appropriate for the manufacturers of such tests to include some commentary about the temperature range for which the printed chart is considered appropriate and

Table 2 Mean blue values for reactions run for 20, 30, 40 min at 20 °C and for 20 min at 35 °C

As(III) concentration $\mu\text{g L}^{-1}$	Hach colour chart	20 min	30 min	40 min	24 h	35 °C
0	255	255	255	255	255	255
10	223	249	241	233	154	245
25	137	240	210	182	128	215
50	101	211	161	140	110	145
100	65	187	132	106	69	129
250	53	157	118	95	24	61
500	44	124	101	85	8	19

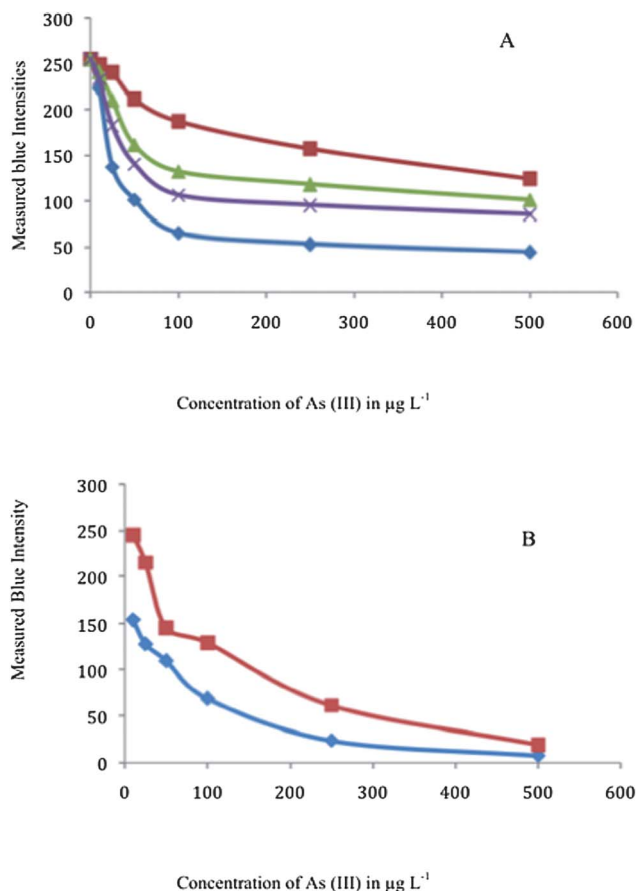


Fig. 2 (A) A comparison of the measured blue intensities *versus* concentration of As(III) in $\mu\text{g L}^{-1}$ at 20 min (square), 30 min (triangle), 40 min (X) (all experiments at 20 °C) and the Hach color-chart (diamond). (B) A comparison of the measured blue intensities *versus* concentration of As(III) in $\mu\text{g L}^{-1}$ at 35 °C and 20 min (square) and 24 h and 20 °C (diamond).

maybe print more than one version of the chart corresponding to different temperatures. The same comment is relevant to the effect of reaction time. We suggest that it might be possible to create an algorithm that could be applied to correct for the effects of time and temperature and allow a more accurate match between the measured blue intensity and the intensities in the printed chart.

Precision

The standard deviations in blue intensity values (s_B) and the corresponding standard deviations in concentration (s_C) from

values from scanned strips run at 20, 30, 40 min, 24 h at 20 °C and for 20 min at 35 °C are given in Table 3. It may be seen that the standard deviations in both domains increase as concentration increased as predicted by the Thompson and Howarth model.¹⁴ A plot of s_C as a function of C for 20 min reaction at room temperature is shown in Fig. 3; while these data show reasonable agreement with the linear model, inspection of the data for other reaction conditions shows that the relationships are not all linear. The nature of the response curves, especially those in Fig. 2A, is such that there are two fairly distinct response regions, below 100 $\mu\text{g L}^{-1}$ and above 100 $\mu\text{g L}^{-1}$. In the latter region, the slope of the response plot is much smaller than that in the former and so comparable variations (\pm one standard deviation) in blue intensity would give rise to much larger variations in C . Better precision would probably be obtained for the determinations of concentrations above 100 $\mu\text{g L}^{-1}$ if the green intensity values were used as the measure of concentration rather than the blue intensity values. However, as the most important consideration is whether the concentration in a ground water sample is above or below 50 $\mu\text{g L}^{-1}$ or 10 $\mu\text{g L}^{-1}$, depending on the part of the world in which the test is conducted, strategies for improving the precision at above 100 $\mu\text{g L}^{-1}$ are less important than considerations of precision (and accuracy, discussed below) at lower concentrations. For most of the reaction conditions, the relationship between s_C and C was better described by an exponential function than a linear function (results not shown).

The values of s_C estimated by Kinniburgh and Kosmus for the Hach test kit at 10 $\mu\text{g L}^{-1}$ and 50 $\mu\text{g L}^{-1}$ are 10 $\mu\text{g L}^{-1}$ and 22 $\mu\text{g L}^{-1}$, respectively.⁶ These values are given in Table 4, together with the values calculated from the standard deviations of 5 replicates of the responses to these two solutions at the various times and temperatures. The precisions at 30 min, 40 min and 24 h were significantly smaller than the Kinniburgh and Kosmus values, based on a one-tailed F -test at the 95% confidence level. On this basis, the values for 20 min reaction time at both room temperature and 35 °C are *not* significantly better. For the digital image analysis results, the precisions at 30 min, 40 min and 24 h for the 10 $\mu\text{g L}^{-1}$ solution are significantly smaller than the value for 20 min; however, for the 50 $\mu\text{g L}^{-1}$ solution, only the precision at 40 min is significantly better than that for 20 min. We conclude that the use of digital image analysis coupled with increased reaction time will give rise to significantly improved precision at these two critical concentration values and thus would decrease the number of false positive and false negative outcomes compared with the numbers obtained by visual comparison of the developed test strips.

Table 3 Standard deviation in blue intensity values (s_B) and the corresponding standard deviation in concentration ($\mu\text{g L}^{-1}$) values (s_c) from scanned strips run for 20, 30, 40 min, 24 h at 20 °C and for 20 min at 35 °C ($n = 5$ for all experiments)

Concentration of arsenic ($\mu\text{g L}^{-1}$)	20 min s_B	20 min s_c ($\mu\text{g L}^{-1}$)	30 min s_B	30 min s_c ($\mu\text{g L}^{-1}$)	40 min s_B	40 min s_c ($\mu\text{g L}^{-1}$)	24 h s_B	24 h s_c ($\mu\text{g L}^{-1}$)	35 °C s_B	35 °C s_c ($\mu\text{g L}^{-1}$)
10	3.8	6.2	12.1	1.5	6.3	2.3	16.0	2.4	12.9	7.2
25	5.0	6.3	2.6	1.3	9.6	3.5	5.7	4.1	18.2	13
50	13.3	15	9.8	6.5	4	4.0	4.9	6.6	18.3	9.4
100	18.6	45	13.5	43	13	35	33.5	— ^a	19.1	55
250	11.4	72	18.5	230	11.8	158	11.5	52	16.2	30.5
500	20.2	155	24.9	— ^a	8.6	219	2.4	40	4.24	22.1

^a — value could not be computed as quadratic function could not be fitted to points.

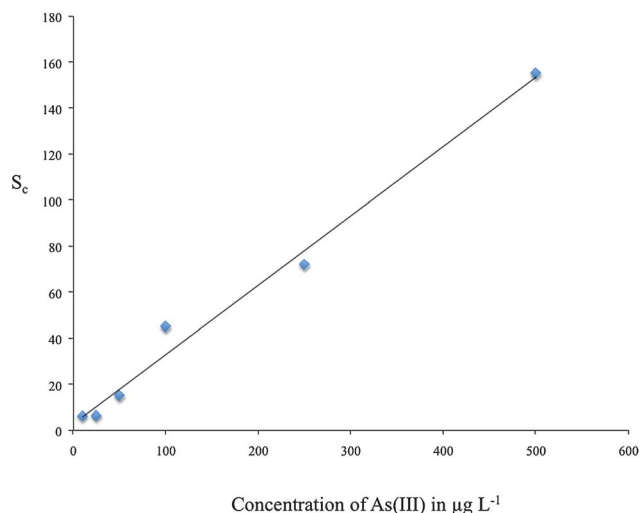


Fig. 3 Plot of S_c versus concentration of arsenic in $\mu\text{g L}^{-1}$ for 20 min reaction time. The line is the best fit by the method of least squares.

Table 4 Standard deviations in concentration units at 10 and 50 $\mu\text{g L}^{-1}$, s_{10} and s_{50} , at various times at room temperature and for 20 min at 35 °C

	Kinniburgh and Kosmus model ⁶	20 min	30 min	40 min	24 h	35 °C
s_{10}	10.3	6.2	1.5 ^{ab}	2.3 ^{ab}	2.4 ^{ab}	7.2
s_{50}	22	15	6.5 ^a	4.0 ^{ab}	6.6 ^a	9.4

^a Value significantly smaller than that of the Kinniburgh and Kosmus model. ^b Value significantly smaller than that for 20 min reaction time.

Accuracy

The effects of reaction time and temperature are clearly quite marked, but with the possible exception of the results for the colours developed after 24 h, the colours for all concentrations are “lighter” than those printed in the chart supplied by the manufacturer. Visual observation under normal laboratory lighting conditions (a mixture of diffuse daylight and fluorescent strips) supports this general observation. This raises the issue about the accuracy of the test as performed under normal laboratory conditions according to the manufacturer’s instructions (20 °C for 20 min). One possible reason for the discrepancy is that the light source in the scanner (a cold cathode fluorescent tube) has a spectral output that produces responses to the printed chart and an “exposed” test-strip that are different from those

that would be observed in daylight (assuming that this is what the manufacturer has in mind). We have not, as yet, investigated the effect of the light source on the image characteristics. However, as the responses for exposure for 24 h and at 35 °C are much closer to those of the printed chart, it seems unlikely that the light source is a major source of inaccuracy, even though the chemical species responsible for the colour on an exposed strip and on the printed chart are quite different.

Conclusions

Compared with visual comparison of the exposed strips with the printed calibration chart, analysis by the evaluation of the digital images created with a flat-bed scanner is more precise and thus leads to a methods that will give rise to fewer false positive or false negative values at the critical decision values of 10 and 50 $\mu\text{g L}^{-1}$. The intensities of the colored spots are functions of reaction time and temperature, both of which affect the accuracy of the kit. The results indicate that under the recommended operating conditions, the reactions responsible for the colour formation are not complete. The experiments reported here do not allow a distinction between the processes of (a) generation and evolution of arsine into the head-space and (b) reaction of arsine with the mercuric bromide in the strip. Experiments currently in progress suggest that both processes are slow, but that the evolution is slower than the reaction between arsine and the mercuric bromide. Preliminary results also indicate that the rate of evolution is strongly dependent on the nature of the agitation of the contents of the reaction vessel. We suggest that when reporting on the performance of such test kits, information is provided about the reaction temperature, exposure time of the strips, and the lighting conditions under which comparisons were made. The quite large deviations from the colours in the printed chart that were observed are cause for concern, and we suggest that a better strategy would be to calibrate at the time of analysis by measuring solutions of known concentrations. This, of course, raises practical difficulties for genuine field deployment of the test and requires operators to have access to the supplies and facilities necessary to prepare the appropriate standard solutions. There is also the issue of the instability of the colours once formed (especially if exposed to light) and so replacing the printed chart with a set of exposed strips is not a viable strategy. We suggest that this calibration strategy be combined with digital image analysis and applied to every batch of reagents (the Hach kits are supplied with enough reagents to perform 100 tests). We

also propose that the scanner could be replaced by a digital camera. Calibration in duplicate based on 5 standards, chosen to match the likely range of concentrations encountered, would consume 10% of the analytical capability of the batch or reagents raising the cost (at the time of writing) by just under \$0.04 per test. If the target samples are likely to contain concentrations around the WHO critical value of $10 \mu\text{g L}^{-1}$, we suggest that the 24 hour version of the test be adopted. We realize that this probably means taking samples back to a laboratory of some sort, and almost certainly requires that more reaction vessels be available, as well as limiting the numbers of samples that can be processed in any given time. We expect that the 24 hour version of the test would have a lower detection limit, though it will be necessary to establish the mathematical relationship between colour intensity and concentration.

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