Lab on Paper: Adapting Quantitative Chemical Techniques for Use in Low Resource Areas

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LAB ON PAPER: ADAPTING QUANTITATIVE CHEMICAL TECHNIQUES FOR USE IN LOW RESOURCE AREAS

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Abstract

by

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People need high quality fortified foods and medicines to protect their health. However, bad quality products are on the market. Regulatory agencies can remove bad products from the market but only after the quality is confirmed with expensive testing techniques. As a result, bad products persist in the marketplaces of low- and middle-income countries (LMICs). Analytical chemistry can solve this problem, but monetary and infrastructure problems prevent it from doing so. Labs are expensive to build, maintain, and employ with trained personnel. Labs are also reliant on a constant supply of electricity. The goal of my project was to design chemical analyses to work within the financial and infrastructure constraints of LMICs, thereby increasing testing capacity. I addressed this need for analysis outside the lab by adapting quantitative chemical tests to paper platforms, resulting in inexpensive technologies that require no technological infrastructure.

During the course of my thesis research, I engineered three test cards. Two test cards support universal salt iodization programs by quantifying the amount of iodate in fortified salt or by measuring urinary iodide levels. The cards can be used during surveillance studies to see if iodized salt is in the marketplace and whether the iodine is making its way into people's diets. I made another card to quantify beta-lactam antibiotics in finished pharmaceutical pills, so the user can determine if the medicine contains the dosage stated on its label. I have taken these new technologies through validation studies to establish how well they work. All of them have about 90% accuracy or greater, and in some cases rival the performance of traditional analysis techniques.

These paper test cards could have real impact in LMICs. They are inexpensive, field- and user-friendly, not dependent on power or specialized instrumentation, and enable critical analyses to be performed in LMICs. Breaches in compliance systems could be detected immediately with paper analytical devices and texting the results to a database would isolate the geographic location. After confirming the bad results, public health agencies and law enforcement can be dispatched to remedy the situation, thereby protecting public health from low quality products.

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LIST OF ABBREVIATIONS

aPAD	antibiotic paper analytical device
API	active pharmaceutical ingredient
ASSURED	affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and deliverable to the end user
EFMHACA	Ethiopian Food, Medicine and Health Care Administration and Control Authority
FDA	Food and Drug Administration
GAIN	Global Alliance for Improved Nutrition
GMPs	good manufacturing practices
HPLC	high performance liquid chromatography
ICP-OES	inductively coupled plasma – optical emission spectroscopy
IR	infrared or infrared spectroscopy
LMICs	low- and middle-income countries
µPAD	micro-paper analytical devices
PADs	paper analytical devices
ppb	parts per billion
ppm	parts per million
PXRD	powder X-ray diffraction
QC	quality control
ROC	receiver-operator curve
ROI	region of interest
saltPAD	salt paper analytical device
SAMRC	South African Medical Research Council
uiPAD	urinary iodide paper analytical device
USD	United States dollar
USP	United States Pharmacopeia or United States Pharmacopeia Convention
WHO	World Health Organization

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Thank you to my advisor Dr. Marya Lieberman. She matured me as a scientist. She taught me how to identify a problem, form a potential solution, acquire research funds, design and conduct meaningful studies, disseminate the results, protect intellectual property, and collaborate with parties that advance or will be the end users of the research. Besides working in the lab, we traveled half way around the world to support the research described in this thesis. We visited pharmacies in the slums of Pabo, Uganda and presented our research in Honolulu, Hawaii. The entire graduate school experience has been challenging and enjoyable, contributing to the best years of my life so far.

Thank you to my thesis committee members Dr. Richard Taylor and Dr. Paul Bohn, to my family and friends, to my lab coworkers Gail, Jamie, Michelle, Madeline, Tracy, and Sarah, and to all of the dozens of undergraduate students and early career scientists that I have mentored and helped me to develop the technologies that you will read about in this thesis.

CHAPTER 1:

QUALITY TESTING IN LOW RESOURCE AREAS

1.1 Chemical testing capacity in low- and middle-income countries

Only 29 labs in low- and middle-income countries (LMICs) are registered with and meet the WHO's requirements for "Medicines Quality Control Laboratories."[1] Labs enrolled in this program are assessed for compliance with "Good Manufacturing Processes" (GMPs) and "Good Practices for Pharmaceutical Quality Control Practices." By comparison, 6,190 American facilities that manufacture, prepare, propagate, compound, or process drugs are registered with the Food and Drug Administration's (FDA) "Drug Establishments Current Registration Site,"[2] and they must comply with the FDA's GMPs, which includes adequate quality control testing. The testing capacity in LMICs is low, so the quality of nutritional products and medicines is harder to ensure. LMICs have trouble supporting analytical chemistry laboratories for many reasons. It is difficult to afford equipment and supplies, employ and retain talented analysts, establish compendial testing methods, and adapt to regulatory requirements for testing and documentation when they change. Considering the scarcity of labs in LMICs, their sample queues can be bottlenecked for years, which slows the discovery of bad products during surveillance studies and prevents regulatory authorities from

quarantining them before sale. The testing can be outsourced to labs across national borders, but logistical or monetary problems prevent this solution from working in most LMICs.

1.2 Paradigm shift for chemical testing of nutritional and pharmaceutical products

Instead of focusing on sample analysis within a lab, some alleviation of the need for chemical analysis can come from field screening tests. Samples that generate suspicious results can be sent to a qualified lab for confirmatory testing. The World Health Organization (WHO) prefers field tests that are semi-quantitative to those that are qualitative because counterfeiters are clever enough to include small amounts of active ingredient in their formulations to trick the tests.[3] The WHO also states that field technologies should be viewed as complimentary to gold-standard analyses, and that regulatory action cannot take place until a pharmacopeial method has assessed the quality of a product.[3] Ultimately, field-usable analytical technologies that do not require as much technological infrastructure as a chemical laboratory could help to solve many pressing analytical problems in LMICs.

1.3 The end users and their settings

To better understand the limitations that exist in LMICs, I traveled to East Africa and visited laboratories and settings where field technologies could be used.

In 2013, Dr. Lieberman and I went to Kensalt, a salt fortification plant in Mombasa, Kenya. We toured the manufacturing plant and the quality control laboratory

(Figure 1.1). The titration lab contained only supplies needed for iodated salt analysis: analytical balance, buret, flasks, chemical reagents, and a trained chemist. The lab did not have a functioning water purification system at the time of our visit, so packaged distilled water had to be bought from the marketplace. There was a computer, internet, and thermometer, but no other analytical instrumentation.

Figure 1.1. Kensalt's quality control laboratory, Mombasa, Kenya, 2013.

In Eldoret, Kenya, we visited a hospital and saw a lab where human specimens are analyzed (Figure 1.2). The most advanced instrument was a microscope. The water bath had a “not in use” sign attached. The space was cramped. The electricity went out while we were there.

Figure 1.2. Hospital laboratory, Eldoret, Kenya, 2013.

In Pabo, Uganda, 2014, we went into a slum filled with small shops and found chemists selling medicines (Figure 1.3). The shops had registration numbers from the Ugandan government that permitted them to sell pharmaceuticals. Some customers purchased portions of pill packets cut up by the chemist, so the customers did not receive full courses of antibiotics.

Figure 1.3. Chemist selling pharmaceuticals in Pabo, Uganda, 2014.

To function in settings like these, field technologies should not rely on electricity, advanced instrumentation, highly trained specialists, or spacious operating quarters, and since testing may take place in remote locations accessible only by hours of driving or walking, compact and lightweight kits are necessary.

1.4 Guidelines for developing field tests

The World Health Organization has a mnemonic to help inventors create medical diagnostic technologies effective for use in LMICs. The ASSURED criteria are: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and deliverable to the end-user.[4] It is appropriate that affordability comes first as LMIC's are economically restricted to use the least expensive technology available no matter how inferior its analytical performance. For instance, even though the Rapid Test Kit claims to detect iodate in fortified salt, it has a false positive rate of 60%.[5] However, small-scale salt manufacturers in LMIC's still use it because it only costs a penny to analyze a sample. Competing technologies cost several dollars per assay (presented in Table 2.2). Field technologies have to be sensitive and specific to the analyte of interest. The metrics need to be appropriate for the application. Counterfeiters have been known to trick field tests by including small amounts of active ingredients in formulations, so quantitative technologies are preferred over qualitative ones. Field tests have to be user-friendly. The technology should be usable by a person of any skill level with minimal training. It is not reasonable to expect technical specialists to be readily available. Tests also have to be rapid and robust. Field-tests are used as initial screening

devices, so testing more samples increases the likelihood of detecting a problem. The tests may be performed in a true field setting, so they cannot be sensitive to temperature, humidity, sunlight, or chemical or physical interferences that may be present in the sample. Field tests should be equipment-free, especially of equipment needing electricity. Power outages are common in LMICs, and they can be lengthy. Dr. Lieberman and I traveled to Gulu, Uganda, and we arrived on the 19th day of an outage. Equipment can also break and be expensive or time consuming to replace or repair. Field tests have to be deliverable to end-users. The technology must be shipped to LMICs, arrive in working condition, and be received in a convenient geographic location. The ASSURED criteria can be tested for a new field-technology by performing an external validation with people of various skill levels located in different environmental conditions.

There are no formal guidelines for assessing the ASSURED-ness of a product, so different studies favor different criteria. A recent comparative study ranked 5 technologies that analyze iodized salt according to ASSURED criteria, and the study's authors decided to give twice as much weight to sensitivity and specificity as to user-friendliness, rapidness and robustness, and independence from equipment.[6] They did not quantify the technologies' deliverability to end-users.

1.5 Paper devices for chemical analysis in LMICs

In 2007, the Whitesides group introduced micro-paper analytical devices (μPADs) as powerful tools for solving chemistry problems in low resource settings.[7] PADs can

work well with the confines of an LMIC.[8] Paper is an inexpensive platform that can be easily adapted to analyze many targets.[8] PADs can be engineered so operators of various skill levels can use them. PADs have colorimetric readouts that reveal the presence and identity of invisible or unknown chemicals for visual interpretation, and the colors usually develop within a couple of hours at ambient temperature. They pose little hazard to the analyst or to the environment after disposal because only tiny amounts of chemicals are deposited onto them. PADs can also be shipped via normal mailing systems making them deliverable to most anyone. Manufacturing processes already exist for paper test strips, so product scale-up is possible for successful embodiments of PADs. The biggest challenge with the technology is thoroughly testing its robustness for use in LMICs. There are hundreds of publications describing PADs or other microfluidic paper technologies,[9–15] and only a few of them include field validation studies.[13]

1.6 Current paper devices for chemical analysis

Two classes of paper devices for chemical analysis are commercially available: dip tests and lateral flow assays. A dip test is submerged into a test solution, and it turns colors to indicate the presence of an analyte. Urine and pH test strips are the most well known.[8] In some cases, dip tests can be semi-quantitative. For example, urine test strips are used diagnostically in clinics[16] and short-range pH paper can distinguish proton concentrations that differ by 0.1 pH units.[10] A lateral flow assay, such as a home pregnancy test, usually contains biomolecules that detect an analyte as a solution wicks

from the sample loading zone to a test result line. Some can be semi-quantitative or even quantitative with astonishingly low detection limits of 0.1 ppb if they are coupled with an electrical, optical, or magnetic reader.[9] Gaining the quantitative information, however, usually does not have a practical application (i.e., different levels of viral or bacterial load won't change the treatment). The PADs mentioned in section 1.5 are not commercially available, but they are reported in the scientific literature.[9–15] PADs can perform a quantitative, non-chemical task (examples in Table 1.1) or a colorimetric, semi-quantitative chemical assay (examples in Table 1.2). Some of the chemical assays provide quantitative results because they incorporate a quantitative chemical technique, such as pH titration[17] or the method of standard additions.[18]

TABLE 1.1.

EXAMPLES OF QUANTITATIVE TASKS PERFORMED ON PADS

Task	Interpretation	Analysis range	Accuracy	Precision
Thermometer[19]	reflectance	25-140 ° C	ND	ND
Timer[20]	visual	1-120 min	97%	90%
Filter[7]	visual	ND	ND	ND
Reagent addition*[21]	visual	ND	ND	ND

Note: *Timed, sequential addition of reagents. ND = no data

TABLE 1.2.

EXAMPLES OF SEMI-QUANTITATIVE AND QUANTITATIVE CHEMICAL ANALYSES

PERFORMED ON PADS

Analyte	Test type	Interpretation	Analysis range (ppm)	Error (ppm)	Precision (ppm)
Hg^{2+}	colorimetric[22]	visual	10-400	ND	ND
Fe^{2+}	colorimetric[23]	software	45-300*	7*	10*
Ni^{2+}	colorimetric[23]	software	30-600*	10*	15*
Cu^{+}	colorimetric[23]	software	30-500*	10*	15*
Cr^{3+}	colorimetric[24]	software	3-100*	0.25*	2.5*
Phosphate	colorimetric[25]	software	0.05-10	0.01*	0.2*
Cyanide	colorimetric[26]	software	0.01-1*	0.01*	0.054*
Sarin	colorimetric[27]	software	1000-70000*	900*	1100*
TNT	colorimetric[28]	software	25-200*	ND	ND
H^{+}	quantitative[17]	visual	400-800*	2*	ND
OH^{-}	quantitative[17]	visual	1700-17000*	ND	170*
Glucose	quantitative[18]	software	0-900*	32*	42*

Note: *The units reported in the respective publications were converted to approximate ppm so the values can be easily compared in this table. ND = no data

The number of PADs with a built-in functionality that allows quantitative analysis is very limited compared to qualitative colorimetric detection. The quantitative abilities of PADs need to be expanded, so people are better equipped to test samples that may be encountered in the field.

1.7 Technical challenges for PADs

When it comes to quantification on paper, there are a number of technical challenges that must be overcome to ensure reliable analytical measurement. Reagents must be compatible and stable for a long period of time while being in intimate contact with paper. This has been addressed with flow-based techniques, which involves storing reagents on the same plane or in multiple planes of a porous medium and combining them by flowing solvent laterally through the substrate[14] or transversely across several layers[29] or folds of the paper.[30] Another challenge is that wetting of dry paper is a non-equilibrium process; the amounts of analyte and reagents change over space and time.[31]

PADs need to be robust. Chemical tests are usually developed in sophisticated labs, and this well-controlled environment limits variations in temperature or humidity, allowing the best accuracy and precision metrics to be attained. As a result, the robustness for chemical tests can be justifiably underdeveloped. This is the opposite of what is needed for field tests; the technology must be robust enough to work in unpredictable conditions and rarely needs superior accuracy and precision. In order to increase testing capacity in LMICs, chemical tests on paper need to be engineered to

work effectively outside of a well-equipped lab and provide quantitative responses with minimal costs.

1.8 Overarching goal

The goal of my thesis was to adapt robust quantitative chemical analyses to paper platforms. I evaluated their performances through internal and external validation studies.

1.8.1 Iodine analysis

In 1993, Notre Dame started the Haiti Program with the goals of eliminating lymphatic filariasis and achieving universal salt iodization in Haiti (https://haiti.nd.edu/). These goals could be achieved by co-fortifying food-grade salt with diethylcarbamazine citrate and potassium iodate, and then mass distributing it to the population. Notre Dame built a fortification plant to make the salt product. The quality control lab at the manufacturing site had problems establishing testing methods to analyze the quality of the fortified salt, so they sought help from Notre Dame to conduct the chemical analyses. The Haiti Program was directed to Marya Lieberman, a chemist in the Department of Chemistry and Biochemistry, who helped with the analyses. She realized the manufacturer needed a low-tech analysis method to test the quality of the product in Haiti. She searched the literature, found a publication by George Whitesides's research group about μPADs, and wondered if iodometric titration could be transferred to this platform. A test card for iodized salt would enable the manufacturer to analyze the iodine level of their salt product on site. During this time, I entered the Ph.D.

program in the Department of Chemistry and Biochemistry at Notre Dame. I interviewed with Dr. Lieberman, she explained the above problem, and we determined I could create a Ph.D. thesis on the subject. I was confident I could solve this problem as I had just finished a 2 year stint as a quality control technician at a pharma company in Grand Haven, Michigan. I accepted the challenge of engineering and validating paper-based quantitative laboratory techniques in hopes that the test cards could be used to increase the chemical testing capacity in LMICs. This was the birth of the PAD project at Notre Dame. Since then, I have developed two test cards that support universal salt iodization programs in achieving better iodine nutrition.

1.8.1.1 Iodometric titration on paper for assessment of fortified salt

Salt is beneficial if it contains at least 15 ppm iodine at the time of consumption.[32] Nearly all countries set regulatory specifications[33] that salt must contain 20-100 ppm I at the time of production.[34] Only about 15 countries have voluntary salt iodization, and about 15 countries have no laws regarding it.[33] Despite regulatory requirements, salt with improper iodine levels is often found in markets and households. For example, in India, only 79% of household salt samples in 2014 were adequately iodized even though India has mandated salt to be iodized since 2005.[35] Children and pregnant women are the most at risk if adequate amounts of iodine do not reach the population because iodine is incorporated into growth hormones. Most countries have set up universal salt iodization programs to ensure iodine is reaching the population. I have developed two test cards that can support universal salt iodization

programs in achieving better iodine nutrition. I describe a test card that assays part per million levels of iodate in fortified salt in Chapter 2, and I review its performance in several field tests and external validation studies in Chapter 3. Manufacturers can test the quality of their iodized salt products, regulatory agencies can test iodized salt on the market for compliance, and public health agencies can see if household salt samples have a nutritional benefit.

1.8.1.2 Sandell-Kolthoff kinetic assay on paper for assessment of population urinary iodine health

In 2006, Viet Nam's population had healthy urinary iodide levels so salt iodization was deregulated.[36] By their next population survey in 2009, the urinary iodide level was insufficient, so children and pregnant women were at risk for cognitive and physical impairment.[36] This demonstrates the need for continual monitoring by universal salt iodization programs. The target level for population urinary iodine is 100-300 ppb.[32] Only about 50 labs in the world can perform this sensitive analysis accurately,[37] so countries get their iodine health status assessed about every 5-10 years because of limited testing capacity and expense.[38] Five years is too long to wait to perform this critical public health evaluation. Iodization programs can modify their delivery tactics to get iodine to the people that need it, so they should monitor continually to best protect public health. The iodometric titration test card does not have a low enough limit of detection to be useful for urinary iodine analysis. In Chapter 5, I detail a paper test card I created that quantifies urinary iodine levels. The test card

relies on a redox reaction between Ce(IV) and As(III) that is catalyzed by part per billion levels of iodide.[39] These tiny levels of iodide are found in urine, so the reaction can be used to assess a population's iodine health status. The assay requires arsenic, so I had to consider the environmental risks of the test card. I used a "green design" process and built a remediation function into the card that binds the arsenic to iron oxides. This prevents arsenic from leaching into groundwater after the card is landfilled, and may ease some worries about the poisonous assay reagents.

1.8.2 Iodometric back-titration on paper for assessment of finished beta-lactam pharmaceuticals

Iodometric titration is a fundamental technique used by analytical chemists to assay redox active species. Iodometry is non-specific, so there are many applications it can be used for. While I was developing the test card that performs an iodometric titration, Abigail Weaver, a coworker of mine, was developing paper tests to identify beta-lactam antibiotics.[40] I realized her qualitative system could be paired with the iodometric test card to both identify and quantify beta-lactam antibiotics.

Amoxicillin and amoxicillin/clavulanic acid medicines are commonly used to fight infections, especially in LMICs such as Kenya.[41] When a person buys medicine, they expect a therapeutic benefit from it. If a person consumes a substandard antibiotic, their infection may not be cured, and in the worst case they could die. Survivors may lose trust in the healthcare system.[42] Low quality antibiotics can also contribute to the development of resistant strains of bacteria.

Finished pills of beta-lactam antibiotics must contain 90-120% of the amount stated on the label according to the United States Pharmacopeia (USP).[43] About 140 countries in the world use the specifications set forth in the USP for regulatory enforcement. Since 1999, there have been 9 publications about the prevalence of bad quality amoxicillin and ampicillin pharmaceuticals in LMICs, and the rates have ranged from 0-100% (see Table 4.1). The reported rates are highly variable and may be due to an ebb- and flow- effect in which bad quality drugs come onto the market. An unscrupulous manufacturer can flood the market with a bad product that persists on shelves, which is followed by a lag period until another bad batch of product is released. The WHO recommends that countries set their own strategies for monitoring medications,[3] but the national regulatory authorities in low-income countries are usually ill equipped and lack the funding to incorporate systematic and routine surveillance of pharmaceuticals. In Chapter 4, I explain how I developed a test card that assays beta-lactam antibiotics in finished pharmaceutical pills. The test card relies on iodometric back-titration for the chemical analysis. Governmental agencies can establish their own monitoring systems for beta-lactam antibiotics, and use the test card to help achieve safe medicines for their population.

1.9 Foreshadow

Throughout this thesis, I will demonstrate not only the development of new quantitative PADs, but also their performance in the field. The test card for antibiotic analysis even detected low quality pills during a validation study at Notre Dame. The results were confirmed by HPLC analysis and shared with the Kenyan Poisons and Pharmacy Board, who initiated a regulatory investigation. This is an example of how test cards can help solve problems of product quality and regulatory compliance in LMICs.

CHAPTER 2:

LAB ON PAPER: IODOMETRIC TITRATION ON A PRINTED CARD

2.1 Overview

Reprinted (adapted) with permission from Myers, Nicholas M.; Kernisan, Emalee N.; Lieberman, M. *Anal. Chem.* **2015**, *87*, 3764-3770. Copyright 2015 American Chemical Society. ML originated the concept of transferring iodometric titration to a paper substrate. NM created the physical embodiment of iodometric titration on paper. NM, EK, and ML validated the device. Abigail Weaver and Michelle Pillers served as card readers. NM and EK conducted the stability and robustness studies. Doa'a Aldulaimi fabricated the devices for routine analysis. Emily Mediate, Kellie Raddell, and Evan Graham screened early versions of the test card.

2.2 Background

Salt for human consumption is typically fortified with potassium iodate to levels between 15 and 100 ppm iodine (expressed as mg iodine atoms/kg salt), depending on local regulatory requirements.[44] Many small salt producers do not have access to a lab that can perform iodometric titration on the iodate fortificant, so we decided to design a test card to carry out the analytical task. Iodometric titration requires sequential addition of multiple reagents that are unstable when stored together, making it a

challenge to translate to a paper substrate. Accomplishing this goal adds redox titration to the toolbox of techniques that chemists can select from when analyzing samples in low resource areas.

Analytical metrics of the redox titration on paper were established using saline solutions spiked with iodate. Most developing countries in tropical climates use potassium iodate as the iodization source.[34] During analysis by iodometric titration, excess iodide reacts with iodate, and in the presence of acid, triiodide forms (see Figure 2.1). Triiodide is then titrated with thiosulfate using a starch solution as an indicator. If the amount of triiodide exceeds the reducing capacity of the thiosulfate, the indicator turns blue; if the amount of triiodide is smaller than the reducing capacity of the thiosulfate, the indicator remains uncolored.

$$IO_3^- + 6H^+ + 8I^- \rightarrow 3I_3^- + 3H_2O \quad \text{(Reaction 1)}$$

$$I_3^- + 2S_2O_3^{2-} \rightarrow 3I^- + S_4O_6^{2-} \quad \text{(Reaction 2)}$$

$$I_3^- + \text{starch} \rightarrow \text{blue complex} \quad \text{(Reaction 3)}$$

Figure 2.1. **Reaction 1.** In acidic conditions, excess iodide reacts with iodate to produce triiodide. **Reaction 2.** Triiodide is reduced to iodide by thiosulfate. **Reaction 3.** Any triiodide present will form a blue complex with starch. For a glassware titration, the endpoint is reached when the solution turns clear.

2.3 Results and discussion

2.3.1 Reagent storage and surface tension-enabled mixing (STEM)

The reagents for the iodometric titration of iodate cannot be stored together on a paper substrate. At acidic pH, iodide is readily oxidized to iodine by air (leading to a false positive), and starch indicator[45] is degraded by both acid and iodine. These reactions occur both in solution and in reagents dried on paper. Thus, strict isolation of the stored reagents before use is necessary. Wax patterning[46] via rapid printing methods developed in the Whitesides group[47,48] was used to create zones on paper that could separate the reagents and allow them to recombine during the titration. We initially tried spotting the reagents at different positions in a chromatographic lane[40] which was then developed in a solution of the salt sample, but the inhomogeneity of mixing in the lanes was so large that quantification could not be reliably achieved. The best precision was obtained by adding all of the reagents at once within a well-defined reaction area. The backside of the paper is impregnated with wax, and 12 large square frames are printed on the front side of each test card. Heating the paper melts the wax and creates 12 reaction areas. Narrow wax lines subdivide each of the 12 frames to form five loading zones (Figure 2.2). Solutions of the titration reagents are deposited into the loading zones and then dried for storage. In order to determine the optimal line widths, dye solutions were deposited into a series of frames and loading zones created with various line widths. The best as-printed line widths were at least 1.75 mm for the frames surrounding the reaction areas, and 0.30 mm for the thin lines that define the loading

zones (Figure A.1). To use the card, a test solution is added to the reaction area, and the card is gently shaken by placing it on a flat surface and moving it back and forth about 1 cm at a rate of 2 Hz. The solution can pass over the narrow wax barriers separating the loading zones, dissolving the dried reagents, but the solution meniscus is pinned at the edge of the thick wax frame. We call this process surface-tension enabled mixing, or STEM (Figure 2.2).

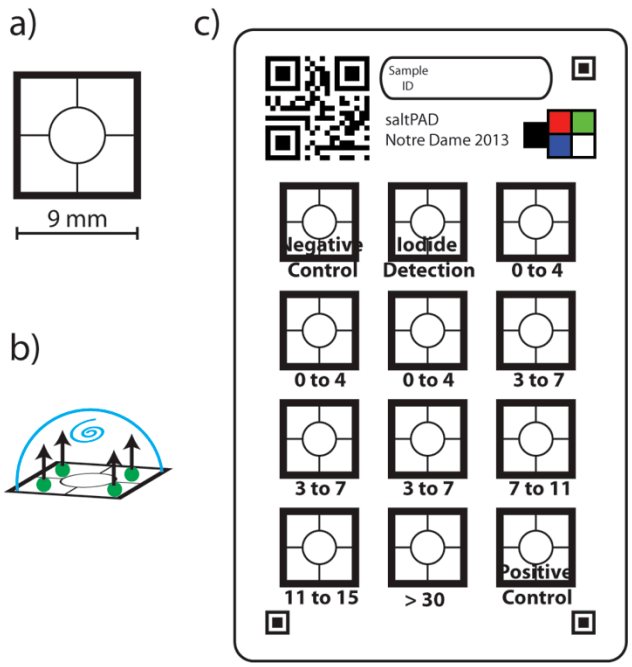


Figure 2.2. Design of titration test card. a) Black lines represent hydrophobic barriers. Up to five reagents can be stored separately in the loading zones. b) Surface tension-enabled mixing (STEM). When liquid is applied to the reaction area, it forms a dome confined by the solution meniscus, and it is pinned at the border of the thick square. The reagents stored in the five loading zones dissolve and mix. c) The different reaction areas perform points in an iodometric titration, a limit test for iodide, or a positive or negative control. The numbers under the squares represent ppm iodine levels that can be quantified (in units of mg iodine atoms/L) except for the square labeled > 30, which is a limit test for over-iodized salt.

2.3.2 Part per million titration on paper

In a regular iodometric titration, aliquots of thiosulfate are titrated into the sample until the equivalence point is reached. In the paper test card implementation, the zones in each reaction area are loaded with different amounts of sodium thiosulfate, along with excess potassium iodide, tosic acid, and starch indicator (which is in the central detector area). The complete fabrication recipe is in the appendix (Table A.1 and Figure A.2) along with a description of how the optimal thiosulfate amounts were selected (Figure A.3). An additional recipe is included in the appendix (Table A.1 and Figure A.2) showing how to adjust the card to measure iodine levels typically used during production (15-55 ppm I in the solid salt sample). Iodide is further stabilized for storage as a Cd(II) salt.[49] Aliquots of 0.125 mL test solution are applied to each reaction area to activate the titration. The paper test card is not like a traditional titration, which performs best with a sharp endpoint indicator. Instead, each reaction area gives no color unless the amount of iodate in solution overwhelms the amount of thiosulfate on the reaction area. At this point, increased iodate content in the reaction area causes increased color production from the indicator until the response becomes saturated. See Figure A.4. Multiplexing the reaction areas allows 12 tests to occur simultaneously. Thus, the paper card combines features of limit tests and of a colorimetric quantification assay.

The layout of the twelve reaction areas is detailed in Figure 2.2. The unit of measurement for all the iodine detection squares is ppm I, defined as mg iodine atoms/L solution. Reading from left to right and top to bottom, the first square is a

negative control containing only acid and starch; there should be no response when a test solution is applied. The second square is a limit test for iodide, which is an iodizing agent used mostly in temperate countries; it is included to detect iodized salt that may have been imported to the markets of low- and middle-income countries. Iodide gives false negatives with the reactions used for a standard iodometric titration; instead, it must first be oxidized to triiodide using nitrite, which is then detected with starch indicator (Figure A.5). The limit test on the PAD detects iodide in concentrations greater than 20 ppm I (Figure A.6). Squares 3-5 quantify iodate in the range of 0-4 ppm I; squares 6-8 quantify iodate in the range of 3-7 ppm I; square 9 quantifies iodate in the range of 7-11 ppm I; and square 10 quantifies iodate in the range of 11-15 ppm I (see Figure 2.4 for calibration curves). Square 11 is a limit test for iodate solutions that contain > 30 ppm I (Figure A.6). This level of iodine corresponds to over-iodized salt that could present a health risk to some consumers. Square 12 is a positive control containing potassium iodate, potassium iodide, tosic acid, and starch to produce a blue response no matter what test solution is applied. Additional features on the test card include a QR code, fiducial marks, and color standards to facilitate computer image analysis.

2.3.3 Calibration of device

Iodate solutions in a 3.7 M sodium chloride matrix were run on the test card in order to quantify iodine levels in fortified table salt (Figure 2.3). Quantification by color measurement is particularly challenging if there are variations of images captured in

sun, shade, incandescent or fluorescent lighting, and so on. Images were therefore acquired in a lightbox that provided consistent illumination (see “How to make a lightbox” in section A.6). The blue color in the central circle of each reaction area was measured by computer image analysis, see Figure 2.4. Each reaction zone displays a sigmoidal increase in color with increasing iodate concentration (Figure A.4). The pseudo-linear portion of each curve, which covers a range of roughly 4 ppm per reaction zone, is quantitatively useful (Figure 2.4 and Table 2.1). In general, the triplicate reaction areas at 1-7 ppm levels of iodizing agent give superior precision over the single-point measurements at higher concentrations. This range of iodine concentration is particularly important because after accounting for a five-fold dilution of the salt sample during preparation, the 3 ppm level differentiates salt that is properly iodized at 15 ppm (expressed as mg iodine/kg salt) from salt that is under-iodized. Using the slope of curve 3,4,5 in Figure 2.4 and the SD of 10 blank samples, the limit of detection (LOD) was determined to be 0.8 ppm I (3 * SD/slope) and the lower limit of quantification (LLOQ) was estimated to be 2.4 ppm I (10 * SD/slope).

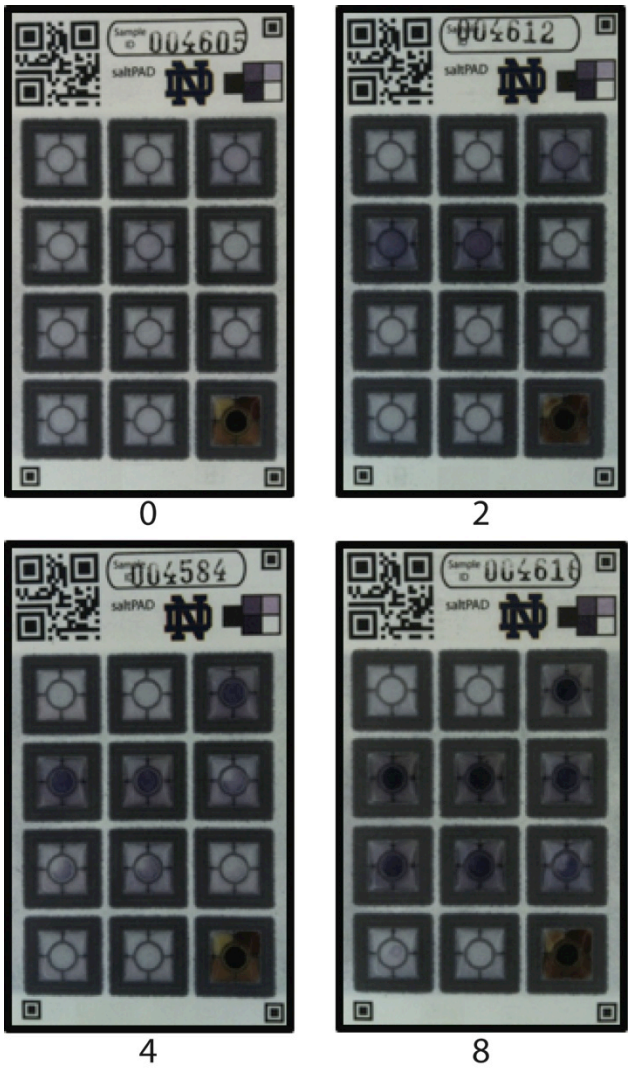


Figure 2.3. PAD response to various levels of iodate in solution. The numbers below the images are expressed as ppm of iodine atoms in the solution applied to the test card; the user must multiply the value by the dilution factor to get the iodine concentration in the solid salt sample. A greater number of indicator circles turn blue as the iodine concentration increases, and the intensity of the blue color increases in a specific reaction zone (e.g., reaction zone 3 becomes darker in each consecutive image). More standard images can be found in Figure A.7.

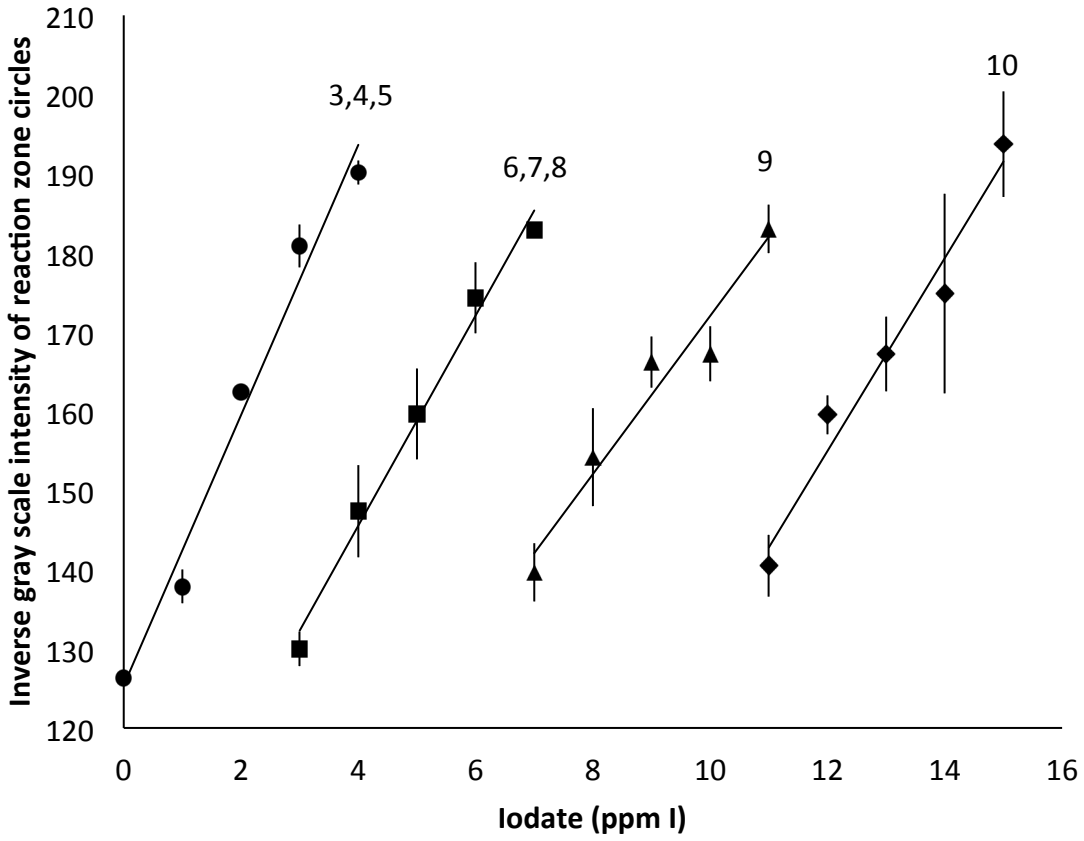


Figure 2.4. saltPAD calibration. Overlapping ranges allow quantification of iodine over a working range of 0-15 ppm I in the test solution. Each calibration line represents a reaction zone (number above line) that contains a different amount of thiosulfate, as shown in Table 2.1. Each data point is an average from 36 reaction zones with ± 1 SD error bars. The concentration must be multiplied by the dilution factor to get the concentration on the solid salt sample.

TABLE 2.1.

CALIBRATION EQUATIONS FOR DIFFERENT REACTION ZONES ON THE SALTPAD

Reaction zones	$S_2O_3^{2-}$(nmol)	Slope	y-intercept	R^2
3, 4, 5	6.0	17.1 ± 1.4	125.4 ± 3.5	0.980
6, 7, 8	33	13.3 ± 0.8	92.5 ± 4.3	0.989
9	60	9.9 ± 1.3	72.1 ± 11.9	0.951
10	87	12.2 ± 1.3	8.8 ± 17.2	0.966

2.3.4 Internal validation

Using a blinded methodology, 2 expert users each analyzed 55 solutions. Simply comparing the number and intensity of colored reaction areas by eye to the standard images gave a good estimate of the concentration of the iodate (Figure 2.5). The experts read the test cards with an accuracy of 0.5 ppm on average, and with an inter-operator precision of 0.5 ppm (see Figure A.8) over the visual analysis range of 0-15 ppm I in solution. The average absolute inter-operator precision based on visual analysis was 0.5 ppm I, as evaluated by equation (1) below:

$$\frac{\sum_{i=1}^{n} |\bar{X}_1 - \bar{X}_2|}{n} \qquad (1)$$

where $\bar{X}_1$ is the average response of 5 PADs from expert 1, $\bar{X}_2$ is the average response of 5 PADs from expert 2, and *n*, the total number of solutions containing a different concentration, is 11. The accuracy was calculated by equation (2),

$$\frac{\sum_{i=1}^{n} |X_{real} - X_{measured}|}{n} \quad (2),$$

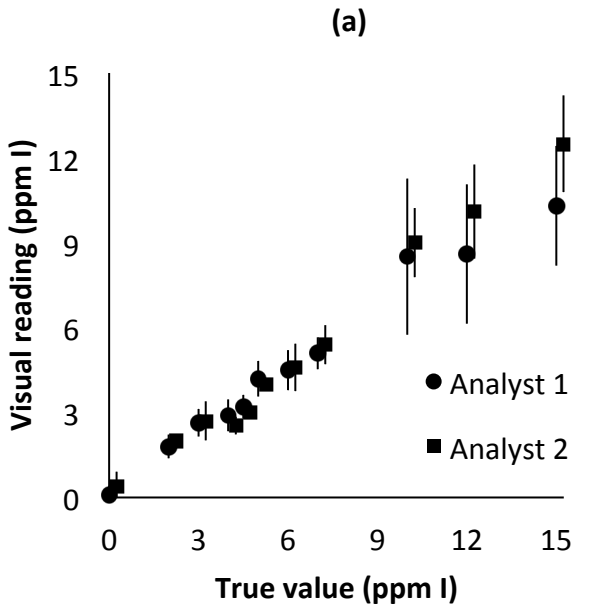
where X_{real} is the known concentration, $X_{measured}$ is the saltPAD response as measured by eye, and n is the number of saltPADs used (n=110).

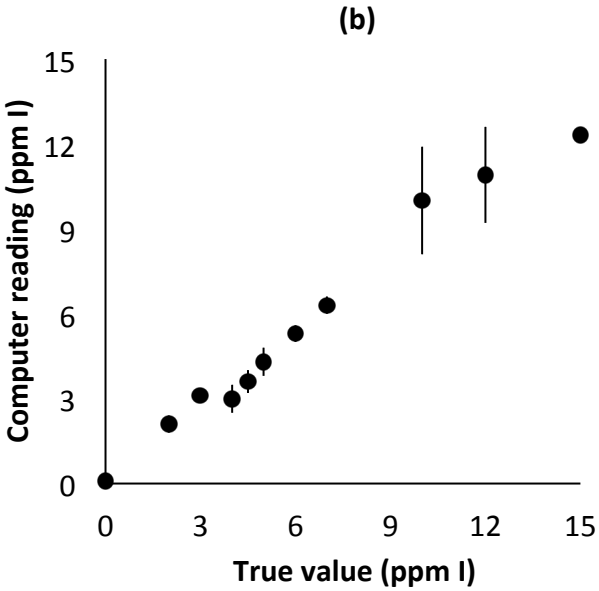
When 2 newly trained users analyzed the same 110 images, they achieved an average accuracy of 1.4 ppm I and an average precision of 0.9 ppm I (Figure 2.5a). The precision was determined by using equation (3),

$$\frac{\sum_{i=1}^{n} SD}{n} \qquad (3),$$

where SD is the standard deviation of the 10 replicates per sample and n is the number of unknown concentrations (n=11).

Figure 2.5. (a) Visual analysis of the saltPAD. Each data point is the average of the visual reads of 10 images by one of two newly trained analysts. Error bars show one SD. The data set is the same as the one used for computer image analysis. The true values for both analysts were identical, but the average and error bars for Analyst 2 are offset slightly along the x-axis to ease the reading of the data. Analyst 1 had an accuracy of 1.7 ± 1.0 ppm I and Analyst 2 had an accuracy of 1.2 ± 0.7 ppm I. (b) Computer analysis of the saltPAD. Each data point is the average of 8-10 replicates per concentration. Error bars show one SD. 4 PADs gave discrepant results and are not included; 4 samples gave an absolute error > 3 ppm but are included in the analysis.


(a)
Visual reading (ppm I)
15
12
9
6
3
0
0
3
6
9
12
15
True value (ppm I)
Analyst 1
Analyst 2


(b)
Computer reading (ppm I)
15
12
9
6
3
0
0
3
6
9
12
15
True value (ppm I)

In the computerized image analysis, the intensities of the colors in the indicator dots were measured using ImageJ and concentrations were calculated using the calibration curves shown in Table 2.1. Four of the 110 saltPADs gave discrepant results (color intensities that did not fall within any of the calibration ranges) and could not be quantified. Based on the appearances of these cards, we believe these discrepant PADs contained fabrication errors (e.g., wax lines that failed to seal properly or reagent spotting errors). In a field analysis situation, these samples would have had to be re-assayed on new cards. Four other samples assayed at greater than an absolute error of 3.0 ppm I (about 3 * SD of the method); these samples were retained in the determination of the analytical metrics. The average absolute accuracy for the computer image analysis of the PADs was 0.9 ppm I as calculated by equation *(2)* where X_{real} is the known concentration, $X_{measured}$ is the saltPAD response as measured by ImageJ, and n is the number of saltPADs used (n=106). The main source of inaccuracy is systematic underestimation of the iodine concentration at high iodine concentrations (Figure 2.5; see Figure A.9 for a Bland-Altman plot). In the high concentration ranges, which are less critical for analysis of minimum iodine content in market samples of salt, there is only a single point measurement, not a triplicate measurement as for the lower concentrations. Another cause of this systematic error is the camera software. Camera software often adjusts the exposure time of images to make them pleasing to the eye. When many dark blue circles appear, the software adjusts the coloration of the image (compare the 0 ppm image with the 13 ppm image in Figure A.7), which lowers the

apparent iodine concentration. Using a camera with manual exposure could help avoid this issue.

The average precision of the test card (method precision) for computer image analysis was determined using equation *(3)*. For samples in the full range of 0-15 ppm I, the average precision is 0.9 ppm I, and for samples in the range of 0-7 ppm I (where the test card includes triplicate reaction zones), the average precision is 0.3 ppm I. These measurements show that the computer image analysis is more accurate and precise than visual estimates of concentrations by newly trained users, but that expert readers can surpass the accuracy and precision of the computer image analysis program.

There are advantages and disadvantages to visual interpretation and computer image analysis of the cards. Visual analysis increases the usability in low resource settings, but introduces human error in reading or recording results. Capturing the data with a cell phone camera and interpreting the data electronically preserves the primary data and facilitates centralized record keeping through the mobile phone network. This would empower monitoring agencies to track the quality of salt in different geographical areas over time.

2.3.5 Robustness

The robustness of the PAD was tested to see if its age or water source affected the response. SaltPADs were wrapped in aluminum foil to exclude light, vacuum packed in Ziploc baggies, and stored in a 40˚C convection oven to accelerate the aging of the test cards.[50] The saltPAD response was tracked over time by analyzing low, moderate,

and high concentration standards as seen in Figure 2.6. Of 23 data points, 18 assayed within 2 ppm of the correct value by ImageJ analysis. To test how alternate water supplies may affect the analysis, an 8.0 ppm I (from iodate) standard was made up in a matrix of 3.7 M salt using tap water with high mineral content (170 ppm calcium) and also in a matrix of water from St. Mary's Lake on Notre Dame's campus (high content of natural organic matter). More information about the composition of these water samples is listed in Table A.2**.** The solutions were then run on the PADs. The standards made in hard water had an error of 8% (n=2) while the standards in the lake water produced an error of 17% (n=2).

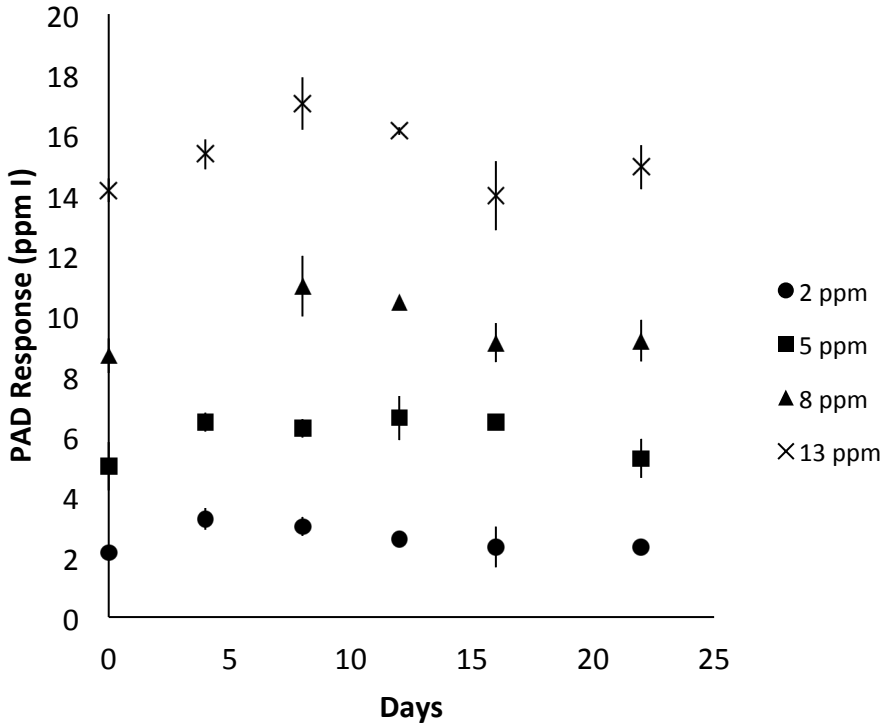


Figure 2.6. SaltPAD stability testing. SaltPADs were stored at 40°C and then tested with standard solutions of 2, 5, 8, and 13 ppm I. Each point and error range shows the average response of 3 test cards ± 1 SD.

2.3.6 Comparison with other field assays for iodized salt

Current methods for iodine measurement in salt include glassware titration, rapid test kits (RTKs), and spectroscopic test kits. Each method has trade-offs in terms of accuracy, cost, and ease of use. When conducted by a skilled operator in a well-controlled lab setting, titration with a buret gives excellent accuracy and precision. However, under field conditions in developing world settings, the accuracy and precision are compromised[51] by practices such as use of old or impure reagents, lack of calibration or service for analytical balances, and use of over-concentrated titrant. Rapid test kits, in which test reagents are applied directly from dropper bottles onto solid salt to detect the presence of iodate, are very inexpensive and easy to use, but an external validation study conducted by the World Health Organization showed that the kits do not give reliable quantification.[5] Distinguishing adequately iodized salt (≥15 ppm I) from inadequately iodized salt was problematic as it only had a 40% specificity for multiple observers.[5] When determining just the presence of iodate on salt, the test kit only had 14% specificity for multiple users. The World Health Organization does not endorse the RTK. In a different study undertaken by the South African Medical Research Counsel (SAMRC) in collaboration with WHO (Geneva), UNICEF (New York, USA), CDC (Atlanta, USA), and the University of Saint Pierre, Brussels, ten existing RTKs for testing iodated salt were evaluated. Progressive color intensities were observed with increasing iodine concentrations. However, these color reactions were crude approximations of the iodine concentrations compare to the titration method.[52] At best the existing rapid test kits used in household and market surveys can only be used to qualitatively estimate

broad categories of iodine concentrations.[5,52] Despite this, they are still used in some LMICs to check salt in the household or at the manufacturing plant. In the past decade, spectrophotometric assays using the characteristic blue/purple color of the starch-triiodide complex have been used to monitor salt quality. These assays show good analytical metrics but have a limited quantification range and require purchase of both a specialized reader and kits of reagents and vials. [51,53] Table 2.2 summarizes the reported analytical metrics, costs, and technical expertise required to use each of these methods and the saltPAD. It is important to independently assess these criteria, so Chapter 3 details many external validation studies conducted on the saltPAD.

TABLE 2.2.

SUMMARY OF QUANTITATIVE METHODS FOR IODINE MEASUREMENT IN IODIZED SALT

Method	Accuracy	Imprecision*	Cost (US$)	Technical Expertise
saltPAD	86%	12%	no capital investment $0.59 per card	Low
Titration under field conditions[51]	86%	10%	$4000 initial investment + $0.05 per sample	Need chemical technician and lab setting
Rapid test kit[5]	72%	NA#	$0.01 per sample	Low
BioAnalyt iCheck[51]	91%	1%	$3500 initial investment + $3 per sample	Low but need to use syringe and needle
WYD iodine checker[53]	97%	6%	$400 initial investment + $1 per sample	Need chemical technician

Note: *See Table A.4 for calculations of imprecision. #The study was conducted as a categorical response and it is unclear if replicates of the same test solution were used, so precision data is not definable.

2.3.7 Suitability for use in low-resource settings

The World Health Organization uses ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and deliverable to the end user) criteria to assess devices meant for use in low resource settings. The ASSURED criteria were considered at every stage of design of the saltPAD. For our current in-house fabrication process, materials costs are $0.09 USD (see Table A.3 for a cost analysis) and labor about $0.50. This cost is already less than the per-assay cost of kit-based spectrophotometric iodate assays, and we think it could be greatly reduced if manufacturing were automated using a roll-to-roll process. Scienion prototyped a chemical spray deposition process onto the PAD, and the results are shown in Appendix A.2 and the experimental is attached as a supporting document for the thesis. The paper test card performs iodine analysis with accuracy and precision that rivals other technology currently used in salt fortification plants in the developing world. The salt assay is easy to perform; one page of instructions and one page of comparison images can provide sufficient guidance even for first time users. For salt analysis, the user does not have to handle any chemicals other than salt and water. The test could be used in conditions (high heat, humidity, small amount of space) likely to be found in a small salt fortification plant. The sample can be prepped and run on the test card in about 5 minutes. The test cards can be easily transported or mailed to the end user and even withstand the conditions of a warm shipping van. Finally, the test cards can be analyzed using computer image processing of photographs taken with mobile phones, which enables data collection and archiving. While a full assessment of how well the paper

analytical device meets the ASSURED criteria will require field-testing of manufactured products, the results from the prototyped device suggest that adequate analytical performance for analysis of fortified salt can be obtained with this robust and easy-to-use paper test card.

2.4 Experimental

2.4.1 Materials

2% Starch indicator (BDH-VWR International), p-toluenesulfonic acid (tosic acid, Sigma Aldrich), potassium iodide (Alfa Aesar), cadmium chloride (Alfa Aesar), sodium nitrite (Mallinckrodt), anhydrous sodium thiosulfate (J.T. Baker), secondary standard of potassium iodate, 100.2% (J.T. Baker), sodium chloride (Macron), 0.01N iodine solution (Alfa Aesar), hydrochloric acid (Fisher), sodium hydroxide (Fisher). The paper source was Ahlstrom 319 (Midland Scientific). Pad.crc.nd.edu has a program that generates automated serialization of the PADs onto its LaserJet printing layer. The wax printing was done with a Xerox ColorQube 8570N. A Biomek FX (Beckman Coulter) liquid transfer robot was used to create the saltPADs. A convection oven (GCA Corporation) was used to perform stability studies.

2.4.2 Fabrication of saltPAD

SaltPADs were created by printing onto Ahlstrom 319 paper using solid wax ink.[46,47,48] The designs were created in and printed from Adobe Illustrator. Labels and alignment marks were printed with a laser printer, and then the thick and thin lines for

the reaction zones and the back side hydrophobic barrier were printed on the wax printer. The layers of wax were melted by baking at 100°C for 14 minutes to form hydrophobic barriers. A solution deposition robot (Biomek FX) deposited 60 aliquots of reagent solutions into the loading zones of each test card at a rate of 30 test cards per hour. The spotting error by the robot was approximately 1%. Any missed spots (visible as whiter areas against the grey background of damp loading zones) were pipetted by hand.

2.4.3 Running and analyzing the test cards

To create a test solution, the user diluted 3.25 g of salt into 15 g of water, which is a 1:5 dilution after accounting for the solution's density. Test cards were dosed with 125 µL of solution per reaction zone using an Eppendorf pipet, and then they were gently shaken by hand for 3 minutes. This requires some practice because if the paper is shaken too vigorously solution will spill out of the reaction zones. Using an orbital shaker with a 22 mm diameter at 4 revolutions per second provided great mixing, but at 5 revolutions per second the solution spilled. Images were taken in a lightbox equipped with two strings of plug-in 3 Watt LED lights that provided a light output of 162 Lumens and were rated 82 on the Color Rendering Index. The LED light output has a color rating of 3500K on the Correlated Color Temperature scale. Images were taken with an iPhone4 and imported into ImageJ for analysis.[54] The images were inverted and the average unweighted gray value over the entire reaction zone circle was measured.

2.4.4 SaltPAD calibration curves

Working iodate standards ranging from 0-15 ppm I in 3.7M NaCl were created by dilution from a stock potassium iodate solution. The working standards were pipetted on the saltPADs, and the blue color of the PAD response was measured with ImageJ to create calibration curves.

2.4.5 Internal lab validation

After construction of the calibration curves in Figure 2.4, an in-lab study was conducted using image analysis as a tool to assess the accuracy and precision of the PADs. 3.7 M sodium chloride brine was spiked with eleven levels of iodate, and the coded samples were analyzed blind by two operators, who each independently applied test samples, ran test cards, visually interpreted the test card by comparison to printed standard images, and then used ImageJ to evaluate test card results for 5-fold replicates. Four of the resulting 110 images gave discrepant results (e.g., one or more of the low range dots was uncolored and higher range dots were colored) which meant that they could not be quantified; four other cards gave results with absolute errors greater than 3 ppm. The card images were also evaluated by two newly trained readers in a blinded study. Each reader watched a Powerpoint presentation that explained how to evaluate the different reaction areas including the controls, the triplicate reaction areas, and the single range reaction areas. Before evaluating the blinded images, the readers then took a quiz in which they classified ten unknowns. Each reader scored at least 8/10 correct on the quiz.

2.4.6 Stability testing

Accelerated aging of the test cards was simulated by storage at 40˚C in a convection oven.[50] The cards were run with standard iodate solutions.

2.5 Conclusion

A paper card that stores multiple reagents and recombines them through surface-tension enabled mixing (STEM) can carry out an iodometric titration. This paper analytical device performs accurate and precise measurement of iodate in iodized salt. The test outcomes can be read directly by eye with an accuracy and precision of under 1.7 ppm, or by electronic image analysis with an accuracy and precision of under 1 ppm I (expressed as mg iodine atoms/L) over a range of 0.8-15 ppm I. Four out of 110 cards gave discrepant readings, probably due to fabrication errors. In addition to performing the iodometric titration, the card can detect analytes such as iodide and contains positive and negative controls. The iodometric titration test card could be used to assay other redox active analytes, including analytes in solutions of lower ionic strength than the iodized salt solutions.

The Joint UNICEF/WHO Committee on Health Policy recommends that all food-grade salt, used in household and food processing, should be fortified with iodine as a safe and effective strategy for the prevention and control of iodine deficiency disorders (IDD).[55] Thanks to global efforts by national governments, salt industries, international and national non-governmental organizations and scientists over the last two decades, iodine deficiency is no longer a common public health issue in many parts of the world

as 76% of the global population has access to adequately iodized salt.[56] The goal is to reach 90%. There will be a need for field technologies like the saltPAD that support this goal and maintain the progress already made.

2.6 Acknowledgments

The Notre Dame Faculty Research Support Program and the Global Alliance for Improved Nutrition (GAIN) provided financial support. A special thank you to our main contacts at GAIN, Ms. Rebecca Spohrer and Mr. Rizwan Yusufali.

CHAPTER 3:

SALTPAD: A VALIDATED TOOL FOR MONITORING SALT IODIZATION

3.1 Overview

Reprinted from Myers, N. M.; Strydom, E. E.; Sweet, J.; Sweet, C.; Spohrer, R.; Dhansay, M. A.; Lieberman, M. *Nanobiomedicine*. **2016**, *3*, 1 under Creative Commons CC-BY license found at https://creativecommons.org/licenses/by/3.0/. RS, MD, and ES created the study design for the external validation performed at the South African Medical Research Council (SAMRC), and operations were conducted with the aid of S Sherriff, IR Rix, T Williams, E Du Toit, and L Snyders. The study's data was shared with NM for processing. JS and CS developed the software to analyze images of the test card, and NM acted as a beta-tester. For the usability study conducted at Kensalt, NM and ML worked with Richard Mbaru, a quality control technician at the salt fortification plant. Doa'a Aldulaimi fabricated the saltPADs.

3.2 Background

3.2.1 Necessity of validation testing and regulatory oversight

A network of technological and business infrastructure supports laboratories and the equipment within them. In low- and middle-income countries, these systems are

often hindered by resource limitations, lack of trained staff to operate or fix equipment, rapid staff turnover, power outages, no refrigeration, inadequate bench space, and slow or unreliable mail delivery services. Because of these differences, new technologies that are developed in well-equipped laboratories need to be tested for implementation in resource-limited areas.

The studies of new technologies that perform quality checks of nutritionally fortified food should include internal process control at the manufacturing facility, regulatory monitoring of the facility, quality checks of imported products at borders, and marketplace surveillance. For salt iodization, national regulations vary but usually call for 20-100 micrograms of iodine per gram of salt,[44] which complies with the World Health Organization's (WHO's) recommendations for populations consuming 5-10 grams of salt per day.[34] The bodies responsible for regulation differ by country and sometimes by level of monitoring. For example, at factory level, regulation could be implemented by food and drug regulatory authorities, bureaus of standards, or dedicated bodies within Ministries of Trade. It is also necessary to test imported fortified salt, which can involve customs officials. Sampling in markets is necessary to prevent the fraudulent practice of packaging non-iodized salt with iodized salt labels, as a check on production quality control, and because iodine content of properly iodized salt may degrade over time. Once regulations are in place, they should not be relaxed as it can cause serious harm in a short time. For example, in 2006, Vietnamese lawmakers deregulated salt iodization after healthy levels had been attained in the population's diet. As a result, large quantities of non-iodized salt entered the market, and the population became

iodine deficient by 2009.[36] Provision of high-quality fortified salt must be treated as a long-term nutritional intervention and endowed with sustainable oversight, regulation, and technologies. The saltPAD must be tested at as many of the above-discussed points in the supply chain as possible. We were able to support many case studies during my Ph.D. tenure, including an external validation study that used salt samples collected from the marketplace and a usability study at a salt fortification plant in Kenya. GroundWork, a public health group that provides technical support to Ministries of Health and UNICEF, conducted a comparative study in Burkina Faso that assessed the saltPAD's performance against other commercially available technologies.

3.3 External validation at the South African Medical Research Council

3.3.1 Experimental

The Global Alliance for Improved Nutrition (GAIN) set up and funded an external validation study at the South African Medical Research Council (SAMRC). The purpose of the study was to independently establish the analytical metrics of the test card. SAMRC decided to use many salt samples collected from the marketplace, so the study also addressed how well the test card could work when analyzing manufactured fortified table salt collected from the supply chain. The salt samples included fine, medium, and coarse-grained salts. A total of 287 salt samples were tested with the saltPADs in a blind fashion using titration as the reference method. All samples were analyzed in duplicate by each method and 3 people read every test card image. SAMRC reported the titration results to GAIN, and SAMRC gave test card images to me for processing. I reported

visual and software analysis results to GAIN, who then unblinded the true values and provided me with all the raw data to calculate the analytical metrics.

The test cards were made at the University of Notre Dame (UND), vacuum-sealed in plastic, and shipped to the SAMRC. The typical transit time was less than a week, and the cards were used at 1-3 months of age. The true iodine concentrations of the salt samples were unknown to the analysts running the saltPADs. For 108 samples, portions of non-iodized and iodized salts were mixed together in various ratios to get final concentrations in the 5-50 ppm I range. The remaining 179 samples were collected directly from the marketplace and contained 0-120 ppm I (as iodate). Two 10 g portions of each sample were iodometrically titrated, and the average concentration was used as the true value for the study. The average RSD for titration was 5%, which reflects a combination of the sample heterogeneity and the titrimetric precision. Each salt sample was prepared for PAD analysis using 6.5 g of salt and 30 mL of deionized water and tested on 2 saltPADs. A picture of each saltPAD was taken in a lightbox with a Nokia Lumia 920 phone (Figure 3.1). Two novice users at the SAMRC and an expert reader at UND analyzed all of the images by visual comparison to standard images (A.7).

3.3.2 Results and discussion

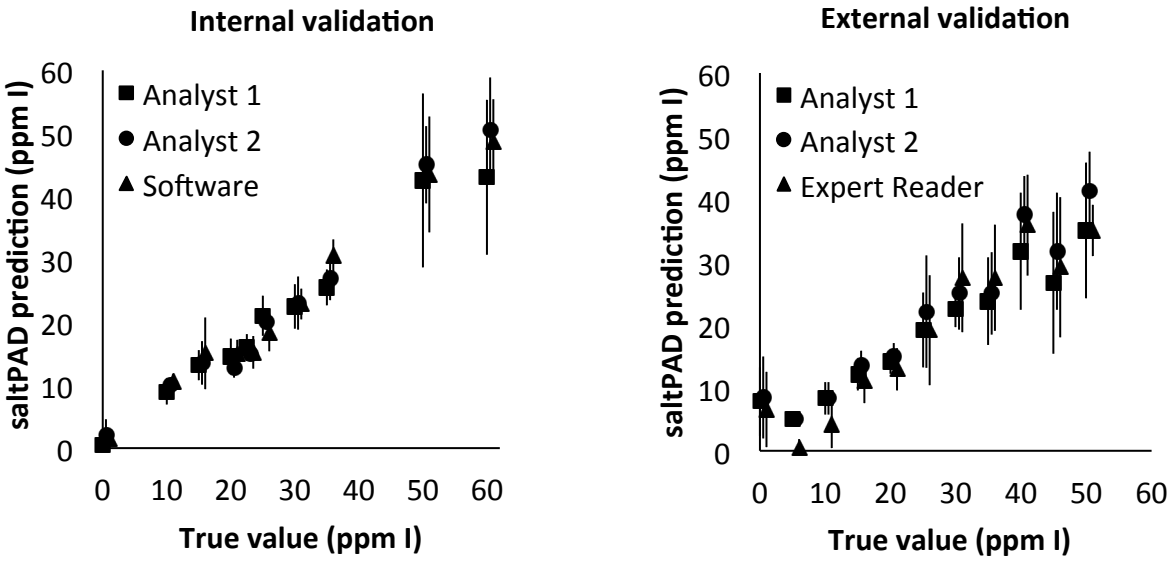
The first challenge at SAMRC was to build a lightbox as detailed in A.6, so the raw data could be captured with a cell phone camera. Their setup is shown in Figure 3.1. They were able to find and purchase all of the equipment needed, including a light strip that provided good illumination for the test cards. Numerous emails were exchanged to

ensure this was constructed correctly, so it may have been easier to mail a lightbox to SAMRC.

Figure 3.1. Illustration of the lightbox set up at SAMRC and the positioning of the saltPAD in the box.[57]

SAMRC ran standardized iodate solutions in 3.7 M NaCl on the test cards to create calibration curves from images of the cards, similar to those in Figure 2.4. All unknown samples were compared to the SAMRC calibration set. Three analysts, with me acting as the "expert reader," each read 568 test card images for a total of 1704 reads. The accuracy (the average of all absolute errors) was determined for each analyst and is shown in Figure 3.2; the average accuracy for all 3 analysts was 7.0 ppm I, expressed as mg I per kg salt. The method precision for each analyst was calculated by taking the standard deviations of the duplicate analyses and averaging them; the average precision

for all 3 analysts was 4.9 ppm I. These analytical metrics are basically the same as the metrics obtained in the internal validation study (also shown in Figure 3.2), showing that the saltPAD could function just as well in an external laboratory setting.



Internal validation metrics

	Accuracy	Precision	n
Analyst 1	8.5	5.0	55
Analyst 2	6.0	3.5	55
Software	4.5	4.5	110

External validation metrics

	Accuracy	Precision	n
Analyst A	5.8	4.5	538
Analyst B	6.9	4.7	540
Expert	8.3	5.6	541

Figure 3.2. Internal and external validation of saltPAD. Accuracy and precision are expressed in units of mg of I atoms per kg iodized salt. The true values on the graphs have been slightly offset to ease the reading of the data. Since there are so many data points for the external validation, and to help ease the reading of the graph, only the data points with true values that are a multiple of 5 ppm I have been displayed. The metric values in the table include all data points.

The saltPAD design used in the SAMRC study was optimized to distinguish adequately iodized salt (≥15 ppm) from inadequately iodized salt (<15 ppm), a cutoff usually used during market surveillance studies. It performed triplicate measurements in the 0-35 ppm I range and single measurements at higher concentrations. To see how well the 15 ppm I limit test could work, the quantitative reads were grouped accordingly. Of 486 reads that should have predicted a sample to be under-iodized, 440 (90.5%) did so (Table 3.1). For 174 reads that should have fallen in the slightly under-iodized region of 10-15 ppm I, 149 (85.6%) did so, demonstrating a combination of good accuracy and precision. For 1218 reads that should have predicted sufficient iodine levels, 1122 (92.1%) did so. Figure 3.3 shows the receiver operator curve (ROC) for distinguishing these levels. The calibration curves generated at SAMRC were used to set the 15 ppm threshold; no adjustment was made using a second set of images. The area under the curve is 99.4%, so the test card is extremely good at discriminating properly iodized salt from under-dosed salt.

TABLE 3.1.

ACCURACY TABLE OF THE SALTPAD USED AS A MARKET SURVEY TOOL

	saltPAD (ppm I)	
Titration (ppm I)	<15	≥15
<10	92.9% (N=290)	7.1% (N=22)
10<x<15	86.2% (N=150)	13.8% (N=24)
≥15	7.9% (N=96)	92.1% (N=1122)

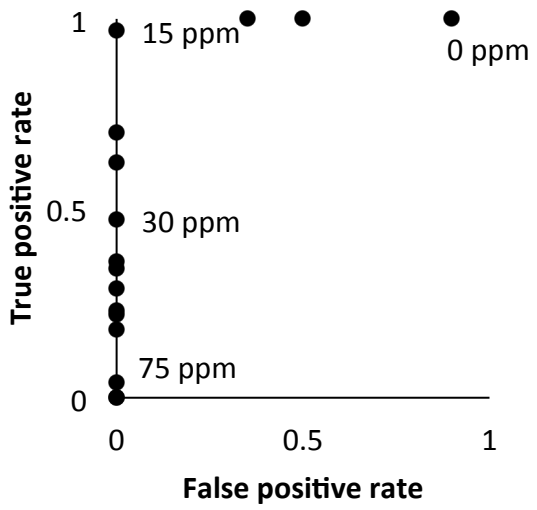


Figure 3.3. Receiver-operator curve for salt adequately iodized at 15 ppm I or above. The area under the curve is 99.4%.

Although the saltPAD tested in the SAMRC study was optimized for detection of market-level iodization (with an analytical "sweet spot" around 15 ppm I), we evaluated if this card could be suitable for quality control (QC) applications in a low resource setting such as Kenya, where a usability study had already occurred (see section 3.4). Regulations in Kenya dictate that an iodized salt sample must be between 30-50 ppm I at the time of production in order to be sold in Kenyan marketplaces, so the process control requires detection of both a minimum and maximum level of iodine. The goal for the test card is to provide an accurate result that correctly predicts if an adjustment to the iodization machinery is necessary. This can happen in near-real time instead of waiting for the titration lab to relay a decision, and a large batch of bad product can be avoided, instead of being thrown away or reprocessed. For the 1704 card images in the SAMRC study, only 1298 (76.2%) of the visual interpretations produced correct categorization of the salt samples according to these QC levels (Table 3.2).

TABLE 3.2.

ACCURACY TABLE OF THE SALTPAD AS A QUALITY CONTROL TOOL

Titration (ppm I)	saltPAD (ppm I)		
	<30	30-50	>50
<30	97.6% (N=996)	2.4% (N=24)	0% (N=0)
30-50	56.6% (N=292)	40.5% (N=209)	2.9% (N=15)
>50	7.7% (N=13)	36.9% (N=62)	55.4% (N=93)

Note: Overall, 76.2% (1298/1704) of the visual reads predicted the correct categorization for the salt sample.

Most of the errors are due to low readings. However, only 2.4% of samples containing < 30 ppm I were predicted to have sufficient levels for sale, so it is unlikely that salt with no health benefit (< 15 ppm) would make its way to the market. The performance of the saltPAD could be improved by redesigning it with replicate measurements in the 30-50 ppm I range.

3.4 Usability study at Kensalt

Another possible use for this field-screening tool is to monitor the iodization process during salt fortification. To assess the user-friendliness of the test card when used by a quality control operator, Dr. Lieberman and I traveled to Mombasa, Kenya and conducted a usability study at Kensalt, a salt fortification plant. The plant uses spray deposition of potassium iodate solution, followed by drying and packaging. The spray

rate must be adjusted based on many factors, including the temperature, the particle size of the salt, and the conveyer belt speed, but the decision is based upon a result obtained by glassware titration. Every 30-60 minutes, a technician grabs a sample directly after the iodization spray nozzle and titrates it to see if the iodine content is within the regulatory specification of 30-50 ppm I. If the iodization level is below 32 ppm I, the technician increases the spray rate, and if the iodization level is above 46 ppm I, the technician decreases the spray rate. The technician may also make an adjustment if the results are trending out of bounds. The test card was used by the quality control technician in the plant's lab to see if the test card's results agreed with glassware titration, thereby predicting the correct process control adjustment.

3.4.1 Experimental

We trained Richard Mbaru, a quality control technician at Kensalt, to use the saltPAD in 31 minutes (Figure 3.4). This included sample preparation, running it on the test card, and interpreting the response by comparison to electronic standard images.

Figure 3.4. Richard Mbaru, Kensalt quality control technician, using the saltPAD. Mombasa, Kenya, 2013.

He collected 14 grab samples from Kensalt's manufacturing line. These samples were analyzed with saltPADs in the plant's titration lab. The temperature was 25-30°C, and the humidity was high; the lab had a thermometer but not a hygrometer. Mr. Mbaru, Dr. Lieberman, and I visually interpreted the test card responses by comparing them to standard images generated at the University of Notre Dame. A total of 84 visual reads were performed. The salt samples were also analyzed by glassware titration for a reference method, but the results were kept blind until the saltPAD interpretations had been completed.

3.4.2 Results and discussion

We evaluated the process changes the saltPAD readings would require the technician to perform, and compared them to the process changes required by the results of the gold standard titration analysis (Table 3.3). The iodine level determined by titration of two of the salt samples was above 50 ppm, and would require turning down the spray rate. Of the 12 reads, only 4 (33.3%) of the corresponding saltPAD readings indicated that the iodine level was too high. The iodine level for five of the salt samples was in the correct range of 30-50 ppm I and would not require any change in spray rate; 25 (83.3%) of the corresponding saltPAD readings indicated that the iodine level was in the correct range. The iodine level for seven of the salt samples cases was below 30 ppm I, and would require turning up the spray rate; 36 out of 42 (85.7%) saltPAD readings indicated that the iodine level in these samples was too low. Overall, 77.4% of the saltPAD-based process actions matched those required by the titration results.

TABLE 3.3.

THE PERFORMANCE OF THE SALTPAD AS A PROCESS CHANGE TOOL

Titration (ppm I)	saltPAD (ppm I)		
	<30	30-50	>50
<30	85.7% (n=36)	16.7% (n=6)	0% (n=0)
30-50	6.7% (n=2)	83.3% (n=25)	10.0% (n=3)
>50	0% (n=0)	66.7% (n=8)	33.3% (n=4)

The average of the differences between the saltPAD results that gave a semi-quantitative reading and the titration results was 2 ± 6.5 ppm, which is statistically indistinguishable from zero (paired t-test, 95% CL). Performing 28 saltPAD analyses took Mr. Mbaru 3 hours, which was the same time required to perform 14 glassware titrations. This does not include the time required to prepare and standardize the titration reagents. The saltPAD analyses generated less than 500 mL of waste (salt solutions plus the used saltPAD cards) while the titrations created over 5L of waste.

The outcome of this study in a fortification facility was similar to the outcome of the SAMRC study, which was performed in a controlled laboratory environment, so this suggests that the limited environmental control in the LMIC lab did not affect the saltPAD.

3.5 Comparative study conducted by GroundWork

A study was carried out by GroundWork in Burkina Faso to compare the performance of 5 quantitative field analysis technologies in a developing world setting (Table 3.4 and Figure 3.5).[6] Nobody from the University of Notre Dame was involved in the study other than to provide test cards and to answer questions about how the test cards work. We received many emails about the construction of the lightbox needed to capture images of the test card, and a light strip had to be hand delivered from a developed country; after the study, GroundWork stated a preconstructed lightbox would have been preferred. Replicate analyses of spiked saline solutions and 59 salt samples collected from several different countries were conducted in a laboratory setting and in a field setting, and by experienced lab technicians and non-technicians. For each technology, analytical metrics such as accuracy and inter-device and inter-operator precision were determined. In addition, the usability and field-friendliness of each system was evaluated. Technologies were rated lower if they were difficult to perform correctly, required many steps or access to other lab equipment such as an analytical balance, generated hazardous waste, or required a controlled laboratory environment. All of the technologies except the saltPAD were commercially available systems based on spectrophotometric analysis methods. The commercial systems require that the user obtain and maintain the spectrometer as well as reagent solutions. For some of the spectrophotometric systems, the reagents must be prepared by the user, and in other cases, they are available as pre-measured portions for analysis of a

single sample. The saltPAD was less accurate than some of the spectrophotometric systems, but was rated highly for being field- and user-friendly.

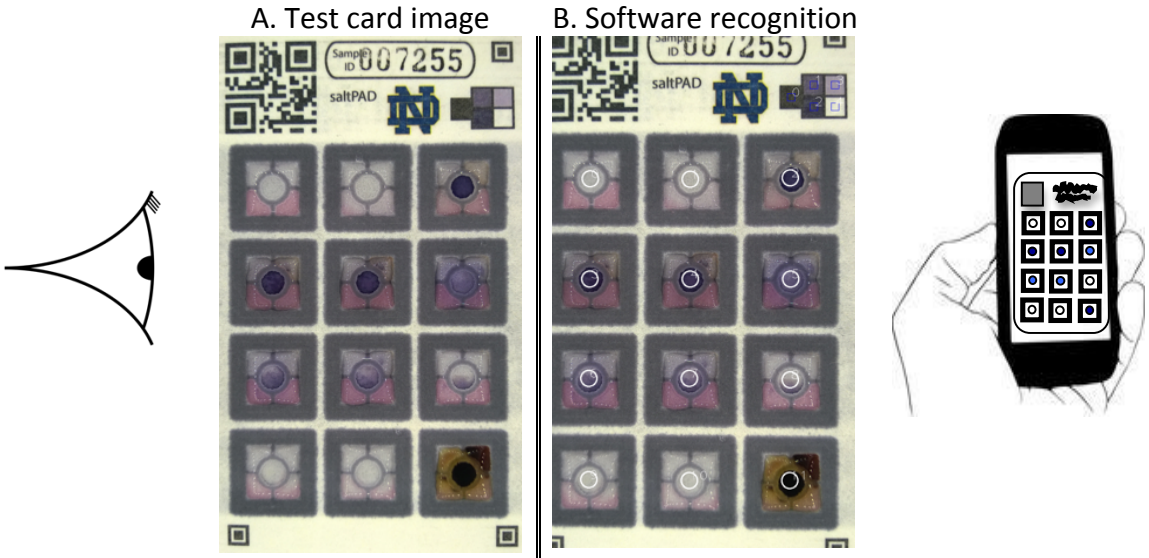
TABLE 3.4.

A COMPARATIVE STUDY BY GROUNDWORK RANKED 5 TECHNOLOGIES FOR OVERALL PERFORMANCE

Method	Rank	Strengths	Weaknesses
I-Reader	1	Analytical metrics, user-friendly, field-friendly, compatible with low resource settings	Does not recommend appropriate sample preparation for heterogeneous samples
iCheck	2	Analytical metrics, user-friendly, field-friendly	Cost, need computer, glass and sharps waste
saltPAD	3 (tied)	Field-friendly, user-friendly	Need test kit development and automated image analysis
WYD	3 (tied)	Analytical metrics, compatible with low resource settings	Need lab setting and skills
ID-ERTK	5	Compatible with low resource settings	Need lab setting and skills

The saltPAD was the only device in the GroundWork study whose accuracy and precision improved when it was used by non-technicians: this was because two of the technicians in the central lab did not read the saltPAD results correctly. In order to eliminate this source of inter-operator variability, software was written to calculate a sample's iodine concentration from a cell phone image of the test card. A repository with the open source code for this image comparator is available at https://github.com/PaperAnalyticalDeviceND/SaltPad. This computer image analysis method makes use of fiducial marks and color standards printed on the saltPAD. First, the locations of six fiducial marks are determined and a geometrical correction is applied to remedy image scaling, tilt, and keystoning. The image is then cropped along the fiducial marks, and the white balance is adjusted. A mask alignment step, shown in Figure 3.5, is used to identify the twelve reaction zones with their central indicator circles; these circles where color development occurs are the regions of interest (ROI) for the quantitative analysis of iodine concentration. The greyscale intensity integrated over each ROI is measured and then fitted to the appropriate calibration curve for that reaction zone, stored in program memory. The program ignores any spots with color intensities that fall outside the linear calibration ranges, and rejects the card altogether if the positive or negative control spots give incorrect readings. The measured concentration and ROI data are output to the user in spreadsheet format. When this program was applied to the images from the Burkina Faso study, it improved the sensitivity greatly from 45.0% to 87.5% and slightly reduced the specificity from 100.0%

to 89.5%,[6] eliminating erroneous reads from operators who did not understand the visual interpretation.



	Visual analysis	Software analysis
Sensitivity	45.0%	87.5%
Specificity	100.0%	89.5%
Cohen's kappa	0.41	0.74

Figure 3.5. Automated software analyzes images of the saltPAD. A. A picture of a saltPAD taken with a cell phone. B. The input image is analyzed by the program which locates the regions of interest designated by the white circles. These coincide with the indicator spots. The greyscale intensity is measured, fitted to calibration curves stored in the program memory, and the resulting iodine concentration is reported to the user.

3.6 Conclusion

The saltPAD is an environmentally friendly quantitative rapid test kit and is ideal for settings where sophisticated equipment is not available. The external validation studies prove its robustness, see Table 3.5 for a quick summary of analytical metrics.

TABLE 3.5.

ANALYTICAL METRICS FOR THE TEST CARD IN VARIOUS VALIDATION STUDIES

Validation type	n	Accuracy (ppm I)	Precision* (ppm I)
Internal lab[58]	107	4.5	4.7/2.4
External lab**	1619	7.9	1.4/2.3
Usability	84	2.0	NA
Comparative[6]	203	7.1	1.6/6.2

Note: All units are expressed as ppm iodine atoms in solid salt. *Inter-device/inter-operator precision. **Study conducted by the South African Medical Research Council, not yet published by them.

The utility of the saltPAD has been demonstrated for market surveys and for QC applications in salt fortification facilities. The saltPAD's analytical metrics provide enough power to serve several important applications in low resource settings, where the portability and low cost of the saltPAD could provide an advantage over other analytical methods. In particular, the saltPAD is well suited for quantitative household and market level surveys, where salt iodized at ≥ 15 ppm must be distinguished from

salt iodized at lower levels. The saltPAD was able to correctly categorize more than 90% of the samples in the SAMRC study according to this criterion. Although titration is the "gold standard" for analysis of iodized salt, transporting household or market salt samples to a laboratory can introduce logistical delays of weeks or months, and salt samples can also be lost or damaged, particularly if they are poorly packaged. An on-the-spot quantitative assay would eliminate these sources of delay and error. It would also provide an opportunity to discuss the results immediately with the market vendor or householder, which could be useful in supply chain surveillance or public health education contexts. This assay has been shown to produce reliable results in an internal study, an external laboratory, and a fortification facility environment. However, it has not been tested under field conditions particular to health related surveys and regulatory monitoring at border posts. Nevertheless the currently designed saltPAD could replace qualitative rapid test kits currently used for many market and household surveys, providing better data about whether there is sufficient iodine present in salt samples.

The saltPAD offers two features that are suited for low-resource settings. The cell-phone based image analysis program can provide a mechanism for collecting the test data centrally and allows a wide range of users to report accurate test results. Regulatory agencies and health organizations could use this feature to monitor iodized salt quality across a region in real time. Archiving, collating, and comparing data could help to improve the transparency of monitoring efforts, as well as guard against bad practices such as dry labbing. Second, the capital cost for setting up saltPAD testing is

very low, about $20 USD, and there is no requirement for the user to prepare reagents or provide any consumables other than the cards themselves. Although the cards are not yet available commercially, the cost is projected to be under $1 USD per card.[58] This is in contrast to the $3000 USD cost of establishing a titration lab, which requires suitable laboratory space, an analytical balance, lab equipment, and chemicals, or the $400-$4000 USD cost for a spectrophotometric reader. These laboratory analysis methods come with continuing costs of reagent preparation in a lab setting or purchase of $1-4 USD per sample reagent vials.

The saltPAD design allows for rapid modifications to meet the specifications of different analytical tasks. The saltPAD's analytical "sweet spot," which is the concentration range of greatest accuracy and precision, is determined by the number of replicate reaction areas provided in different iodine concentration ranges. For surveys whose only question is whether the salt sample is adequately iodized or not, the saltPAD could be redesigned with four sets of triplicate reaction areas that cover the 10-20 ppm range. Four samples could be analyzed on a single card, further reducing the cost per analysis. Alternatively, to accommodate fortification facilities and regulatory authorities, a saltPAD with better accuracy/precision in the 30-50 ppm range (or other ranges as desired) can be designed, validated, and manufactured. The recipe can be found in Figure A.2. These options for modification of the sensitivity of the saltPAD could allow fortification facilities, without an established titration laboratory onsite, to monitor and improve the quality of iodized salt products.

Regardless of the card design, it would be desirable to fully automate the saltPAD reader as a software application that can be used on inexpensive smartphones now becoming available throughout the developing world. The smartphone would read the card's unique QR code, look up the appropriate calibration data, and provide an immediate reading of the iodine concentration in the sample. It would also communicate the result to a central database, add timestamp and location data, and prompt for direct entry of sample metadata. With this information, regulators and health authorities can target micronutrient interventions toward populations most in need.

3.7 Acknowledgments

The Global Alliance for Improved Nutrition (GAIN) funded the internal and external validation studies. Cerebos (Pty) Ltd and Saltcor Royal Co (Pty) Ltd donated salt samples for the study at SAMRC. Kensalt Ltd provided salt samples and access to their fortification facility.

CHAPTER 4:

LAB ON PAPER: ASSAY OF BETA-LACTAM PHARMACEUTICALS BY REDOX BACK-TITRATION

4.1 Overview

Nicholas Myers originated the idea of performing iodometric back-titration on a paper substrate. NM created the physical embodiment of iodometric titration on paper. NM, Jalen Carpenter, Jamie Luther, and Doa'a Aldulaimi validated the device for amoxicillin and ampicillin analysis. DA fabricated the devices for routine analysis. Marya Lieberman and Galen Brown read the test cards to assess operator variability in reading. ML performed infrared spectroscopy on the unknown powder isolated from the bad quality amoxicillin pill. Dr. Allen Oliver performed powder X-ray diffraction on the unknown substance isolated from the bad quality amoxicillin pill.

4.2 Background

Falsified and substandard drugs jeopardize maternal and child health systems, killing more than 120,000 patients in Africa each year, most of whom are children under 5 years old.[59] In Kenya, 26% of the deaths of children living in slums are due to pneumonia.[60] The bacterial form of pneumonia responds well to treatment with inexpensive amoxicillin, which is the standard of care in Kenya for community-acquired

pneumonia in children.[41] The quality of amoxicillin is a matter of life or death. However, it is hard to find data about antibiotic quality in LMICs. Some researchers have recognized the importance of these inexpensive antibiotics and have reported results of post-market surveys in several countries. The prevalence of bad quality amoxicillin and ampicillin containing pharmaceuticals has been measured as 0-100%. The geographic locations, analytes, sample sizes, and rates are listed in Table 4.1. I did a literature survey using the Web of Science.™ I searched for "pharmaceutical quality," and then refined the search using "amoxicillin" or "ampicillin." I found a review published by Kelesidis in 2015 that directed me to all of the studies in Table 4.1 listed with publication date between 1999-2014.[61] I found the other studies listed in Table 4.1 but published after 2014, through the literature survey already described. I only included studies in the table if HPLC was the analysis method. The total number of studies that met all of these criteria was 9. Many of these studies did not use random sampling and many have a small sample size, so prevalence rates of bad pharmaceuticals on the market cannot be ascertained. All but one of the studies followed compendial assay specifications. Pharmacopeias state that beta-lactam pharmaceutical products must contain 90-120% of the labeled amount of the pharmaceutical.[62] Some pharmacopeias give a tighter range. Most studies report only the failure rate without describing the distribution of results and almost never report what is in the bad quality samples.

TABLE 4.1.

PEER-REVIEWED PREVALENCE STUDIES OF AMOXICILLIN AND AMPICILLIN

PHARMACEUTICAL PRODUCTS

Leading author	Publishing year	Location	API	Sample size	Bad quality rate (%)
Fadeyi[63]	2015	Ghana	Amoxicillin	8	0
Fadeyi[63]	2015	Nigeria	Amoxicillin	4	25*
Yong[64]	2015	Cambodia	Amoxicillin	16	13+
Yong[64]	2015	Cambodia	Ampicillin	15	53+#
Yong[64]	2015	Laos	Amoxicillin	6	83+
Yong[64]	2015	Laos	Ampicillin	5	100+
Hetzel[65]	2014	Papua New Guinea	Amoxicillin	47	2
Baratta[66]	2012	Many African countries, Brazil, India	Amoxicillin	24	46
Hadi[67]	2010	Indonesia	Amoxicillin	20	20
Kyriacos[68]	2008	Lebanon, Jordan, Egypt, Saudi Arabia	Amoxicillin	111	56
Kayumba[69]	2004	Rwanda, Tanzania	Amoxicillin	7	0
Taylor[70]	2001	Nigeria	Amoxicillin	37	27
Taylor[70]	2001	Nigeria	Ampicillin	46	61
Wondemagegnehu[71]	1999	Myanmar	Amoxicillin	18	11
Wondemagegnehu[71]	1999	Myanmar	Ampicillin	13	15
Wondemagegnehu[71]	1999	Viet Nam	Amoxicillin	10	30
Wondemagegnehu[71]	1999	Viet Nam	Ampicillin	6	33

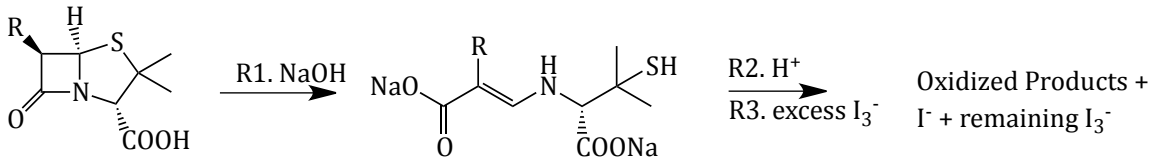
Note: *The one failure had no expiry date listed on the package. +The authors used 85-115% as the “good quality” criteria, which deviates from compendial standards. #In one failing pill, the low ampicillin content had been replaced with amoxicillin, even though amoxicillin was not stated on the package.

Besides the scientific literature, there is non-peer reviewed information available about the prevalence of bad quality pharmaceuticals. The United States Pharmacopeial Convention runs "Promoting the Quality of Medicines (PQM)" in collaboration with countries in Africa, Asia, Europe, and North and South America to create monitoring programs that track the quality of medicines. The results of the monitoring are publicly shared in the "Medicines Quality Database." I searched the database for amoxicillin and ampicillin samples from 2003-2015 that had been analyzed by compendial methods. I grouped the data by year and country, and I only considered the aggregated results that had 20 or more samples. There were data sets from Peru, Cambodia, Viet Nam, and Mozambique that met these conditions. The prevalence rates for substandard amoxicillin and ampicillin pharmaceuticals ranged from 0-13%. I accessed the data on October 6, 2016.

Prevalence studies are expensive to conduct, so they happen infrequently. Samples have to be shipped to a certified lab for compendial testing. Skilled workers spend many hours using sophisticated techniques to assay active pharmaceutical ingredients (API), measure dissolution rates, detect impurities, and perform whatever other tests are listed in a product's monograph. The lab analysis is the most expensive part of this process, and it can cost several hundred dollars per sample. A less expensive way to conduct prevalence studies is to screen pharmaceuticals in the field and send suspicious samples to a lab for further testing. Some technologies that enable fast screening are handheld Raman,[72] infrared,[73] and X-ray fluorescence spectrophotometers.[74] They all have an initial investment of $10,000 USD or more.

Handheld Raman spectrophotometers cannot detect low quality pharmaceuticals.[75] A different recommendation from the World Health Organization for fast quality control checks of pharmaceutical products in locations where no labs are available is to perform packaging analysis.[3] Errors in spellings, seals, and printed features (e.g., quality stamps, color of ink, or misalignments) flag the product as suspicious. The manufacturer, brand name, batch, and expiry date can be checked against a National Regulatory Authority's records of approved products. Some packages are printed with holograms or other security features to discourage counterfeiters from duplicating them.[76] However, the packaging analysis and handheld spectrophotometers reveal nothing about the therapeutic dose the medicine contains, which is a necessary criterion in determining the quality of a medicine. An inexpensive field- and user-friendly technology is needed that can quantify antibiotics in finished pharmaceutical products.

The United States Pharmacopeia contains a relatively low-tech method, <425> "Iodometric assay- antibiotics," that quantifies beta-lactam antibiotics by a back-titration (Figure 4.1).[43] This analysis is currently limited to a lab because titration grade reagents and glassware are needed. To conduct the analysis, a beta-lactam antibiotic is degraded in base for 15 minutes to generate redox active species. (The complete degradation pathways are complex,[77] so only one product is shown in Figure 4.1 to simplify the explanation of the assay.) The solution is then acidified, and an accurately known excess of triiodide is added to oxidize the degradation products. The solution reacts for another 15 minutes, and whatever triiodide remains is titrated with thiosulfate using starch as an endpoint indicator.



R4. I_3^- + $Na_2S_2O_3$ ←→ I^- + $Na_2S_4O_6$

R5. I_3^- + starch ←→ blue complex

Figure 4.1. Back-titration of beta-lactam antibiotics. R1. A user prepares an aqueous solution of the antibiotic and degrades it with base to produce redox active species, reaction time = 15 min. R2. The solution is acidified. R3. A known excess of triiodide is added, reaction time = 15 min. R4. The remaining triiodide is quantified via iodometric titration. R5. The starch indicator will be colored blue if the oxidizing capacity of the triiodide exceeds the reducing capacity of the thiosulfate. If not, the indicator will be colorless.

I developed a test card that performs an iodometric titration (Chapter 2) and thought it could be modified to quantify beta-lactam antibiotics. Accomplishing this goal would add redox back-titration to the chemist's toolbox of techniques employable in field settings. There is an immediate use for a technology that analyzes the quality of beta-lactam antibiotic medicines in the field so that bad products can be reported to regulatory authorities.

We have a collaboration with the pharmacovigilence department at Moi Teaching Hospital in Eldoret, Kenya. A team of pharmacists monitor patient reactions to medications that come into the hospital. The pharmacovigilence unit also has secret shoppers who go into the marketplace of 16 nearby towns to buy medicines from multiple pharmacies. When the secret shoppers are offered a product, they ask for a

cheaper version. In 2012-2013, the secret shoppers collected 465 units of amoxicillin, amoxicillin/clavulanate, or ampicillin medications and mailed them to Notre Dame for analysis and use in validation studies.

4.3 Results and discussion

4.3.1 Initial determination of the quality of Kenyan beta-lactam pharmaceuticals

We performed HPLC analysis on pharmaceutical products collected in Kenya in 2013-2014. 189 units of amoxicillin, amoxicillin/clavulanate combo pills, and ampicillin were tested, and 46 of them failed for API content (24%). See Table 4.2. 32/46 (70%) of the failures occurred because the medications contained 80.0-89.9% of the labeled API.

TABLE 4.2.

INITIAL DETERMINATION OF THE QUALITY OF KENYAN BETA-LACTAM PHARMACEUTICALS

Pharmaceutical	Bad samples[*]	< 80.0%	80.0-89.9%	> 120.0%
Amoxicillin	31/128	3	28[#]	1[#]
Amoxicillin/ Clavulanate	2/54[+] 14/54[+]	1 11	1 3	0 0
Ampicillin	1/7	0	1	0

Note: [*]Using the USP's assay specification that pills must contain 90.0-120.0% of the labeled amount. [#]For one unit, 1 pill tested 80.0-89.9%, a second pill tested as 90.0-120.0, and a third pill tested as > 120.0%; since 2 out of the 3 pills failed, we classified the unit as failing, and both categories are tallied in the table. [+]54 combination pills were analyzed in total and 2 pills failed for both amoxicillin and clavulanate content. 46 out of 189 units (24%) failed.

4.3.2 Design of antibiotic paper analytical device (aPAD)

Considering that our initial determination of pill quality in Kenya revealed that deficient levels of antibiotics are more likely than excessive levels, I decided to engineer the test card to attain the best accuracy and precision at 90.0% API. This can be accomplished by centering the quantification capabilities of the test card around the 90% API regulatory threshold. A yes/no test using 90.0% API as a cutoff would allow only categorical analysis. I designed the test card to quantify in the ~80-110% range to include both a portion of the regulatory specifications but also the most common levels we encountered for bad quality samples. The aPAD was modeled after the saltPAD but with a couple of modifications. The controls were removed from the saltPAD design for this study because the calibration card would reveal fabrication and reagent deposition errors. The controls can be reincorporated into the design of the technology for field validation studies. The iodide detection zone was also removed from the saltPAD to create the aPAD because the chemistry in that zone is not useful for the analysis of beta-lactams. Each column of squares on the test card performs a titration at different concentration ranges, so each test card performs a triplicate measurement. A user picks the standard image that best matches their test card response after performing the "Test card analysis" detailed in section 4.4.9. The remaining features printed onto the test card are a QR code and fiducial marks to enable automated image analysis if a picture of the card is taken with a cell phone. This could help field-testing because metadata and results can be logged automatically. The entire cost of materials for the

aPAD analysis is about $0.18 USD compared to $2.60 USD for HPLC analysis. See Table B.3.

The analysis requires a sample preparation followed by degradation and analysis on the test card. For the internal validation study, an analytical balance and an automatic pipet were used. For field-testing purposes, an analytical balance cannot be used because it requires electricity, and a stable surface to sit on, and it is not portable. There are two ways the pill solution can be prepared without using an analytical balance. The pill's contents can be emptied into a liter bottle and 1 mL of water added per mg of antibiotic stated on the label (i.e., a 500 mg pill diluted with 500 mL water) or a portable milligram balance and an automatic pipet can be used. All of these items are small enough to include in a test kit. A sufficiently accurate and portable milligram balance costs about $20 USD. See section B.5. An automatic pipet costs about $200 USD; if that is too expensive, the milligram balance can be used to measure solutions by mass. The reagents needed to degrade and acidify the sample (1 M NaOH, 0.0050 M triiodide, and 1.2 M HCl) were added at the time of analysis for the internal validation study. They could be stored in glass vials for a field test kit. The back-titration part of the analysis has already been incorporated into a paper platform.[58]

4.3.3 Calibration of the test card

USP <425> is validated for about 12 antibiotics,[43] so the aPAD should be able to analyze all of them. I calibrated and validated the test card using amoxicillin and ampicillin since they are common antibiotics with evidence of quality problems (Figure 4.2 and Figure 4.3).

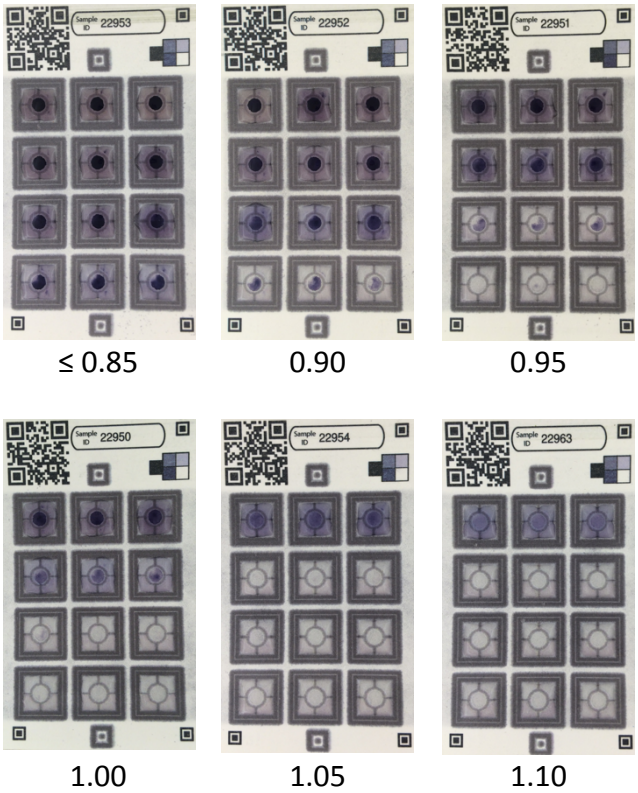


Figure 4.2. Amoxicillin standard images. Units are mg amoxicillin/mL.

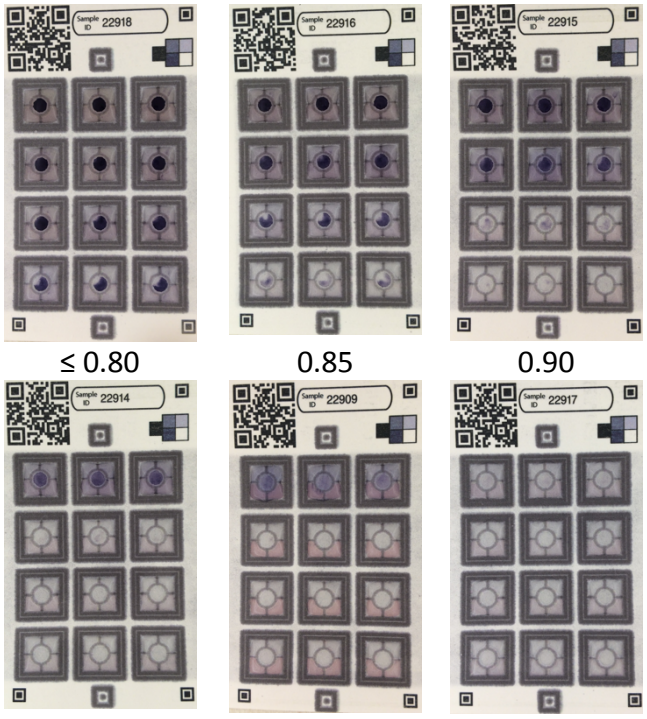


Figure 4.3. Ampicillin standard images. Units are mg ampicillin/mL.

Varying the concentration of antibiotic by just 0.05 mg/mL achieved good visual distinction on the test card over the 0.80-1.10 mg/mL range. The number of blue circles and their intensities vary from one standard image to the next. The responses become saturated at the ends of the range, so the amoxicillin response is interpreted as ≤ 0.85 mg/mL (≤ 85% of labeled dosage amount) or ≥ 1.10 mg/mL (≥ 110% of labeled dosage amount) while the ampicillin response is interpreted as ≤ 0.80 mg/mL (≤ 80% of labeled dosage amount) or ≥ 1.05 mg/mL (≥ 105% of labeled dosage amount).

The card could be calibrated for the other beta-lactam antibiotics listed in USP <425>, but 1.00 mg/mL solutions of antibiotics may produce different responses on the test cards. This is due to the units not being expressed in molarity and possibly due to differences in stoichiometry between triiodide and an antibiotic's degradation products. The stoichiometry of the triiodide and antibiotic reaction at 15 minutes was determined by glassware titration following the USP <425> method. The back-titration revealed that one mole of degraded amoxicillin or ampicillin reacted with about 5.7 moles of triiodide whereas one mole of degraded clavulanic acid reacted with about 0.3 mole of triiodide. See section B.7.

Only monotherapy products can give an accurate response on the test cards; the APIs in combination pills, such as amoxicillin and clavulanic acid capsules, would be jointly assayed. This makes the specificity of the analysis low. If a user must identify the antibiotic (sample is truly blind), Weaver et. al. designed a test card that can do so with greater than 90% specificity.[40] Finding product with API substitution is a relatively rare event compared to finding substandard medicine, so using the test card that quantifies

the API will catch more bad quality products. Of the studies shown in Table 4.1, only in Yong's study were the bad quality samples further analyzed to determine the unknown substance that made up the remainder of the pill's contents. Yong's study found 20 bad samples and only one API substitution was identified (an ampicillin sample contained amoxicillin).

4.3.4 Internal validation

A blind internal validation study was performed to establish the analytical metrics of the test card. See section 4.4.10 for details. An additional 120 units of amoxicillin and ampicillin pharmaceutical samples were collected in Kenya from 2014-2016 and 81 of them were used for the validation study. At the start of the validation study in 2015, there were not enough samples with true values between 0-80% API. The number of poor quality samples were increased by thermally degrading authentic samples in an oven or by diluting them with talc (see section 4.4.7). The test card's predictions were compared against HPLC results, shown in Figure 4.4.

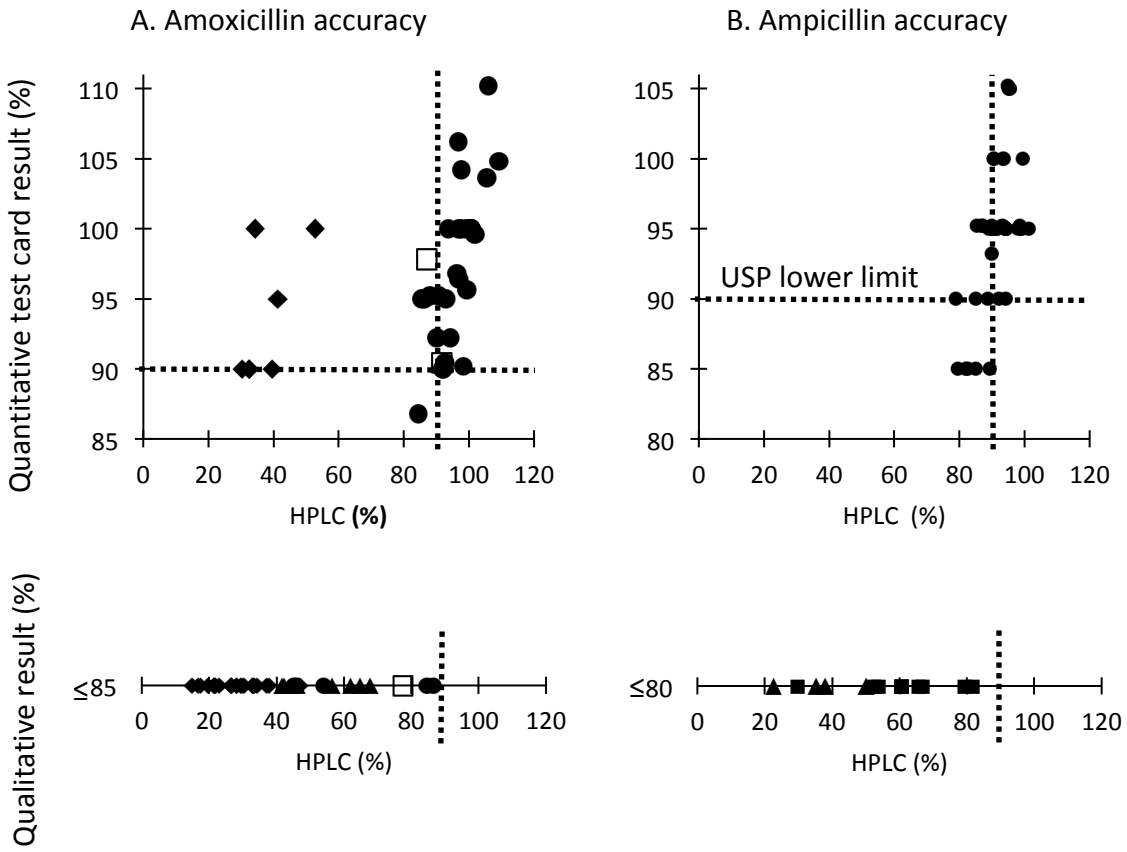


Figure 4.4. Accuracy plot for amoxicillin and ampicillin analysis. The results are expressed as % of the dosage amount stated on the packaging. The card's ability to quantify antibiotics ends at a threshold of 85% for amoxicillin and 80% for ampicillin, so results below these thresholds cannot be distinguished quantitatively. The USP lower limit is 90.0% and is designated by the dotted lines. Samples: (●) = unexpired, (■) = expired, (□) = no expiry date listed, (◆) = purposefully degraded with heat in lab to increase the sample size of the bad quality pills, (▲) = purposefully diluted with talc in lab to increase sample size of bad quality pills. For amoxicillin, n = 80. For ampicillin, n = 56.

4.3.5 Quantitative analysis

The test card can semi-quantitatively assay the amount of antibiotic present in a sample. The readouts are in steps of 5%, from about 80-110% of the amount stated on the product. For the following data analysis, only samples that gave a semi-quantitative readout were considered, including the false positives. The error (average value of absolute errors), system bias (average value of errors), and precision (one sample was analyzed 5 times) are reported in Table 4.3.

TABLE 4.3.

THE TEST CARD'S PERFORMANCE OF QUANTIFYING ANTIBIOTIC SAMPLES

Analyte	Amoxicillin	Ampicillin
Error (%)	13.0 (n=41)	4.7 (n=40)
Bias (%)	11.2 (n=41)	3.2 (n=40)
Inter-device precision (%)	0* (n=5)	2.2# (n=5)

Note: The units are expressed as % of amount stated on the product. HPLC was the reference method. *One sample with a true value of 100.6% was run 5 times and all test cards were interpreted as 100%. #One sample with a true value of 94.2% was run 5 times and the test cards were read as 95%, 95%, 95%, 95%, 90%.

The error for the ampicillin analysis was 4.7%. The error for the amoxicillin analysis is much higher at 13.0%. This high error rate was caused by our attempt to degrade samples for analysis. A subset of the amoxicillin samples underwent a degradation process at 85°C, which is a very unlikely temperature for pharmaceuticals

to encounter in the supply chain, and these samples gave anomalously high readings on the card. The error and bias were recalculated for the amoxicillin analysis ignoring all thermally degraded samples, and the metrics improved to 4.0% for the error and 2.1% for the bias(n=34). Since the false positive rate was high from the thermally stressed samples, a subset of the good quality samples and thermally degraded, bad quality samples were analyzed by the glassware titration method to ascertain if the chemistry or the test card was at fault. The glassware titration had high agreement with HPLC when good quality samples were analyzed (9% error), but this did not hold true for thermally degraded, low quality samples (28% error). Additionally, the glassware titration overestimated every bad quality sample analyzed (n=6) even though a blank titration was performed to account for antibiotic degradation that happened prior to the analysis. See “Agreement of USP method <425> with HPLC” in section B.8. Since the titration chemistry does not quantify thermally degraded, low quality samples well, the test card also fails.

Since reading the test cards by visual analysis is subjective, a study was performed to calculate the variation that arises when different operators read the test cards (Table 4.4). This study was conducted on images that had been collected through November 2015, which is only a subset of all images collected throughout the entire validation study. The true values were blinded and the operators read the test cards independently. The analysts had skill levels that were assumed to be different from one another. Analyst 1 developed the technology, analyst 2 was familiar with it, and analyst 3 had never seen the test card. All analysts achieved similar errors, which were close to

2%. The inter-reading precision among all analysts was 0.6%. The standard deviation of the 3 reads for each test card (n=15) was calculated, and then all 15 standard deviations were averaged to determine the inter-reading precision. All analysts achieved similar errors in reading the test card, so a newly trained user can interpret the test card nearly as well as an expert reader.

TABLE 4.4.

VARIABILITY OF VISUAL TEST CARD INTERPRETATION

Analyst	1	2	3	n
Error (%)	1.7	2.0	2.5	15
Bias (%)	0.4	0.8	1.4	15
Inter-device precision (%)	0	2.2	2.2	5

Note: All samples were amoxicillin.

4.3.6 Categorical analysis

To see if the test card can be used as a tool that rates the medicine as “good” or “bad” quality, the visual reads were grouped using the USP’s 90.0% assay requirement as a limit. A “good quality” response was assigned the positive condition for the metrics in Table 4.5.

TABLE 4.5.

THE TEST CARD'S PERFORMANCE FOR CATEGORIZING THE QUALITY OF ANTIBIOTIC SAMPLES

		Amoxicillin		Ampicilllin	
		HPLC		HPLC	
		≥ 90.0%	< 90.0%	≥ 90.0%	< 90.0%
Test card	≥ 90.0%	39	11	21	7
	< 90.0 %	0	30	0	28
Correctly categorized		86% (69/80)		88% (49/56)	
False positive rate		22% (11/50)		25% (7/28)	
False negative rate		0% (0/30)		0% (0/28)	

For amoxicillin, the test cards and HPLC predicted the same quality status for 86% (69/80) of the samples. All 11 mis-categorized samples were false positives, meaning the samples tested as deficient by HPLC, but the test card predicted the medicine to contain at least 90% of the labeled API amount. Seven of these samples were thermally degraded in lab to increase the study's "bad quality" sample size. The remaining 4 samples had a true value of 85-89%. For ampicillin, 88% (49/56) of samples were correctly categorized, and all 7 mis-categorized samples were false positives. Of the false-positive results, 6 had a true value of 85-89%. There were no false negatives for either amoxicillin or ampicillin, and Cohen's kappa was 0.73 and 0.75 respectively, showing good agreement between the methods.

If these results hold true in a field-setting, at least three-quarters of bad samples would be detected by the test card and then sent to a certified lab for additional testing. During the investigation, the sample's suspected low API content would be confirmed. Despite the high false-positive rate, the card could help to get bad product off the market. Conversely, a high false negative rate would trigger many investigations that conclude the medicine is good quality; this would add an expensive and unnecessary burden to the regulator or health agency, and they may advocate against the test card.

For HPLC method development, acceptable error and precision criteria are commonly set at 2.0%. The test card's error is only about double that and the precision is comparable. While its analytical performance is worse than HPLC, the metrics are more than adequate for a field-screening technology.

There are many instances where HPLC analysis is not feasible, and the test card could be used instead. Regulatory and health agencies can test products at any point in the supply chain. Buyers or sellers can check pharmaceuticals during the exchange of product. Production operators can use the card to analyze pull-samples as product is being made and make a correction to the manufacturing process in near real time instead of waiting for results from the quality control lab. In all of these cases, the test card is helping to protect the quality of life saving medicines.

4.3.7 Identification of bad quality market samples

During the validation study, 3 real amoxicillin samples analyzed as < 85% by the test card and all were confirmed by HPLC analysis to contain approximately 50% API. The stated brand and manufacturer on the packaging of all three samples was Caremox by Shandong Shenglu Co., Ltd (Figure 4.5).

Figure 4.5. Low quality amoxicillin medication labeled as Caremox-500 with Shandong Shenglu Co., Ltd stated as the manufacturer.

The samples prepared for HPLC analysis were very cloudy. One sample was filtered to recover the unknown material and extensively washed with water. It was insoluble in 1 M NaOH, 0.5 M HCl, methanol, acetonitrile, acetone, toluene, 1-propanol, and hexane. By mass, the insoluble materials comprised 45% of the capsule contents. A portion of the insoluble material was analyzed by infrared spectroscopy (IR) in the 4000-500 cm^{-1} range. The material contained no organic functional groups, and featured bands at 1004.7 and 668.6 cm^{-1}. See Figure 4.6. The IR spectrum was consistent with a layered magnesium silicate mineral, such as talc, and a reference spectrum is shown in Figure 4.7.

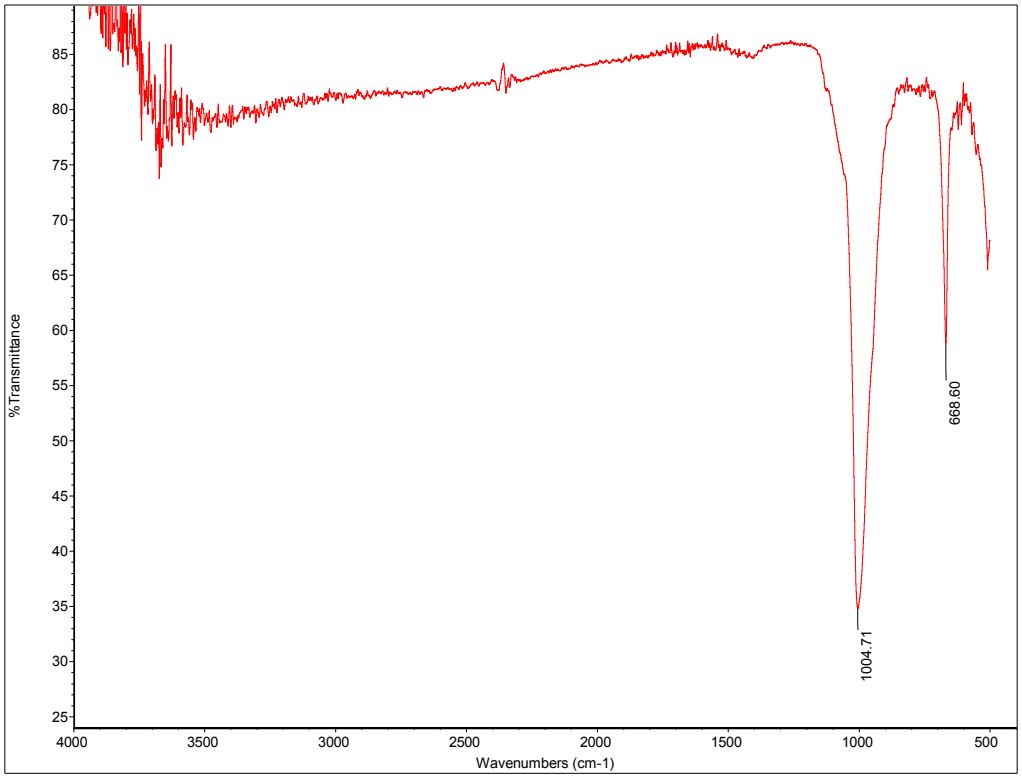


Figure 4.6. ATR-IR spectrum of insoluble material isolated from a bad quality amoxicillin capsule.

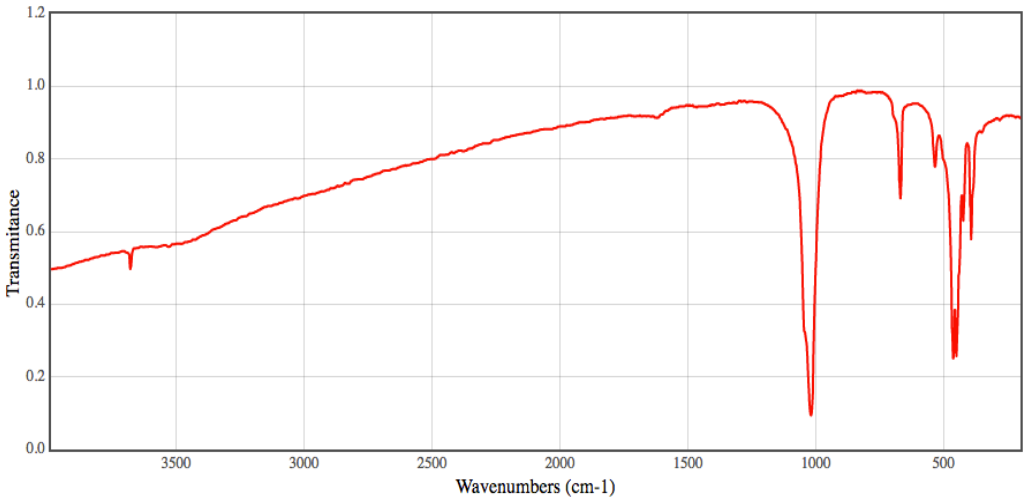


Figure 4.7. Reference spectrum of talc from the National Institute of Standards and Technology.

In order to confirm the identity of the magnesium silicate mineral, a powder x-ray diffraction (PXRD) pattern was recorded on a representative specimen taken from the bulk unknown powder (Figure 4.8). Computer fitting of the diffraction peaks to common talc phases showed the material to be about 86% triclinic talc and 14% monoclinic talc. Other common insoluble minerals (SiO_2, TiO_2 or $CaCO_3$) did not match the observed diffraction peaks.

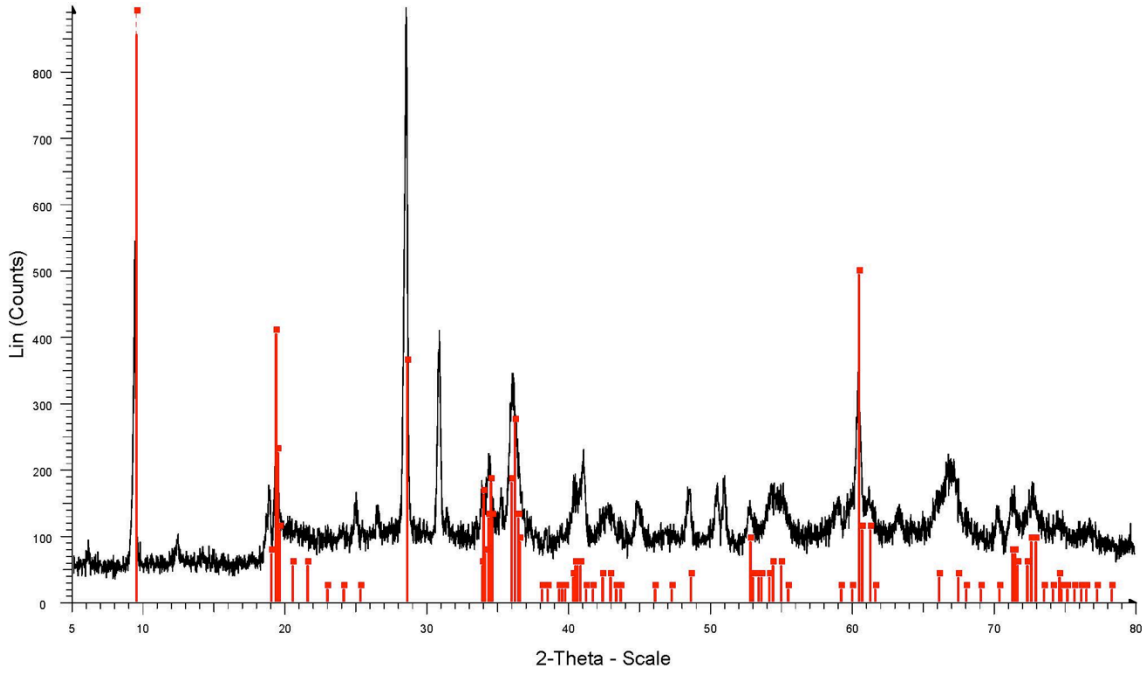


Figure 4.8. Powder X-ray diffraction of insoluble material isolated from bad quality amoxicillin capsule. The black trace of the diffraction pattern is overlaid by monoclinic and triclinic talc reference spectra represented by red sticks.

The IR and PXRD confirm the insoluble material isolated from the capsule is talc. The supposed Caremox product is not only terribly deficient in the amount of amoxicillin it claims to contain, but also the levels of talc are so high that the correct amount of amoxicillin never could have been placed into the pill. This pharmaceutical product was purposefully adulterated. We filed a report with the Kenyan Pharmacy and Poisons Board (KPPB), and included all of our investigatory data and results. We also included the results for the low quality amoxicillin/clavulanate pills reported in Table 4.2 KPPB is currently investigating the situation.

4.4 Experimental

4.4.1 Materials

Starch (J.T. Baker), p-toluenesulfonic acid (Alfa Aesar), potassium iodide (Amresco), cadmium chloride (Acros), anhydrous sodium thiosulfate (J.T. Baker), secondary standard of potassium iodate, 100.2% (J.T. Baker), primary standard of amoxicillin (USP), secondary standard of amoxicillin (Sigma Aldrich), primary standard of ampicillin (USP), secondary standard of ampicillin (Sigma Aldrich), standardized 0.005 M iodine solution (Alfa Aesar), hydrochloric acid (Fisher), sodium hydroxide (Fisher), Ahlstrom 319 (paper source, Midland Scientific), ColorQube 8570N (wax and printer, Xerox)

4.4.2 Fabrication of test card

The test card was created by printing wax onto Ahlstrom 319 paper. The printing pattern is provided as a supporting material file in the saltPAD publication.[58] The cards were baked at 100°C for 14 minutes. The seal was tested on a small number of the cards by placing water into a zone, waiting 30 seconds, and seeing if water leaked into an adjacent zone. If it did, then the card was baked for 3 more minutes, retested, and repeated until the seal was good. The reagents were deposited by hand into the locations specified in section B.1.

4.4.3 Pharmaceutical sample collection

Secret shoppers purchased pharmaceutical samples in western Kenya, and the samples were shipped to the University of Notre Dame for analysis. At the time of purchase, secret shoppers would ask the clerk if a cheaper version of the product was available. The samples were stored in a 4°C refrigerator.

4.4.4 Stock solution preparation for test card and HPLC analysis.

A nominal 2.0 mg/mL stock of amoxicillin or ampicillin was prepared in deionized water. An aliquot of the stock was used to create a 1.00 mg/mL solution for the test card analysis. A separate aliquot was taken from the stock to create a 0.5 mg/mL solution for HPLC analysis. This was done so pill heterogeneity could not cause different measurements between the methods.

4.4.5 Standard solution preparation for test card analysis

Standard solutions were prepared using deionized water and primary or secondary grade reference materials traceable to USP standards. The concentration of amoxicillin or ampicillin in the solutions varied from 0.80 to 1.10 mg/mL in 0.05 mg/mL increments.

4.4.6 Purposefully degraded amoxicillin samples

The number of bad quality units collected in Kenya was too low to perform a validation study, so they had to be mocked-up in lab. The pill contents were poured into a plastic baggie and left open inside of a 55°C oven. The antibiotic content of 2-3 pills was assayed periodically by HPLC analysis to see if the powder had degraded. After three weeks, there was no substantial degradation. To speed it up, two drops of water were added to each baggie, and the oven temperature was increased to 85°C. After 2 days, all samples contained 10-60% of the labeled amount of amoxicillin and were then stored in a 4°C refrigerator. The samples were prepared as noted in section 4.4.9 and tested by an analyst different than the one who performed the HPLC analysis.

4.4.7 Mocked-up amoxicillin and ampicillin samples

The number of bad quality pills collected in Kenya was too low to perform a validation study, so they had to be mocked-up in lab. Accurately massed portions of pharmaceutical samples and talc were placed into a glass vial and mixed thoroughly using a vortexer. The adulterated samples were analyzed on the test cards, and the analyst who did the adulteration did not perform the test card analysis.

4.4.8 HPLC analysis

For each of the HPLC sample solutions, a nominal 0.5 mg/mL amoxicillin or ampicillin solution was prepared from a 2.0 mg/mL stock. See section B.2 for the HPLC methodology.

4.4.9 Test card analysis

For capsules, the powder contents were massed by difference using an airstream to blow out the capsules until no residual powder could be seen. Tablets were ground to a fine powder using a mortar and pestle. An accurately known portion of the medicine was taken to create a 2.0 mg/mL stock solution, which was then diluted to nominal 1.00 mg/mL in deionized water for analysis on the test card. The leftover powder was refrigerated in a sealable plastic baggie. See section B.3 for an example of how to create a nominal 1.00 mg/mL solution. The following steps were performed in a capped scintillation vial, but any glass container with a lid should work. To 4.0 mL of the nominal 1.0 mg/mL sample solution, 2.0 mL of 1.0 M NaOH was added and allowed to react for 15 minutes. Then 2.0 mL of 1.2 M HCl and 10.00 mL of 0.0050 M triiodide was added and allowed to react for an additional 15 minutes. 125 µL of the test solution was pipetted onto each of the twelve squares of the test card. Using the pipet tip, the solution meniscus was drawn across all 5 subsections of the square to cover it completely. The card was left on a flat surface and gently moved back and forth about 1 cm at a rate of 2 Hz for 3 minutes. Then, a picture of the paper test card was taken in a lightbox and the image was read by visual comparison to standard images. The sample

preparation allows the units to be converted directly from “mg/mL” to “% of labeled amount” (e.g., if a sample matches the 0.90 mg/mL standard image, the sample contains 90% of the labeled antibiotic amount). See section B.4 for how the conversion works.

4.4.10 Internal validation

Analyst 1 prepared the stock samples for analysis on both HPLC and the test card. Analyst 1 performed only the HPLC analysis and kept the results a secret from Analyst 2. Analyst 2 performed the test card analysis and reported the results to Analyst 3, after which time Analyst 1 unblinded the results.

Solutions of 1.0 M NaOH and 1.2 M HCl were stored in polyethylene bottles, and 0.0050 M triiodide was stored in glass vials with Teflon® caps. Aliquots were transferred to another glass vial to perform the degradation and acidification. The titration takes place on the test card. Step-by-step instructions are in section 4.4.9.

4.4.11 Powder X-ray diffraction of insoluble material isolated from amoxicillin capsule

Data were recorded as a series of 360 degree phi rotation photos at 250 K using monochromated Cu radiation with an APEX-II area detector operating in 1Kx1K mode at 15 cm specimen to detector distance, yielding an effective scan resolution of 0.01 deg. Data to 30 degree in 2-theta were recorded for 60 seconds per rotation frame and from 30 to 80 degree at 120 seconds per frame. The images were composited and intensities integrated from the composite image using the APEX-3 suite of software.

4.4.12 IR of insoluble material isolated from amoxicillin capsule

IR was obtained by spreading the sample on an ATR plate. 16 scans at 4 cm^{-1} resolution were acquired and the air background was subtracted. The baseline was corrected using a linear fit; the data are unsmoothed.

4.5 Conclusion

A paper test card that performs an iodometric titration successfully analyzed finished pharmaceutical products of beta-lactam antibiotics. Over a range of about 80-110% of the labeled dosage amount, the card had less than 5% error and 2% precision when analyzing amoxicillin and ampicillin products. If the product was thermally degraded, the error increased to 13%. The test card's performance for categorizing a product as good or bad quality was about 86%; all mis-categorizations were false positives. The material cost to analyze one sample is $0.18 USD, which is at least 10 times cheaper than HPLC analysis.

Iodometry is nonspecific, so the test card cannot differentiate beta-lactams from one another. Another test card reported in the literature can identify antibiotics,[40] and if used with the aPAD, could generate a testing system with high specificity and sensitivity. With further usability and implementation studies, the paper test card could become a method for assaying beta-lactam pharmaceuticals in the field, helping regulatory authorities get bad product out of the pharmaceutical supply chain. During the validation of the aPAD, bad quality amoxicillin capsules, supposedly manufactured by Shandong Shenglu Co., Ltd. were detected by the test card. The falsified medication

contained 45% talc, so the correct amount of amoxicillin was never placed into the pill. A report was filed with the Kenyan Pharmacy and Poisons Board, who launched an investigation. This demonstrates the test card's ability to detect a truly falsified medication collected in the marketplace of an LMIC, and after confirmatory testing, trigger a regulatory investigation.

4.6 Acknowledgments

The United States Pharmacopeial Convention supported me through a fellowship. Thank you to Maura Kibbey, my mentor from USP, and to Ahalya Wise and Lucas Roth. Thank you to the secret shoppers for purchasing pharmaceutical samples.

CHAPTER 5:

GREEN DESIGN OF A PAPER TEST CARD FOR URINARY IODIDE ANALYSIS

5.1 Overview

Content in this chapter is currently undergoing peer review for publication in *PLoS One*. Myers, N. M.; Leung, I. C.; McGee, S. W.; Eggleson, K.; Lieberman, M. ML originated the idea of performing the assay on paper, and NM and IL formulated and validated the test card. ML, KE, NM, SM, and Ashley Berding contributed to the conception of the remediation module, and NM, AB, and SM developed the final design and validated it. AB collected SEM images shown in Appendix C.

5.2 Introduction

Millions of children are at risk for cognitive impairment that can be prevented with iodized table salt at a cost of $0.05 per person per year.[78–81] Universal salt iodization programs must make sure that the iodine is consumed by the people who need it. This is done by measuring the iodide levels in urine samples collected from a representative subset of a population.[82] The technical difficulty of analyzing trace amounts of iodide in urine makes it difficult for low and middle-income countries (LMICs) to conduct surveys frequently and hinders the monitoring and evaluation of

iodine supplementation programs. The World Health Organization deems a population iodine-deficient if the median urinary iodide value obtained during a survey is less than 100 parts per billion iodine (ppb I), adequate if it is 100-299 ppb I, and excessive if it is greater than or equal to 300 ppb I.[32] Only about 100 labs worldwide are registered with the Centers for Disease Control and Prevention's Ensuring the Quality of Urinary Iodine Procedures (EQUIP) program, which provides standardized urine samples to check that a lab's in-house method is producing accurate results for iodine concentration.[83,84,37] Approximately 80% of countries in Africa or South America do not have a participating lab.[83] Population surveys are performed about every 5-10 years within many countries,[38] so a whole generation of children may be affected by iodine deficiency before a problem is detected.

We designed a paper test card to accurately measure physiologically relevant iodide levels in simulated urine. The card gives a colorimetric readout that a person can interpret by eye to obtain categorization of urine samples or can image with a cell phone camera for quantification by computer image analysis. The colorimetric reaction relies on the Sandell-Kolthoff (SK) reaction, a kinetically slow reaction between Ce^{4+} and As^{3+} that is catalyzed by iodide (Figure 5.1).[39] The fading of the yellow color from Ce^{4+} is difficult to monitor by eye, particularly in urine samples that are strongly colored, but ferroin can be used as a redox indicator to enable visual readout. Previous laboratory methods make use of this indicator to allow visual analysis or digital readout by a plate-reader.[85–87] Only one field-friendly technique for urinary iodide assay is reported in the

literature, but the specificity obtained during field validation was 61%.[88] In this assay, iodide catalyzes the oxidation of 3,3',5,5'-tetramethylbenzidine by peroxide.[88]

$$4\ Ce^{4+} + 4\ I^- \rightarrow 2\ I_2 + 4\ Ce^{3+} \quad (1)$$

$$5\ H_2O + 2\ I_2 + As_2O_3 \rightarrow 10\ H^+ + 4\ I^- + 2\ AsO_4^{3-} \quad (2)$$

$$Ferroin^{2+}\ (red) + Ce^{4+} \rightarrow Ferriin^{3+}\ (blue) + Ce^{3+} \quad (3)$$

$$4\ Ferriin^{3+}\ (blue) + As_2O_3 + 5\ H_2O \rightarrow 4\ Ferroin^{2+}\ (red) + 2\ AsO_4^{3-} + 10\ H^+ \quad (4)$$

Figure 5.1. Reaction scheme for uiPAD. (1) Ce^{4+} oxidizes the iodide in urine to iodine. (2) Iodine is reduced back to iodide by arsenite. The iodide-catalyzed reaction between Ce^{4+} and As^{3+} is known as the Sandell-Kolthoff reaction.[39] (3) The reaction is tracked visually using ferroin, $[Fe(o\text{-phen})_3]^{2+}$. The solution is blue while Ce^{4+} is present. (4) Excess arsenite regenerates ferroin. The solution turns red and signals the completion of the SK reaction.

Since the Sandell-Kolthoff method requires arsenic, disposing of its waste is problematic in the low resource settings where the card would likely be used. A likely fate of the cards would be a landfill with no barriers or monitoring. To responsibly develop this technology, we set a goal to reduce the amount of leachable arsenic below levels set by regulatory standards. The Environmental Protection Agency's toxicity characteristic leaching procedure (TCLP) for solid waste defines an acceptable limit to be less than 5 parts per million arsenic in a volume of leachate determined by a function of the waste material's weight.[89] Even though this is a regulatory requirement for the United States, it is a useful guideline in the development of this technology for

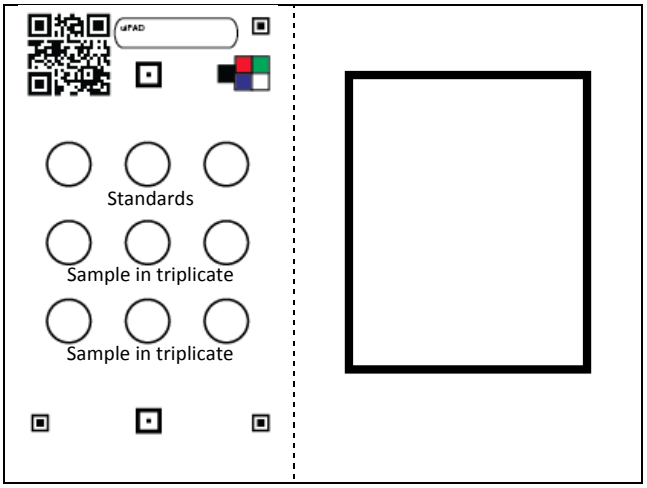
application in LMICs, where environmental regulations may not address arsenic waste. The goal of this upstream intervention in the product design is to reduce the harm at the end of the product lifecycle without compromising its performance.[90] Devising a way to perform the Sandell-Kolthoff kinetic assay with a built in remediation of its toxic waste will expand the toolbox of techniques chemists can use in low resource areas.

5.3 Results and discussion

5.3.1 Test card design

The test card is divided into an assay module and a remediation module (Figure 5.2). The assay module has nine analysis areas which are defined by wax;[7] three are used for standards and six are used for samples. At the time of analysis, the user prepares the test card by pipetting onto each circle solutions that are provided in a test kit. The small volumes of solution give a 25 fold reduction in the amount of arsenic needed to perform the analysis when compared to the gold standard UV-vis method (Table C.1).[91] The defined reaction areas confine the assay solutions and produce superior reproducibility in color production when compared to borderless reaction areas.[46] When the user pipets the last solution, which contains arsenite, the SK reaction is initiated. The final reagent can be loaded into all 9 circles in 30 seconds using a single channel automatic pipette, or a multi-channel pipet can be used to initiate the nine reactions in 10 seconds. After 3 minutes, the 0, 100, and 300 ppb standards attain distinctive colors. See Figure 5.3 and Figure 5.4. The results from the triplicate measurement of the sample can be interpreted visually or the card can be

photographed to record the data for later analysis. The final step is to render the test card nonhazardous with the aid of the remediation module. The user does this by adding goethite to the remediation module, which is preloaded with Oxone®, an oxidant. The remediation module is then pressed face-to-face with the assay module, and they are folded together. The arsenic becomes oxidized and binds tightly to the goethite, immobilizing the arsenic species and preventing it from leaching into groundwater. At this point the PADs can be placed into the trash. The cost of the materials for the assay and remediation modules is about $0.40 USD. See Table C.2 and Table C.3 for a cost analysis. The card can test two samples in triplicate or six samples one time.



A. Assay module B. Remediation module

Figure 5.2. Layout of the test card. A. Assay module. The 0, 100, and 300 ppb internal iodide standards in the top row are used to determine the concentrations of the samples in the bottom rows. Fiducial marks and color standards are printed on the card to facilitate automated image analysis. B. Remediation module. The rectangular area is loaded with reagents to bind arsenic so it cannot leach into groundwater. After the assay is complete, the remediation module is folded on top of the assay module to render the test card non-hazardous.

The formulation of the device was optimized to produce a readable output in a short period of time that could be analyzed by a computer image analysis program. Several colored species (Ce^{4+}, ferriin, and ferroin) are present during the course of the reaction, so different ways for measuring the reaction progress were evaluated. For image analysis, single channel measurements (Figure C.1 and Figure C.2) were not as effective as the difference between the red and blue channels (Figure 5.3). This metric gives the best distinction amongst solutions that contain the most important iodide levels of 0, 100, and 300 ppb I at a reaction time of 3-5 minutes. The exact time doesn't matter for categorization and should give some leeway for temperature variations, solution stability, and minor pipetting errors.

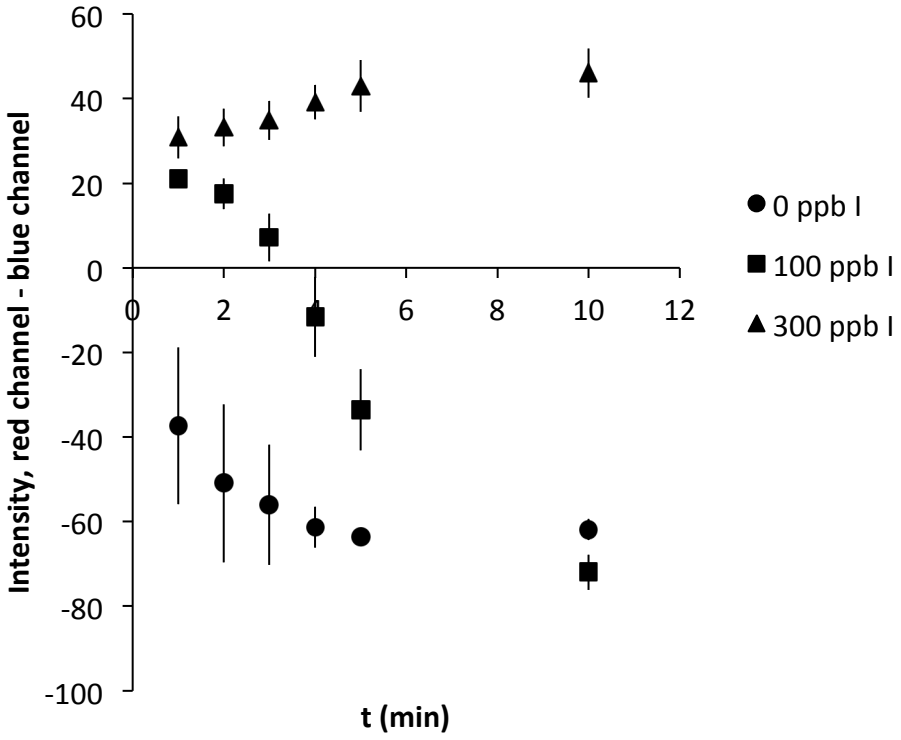
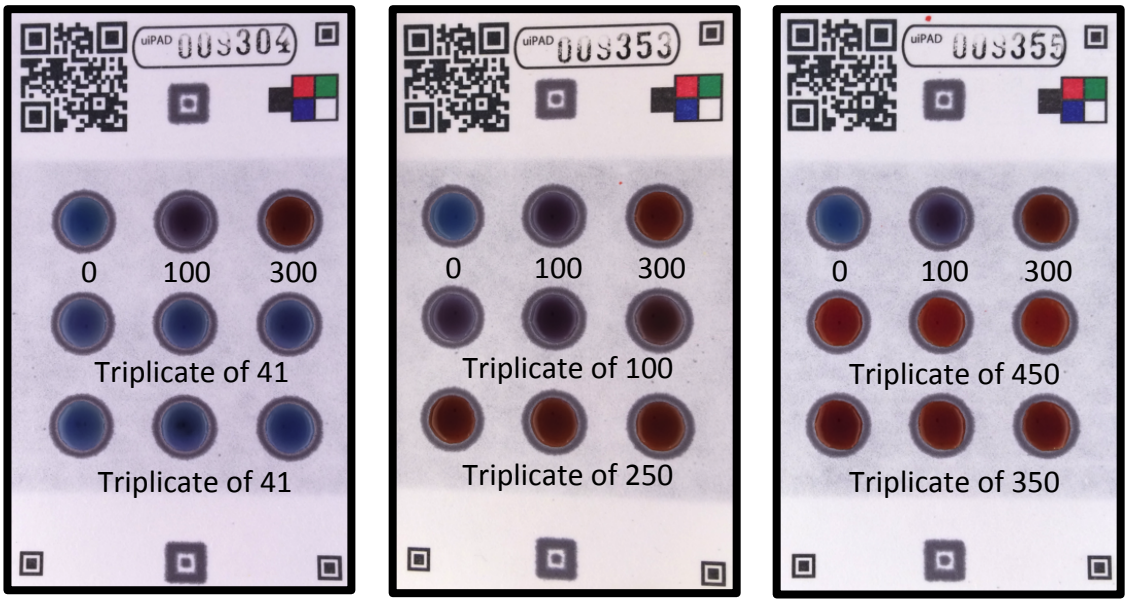


Figure 5.3. Progression of the Sandell-Kolthoff reaction on paper. The test card's internal standards change color over time and are distinguishable by ImageJ analysis of cell phone pictures taken 1-10 minutes after the start of the reaction. The best distinction is achieved 3-5 minutes into the reaction. Error bars show the SD of 3 test cards.

The 100 ppb I test solution exhibited an unexpected trend in color development, becoming bluer over time instead of redder. Ceric arsenate precipitates during the preparation of the test card because the solution is not sufficiently acidic (i.e., a basic arsenic solution is pipetted into an acidic cerium solution).[82] The original pH of the test solutions keeps the test reagents soluble for deposition. The ceric arsenate dissolves over time. During mixing, the ceric ion dissolves faster than the SK reaction can consume it, the solution stays blue. For the solution containing 300 ppb I standard, the ceric ion is consumed quickly, and the excess As(III) reduces ferriin to ferroin, giving the solution a red color.

5.3.2 Validation of the assay module

The analytical metrics of the assay module were determined by a blind internal study using 30 different solutions of artificial urine containing physiologically relevant concentrations of iodide, urea, chloride, sodium, and potassium.[92] The preparer coded the solutions and gave them to two different researchers to analyze on the test cards. The researchers ran the solutions independently and took pictures of the test cards in a lightbox using an iPhone 5s (see “How to make a lightbox” in section A.6). They read the device by visually comparing the color of the samples to that of the standards after 3 minutes of reaction time (Figure 5.4).



Deficient samples Adequate samples Excessive samples

Figure 5.4. The test card response to various levels of iodide at 3 minutes. The blank standard appears blue while the 100 ppb I standard looks purple, and the 300 ppb I standard is red. Each unknown was applied to three circles in a row; the ppb I concentration in the sample solution is shown below the row. The samples are visually categorized to contain < 100, 100-299, or ≥ 300 ppb I.

Pooling all reads together, 56 out of the 60 test card reads (93%) were correctly categorized as deficient, adequate, or excessive in iodide content (Table 5.1). All 20 deficient samples were identified correctly. Two of the mis-categorizations were borderline errors (i.e., a 300 ppb I sample was categorized as adequate rather than excessive, and a 110 ppb I sample was categorized as deficient rather than adequate). This data gives a weighted Cohen's kappa value, K, of 0.926, meaning there is good categorization of the data even when chance agreement is accounted for.[93] The accuracy of visual categorization means that the cards could be used in field settings where electrical power and internet connectivity are not reliable.

Computer image analysis has several potential advantages over visual analysis, including greater objectivity, less dependence on the visual acuity of the operator, and greater ability to archive and share raw data and test results. Pictures of the cards were acquired in a home-built lightbox and analyzed in ImageJ.[54] The internal standards on each test card were used to create a calibration curve against which the samples were compared. A quantitative readout was generated and each sample was categorized as deficient, adequate, or excessive. 53 out of 60 (88%) categorical predictions were correct. See Table 5.1. The weighted K value was 0.825, which again shows good categorization for the technique. Nearly all mis-classifications happened when the true value was near a cutoff boundary (Figure 5.5). The visual categorization out-performed the computerized categorization, but both yielded good results. This suggests that there are additional color parameters that could be taken into account by the computerized analysis to bring its performance level up to that of the human eye.

TABLE 5.1.

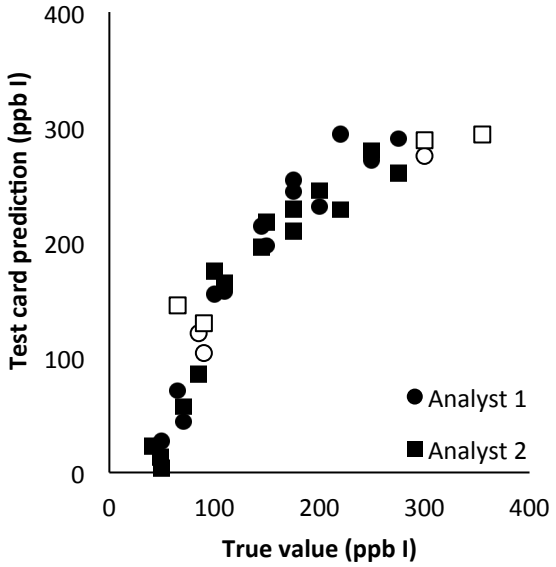
VISUAL AND COMPUTER IMAGE ANALYSIS OF THE TEST CARDS

	Visual analysis, ppb I			*Computer image analysis, ppb I*		
True values, ppb I	< 100	100-299	≥ 300	< 100	100-299	≥ 300
< 100	20	0	0	16	4	0
100-299	1	17	2	0	20	0
≥ 300	0	1	19	0	3	17
Cohen's kappa, K	0.926			0.825		

Note: For the visual analysis, 56 out of 60 samples (93%) were properly categorized, and all iodide deficient samples were recognized. For the computer image analysis, 53 out of 60 samples (88%) were properly categorized. The weighted Cohen's kappa values show both analysis methods categorize samples correctly.

The quantitative readouts produced by ImageJ analysis can be assessed directly instead of forcing them into a qualitative response. Only the 35 card responses that fell into the calibration range of 0 to 300 ppb were used in the following metrics. 17 excessive results were disregarded as well as 8 results with negative values (the negative values were within the error of the y-intercept). The accuracy (average of absolute errors), system bias (average of errors), inter-device precision (standard deviation of a 150 ppb I standard, n=5), and inter-operator precision (the average difference in results obtained by two analysts when using the same solution, n=16) were calculated (Figure 5.5). Both analysts performed the same on accuracy (40 ppb) and inter-device precision (20 ppb I), and both over-estimated the iodide content of the test

solution (on average, Analyst 1 was 31 ppb I high and Analyst 2 was 18 ppb I high). Inter-operator precision was 27 ppb I on average when each operator analyzed the same solution (Figure C.3). The test cards tend to under-estimate solutions containing less than 100 ppb I, but over-estimate solutions containing 100-300 ppb I (see Figure C.4 for a residual plot). The sensitivity of the assay is highest in the 50-300 ppb iodide range, which spans both threshold values for the WHO's "adequate iodine" category. Above and below these concentrations there is little variation in the color of the indicator at t=3-5 min, so quantification becomes unreliable. Capturing more images over a longer period of time could increase the dynamic range and could be helpful if the WHO ever changes the classification system.



	Analyst	
	1	2
Accuracy (n= 16, 19)	40 ppb	39 ppb
System bias (n=16, 19)	31 ppb	18 ppb
Precision:		
Inter-device (n=5)	20 ppb	17 ppb
Inter-operator (n=16)	27 ppb	

Figure 5.5. Accuracy plot for ImageJ analysis of the test card. Mis-categorized results are shown as empty circles or squares, correctly categorized results as filled circles or squares. All metrics for both users are expressed as ppb I.

The next step will be development of a more field-friendly urine pre-treatment procedure. Urine is a complex matrix with more than 60 chemical species exceeding 10 ppm;[92] some of these species are known to interfere with the SK reaction.[94] When the SK reaction is performed in a lab setting the urine is boiled with strong acids and oxidizers to remove interferences.[91] We are trying to avoid this procedure for field use, so a field-friendly urine pretreatment needs to be investigated. Filtering the urine through activated carbon has been reported to remove interferences from urine.[95] Madeline Smith, a fellow graduate student I am mentoring who will take over the project once I graduate, soaked urine samples over activated carbon for 1 day. Before the soak, 2 out of 3 samples were categorized correctly by visual analysis, but after the soak, all 3 were correct. If this method does not pan out for a larger number of samples, the matrix effects might be compensated for by using the method of standard additions, the feasibility of which has already been demonstrated on a paper substrate.[18]

5.3.3 Development of the arsenic remediation module

The amount of arsenic needed for the paper implementation of the SK assay was reduced by 25-fold when compared to the gold standard UV-vis method (Table C.1) due to the tiny volumes of solutions needed. However, the amount of arsenic on the card still poses a health risk if it can leach into the environment after disposal. A toxicity characterization leaching procedure (TCLP) carried out on the assay module of the card showed levels of 28.8 ± 3.1 ppm As (n=3) in the leachate, which categorizes the assay module as hazardous waste according to EPA regulations. It is well known that arsenic

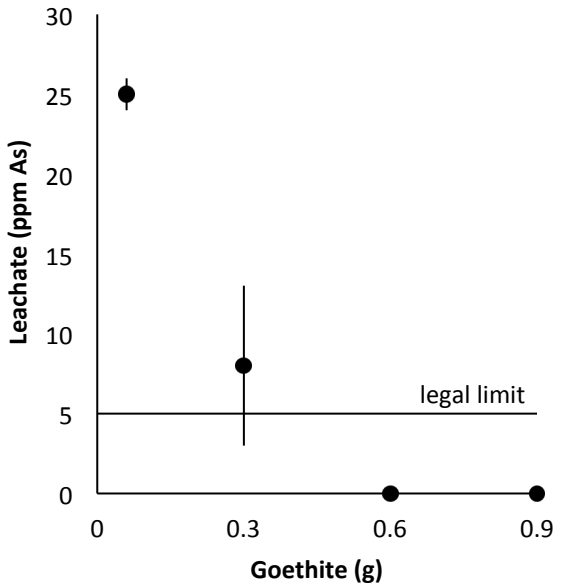
can be put into a non-leachable form by binding it to iron oxide.[96] Arsenic and iron oxides have a high affinity for each other, but the binding works best when the pH is slightly acidic, the arsenic is fully oxidized, and the iron oxide is in a specific mineral phase called goethite.[97] The pH condition was already met by the assay solutions, so we added an oxidizing agent to the remediation module to convert leftover arsenite to arsenate and to combat reducing conditions sometimes found in landfills. To accomplish this, 60 mg of potassium peroxymonosulphate (Oxone®) is stored in the paper. After the assay module is used, goethite is sprinkled over the paper, and the test card is folded over to bring all the remediation reagents into contact with the assay solutions. Iron oxides can be stored in paper fibers (Figure C.5), which allows for a user-friendly system of folding over one module onto the other, but more development work is needed to fit 0.9 g of goethite (Figure 5.6) in the test card area. The cost of the materials to make the remediation module is about $0.20 USD (Table C.3).

5.3.4 Performance of the arsenic remediation module

TCLP measurements were made according to EPA SW-846 Test Method 1311 with arsenic analysis by ICP-OES. The theoretical starting arsenic level in the leachate was 28.82 ppm, and when cards were subjected to the TCLP without any remediation efforts, 28.8 ± 3.1 ppm As (n=3) was measured. This indicates that all of the arsenic readily leaches out of the PAD.

Of 20 test cards subjected to the remediation procedure (Figure 5.6), 16 had undetectable levels of arsenic in the leachate, so the remediation level was estimated

using the method LOD of 0.028 ppm As. More than 99.9% of the arsenic was removed from these samples. See “Arsenic remediation” in section 5.4.7. The average arsenic leached from the remaining four cards was 0.7 ± 0.7 ppm. This corresponds to 97.6% of the arsenic being absorbed. All 20 samples met the regulatory requirement of producing leachate with arsenic levels below 5 ppm, so when the assay module is used with the remediation module, the combined waste is nonhazardous waste by EPA standards.



Distribution for 0.9 g goethite (n=20)			
[As] (ppm)	< LOD	LOD to 5	≥ 5
Occurrences	16	4	0

Figure 5.6. TCLP results. At least 0.6 g goethite must be used to reliably mitigate the leachate to acceptable arsenic levels. For the 0.9 g level, n=20 and for all others n=3. LOD = 0.028 ppm As. All error bars are shown, but some are too small to see on this scale.

5.4 Experimental

5.4.1 Chemicals

Urea (J.T. Baker); sodium chloride (Macron); potassium chloride (Fisher); potassium iodide (Amresco); arsenic trioxide (Alfa Aesar); ceric ammonium nitrate (Alfa Aesar); 1,10-phenanthroline (Amresco); iron sulfate (J.T. Baker); sulfuric acid, trace metal grade (Fisher); sodium hydroxide (BDH); 1000 ± 2 ppm iodide standard, ICP grade (Fluka lot BCBP1989V); goethite, characterized by XRD (Aldrich); trace-metal grade nitric acid (BDH); glacial acetic acid (Fisher); Oxone® (Alfa Aesar); test solutions were diluted with deionized water; 18 M-Ohm water was used for ICP-OES work.

5.4.2 Precautions to prevent iodide contamination

All glassware, reagents, and paper were handled with care to prevent iodide contamination. This included wearing disposable gloves and working in a hood lined with freshly laid out absorbent towels. Glassware was washed with 5% v/v nitric acid.

5.4.3 Simulated urine recipe

Water was spiked with 15,000 ppm urea, 3800 ppm chloride, 1800 ppm sodium, and 1200 ppm potassium to mimic urine. These values are within physiological ranges.[92] This diluent was used to create standards for the validation study.

5.4.4 Fabrication of test card

The test card is designed using Adobe Illustrator with art boards that are 8.5” x 11”, which accommodates printing onto Ahlstrom 319 paper with commercially

available printers. One layer of the art board is used to create the fiducial marks, QR code, color standard, lettering, and serialization zone; all are printed with a laser printer. Another layer of the art board is used to create the reaction zone circles, remediation zone, and backside barrier; all are printed with a wax printer (Xerox ColorQube 8570N). Specific dimensions can be found in the Adobe Illustrator file accompanying the publication. The cards are baked at 100°C for 14 minutes and a subset are tested with water to ensure the barriers are sealed; if the barriers leak, the cards are baked for 3 additional minutes.

5.4.5 Running and analyzing the test card

Solutions are pipetted onto the test card in the following order: 1) In the top row, add to individual circles 50.0µL of 0, 100, and 300 ppb iodide standards (in a synthetic urine matrix); 2) In the second row, add 50.0 µL of sample A to each circle; 3) In the third row, add 50.0 µL of sample B to each circle; 4) Add to every circle 2.0 µL of 0.12 M ferroin; 5) Add to every circle 4.0 µL of 0.4 M ceric ammonium nitrate in a 0.5 M sulfuric acid solution; and 6) Add to every circle 10.0 µL of 0.2 M As_2O_3 in a 0.15 M NaOH solution. Leaving the card lie on a flat surface, move it back and forth about 1 cm at a rate of 2 Hz to facilitate mixing. The shaking requires some practice because if it is too vigorous the solution will spill out of the reaction zones; the user must start over in this case. The reaction area can hold about 75 µL of solution before it escapes due to the shaking motion. After 3 minutes of shaking, a picture of the card was taken in a lightbox with an iPhone 5s. The lightbox was equipped with 2 plug-in strips of white LED lights

that each had an output of 162 lumens and rated 82 on the color-rendering index. The images were analyzed visually by comparing the samples to the internal standards and picking the appropriate category. The images were also analyzed in ImageJ.[54] The images were split into red, green, and blue channels. The average un-weighted gray value over the entire reaction area was measured for each channel. The blue channel was subtracted from the red channel, then for each test card a linear calibration curve was generated from the responses of the standards; the concentration of the samples were determined against the calibration curve.

5.4.6 Blind internal validation

A researcher volumetrically diluted ICP-grade iodide standard to 30 different levels using synthetic urine as the diluent. The samples were coded and given to two analysts who ran them on the test cards independently on different days. The analysts interpreted the test cards by eye and with ImageJ,[54] then submitted the results to the researcher who created the solutions and knew the true concentrations.

5.4.7 Arsenic remediation

The remediation module was wet-loaded with 60 mg Oxone® and dried. Then, the card was loaded with 0.9 g goethite at the time of analysis. It was flipped on top of the assay module, folded together, and placed into a glass jar to perform the Environmental Protection Agency's toxicity characterization leaching procedure (TCLP).[89] The leachate had to be diluted by a factor of ten and the instrument lines washed with 10% v/v nitric acid solution for 3 minutes in between injections to avoid

salt and rust buildup in the ICP-OES. The test solution was filtered and analyzed using a Perkin Elmer Optima 8000 ICP-OES. The plasma was viewed down the axial axis, and the analytical wavelength monitored was As 193.696 nm. The torch was placed at the -4 position. The plasma gas flow rate was 15 L/min, and the auxiliary gas flow was 0.2 L/min. The nebulizer was set to 0.7 L/min, and the power was at 1400 W. The peristaltic pump flowed at 2.0 mL/min. The negative controls had no detectable levels of arsenic (n=3). The LOD for As containing solutions was estimated to be 0.0028 ppm (3*standard deviation of replicate injections of a 0.1 ppm As standard, n=5); a ten-fold dilution was performed on the leachate, so the method LOD was 0.028 ppm. The recovery of the 28.82 ppm starting arsenic level in the leachate was 99.9% (n=3). Quality control samples were performed every 5^{th} injection, and they always analyzed within 3% error.

5.5 Conclusion

We designed an easy-to-use paper test card that quickly measures physiologically relevant iodide concentrations without the need for lab equipment, specialized instruments, or reliable electrical power. Over the 0-500 ppb iodide range in an artificial urine matrix, the test cards correctly classified 88% of samples by computerized image analysis and 93% by visual analysis. Computer image analysis can extract quantitative data from the test card, and the accuracy of doing so was 40 ppb I while the precision was 20 ppb I.

Further development of the automated image analysis for the test cards would facilitate data storage and sharing with nutritional monitoring programs. Analyzing cell phone pictures of paper devices has been demonstrated in low resource settings,[98,100] and the existing cell phone infrastructure in Africa[101] should support this task. When iodine deficiency is detected, monitoring agencies can intervene and make corrective actions to a nation's salt iodization program.

The arsenic levels required for the test card classified it as toxic waste by US regulatory standards, so we addressed the risk associated with the analysis by incorporating a remediation module into the card design. The arsenic level in a leachate of a test card was reduced to such a low concentration by the remediation module that the As could not be detected by ICP-OES in most samples, and all samples tested were well below the EPA regulatory limits. Without compromising the efficacy of the Sandell-Kolthoff reaction, the end product was rendered less hazardous to the person handling its disposal as well as the entire community over time by preventing arsenic from leaching into the environment. These risks could have been ignored by citing public health imperatives or by acknowledging the lack of resources for proper disposal in LMIC. Conversely, concerns about the toxicity of the chemical wastes could have stymied further development of this potentially useful field test. The cost to develop the remediation module was well worth the benefit of eliminating a possible barrier to implementation in an LMIC. The risk associated with this dual-module paper test card has been reduced as much as is reasonably possible, and further development is

ethically justified by its potential use for the identification of a serious public health threat.

5.6 Acknowledgments

The Global Alliance for Improved Nutrition supported NM. Serim Research Corporation and Notre Dame College of Science supported IL. The Center for Nano Science and Technology (NDnano) supported SM. The ICP-OES analysis was conducted at the Center for Environmental Science and Technology (CEST) at the University of Notre Dame.

CHAPTER 6:

FUTURE DIRECTIONS

6.1 Summary

I have added redox titration, redox back-titration, and kinetic assay to the toolbox of techniques that can be performed on a paper substrate. Internal validation studies established that the saltPAD,[58] aPAD, and uiPAD have good accuracy and precision in a lab setting. External validation studies of the saltPAD showed that it can work well in labs of LMICs.[57] The comparative study of the saltPAD demonstrated that it rivals commercially available technologies when all aspects of product development are considered (i.e., the ASSURED criteria).[6] With these additional analyses available, the field-testing capacity in LMICs could be increased. However, there are many implementation issues that still need to be addressed.

6.2 Immediate future directions

6.2.1 External validation studies

External validation is intended to show the accuracy, robustness, and usability of a new technology. Validation studies should be conducted in a stepwise fashion, starting in a laboratory setting with trained personnel and working toward a field setting with untrained personnel. These are critical tests because the end users are unlikely to use the technology with the same level of care as a chemist in a lab. The test cards must pass all of these levels of testing to become an implementable technology.

The saltPAD is nearing the end of the validation series as it has been through internal, external, and even comparative validation studies. Now, it is under consideration by several government regulatory agencies, such as the Nutrition Center of the Philippines. Black Lion Hospital and the Ethiopian Food, Medicine and Health Care Administration and Control Authority (EFMHACA) have been awarded funds to conduct an implementation study of the saltPAD in mid-2017 in Ethiopia. They will use the "quality control" prototype that takes more measurements in the 30-50 ppm I range, since Ethiopia's regulatory standards for iodized salt fall within that range.

The uiPAD and aPAD are at the beginning of the external validation stage. Madeline Smith is taking over the uiPAD project, and she will research how to analyze urine samples on the test card and be the lead researcher for validation studies to come. Sarah Bliese will be conducting a usability study of the aPAD in Eldoret, Kenya. As the developer of the technology, I made sure the tests cards attained suitable analytical

performance in the Research and Development phase, and this will help justify future usability and implementation studies in the future.

6.2.2 Automated image analysis for usability

The test cards can be hard to interpret by eye. Not only must users assess the color intensity in several locations, they must also decide whether the controls worked correctly. During the comparative study for the saltPAD, 2 out of 6 users did not understand how to read the test card.[6] This demonstrates the need for an automated reader. I worked with Professor Chris Sweet and James Sweet at Notre Dame's Center for Research Computing to develop a program that analyzes images of the saltPAD. They developed the code and image recognition software based on my input about how the cards work, and I acted as a beta-tester to check that correct results were produced. The software is almost as accurate as a trained human reader (Chapter 3).[57] The software is currently run with a Mac desktop computer, but it was designed to be easily transferred to an Android cell phone app. We designed the software so it could be adapted to read different types of PADs. The saltPAD, aPAD, and uiPAD all have the same fiducial marks printed on them so the recognition software does not have to be specialized for each card type. The software needs to be programmed so it analyzes the appropriate regions of interest against preset calibration parameters (saltPAD and aPAD) or the internal standards run on the test card (uiPAD). An option will have to be added to the graphic user interface so the user can select the type of test card the software needs to analyze,

or the program could automatically choose the analysis based on the card type designated by the PAD's QR code.

6.2.3 Automated image analysis for data sharing

We chemists have no direct route or legal authority to change the behaviors of unscrupulous manufacturers, but we can share the data attained with the test cards with authorities that do. This puts the onus on them to take investigative and corrective action when the test cards detect a problem. Sharing the data is most effective when the data, both raw and processed, is in electronic form. The image analysis system can be designed to relay results instantaneously and automatically to authorities. An investigation could happen immediately and minimize the time bad quality products are in the market.

6.3 Escaping the ivory tower: Dissemination, certification, and commercialization

Information about the test cards must be as easy as possible to access by those in LMICs since they are the intended end users. Many journals have pay walls effectively cutting off access to people the studies are intended to help. Small-scale salt manufacturers in Africa might be looking for a replacement technology for the ineffective Rapid Test Kit,[5] but they cannot afford to pay the access fee for the internal validation study published in *Analytical Chemistry*. The authors of the comparative study of the salt analysis methods published their work in *PLoS One*,[6] an open access journal. This was a purposeful decision on their part because they have experience working within and disseminating information to people in LMICs. We started the dissemination

process in a different way. In Kampala, Uganda we met with the National Regulatory Authority and in Nairobi, Kenya we met with the Kenyan Poisons and Pharmacy Board. We demonstrated PADs to both of these groups. When we were at Kensalt, we met with the CEO and CFO. They wanted to help in the development process, mostly through external validation studies, because they saw an economic benefit in the PADs' projected cost of < $1 USD. The saltPAD and uiPAD projects were financially supported by the Global Alliance for Improved Nutrition (GAIN), an international organization launched by the United Nations to address malnutrition. GAIN organized the external validation at the South African Medical Research Council.[57] I have also presented the work at Micronutrient Forum and Pacifichem, which are international conferences, and won 2nd place for an elevator pitch competition at the Micronutrient Forum in Cancun, Mexico in 2016. Regulators, private companies, public health agencies, and researchers in the micronutrient field know the PAD technologies are coming down the R&D pipeline.

Besides just knowing about the technology, it is important for the PADs to gain regulatory acceptance as a testing method. Currently, the United States Pharmacopeial Convention's Council of Experts and its Expert Committees evaluates the merit of a testing method on the basis of its rationale, procedure, and data.[102] The rationale for PADs is to provide a field-friendly technology in locations that cannot support complicated compendial analyses, so this is an inlet for PADs to gain regulatory approval. There is no official monograph to assess field-friendly technologies, so the USP is currently writing one. This is an important addition to the compendium because field-

friendly technologies should not be held to the same scrutiny as gold standard analysis methods that occur in a lab. A topic often confounded with regulatory acceptance is certification. Achieving a certification is not related to regulatory acceptance as a valid testing method for enforcement purposes. Achieving a certification could convince potential users to purchase the technology, but there is not an obvious certification that applies to the PADs. One possible certification could be the International Organization for Standardization (ISO) 13485 (denoted by a CE marking on the product), which is usually reserved for medical devices in the European Union (EU). The PADs do not make a point-of-care medical diagnosis on an individual, but they may qualify as medical devices in the EU depending on the legal authority who has to interpret the definition for the most general class of medical devices in the EU: "Any instrument, […], material or other article […] for the purpose of diagnosis, prevention, monitoring, treatment or alleviation of disease […] and does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means."[103]

Throughout the R&D of these PADs, I have kept in mind commercialization, which must include product scalability, patent protection, and investors. The fabrication techniques used for the PADs have been kept as simple as possible with only features that can be printed. There are no electronics, or plastic or moving parts. Patents, either provisional or utility, have been filed on these test cards under filing numbers 14/533,746; 62/079,551; and 61/904,304. Investors consider product-market fit and return on investment, and both of these come down to risk. The saltPAD is far into the "de-risking" stage of product development, so an investor will not have to tolerate a

large amount of uncertainty in the commercialization of the product. The technology is not a diagnostic or medicine, so in most countries, it will not need to be certified or pass clinical trials before bringing it to market. The test cards are a one-time use item, so the product will have return buyers once the analysis system is implemented.

6.4 Toward implementation

Analytical performance is important, but there are many other factors that affect the success of new technologies. Financial managers have to decide whether the new technology is worth purchasing. If there has not been a return on the capital investment used to purchase a lab's existing equipment, replacing the system with PADs does not make sense. In that case, the WHO's recommendations about field-screening technologies being complimentary to gold standard analysis methods can be used to persuade decision-makers. The existing testing system is needed because it meets compendial requirements, but the PADs can enable additional quality control testing at a low cost that will help protect the company's product line or a country's medical supply. A different challenge arises when working with regulators. They may be reluctant to admit quality problems exist within their country under their watch. In those cases, it would be best to focus on improving testing systems going forward, and not fault a country for their lack of testing in the past or the current state of bad products in their marketplace. By making the regulators partners and giving them the data they need to do their job better, the PADs can improve a regulatory system without disrupting it.

6.5 Endgame

There is no benefit to having bad quality products on the market, but it is impossible to achieve a 0% prevalence rate. Instead, there has to be a balance between achieving as low a rate as possible and the cost to do so. PADs are an inexpensive technology that provides a way of finding bad quality products at a cost lower than ever before. PADs are so inexpensive that LMICs could conduct near-constant surveillance on products in the marketplace and in households. Breaches in compliance systems could be detected immediately with PADs that use the mobile phone system to transmit and archive data. Public health agencies and law enforcement can confirm and remedy the situation, thereby protecting public health from bad quality products. The paper test cards do not change the challenges facing regulators and companies in LMICs, but PADs are tools that could make it easier to keep bad quality nutritional products and medicines out of the marketplace.

APPENDIX A:

LAB ON PAPER: IODOMETRIC TITRATION ON A PRINTED CARD

A.1 Determination of wax thickness for saltPAD

The optimal line thicknesses for STEM were determined by printing a grid of 100 reaction areas with the thick wax lines set at line widths of 0.25 to 2.50 mm and thin wax lines set at 0.05 to 0.50 mm. A solid wax layer was printed on the back of the page and it was baked for 14 minutes at 110˚C. 3 µL drops of dyes were deposited in the five loading zones of each reaction area to test the containment provided by the thin wax lines. For each design that contained the loaded dyes, 125 µL of water was added to evaluate surface-tension enabled mixing in the reaction area. The thick outer square has to contain the test solution after being shaken for 3 minutes. Images were taken after 3 minutes for both steps. The inner lines need to be at least 0.30 mm thick and the outer lines need to be at least 1.75 mm thick.

(a) (b)

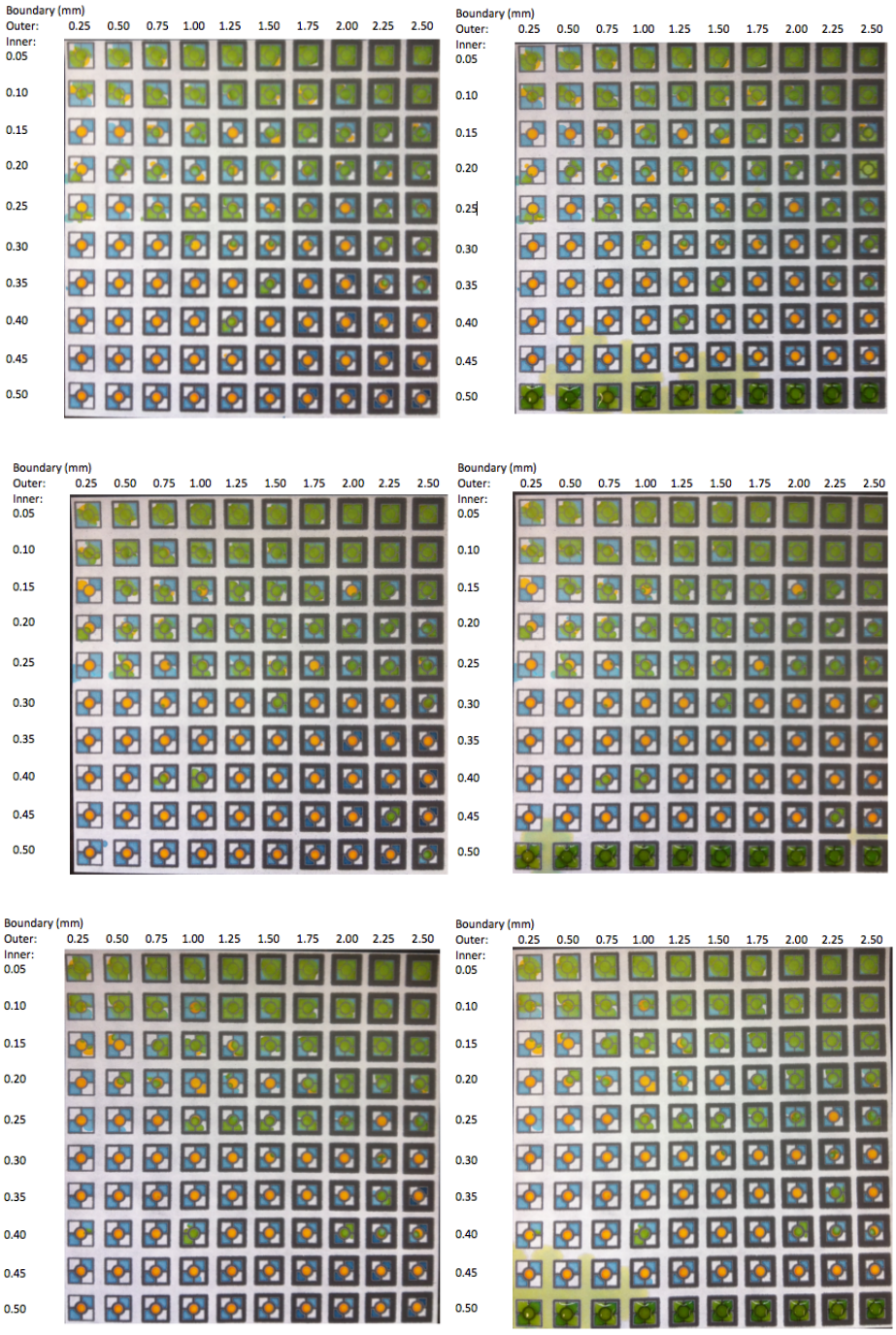


Figure A.1. Determination of wax thickness needed to confine reagents. Green color signifies poor reagent containment. (a) Testing inner lines only. (b) Testing outer lines only in the bottom row of each picture.

A.2 saltPAD reagents and where they are deposited

Reagent solutions (Table A.1) were loaded into 96-well plates. A Biomek® FX Model 717001 robot was used to spot the PADs with 2.0 µL aliquots of reagents into the locations shown in Figure A.2. PADs were allowed to air dry for about 20 minutes, stamped with a serial number, wrapped in aluminum foil, and then vacuum-sealed in Ziploc ® plastic wrap using a food grade vacuum sealer. The robot deposited reagents onto the PAD at a rate of 30 test cards per hour. The spotting error by the robot was approximately 0.5%, and the missed spots were pipetted by hand. The thiosulfate reagent can also be applied by a spray deposition technique developed by Scienion (see supporting file accompanying the thesis), and the remaining reagents applied by hand pipetting or with a 48 pin spoke inoculating tool. Over the 0-13 ppm I range, 94% (n=16) and 93% (n=14) of the test cards gave accurate results by visual analysis, respectively.

TABLE A.1.

REAGENTS USED TO CREATE THE SALTPAD

[I] ppm*	**Location#**	**Chemical**	**Scienion recipe (nL)~**
NA	A	2% Starch	NA
NA	B	1.0 M *p*-toluenesulfonic acid	NA
NA	C	0.5 M KI/0.3 M $CdCl_2$	NA
> 20, from iodide	D	0.42 M $NaNO_2$	NA
0-4	E	3.0 mM $Na_2S_2O_3$	7.00
3-7	F	16.0 mM $Na_2S_2O_3$	38.53
7-11	G	30.0 mM $Na_2S_2O_3$	70.00
11-15	H	43.5 mM $Na_2S_2O_3$	101.5
>30	I	150 mM $Na_2S_2O_3$	NA
NA	J	625 mM KIO_3	NA
NA	Blank	No Reagent	NA

Note: * As expressed in the test solution; multiply by 5 if the suggested sample preparation is followed. #The location where the chemical spotted is shown in Figure A.2. ~857.1 mM $Na_2S_2O_3$

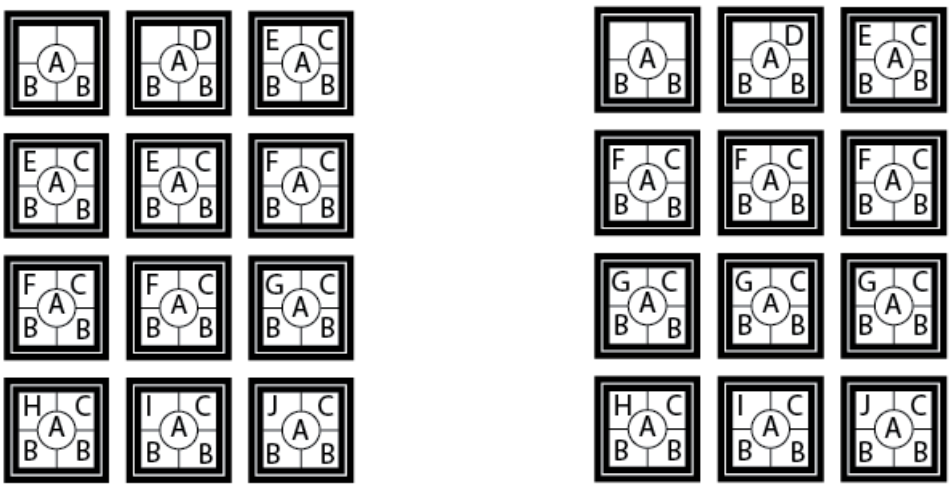


Figure A.2. Chemical deposition locations to create the saltPAD. 1) The market version with a “sweet spot” of 0-7 ppm I in solution (0-35 ppm I in the solid salt sample). 2) The quality control version with a “sweet spot” of 5-11 ppm I (15-55 ppm I in the solid salt sample).

A.3 Determination of thiosulfate levels

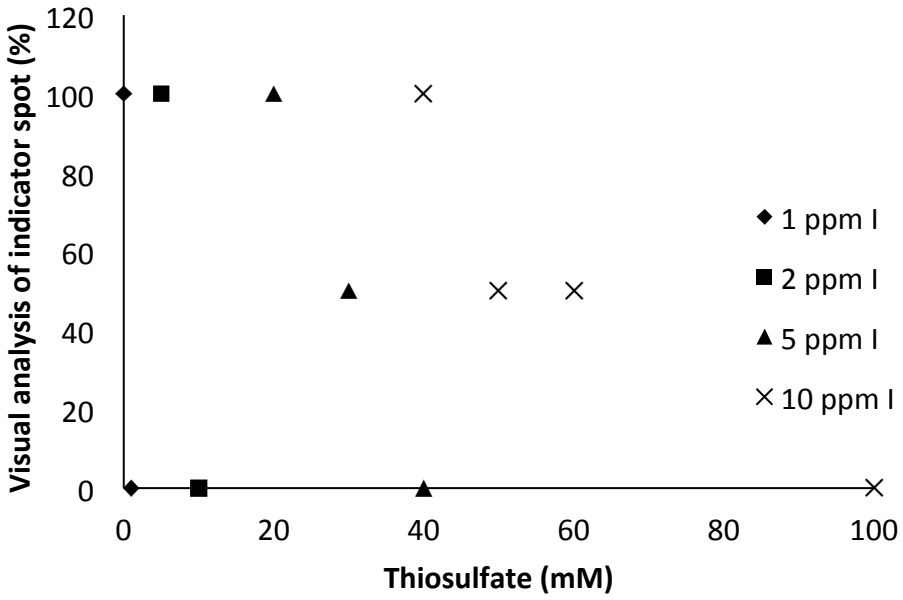


Figure A.3. Determination of thiosulfate for saltPAD. The iodine level was held constant while the amount of thiosulfate placed onto the PAD was varied. The visual response of the indicator spot was recorded as being completely empty (0%), half full (50%), or completely full (100%). The amounts of thiosulfate were scaled to accommodate the test solution volume of 125 µL used on the final version of the saltPAD.

A.4 The test card gives a sigmoidal response

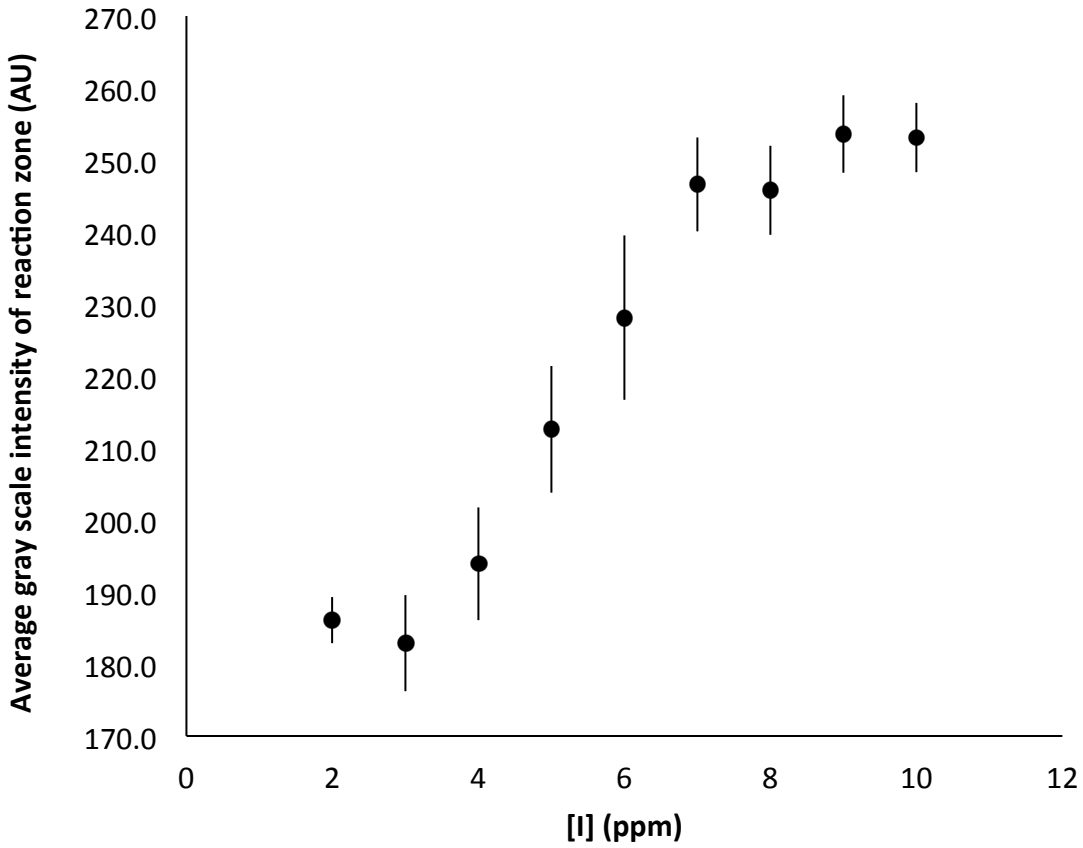


Figure A.4. Sigmoidal response of a test zone on the saltPAD. As the concentration of iodine (from iodate) is increased, the color intensity increases. If the iodine concentration is large enough, the response becomes saturated and the graph exhibits a sigmoidal shape. The pseudo-linear portion of the curve is useful for analytical measurements.

A.5 Detection of high iodine levels

The detection of iodate follows the chemistry provided in Figure 2.1. However, the chemistry must change for the formation of triiodide from potassium iodide, as shown in Figure A.5.[104] The starch indicator then detects the triiodide, and the visual cutoff levels are given in Figure A.6.

$$3\ I^- + 2\ NO_2^- + 4\ H^+ \rightarrow 2\ H_2O + 2\ NO + I_3^-$$

Figure A.5. Formation of triiodide from potassium iodide.

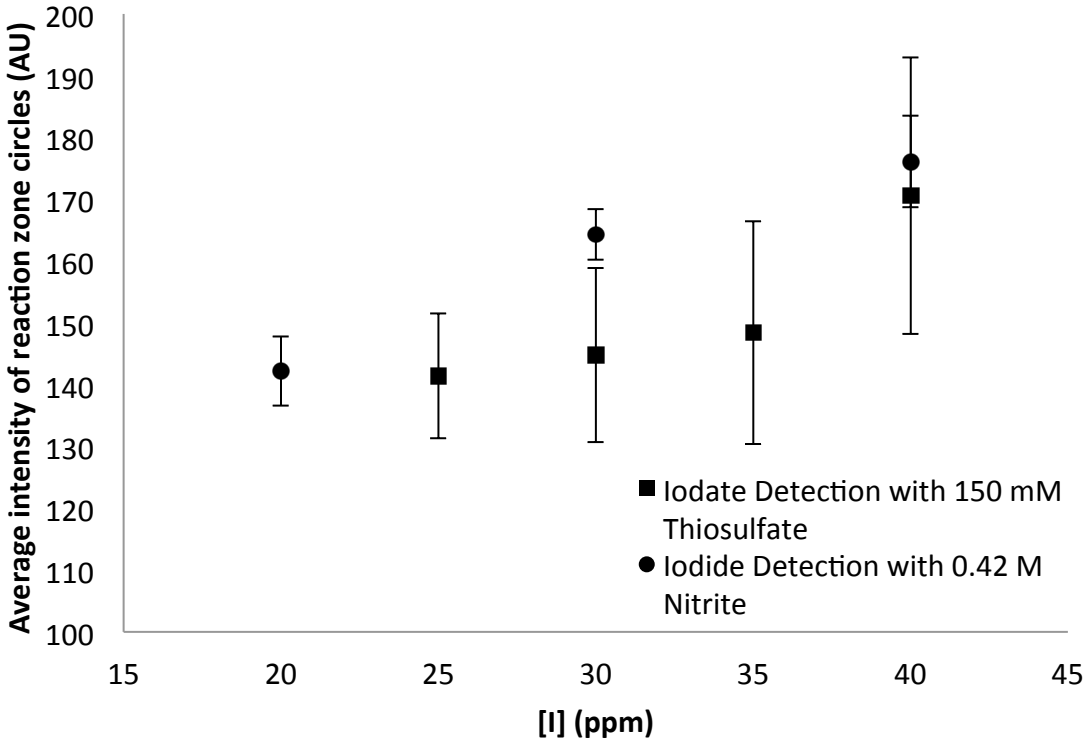


Figure A.6. Detection of high iodine levels. The higher levels of iodine detectable by the saltPAD are set up as limit tests because the signal is too variable for quantification. For iodide detection, a visual response was seen at 20 ppm I, and the response from iodate was noticeable at 30 ppm I.

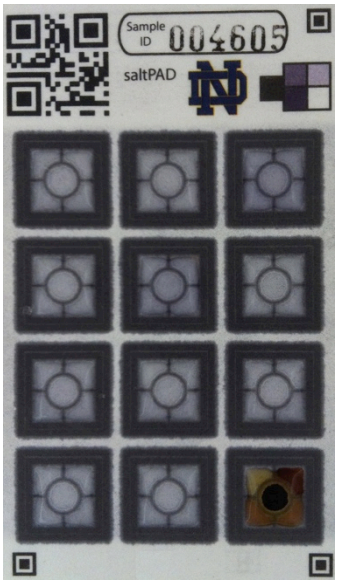
A.6 How to make a lightbox

Use a completely sealed box or shoebox with approximate dimensions of 12” x 8” x 5” to serve as the main structure of the lightbox. It helps if the interior of the box is white to scatter the light. Line the box with 2 strands of LED lights that can be plugged into a power source. Battery-operated lights are highly discouraged as the light intensity will decrease over time. Tape the lights into a permanent position. Do not aim the lights directly at the imaging area. Cover the LED lights with many layers of normal printing paper to diffuse the light. No shadows or gradients of lighting should be seen within the box. Cut a 1 cm diameter hole into the top of the box so that the camera lens can see into the box. Take a practice image to make sure no shadows are present. Adjust the position of the lights and the paper coverings as needed to get even dispersal of light.

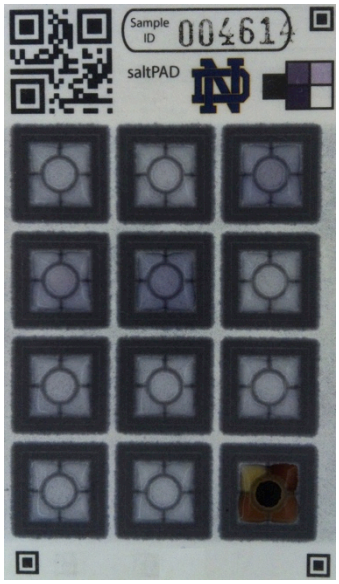
A.7 saltPAD standard images

After running the test, the user visually compares the response of the card to the following standard images:

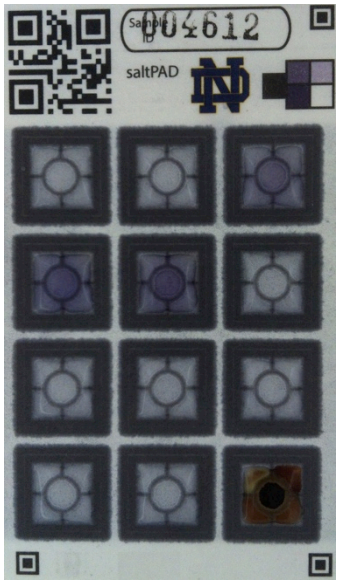
Figure A.7. A set of standard images showing the test card response to various levels of iodine. The numbers under the image represent ppm I from iodate unless otherwise noted. A user must multiply the value by the dilution factor to get the iodine concentration in the solid salt sample. (Pages 146-148.)



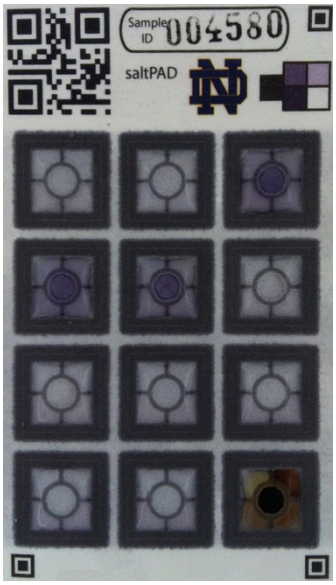
0 ppm I



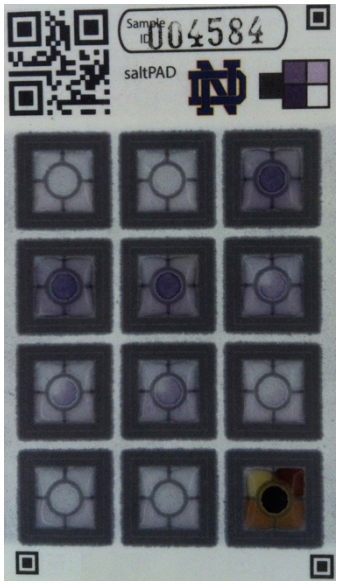
1 ppm I



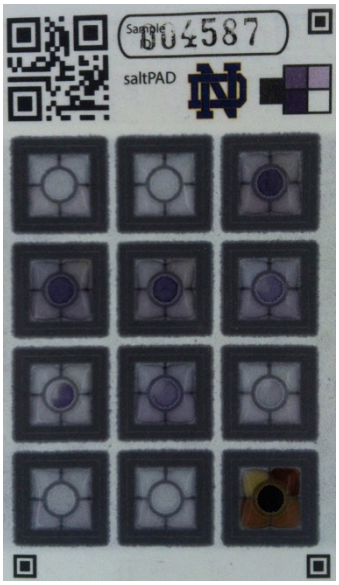
2 ppm I



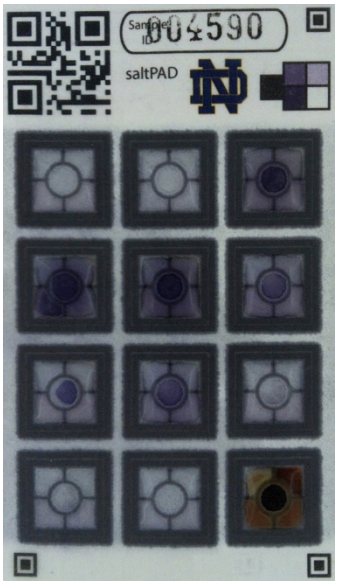
3 ppm I



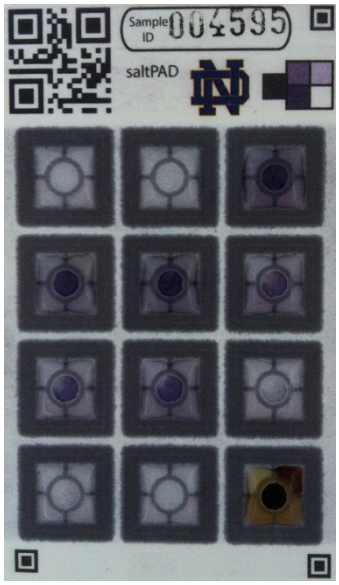
4 ppm I



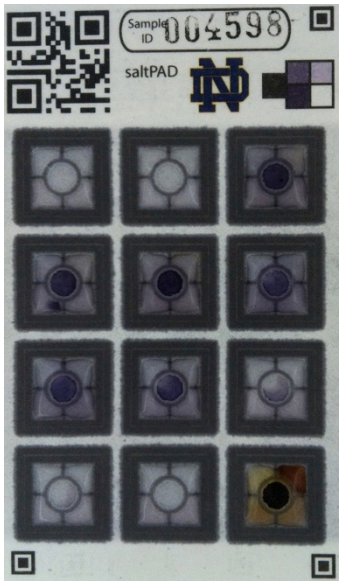
5 ppm I



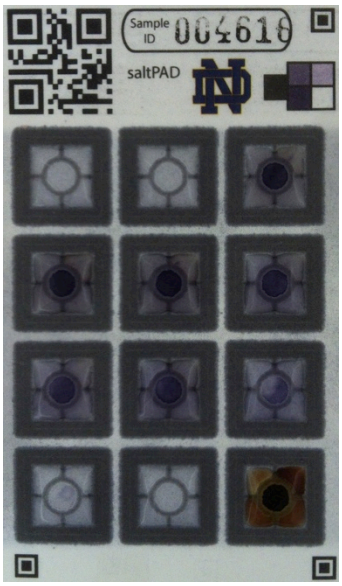
6 ppm I



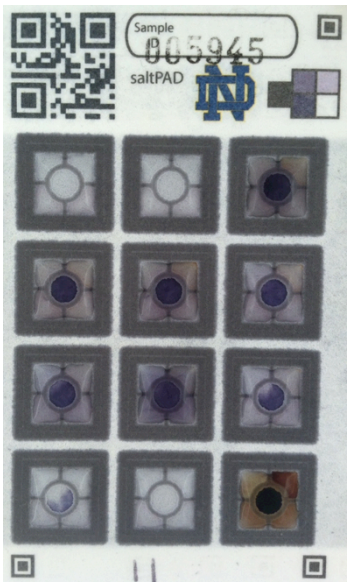
7 ppm I



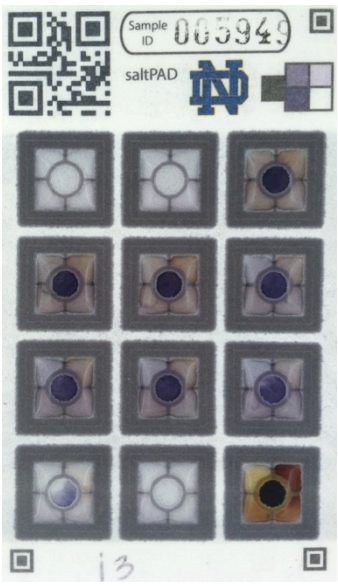
8 ppm I



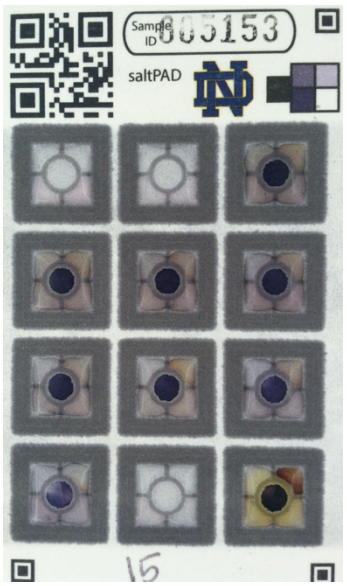
9 ppm I



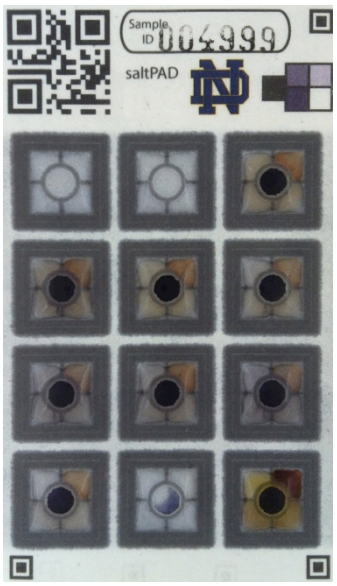
11 ppm I



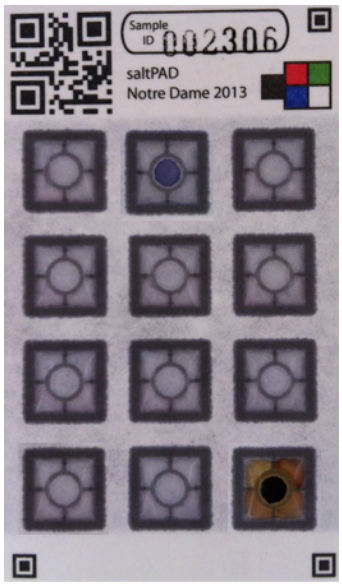
13 ppm I



15 ppm I



> 30 ppm I



> 20 ppm I (from iodide)

A.8 Interpretation of the saltPAD by eye

During the internal validation, analysts were asked to guess the solution concentration by comparing the PAD response to standard images. Each of 11 solutions were run on 5 different test cards. Each analyst interpreted only their test cards, for a total of 55 test cards per analyst. Analyst 1 had an average error of 0.5 ppm I while Analyst 2 had an average error of 0.5 ppm I. The inter-operator precision was 0.5 ppm I. The errors are expressed as ppm values in the test solution, not the solid salt.

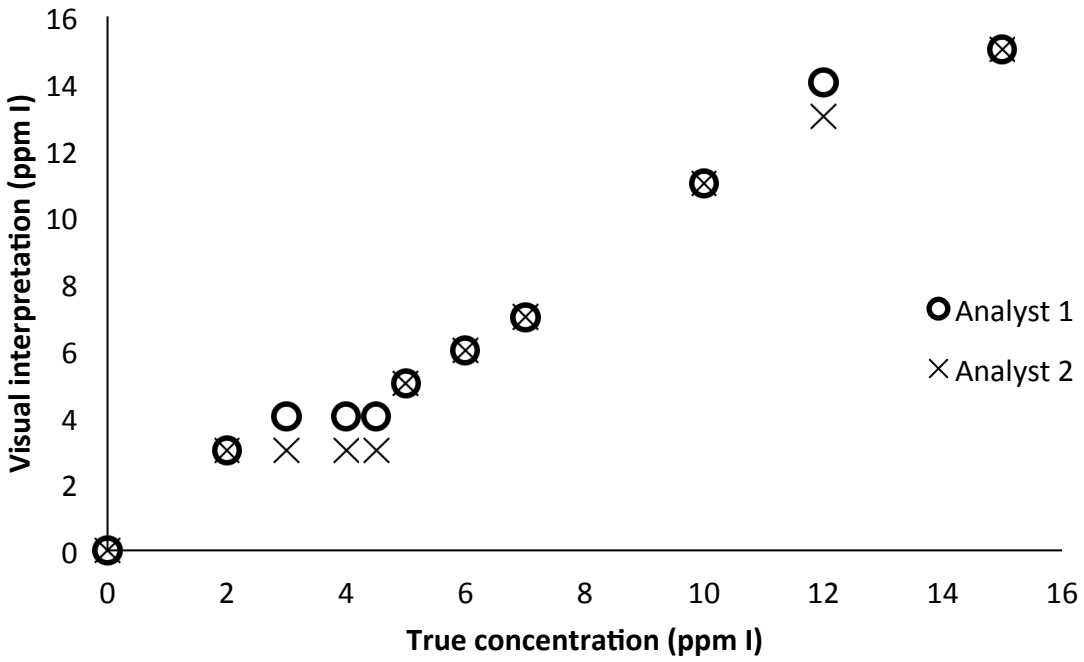


Figure A.8. Interpretation of the saltPAD by eye. Comparing the test card to standard images produced an average error of 2.5 ppm I.

A.9 Systematic bias for the saltPAD

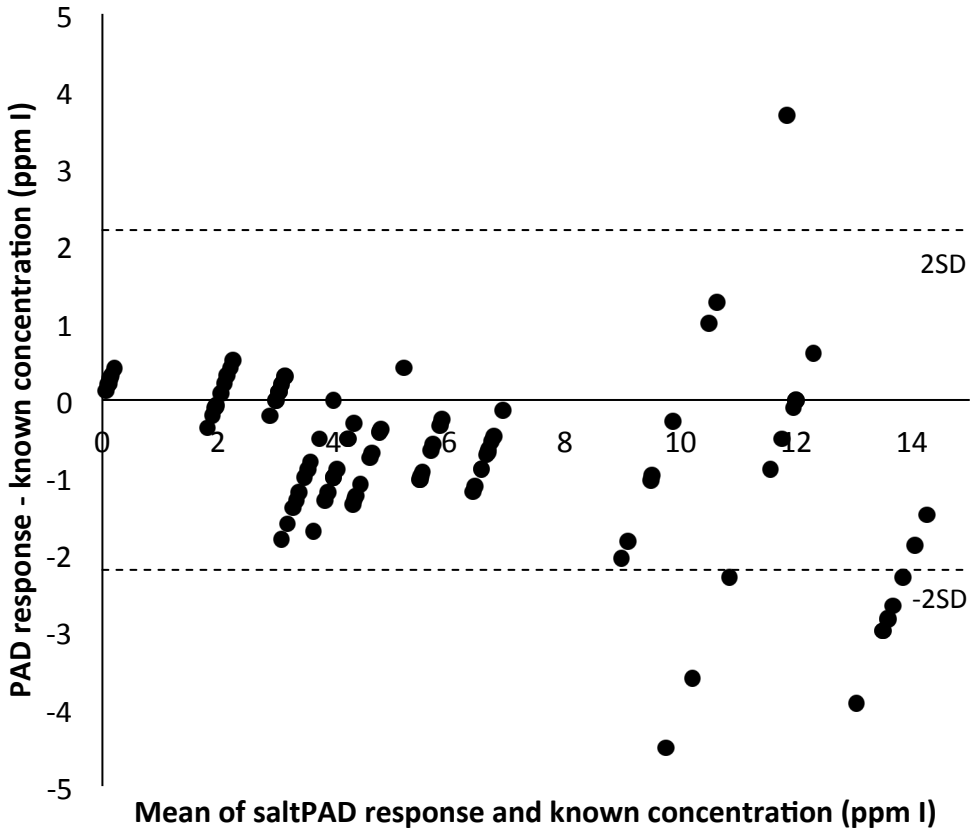


Figure A.9. Bland-Altmann plot comparing the saltPAD response to the known iodine concentration. There is an average bias of -0.7 ppm I which means the saltPAD systematically underestimates the true concentration.

A.10 Different water sources for robustness testing

Solutions of 8.0 ppm I were made using alternate water sources to test the adaptability of this test to water sources other than DI water. Two solutions of 3.7 M NaCl were made using lake water and tap water. These saltwater solutions were then spiked with an iodate stock solution to give two 8.0 ppm I solutions. Each solution was run on two PADs and images were collected for image analysis. The saltPADs run with the tap water produced an error of 8% while the lake water produced an error of 17%.

A.11 Chemical content of tap water at the University of Notre Dame

There is no available data for the chemical or biological content of St. Mary's Lake. The chemical impurities of tap water are available through the University, shown in Table A.2.

TABLE A.2.

CHEMICAL CONTENT OF TAP WATER AT UNIVERSITY OF NOTRE DAME

Chemical	**Highest detected level (ppm)***
Barium	0.14
Nitrate	0.6
Chromium	0.0072
Fluoride	<0.5
Arsenic	0.0041
Sodium	75
Copper	0.550
Lead	0.0059
Calcium	127#
Magnesium	26#

Note: *Highest level detected over the 2011 calendar year. #Analysis performed in an analytical chemistry course; when expressed in mol equivalent of calcium, the hardness level of the water is 170 ppm Ca.

A.12 Cost of the saltPAD

TABLE A.3.

MATERIALS COST ANALYSIS OF SALTPAD

Expenditure	**Cost per test card (USD)**
Ahlstrom 319 Paper	0.05
Wax	0.03
Packaging Materials	0.05*
Chemicals	<0.01

Note: *Assumes 20 cards/pack.

A.13 The error and precision of the saltPAD's competitors

TABLE A.4.

THE ERROR AND PRECISION OF FIELD-FRIENDLY IODIZED SALT ANALYSIS TECHNIQUES

Method	**Iodine range assessed (ppm in salt)**	**How the metrics were calculated**
Titration under field conditions[51]	25-100	Accuracy: average recovery Precision: SD measured at 3 concentrations. These 3 SD were then averaged
Rapid test kit[5]	Categorical: 0, 0-15, >15	Accuracy: categorical agreement with titration Precision: The study did not analyze the same solution in replicate, so precision data is not available
BioAnalyt iCheck[51]	5-100	Accuracy: average of the recovery of 7 concentrations across 5 – 100 ppm I Precision: SD measured at 3 concentrations from 17 – 55 ppm I. These 3 SD were then averaged
WYD iodine checker[53]	25-100	Accuracy: average of the recovery at 2 concentration levels Precision: The SD was determined at 2 concentrations and then averaged

APPENDIX B:

LAB ON PAPER: ASSAY OF BETA-LACTAM ANTIBIOTICS BY REDOX BACK-TITRATION

B.1 Fabrication of the test card

The fabrication of the blank test cards is detailed in section 4.4.2. The chemicals listed in Table B.1 were pipetted into the locations show in Figure B.1. All volumes are 2.0 µL**.**

TABLE B.1.

CHEMICALS DEPOSITED ONTO APAD

Location	Chemical
A	2% Starch
B	1.0 M *p*-toluenesulfonic acid
C	0.5 M KI/0.3 M $CdCl_2$
D	3.0 mM $Na_2S_2O_3$
E	16.5 mM $Na_2S_2O_3$
F	30.0 mM $Na_2S_2O_3$
G	43.5 mM $Na_2S_2O_3$

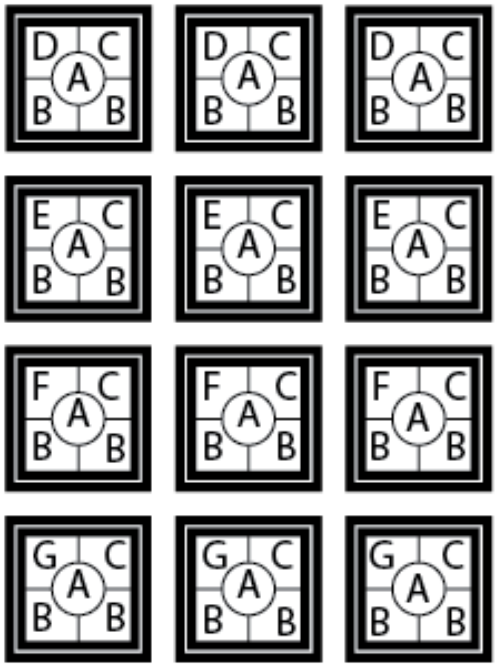


Figure B.1. Location of chemicals. Identities in Table B.1.

B.2 HPLC methodology for analyzing amoxicillin, clavulanate, and ampicillin

HPLC Instrument: Waters 2695

Detector: Waters 2487 Dual λ Absorbance

Amoxicillin and Amoxicillin/Clavulante combination pills

Column: Symmetry C18 5µm, 4.6 x 100 mm column

Run time: 12 min

Peak retention time: 3.3-3.4 min

Wavelength: 220 nm

Injection volume: 18 µL

Flow rate: 1.00 mL/min

Nominal sample concentration: 0.5 mg/mL

Mobile phase: Table B.2

TABLE B.2.

MOBILE PHASE GRADIENT FOR HPLC ANALYSIS OF AMOXICILLIN AND AMOXICILLIN/CLAVULANATE COMBINATION PILLS

Time (min)	Methanol (%)	Phosphate buffer (%)*	Flow (mL/min)	Change#
0.0	5.0	95.0	0.50	hold
0.50	5.0	95.0	0.50	hold
5.00	30.0	70.0	0.50	linear
7.00	90.0	10.0	0.50	linear
8.00	90.0	10.0	0.50	hold
8.50	25.0	75.0	0.50	linear
10.00	10.0	90.0	0.50	linear
11.00	5.0	95.0	0.50	linear
12.00	5.0	95.0	0.50	hold

Note: *20 mM, pH= 4.4 ± 0.1. #The entry describes the change in mobile phase from the previously listed time.

Ampicillin pills

Column: XBridge C18 5µm, 3.0 x 50 mm

Run time: 6 min

Peak retention time: 1.2-1.4 (void time = 0.6 min)

Wavelength: 230 nm

Injection volume: 40 µL

Flow rate: 1.00 mL/min

Nominal sample concentration: 0.5 mg/mL

Mobile phase: 20% Methanol, 80% 20 mM phosphate buffer pH 4.4±0.1 (isocratic)

<u>System suitability for ampicillin</u>

Sample concentration range: 0-0.75 mg/mL, 0.50 mg/mL being nominal 100% level

Accuracy at 50%, 100%, 150% levels are all under 2%

Precision at 50% is 0.7% RSD

Linearity is R^2 = 0.9999 for 0-0.75 mg/mL

Recovery from stressed sample is 99.0%.

B.3 Creating a nominal 1.00 mg/mL solution for the aPAD

Each solution run on the test card had a nominal concentration of 1.0 mg/mL. It is easier to create a 2 mg/mL solution and dilute it to 1.0 mg/mL than it is to create the 1.0 mg/mL directly. Follow these steps:

1. Accurately mass the pill or capsule contents (do not include the plastic shell).
2. Crush or grind up the pill.
3. Accurately mass a ~50 mg portion of the medicine (m_{used}) and dissolve it in 25.0 mL of water.
4. Calculate the nominal purity of the pill (P). Divide the mass of the API stated on the product's label ($m_{labeled\ API}$) by the mass of all the powder in the pill (m_{powder}).

   $P = m_{labeled\ API}\ (mg) / m_{powder}\ (mg)$
5. Calculate the nominal concentration of the 2 mg/mL stock solution (C_{stock}).

   $C_{stock}\ (mg/mL) = m_{used}\ (mg) * P / 25.0\ (mL)$ $\quad m_{used}$ ~50 mg
6. Calculate the volume of stock (V_{stock}) needed to create a nominal 1.0 mg/mL.

   $V_{stock}\ (mL) = 1.00\ (mg/mL) * 4.0\ (mL) / C_{Stock}\ (mg/mL)$

   substituting in variables:

   $V_{stock}\ (mL) = 1.00\ (mg/mL) * 4.0\ (mL) / (m_{used}\ (mg) * P / 25.0\ (mL))$

   $$V_{stock}(mL) = \frac{1.00\ \left(\frac{mg}{mL}\right) * 4\ (mL)}{\frac{m_{used}(mg)}{25\ (mL)} * \frac{m_{labeled\ API}(mg)}{m_{powder}(mg)}}$$
7. Calculate the volume of water (V_{H2O}) needed to bring the solution volume to 4.0 mL.

   $V_{H2O}\ (mL) = 4\ (mL) - V_{stock}\ (mL)$

8. Analyze the solution as given in section 4.4.9.

9. After the visual read, S_{conc} *(mg/mL)*, the amount of antibiotic, $m_{antibiotic}$ (mg), in the pill is calculated:

$$m_{antibiotic}\ (mg) = 25.0\ (mL)\ \text{x}\ S_{conc}\ \left(\frac{mg}{mL}\right)\text{x}\ \frac{4.0\ (\text{mL})}{V_{Stock}(mL)}\ \text{x}\ \frac{m_{powder}\ (mg)}{m_{used}\ (mg)}$$

B.4 Conversion from mg/mL to "% labeled amount"

When determining the amount of solution V_{Stock} to dilute to 1.00 mg/mL, the aliquot is dependent upon the ratio of the labeled amount of API to the total mass of the pill (all variables defined in the section B.3):

$$V_{stock}(mL) = \frac{1.00\ \left(\frac{mg}{mL}\right) * 4\ (mL)}{\frac{m_{used}(mg)}{25\ (mL)} * \frac{m_{labeled\ API}(mg)}{m_{powder}(mg)}}$$

Substituting this into the equation:

$$m_{antibiotic}\ (mg) = 25.0\ (mL)\ \text{x}\ S_{conc}\ \left(\frac{mg}{mL}\right)\text{x}\ \frac{4.0\ (\text{mL})}{V_{Stock}(mL)}\ \text{x}\ \frac{m_{powder}\ (mg)}{m_{used}\ (mg)}$$

$$m_{antibiotic}\ (mg) = 25.0\ (mL)\ \text{x}\ S_{conc}\ \left(\frac{mg}{mL}\right)\text{x}\ \frac{4.0\ (\text{mL})}{\frac{1.00\ \left(\frac{mg}{mL}\right) * 4\ (mL)}{\frac{m_{used}(mg)}{25\ (mL)} * \frac{m_{labeled\ API}(mg)}{m_{powder}(mg)}}}\ \text{x}\ \frac{m_{powder}\ (mg)}{m_{used}\ (mg)}$$

$$m_{antibiotic}\ (mg)$$

$$= 25.0\ (mL)\ \text{x}\ S_{conc}\ \left(\frac{mg}{mL}\right)\ \text{x}\ \frac{4.0\ (\text{mL})}{\frac{4.00\ (mg)}{\frac{m_{used}(mg)}{25\ (mL)} * \frac{m_{labeled\ API}(mg)}{m_{powder}(mg)}}}\ \text{x}\ \frac{m_{powder}\ (mg)}{m_{used}\ (mg)}$$

$$m_{antibiotic}\ (mg)$$

$$= 25.0\ (mL) \times S_{conc}\ \left(\frac{mg}{mL}\right) \times 4.0\ (\text{mL})\ \times \frac{m_{powder}\ (mg)}{m_{used}\ (mg)}$$

$$\times \frac{m_{used}(mg)}{25\ (mL)} \times \frac{m_{labeled\ API}(mg)}{m_{powder}(mg)} \times \frac{1}{4\ (mg)}$$

$$m_{antibiotic}\ (mg) = S_{conc}\ \left(\frac{mg}{mL}\right) \times 1\ \left(\frac{mL}{mg}\right) \times m_{labeled\ API}(mg)$$

$$m_{antibiotic}\ (mg) = S \times m_{labeled\ API}(mg)$$

$$\frac{m_{antibiotic}\ (mg)}{m_{labeled\ API}(mg)} = S$$

$$\%\ of\ Labeled\ API = S$$

B.5 Comparison of commercially available scales

The accuracy and precision of three portable scales were assessed to see which would be the best to use in a test kit. None of the balances achieved the accuracy and precision specifications in USP method <41>, which are expected of an analytical balance in a certified lab. The Gemini 20 had the best performance, and can be used if at least 140 mg of sample is massed.

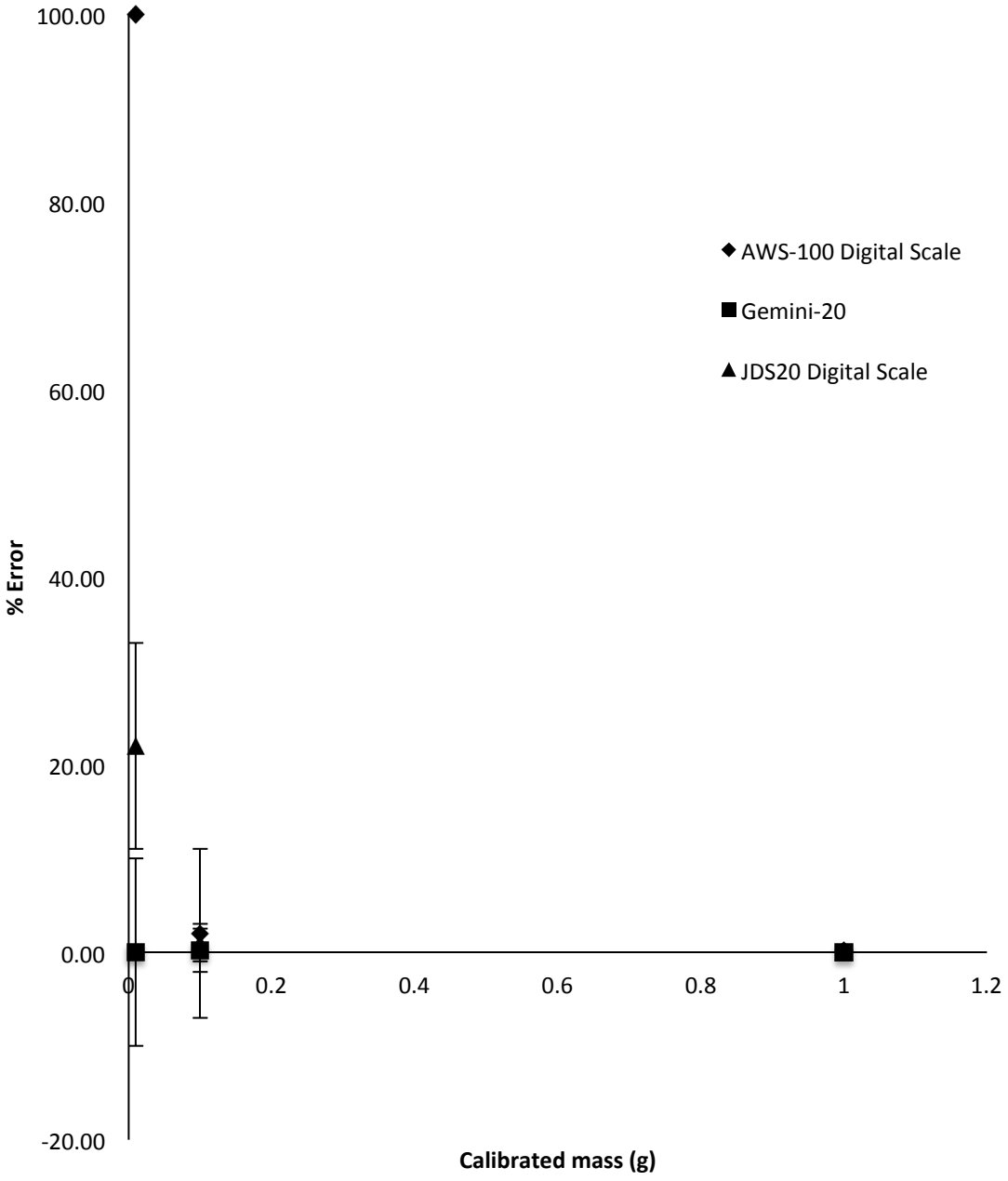


Figure B.2. Accuracy of portable scales. Calibration masses used: 0.01000 g, 0.10000 g, 1.00000 g. The Gemini-20 consistently produced the most accurate result, and it is a milligram scale. n=5 for 0.01000 g and 0.10000 g; n=10 for 1.00000 g.

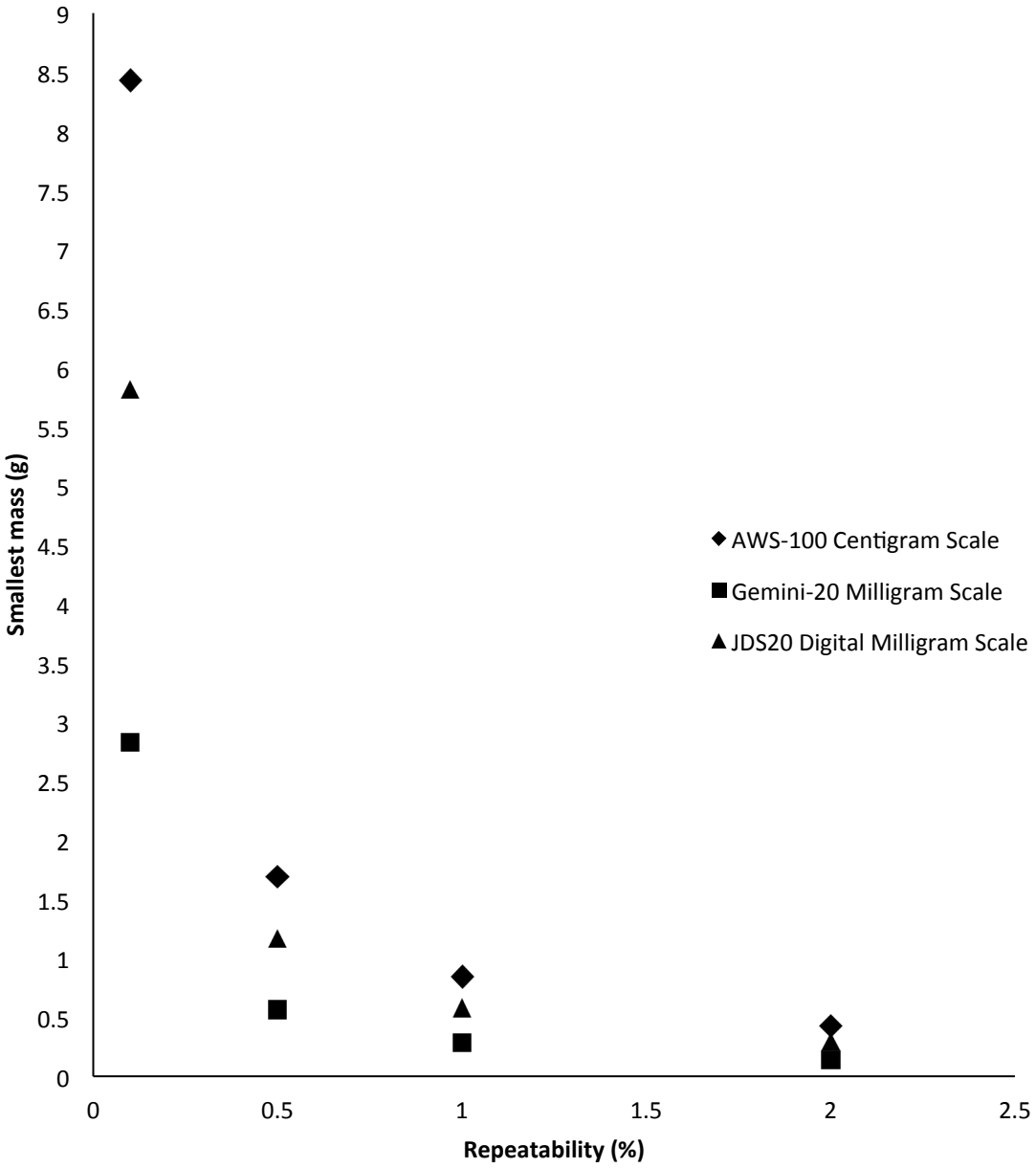


Figure B.3. Repeatability of portable scales. In order to prepare antibiotic pills for analysis that meet the USP requirement for repeatability (not more than 0.1%), at least 3 g would have to be weighed on the Gemini-20. Most pills weigh less than 1 g, so this is not possible. For field analysis, it would be best to anticipate a 2% repeatability since the Gemini 20 can mass ~140 mg reliably at that point. This is the only level that allows 250 mg pills to be analyzed. (n=10 for each balance; the repeatability was set to different levels and the smallest mass calculated).

B.6 Comparative cost analysis for the test card and HPLC consumables

TABLE B.3.

COMPARATIVE COST ANALYSIS FOR THE TEST CARD AND HPLC CONSUMABLES

Expenditure	HPLC analysis (USD)	Test kit (USD)*
Ahlstrom 319 Paper	NA	0.05
Wax	NA	0.03
Chemicals (for test card)	NA	0.01
Plastic wrap to seal test cards	NA	0.05
0.0050 M I_3^-	NA	0.18
Glass scintillation vial for I_3^-	NA	0.10
1.0 M NaOH	NA	0.002
Polyethylene scintillation vial	NA	0.01
1.2 M HCl	NA	0.005
Polyethylene scintillation vial	NA	0.01
Disposable plastic pipets x 3**	NA	0.03
Weigh paper	0.02	0.02
Glass reaction vial x 3***	NA	0.04
Packaging box	NA	0.02
Column#	0.70	NA
UV Bulb##	0.06	NA
Syringe	0.22	NA
Filter	0.79	NA
Autosampler vial	0.27	NA
Autosampler vial lid	0.22	NA
Secondary standard###	0.23	NA
Mobile phase####	0.09	
Total	2.60	0.54

Note: *Cost to analyze 1 sample in triplicate (only need one test card to do so), assuming 20 test cards per pack. **Three are included, one per solution. They should be washed if used for more than one day. ***Three are included so three analyses can happen simultaneously. These would have to be washed between analyses. #Assumes the column costs $700 and 1000 samples can be analyzed before it needs to be replaced. ## Assumes the bulb costs $600, has a 2000 hr life, and each sample has a 12 minute run time; 10,000 samples can be run before it needs to be replaced. ###250 mg of a secondary standard of ampicillin costs $37.10 from Sigma-Aldrich. Assumes 30 mg of the standard is massed for analysis everyday and that 20 unknown samples are analyzed against it. ####Assumes methanol consumed during 12 minute run flowing at 1 mL/min. A single point measurement on a test card is ($0.54 USD / 3) = $0.18 USD.

B.7 Determination of beta-lactam compounds and triiodide reaction stoichiometries by glassware titration

This experiment shows that the test card must be calibrated for each beta-lactam analyzed. The degradation products of some beta-lactams have different stoichiometries with triiodide. The methodology in USP <425> was followed.

TABLE B.4.

STOICHIOMETRIC RATIO BETWEEN AN ANTIBIOTIC'S DEGRADATION PRODUCTS AND TRIIODIDE

AB	$[AB]_i$ (mM)	$[I_3^-]_i$ (mM)	AB_i (mol)	$I_{3\,i}^-$ (mol)	$I_{3\,f}^-$ (mol)	$I_{3\,i-f}^-$ (mol)	I_3^-: AB	avg	sd
Amox	0.16	3.11	2.38E-06	4.66E-05	3.29E-05	1.37E-05	5.76	5.73	0.18
Amox	0.23	3.01	3.58E-06	4.66E-05	2.73E-05	1.94E-05	5.41		
Amox	0.30	2.91	4.77E-06	4.66E-05	1.95E-05	2.71E-05	5.68		
Amox	0.36	2.82	5.96E-06	4.66E-05	1.36E-05	3.31E-05	5.55		
Amox	0.10	3.73	2.38E-06	9.32E-05	7.95E-05	1.37E-05	5.74		
Amox	0.14	3.65	3.58E-06	9.32E-05	7.19E-05	2.13E-05	5.96		
Amox	0.18	3.58	4.77E-06	9.32E-05	6.50E-05	2.82E-05	5.91		
Amox	0.22	3.52	5.96E-06	9.32E-05	5.85E-05	3.47E-05	5.83		
Amp	0.19	3.33	2.86E-06	5.00E-05	3.38E-05	1.62E-05	5.65	5.67	0.65
Amp	0.28	3.23	4.29E-06	5.00E-05	2.66E-05	2.34E-05	5.45		
Amp	0.36	3.13	5.72E-06	5.00E-05	2.01E-05	2.99E-05	5.22		
Amp	0.43	3.03	7.16E-06	5.00E-05	1.59E-05	3.41E-05	4.76		
Amp	0.11	4.00	2.86E-06	1.00E-04	8.00E-05	2.00E-05	6.99		
Amp	0.17	3.92	4.29E-06	1.00E-04	7.45E-05	2.55E-05	5.93		
Amp	0.22	3.85	5.72E-06	1.00E-04	6.68E-05	3.32E-05	5.80		
Amp	0.27	3.77	7.16E-06	1.00E-04	6.02E-05	3.98E-05	5.56		
CA	0.33	3.11	4.88E-06	4.66E-05	4.64E-05	2.50E-07	0.05	0.27	0.15
CA	0.49	3.01	7.31E-06	4.66E-05	4.50E-05	1.60E-06	0.22		
CA	0.63	2.91	9.75E-06	4.66E-05	4.38E-05	2.82E-06	0.29		
CA	0.76	2.82	1.22E-05	4.66E-05	4.16E-05	5.03E-06	0.41		
CA	0.20	3.73	4.88E-06	9.32E-05	9.23E-05	9.25E-07	0.19		
CA	0.30	3.65	7.31E-06	9.32E-05	9.20E-05	1.23E-06	0.17		
CA	0.39	3.58	9.75E-06	9.32E-05	9.00E-05	3.18E-06	0.33		
CA	0.47	3.52	1.22E-05	9.32E-05	8.68E-05	6.38E-06	0.52		

Note: AB = antibiotic, CA = clavulanic acid

B.8 Agreement of United States Pharmacopeia method <425> with HPLC

A small study was conducted to see if the United States Pharmacopeia method <425> has good agreement with HPLC. The regulatory assay requirement for beta-lactam pharmaceuticals is 90.0-120.0% of the labeled dosage.

TABLE B.5.

TITRATION OF AMPICILLIN SAMPLES THAT ARE GOOD QUALITY

Sample*	Titration		HPLC		Error	Dosage
	mg	% of labeled	mg	% of labeled	% of labeled	mg
16-0367 P1	238	95.2	225	90.0	5.2	250
16-0368 P1	528	105.6	467	93.4	12.2	500
16-0241 P1	540	108.0	510	102.0	6.0	500
16-0242 P1	460	92.0	510	102.2	-10.2	500
16-0236 P1	443	88.6	508	101.6	-13.0	500

Note: * Sample numbering system used at ND. Error (%) = 9.3. Bias (%) = 0.2

TABLE B.6.

TITRATION OF AMOXICILLIN SAMPLES THAT WERE THERMALLY DEGRADED TO BAD QUALITY LEVELS

Sample*	Titration		HPLC		Error	Dosage
	mg	% of labeled	mg	% of labeled	% of labeled	mg
14-0676 P1	318	63.6	172	34.4	29.2	500
14-0670 P1	438	87.6	206	41.2	46.4	500
14-0670 P2	280	56.0	264	52.8	3.2	500
14-0664 P1	438	87.6	198	39.6	48.0	500
14-0651 P2	255	51.0	162	32.4	18.6	500
14-0652 P1	277	55.4	163	32.6	22.8	500

Note: * Sample numbering system used at ND. These samples were thermally degraded. Error (%) = 28.0. Bias (%) = 28.0.

APPENDIX C:

GREEN DESIGN OF A PAPER TEST CARD FOR URINARY IODIDE ANALYSIS

C.1 Arsenic waste generated by cuvette vs. paper SK methods

TABLE C.1.

COMPARISON OF ARSENIC WASTE BY METHOD

Method	**Volume (L)***	**Concentration (M)**	**Amount (mg)**
UV-vis	2×10^{-3}	0.025 M As_2O_3	7.5
Test card	10×10^{-6}	0.2 M As_2O_3	0.3

Note: *Volume of arsenic containing test solution per analysis. 7.5/0.3 = 25. It takes 25 times more arsenic by mass to analyze one sample by the UV-vis method than by the test card.

C.2 Cost analysis

TABLE C.2.

COST ANALYSIS OF ASSAY MODULE

Expenditure	Cost per test card ($USD)
Ahlstrom 319 paper	0.05
Wax	0.03
Packaging materials	0.05*
Consumables	0.05*
Chemicals	0.02*
Total	0.20

Note: *Assumes 20 cards/pack.

TABLE C.3.

COST ANALYSIS OF REMEDIATION MODULE

Expenditure	Cost per test card ($USD)
Ahlstrom 319 paper	0.05
Wax	0.03
Packaging materials	0.05*
Consumables	0.02*
Chemicals	0.05*
Total	0.20

Note: *Assumes 20 cards/pack.

Total cost of raw materials for test card system: $0.40 USD

C.3 Red and blue channel analysis of the test card

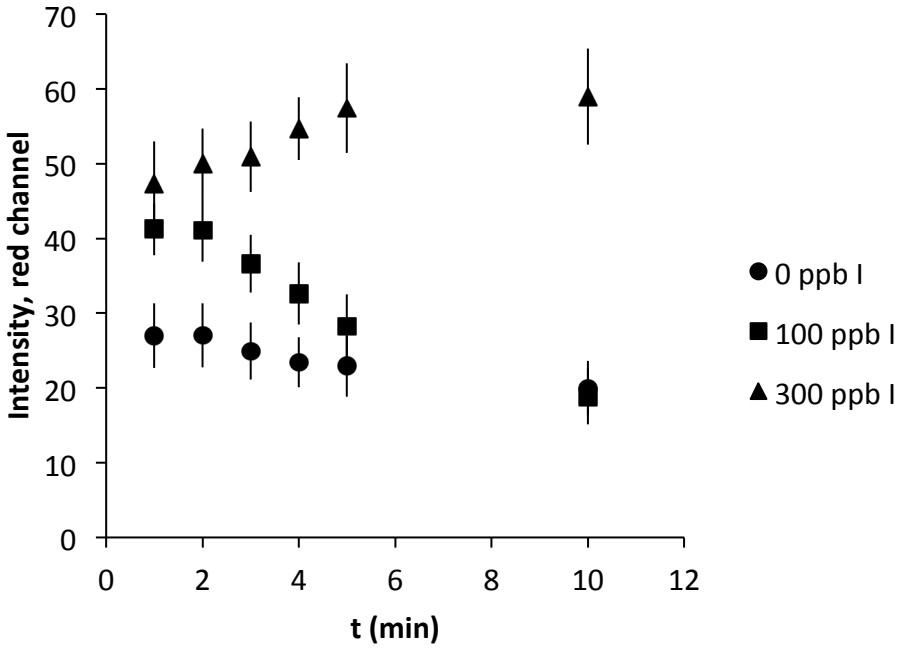


Figure C.1. ImageJ analysis of standards run on the test card. Only the red channel intensity was measured. The error bars are 1 SD of 3 replicate test zones. There is not good distinction at any time (95% confidence level).

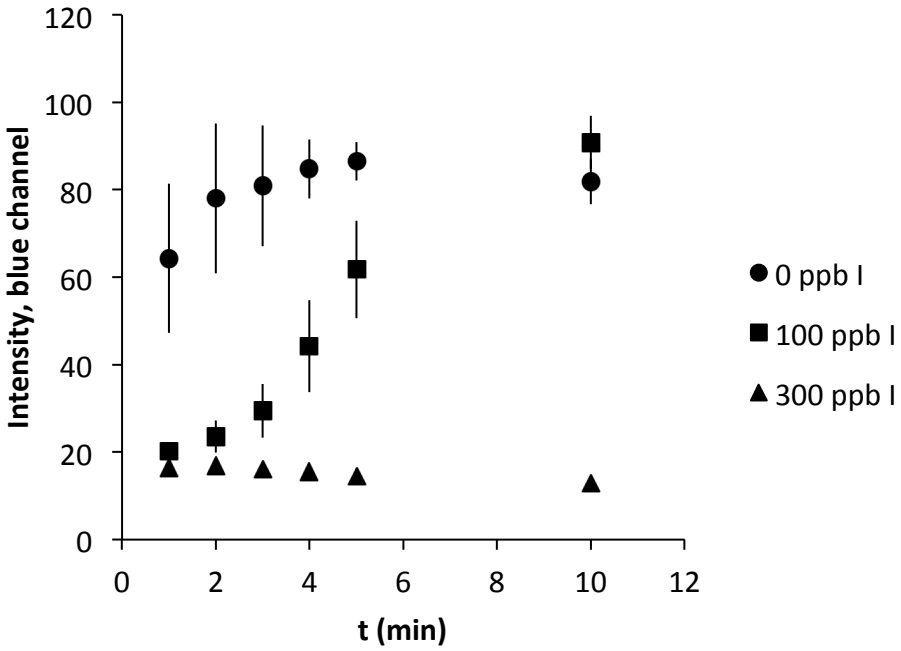


Figure C.2. ImageJ analysis of standards run on the test card. Only the blue channel intensity was measured. The error bars are 1 SD of 3 replicate test zones. There is not good distinction at any time (95% confidence level).

C.4 Inter-operator precision of the test card

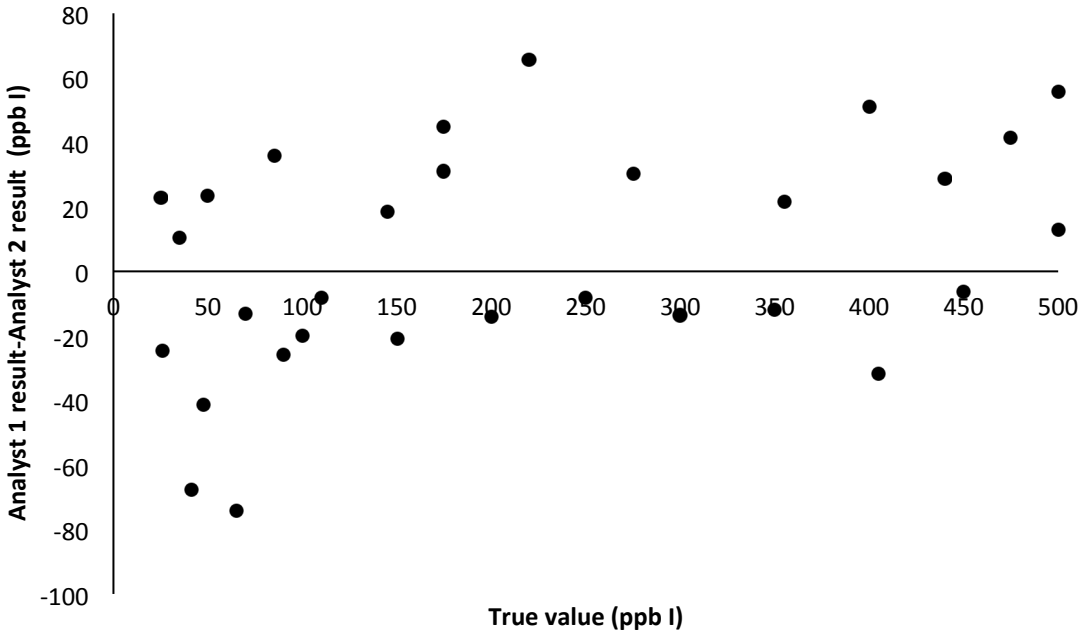


Figure C.3. Inter-operator precision. Each solution was analyzed by 2 people on separate test cards. There is no trend for one analyst to consistently predict higher concentrations than the other. There are 15 points above and 15 points below the x-axis.

C.5 Residual plot for the computerized image analysis of the test card

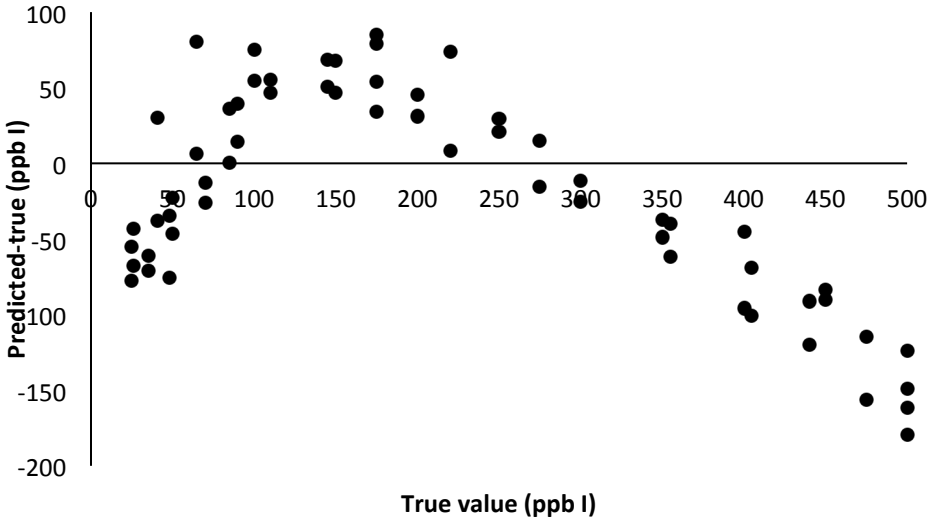
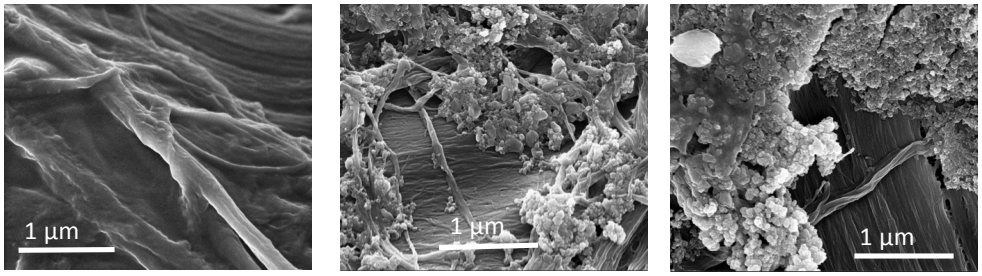


Figure C.4. The residual plot shows a systematic underestimation of iodide solutions that truly contain < 100 ppb I while solutions that contain 100-300 ppb I are overestimated. The readings above 300 ppb I are extrapolated, and the response may be saturated, so the residuals cannot represent a systematic bias.

C.6 Iron oxide storage in paper fibers



A. Paper

B. Paper with iron oxides

C. Paper with iron oxides after exposure to arsenic solution

Figure C.5. Scanning electron microscopy images. All images are to the same scale. A. An individual fiber of paper can be seen. B. When iron oxides are loaded onto the test card, they form clusters around the paper fibers. C. After adding the arsenic containing test solution, the iron oxides remain around the paper fibers.

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