

Paper Millifluidics Lab: Using a Library of Color Tests to Find Adulterated Antibiotics

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Cite This: <https://dx.doi.org/10.1021/acs.jchemed.9b00433>

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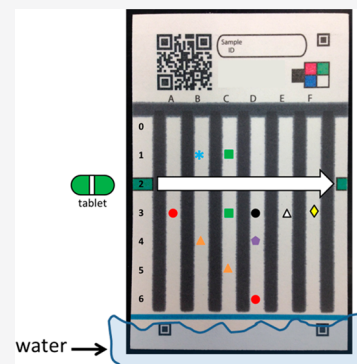
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ABSTRACT: A two to three period analytical chemistry experiment has been developed which allows second year students to explore chemical color tests used to detect adulterated pharmaceuticals. Students prepare several paper analytical devices (PADs) to generate positive and negative controls antibiotics, along with cutting agents such as starch and chalk. These PADs are used to identify the active ingredients and excipients in mystery tablets prepared by their classmates. In the second part of the lab, the students select an individual color test and design an experiment to quantify their mystery pill's active pharmaceutical ingredient (API). Nearly all of the student groups were able to successfully identify adulterants present in their mystery tablets. The quantification of the mystery tablets was also successful with all but one group calculating the correct concentration within 6%. In a postlab assessment, the students identified their largest gains in their ability to analyze data and other information, skill in science writing, and learning of laboratory techniques.

KEYWORDS: Second-Year Undergraduate, Analytical Chemistry, Laboratory Instruction, Collaborative/Cooperative Learning, Inquiry-Based/Discovery Learning, Problem Solving/Decision Making, Applications of Chemistry, Qualitative Analysis, Quantitative Analysis



INTRODUCTION

This two-period lab experiment explores organic and inorganic color reactions that have an important real-world application: detection of adulterated or substandard pharmaceuticals. The students prepare simple paper analytical devices (PADs) to carry out multiple color tests in parallel. In the first 3 h lab period, a total of 20 qualitative and 48 semiquantitative color tests are run to determine the presence or absence of various excipients and the quantity of an active ingredient, ciprofloxacin. Each student group then prepares a mystery pill containing 10–40% (w/w) ciprofloxacin and 90–60% (w/w) excipients. The students exchange mystery tablets and analyze them in the second 3 h lab period. This laboratory does not require instrumentation, which makes it an ideal lab for schools with limited resources.

Paper Analytical Device

Microfluidic devices are used frequently by scientific researchers in a multitude of applications in biology,^{1–6} chemistry and biochemistry,^{7–14} engineering,^{15–18} and physics¹⁹ but have yet to become popular in undergraduate curriculum. This is likely due to traditional microfluidic devices being quite complex to fabricate with hazardous etching materials and the need for photolithography, which limits the feasibility in teaching laboratories. A PAD is advantageous due to their low cost and the safe and easy fabrication. The PADs used in this experiment are the size of an index card (7 cm × 10.5 cm) that can be used to quickly and inexpensively identify an analyte in a complex sample, such as a pharmaceutical. The PADs contain 6 lanes,

separated by hydrophobic wax lines as shown in Figure 1. Different reagents are deposited on the lanes to detect binders, excipients, and active pharmaceutical ingredients (APIs).²⁰ The sample is swiped or rubbed across the PAD at the “swipe line” indicated on the card, and then the bottom of the PAD is placed into a shallow dish of water. As the water travels up the lanes, it brings the reagents in each lane up to a portion of the sample, and this carries out six simultaneous color tests.

Learning Outcomes

The learning outcomes (LOs) for this experiment are as follows:

- I. Gain experience with organic and inorganic color tests.
- II. Design and implement an analytical procedure to qualitatively characterize an unknown.
- III. Design and implement an analytical procedure to measure the linear range and limit of detection for ciprofloxacin.
- IV. Use computer software to collect data, generate calibration curves, and perform statistical calculations.
- V. Write up results (paper should clearly state a testable research question and provide evidence to support or refute the hypothesis).

Received: September 25, 2019

Revised: January 23, 2020

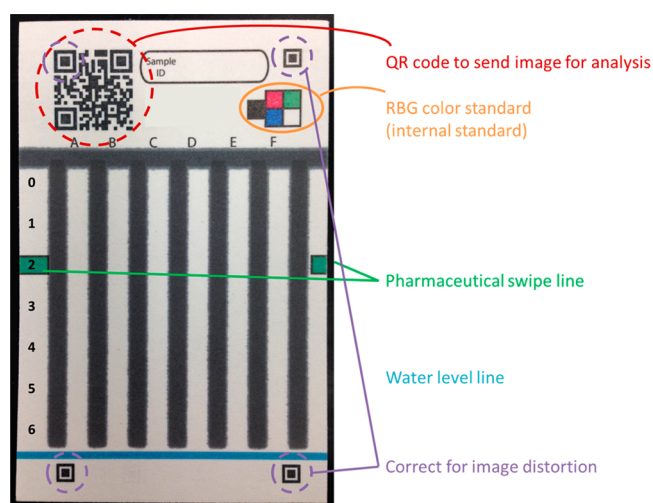


Figure 1. Paper analytical device. The hydrophobic lane outlines are printed with a Xerox ColorQube solid ink printer and then baked in an oven. Reagents are spotted in specific positions on the lanes depending on the colorimetric tests of interest.

MATERIALS

All reagents used to fabricate the PADs were purchased from Sigma-Aldrich. Ahlstrom 319 paper in 8.5 in. \times 11 in. sheets was obtained from Midlands Scientific (Chicago, IL, USA). Solutions needed for spotting the PADs are prepared in water unless otherwise stated: 2 M $\text{Co}(\text{SCN})_2$ (prepared using $\text{Co}(\text{NO}_3)_2$ and KSCN), 1 M tosic acid, 1 M CuSO_4 , 2 M K_2CO_3 , 0.2 M ninhydrin in acetonitrile, 2 M NaOH , 0.4 M NaNO_2 , 0.3 M *o*-nitroaniline in acetonitrile, 0.6 M triiodide in povidone (prepared using 2% povidone solution stirred overnight and added dropwise to I_2 and KI), and 0.3 M FeCl_3 . Amoxicillin, ampicillin, and ciprofloxacin were purchased as secondary reference standards from Sigma-Aldrich.

About 8% of individuals using health care services in the USA report a β -lactam allergy, typically to penicillin.²¹ This lab includes the very common antibiotics ampicillin and amoxicillin as test active ingredients in the first portion of the project. Students can minimize their exposure to β -lactam antibiotics in this lab by use of standard PPE (gloves, goggles, and lab coat),

using aliquots of 0.8 mg/mL ampicillin and amoxicillin rather than dry solid, working with β -lactam antibiotics in a fume hood, and by collection by the instructor of the PAD that contains β -lactam antibiotics (PAD no. 1) for disposal after the students have photographed it. The second part of the lab, where students design a procedure to quantify antibiotics in a mystery pill, uses a fluoroquinolone antibiotic, ciprofloxacin.

Printing and Baking the PADs

Prior to the lab, the instructor prepares blank PADs for the students using the files provided in the Supporting Information (SI). The PADs are printed on a laser inkjet printer to create the color standard box, quick response (QR) code, fiducials, and water level line. The hydrophobic lanes of the PADs are printed on a wax printer (Xerox ColorQube 8570) on both sides of the paper before baking the sheets in an oven at 100 °C for 7 min. Proper sealing of the lanes can be tested by placing a drop of water into a lane and holding the paper up to a light; if the lane is sealed, the water will not leak into neighboring lanes. If the lane is not sealed properly, the PAD is baked another 3 min and tested again. Once cooled the paper is cut to separate the eight PADs printed on each page.

Preparing PADs for Determining APIs

In the first phase of the lab, students carry out color tests to detect several functional groups found in the common antibiotics. They make three PADs to determine what a positive and a negative response look like for several APIs as well as several potential excipients or adulterants. The first card utilizes copper sulfate to detect the presence of the β -lactam ring and ninhydrin to detect primary amines found in both amoxicillin and ampicillin. The second card uses cobalt thiocyanate to detect tertiary amines and iron chloride which detects bidentate oxygen ligands, both of which are found in ciprofloxacin. The third card detects the electron rich phenols found in acetaminophen and salicylic acid using a diazonium salt. Since the diazonium is unstable, it is formed by depositing spots of acid, nitrite, and *p*-nitroaniline that are combined by the capillary action of the PAD, and a spot of base is used to neutralize the excess acid. The spotting guides for test cards 1–3 are shown in Figure 2. In order to observe the positive and the negative color changes, 4 μL of a 0.8 mg/mL solution of the indicated APIs is applied at position 2 on the PAD as shown in

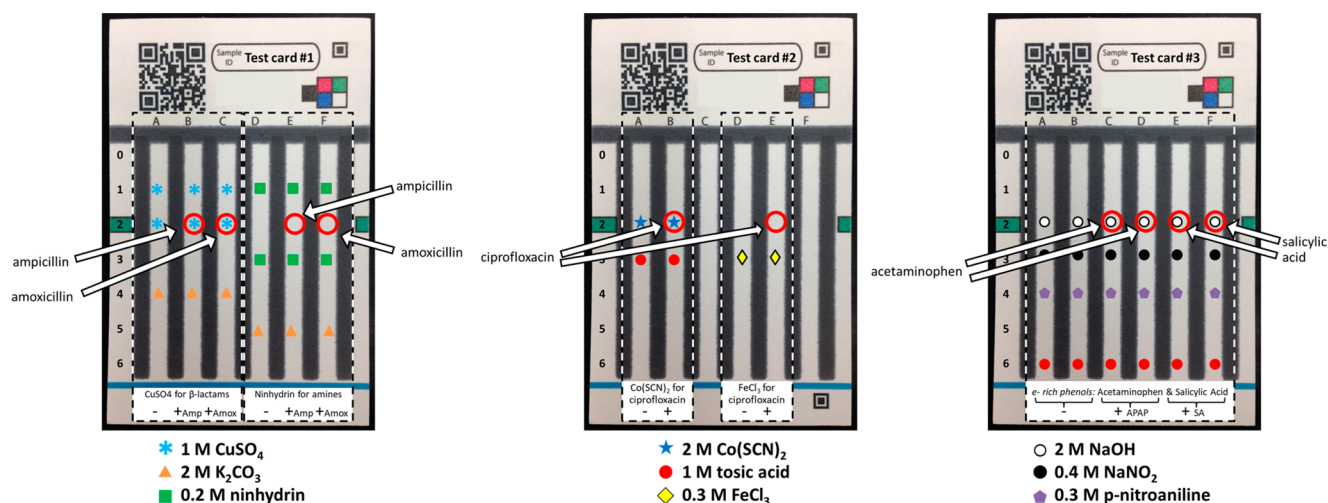


Figure 2. Spotting guide for PADs 1–3 used for testing APIs. All solutions were administered in 4 μL volumes with an automatic pipet. Solutions of API were applied at the positions marked by the red open circle.

Figure 2. Alternatively, a small portion of each of the solid APIs can be applied at position 2 using a clean spatula; as long as the white powder is visible in the lane, the correct lane results will be obtained.

The PADs are placed into a beaker containing approximately 1 cm of water. After 3–4 min, the water reaches the top of the line and the PAD is removed and allowed to dry on a clean piece of paper for 5 min before imaging (Canon PIXMA MG3620 color inkjet multifunction scanner). Alternatively, students could photograph the cards; cell phone cameras give good images.

Preparing PADs for Common Excipients

One PAD (test card no. 4) is set up to produce the positive and negative responses for common excipients such as carbonates and starch. This card uses iodine to detect starch and iron chloride to detect carbonates. The spotting guide for this PAD and its analyte deposition guide is shown in Figure 3.

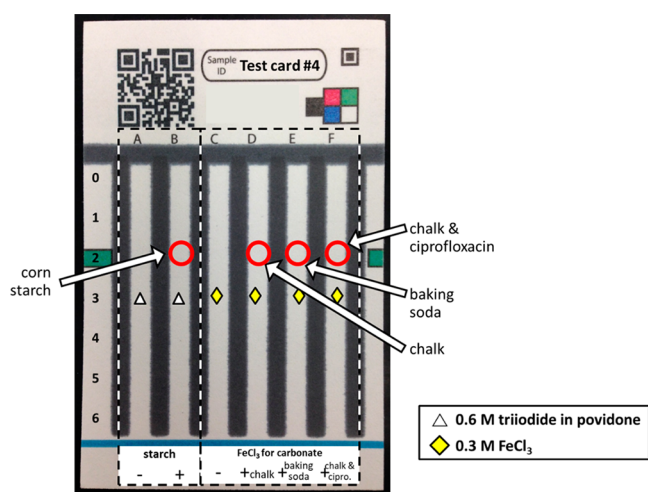


Figure 3. Spotting Guide for PAD 4 used for testing common excipients. All solutions were administered in 4 μL volumes with an automatic pipet. Excipients were applied at the positions marked by the red open circle.

Corn starch, chalk, baking soda, and a 1:1 mixture of chalk and ciprofloxacin were applied to the PAD in the marked locations using a clean spatula. The PAD was run per the procedure listed in the previous section.

Preparing the Complete PAD to Test the Mystery Pill

The complete PAD contained all of the color tests used to determine APIs and excipients in the mystery tablets prepared by the student's classmates. The spotting guide is shown in Figure 4. This PAD was sealed in a zipper-topped bag and stored until the following week (PADs can be stored in the zipper-topped bag for at least 3 months in the student's lab drawer).

Preparing the Mystery Pill

Students prepared a mystery pill containing 10–40% (w/w) of pharmaceutical secondary standard ciprofloxacin hydrochloride (PHR1044, Sigma-Aldrich). The other excipients in the pill were 10% (w/w) poly(vinylpyrrolidone) used as the binder, 5% (w/w) magnesium stearate as the lubricant, and one or two of the following as diluents: lactose, chalk, baking soda, or corn starch. The students prepared a total of 2 g of powder—enough mixture for two 1 g tablets—and mixed the powders together for 3 min in a mortar and pestle before placing 1 g of the mixture in a Parr pellet press (1/2 in. die set). Any debris from the press was removed before pressing their second pill. The tablets were stored until the following week.

Qualitative Analysis of Mystery Pill

To ensure that the correct weight percent of the pill could be calculated, the students weighed their tablets prior to any analysis. The mystery pill was applied by laying a clean piece of wire mesh across the PAD at position 2 and then scraping the tablet across the screen. The screening was then removed and a wooden splint used to press the powder into the paper. The PAD was then run and imaged as described in previous sections. The students compared the results of the mystery pill to the stored images showing their color standards generated using PADs 1–4 to identify the API and diluent.

Quantitative Analysis of Mystery Pill

Ciprofloxacin's 1,3-dicarbonyl group forms an iron(II) chloride complex with a strong orange color that has been used to assay ciprofloxacin in pharmaceutical dosage forms.²² This colorimetric assay can be adapted to the PAD format by spotting

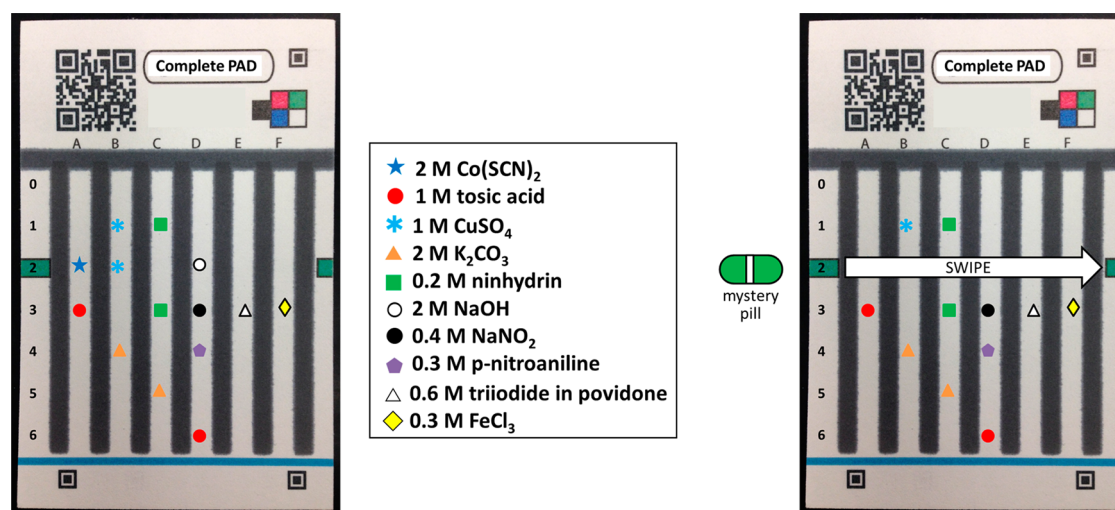


Figure 4. Spotting guide for complete PAD used for testing mystery pill. All solutions were administered in 4 μL volumes with an automatic pipette.

standard solutions of ciprofloxacin onto the iron(III) chloride lane, running the PADs, and analyzing the color intensity with ImageJ and Excel. A blank and five standards allow generation of a good calibration curve.²³ Each pair of students made seven PADs with the iron chloride test in each lane. Six of these PADs were used to generate replicate results for the calibration curve, and the final one was done to test the mystery pill solution. The students chose their ciprofloxacin concentrations for the calibration curve knowing that (a) the mystery pill would contain 10–40% (w/w) ciprofloxacin and (b) the solubility of ciprofloxacin in water is only 0.8 mg/mL. To generate the calibration curves, students could vary the application volume of standard solution (1–7 μL applied) or they would apply up to ten 2 μL aliquots, with each aliquot allowed to dry before application of the next. The PADs were run and imaged per the procedure described previously.

Carbonates from chalk or baking soda can interfere with the quantification of ciprofloxacin. If the qualitative assessment of the mystery pill found carbonates to be present, students were encouraged to prepare their second mystery pill sample in 1 M hydrochloric acid.

Image Analysis

The RGB intensities from the PAD images are used to generate a calibration curve and measure the amount of ciprofloxacin in the mystery pill. Students used ImageJ, the free software developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation.²⁴ The software quantifies the red, green, and blue (RGB) intensity on a scale of 0–255.

Each PAD image was inverted so that a higher color intensity correlated to a more intense color. Each lane was processed two ways: as a fixed rectangular region ($n = 7048$ pixels) that was moved from one lane to the next, or as a polygonal region ($n = 851$ – 5984 pixels) in which only the colored region in each lane was selected. The RGB color standard printed on each PAD was analyzed using a fixed rectangular selection ($n = 1296$ pixels). The RGB color intensities of the black box in the color standard had the lowest relative standard deviation (0.6–2.1%), so it was used as an internal standard to normalize the other color intensities.

HAZARDS

The first lab period involves the use of β -lactam antibiotics. Any allergies should be assessed prior to running the lab. Students who are allergic to β -lactam antibiotics should work in a hood to avoid possible inhalation hazards, and all students should wear gloves when handling the APIs. Students were instructed to collect waste in specific waste containers for proper disposal by the instructor.

For solutions used in preparation of the PADs, copper(II) sulfate may cause skin and severe eye irritation. Cobalt thiocyanate is a possible carcinogen and may be harmful if swallowed or inhaled. Potassium carbonate is harmful if swallowed and causes skin and eye irritation. Sodium hydroxide causes severe skin burns and eye damage. Ninhydrin is harmful if swallowed and causes serious eye and skin irritation. Acetonitrile is highly flammable and harmful upon skin contact or if swallowed. Sodium nitrite is a strong oxidizer and toxic if swallowed. *p*-Nitroaniline is toxic if swallowed, comes in contact with skin, or inhaled. *p*-Toluene sulfonic acid (tosic acid) causes severe skin burns and eye damage. Iodine causes skin, respiratory, and eye irritation and is very toxic to aquatic life.

Potassium iodide is harmful if swallowed and causes serious eye and skin irritation. Iron(III) chloride is harmful if swallowed, causes severe skin and eye irritation, and is toxic to aquatic life.

RESULTS AND DISCUSSION

After completing the qualitative analysis to familiarize themselves with the PADs, students were tasked with designing experiments to answer two questions about their mystery tablets “What excipients are present”, and “How much ciprofloxacin HCl is present?”. They worked in teams of two. The qualitative analysis took one 3 h lab period to complete, and the quantitative analysis took one to two 3 h lab periods, depending on the amount of instructor guidance through the experimental planning process. The experiment was conducted with 74 students over the course of two academic years.

First Lab Period: Qualitative Color Chemistry Tests

In the first part of the experiment the students generated color standards using known samples of pharmaceutical APIs and excipient. Figure 5 shows the expected positive and negative

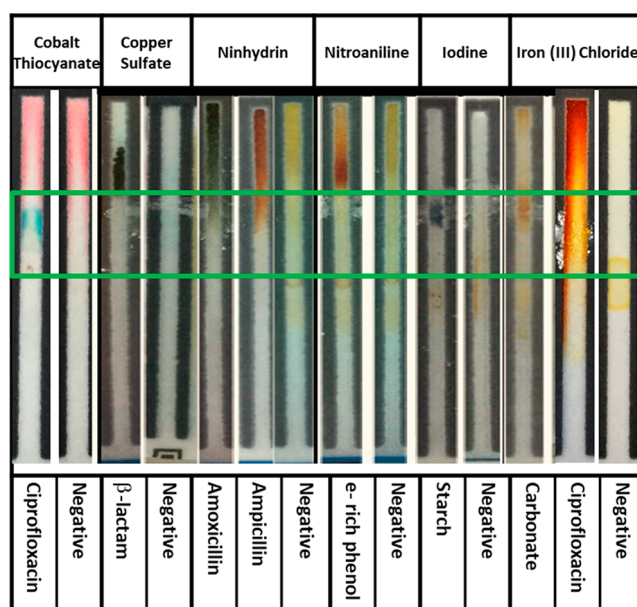


Figure 5. Color standards for qualitative analysis. Using these color standards, the students can discern the API and some of the possible excipients in the mystery tablets. The green box outlines the “swipe line” where the analyte was originally placed.

results for each color test on the PAD. Some of the colored products, such as the iron complex of ciprofloxacin, are chromatographically mobile and move up out of the “swipe line” area, while insoluble products remain at the swipe line. Students should therefore note the location as well as the color of each reaction product they observe. Colors that appear below the swipe line are meaningless.

A positive test for the cobalt thiocyanate lane is indicated with a blue color at the swipe line. The copper sulfate test turns dark green in the presence of β -lactam antibiotics. The ninhydrin test turns green with amoxicillin and red-orange with ampicillin. A positive result for the nitroaniline test is a red to brown color for samples such as acetaminophen and salicylic acid. Starch is noted by the presence of a black color in the iodine lane. Iron chloride turns a faint orange with carbonates and a deep red-orange with ciprofloxacin.

Ciprofloxacin was used as the API for the mystery tablets to avoid any possible hazards for students with β -lactam allergies. The PADs can detect the presence of chalk, corn starch, and baking soda, but not lactose. Chalk and baking soda both interfere in the quantification of ciprofloxacin, so the students were warned to take the presence of these adulterants into account when preparing their mystery tablet for quantification.

Second Lab Period: Qualitative Analysis of Mystery Tablets

Table 1 shows the results of the individual student groups. The two groups that had starch present successfully identified that excipient on the complete PAD.

Table 1. Student PAD Qualitative Data

Student Group	Excipients Present	Positive Carbonate Spot Test Observed	Excipients Observed on PAD
1	Baking soda and starch	Yes	Starch
2	Baking soda and starch	Yes	Starch
3	Baking soda and lactose	Yes	None
4	Baking soda and chalk	Yes	None

A spot test involving a drop of 1 M hydrochloric acid on a small amount of solid mystery pill in a watch plate allowed for screening for the presence of carbonates. The spot test successfully demonstrated the presence of carbonates for all student groups; thus all groups prepared their samples in 1 M hydrochloric acid to remove this interferent.

Calibration Curve Results

The first step in analysis of the colorimetric results was to select a color channel to build the calibration curve. Since the PADs were not all imaged as one JPG file, an internal color standard was necessary to correct for possible inconsistencies in imaging. The data were normalized by dividing the mean inverted color intensity from each color channel of the iron chloride lane by the mean inverted color intensity of the black color standard square. To determine which color channel had the greatest sensitivity, all RGB results were plotted against the ciprofloxacin concentration. The sensitivity curves are shown in Figure 6. The curve with the best linear fit was the normalized blue signal acquired using the fixed rectangular sampling; thus, this was the method used to generate the calibration curve for further analysis.

The calculated limit of detection (LOD) and quantification (LOQ) are 17.1 and 56.8 nmol, respectively, for the instructor data using the normalized blue signal acquired using the fixed rectangular sampling. Equations 1 and 2 show the formula used to calculate LOD and LOQ.

$$\text{LOD} = \frac{3\text{SD}_{\text{blank}}}{m} \quad (1)$$

$$\text{LOQ} = \frac{10\text{SD}_{\text{blank}}}{m} \quad (2)$$

Table 2 shows the results of the individual student groups.

The students' data are fairly linear with most of the R^2 values around 0.9. All student groups reported that the PADs could be used to quantify ciprofloxacin on the basis of the color intensity of their mystery pill. Student groups 1 and 2 used up to 10–2 μL aliquots of a standard ciprofloxacin solution to increase their calibration curve range, while groups 3 and 4 changed the aliquot size from 1 to 7 μL to generate their range. The latter approach gave superior LOD and LOQ metrics.

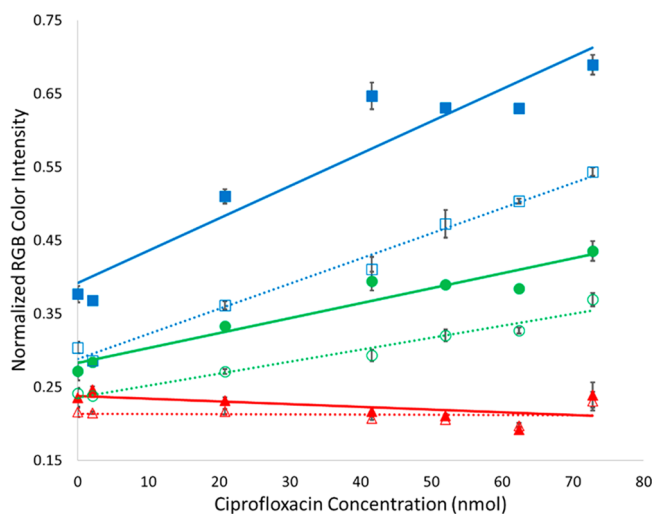


Figure 6. RGB sensitivity curves for Fe-ciprofloxacin test. Fixed (dashed) and polygon (solid) analysis techniques are plotted for the signals in red triangles, green circles, and blue squares. The best-fit regression line was $\text{blue}_{\text{norm}} \pm 0.01_3 = (0.0034_2 \pm 0.0001_8) \text{ nmol}_{\text{cipro}} + (0.28_9 \pm 0.008_2)$, and the coefficient of determination (R^2) was 0.986. Error bars are standard error (s/\sqrt{n}), where $n = 6$.

Table 2. Student PAD Quantification Data

Student Group	Calibration Curve Range, nmol	R^2	Calculated LOD, nmol	Calculated LOQ, nmol
1	10.3–61.9	0.929	19.8	66.1
2	13.0–64.8	0.713	18.1	60.2
3	4.9–17.1	0.875	1.2	4.1
4	3.2–20.0	0.912	2.6	8.5

Quantification of Mystery Tablets

Students dissolved a portion of their mystery tablets to prepare a 1 mg/mL solution in 1 M HCl. Using their calibration curves, the students quantified their mystery tablets. The students convert the calculated concentration from the calibration curve from nanomoles to mass using the molecular weight of ciprofloxacin and the volume of solution applied. The mass of ciprofloxacin in the sample is then divided by the mass of the mystery pill used to prepare the solution to get the weight percent of the unknown sample. Table 3 summarizes the results of their analysis.

Success of the Learning Outcomes

The students designed an experiment generating calibration curves for the iron chloride test, which allowed them to determine the PAD's LOD for ciprofloxacin along with the concentration of their mystery pill. When generating the calibration curves, students utilized the LINEST function in Excel to determine the variation in their results. The students learned quickly that ciprofloxacin does not have good solubility, which limited their ability to generate standard calibration curves. They overcame this issue by finding an appropriate solvent, dilute hydrochloric acid, and pipetting multiple aliquots of the standard solution on the PAD to generate a calibration curve covering their dynamic range. Most of the students' pill results were not significantly different than the expected results. The students learned how to normalize the color intensity data using an internal standard printed on the card. This allowed for discussion about what qualities make a good internal standard. As part of the laboratory assignment, the students were asked to

Table 3. Student Mystery Pill Analysis

Mystery Pill	Calculated Concentration		s_{conc}		Expected Concentration		Error, %
	nmol	% (w/w)	nmol	% (w/w)	nmol	% (w/w)	
1	49.6	37.4	9.2	6.9	52.9	39.9	-2.5
2	29.1	19.3	6.9	4.6	37.7	25.0	-5.7
3	9.9	24.7	1.9	4.7	9.5	23.7	+1.0
4	13.5	41.9	1.4	2.2	9.8	30.2	+11.7

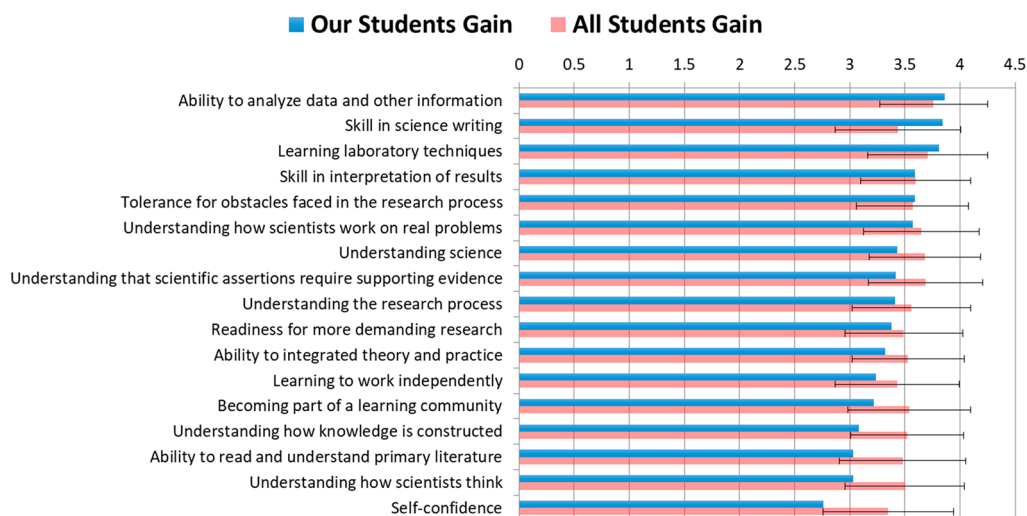


Figure 7. CURE results for student after the experiment. The students were asked their level of gain in each learning outcome with the average score reported. Error bars are standard deviation.

write a research paper that summarized their designed experiment, results, and interpretation.

While this experiment provided great opportunity to demonstrate analytical techniques in experimental design and analysis, the lab gave the students opportunities to further develop their scientific identity. Most of the students in this lab were second or third year undergraduate students that are considering whether to pursue science majors. To determine the students' attitudes toward science before and after the lab, the students took the CURE survey. The CURE survey was developed by Grinnell College, Hope College, Harvey Mudd College, and Wellesley College to measure student experiences in "research-like" or other science courses.²⁵ The students were asked to rate a series of learning gains on a scale of 1–5 with 5 being a large gain. Figure 7 summarizes the results of the learning gains along with a comparison to all students that have taken the CURE survey from Jun. 1, 2015 to May 24, 2016.

Overall, the students claimed to have an average gain in all of the learning gains, which is comparable to all of the students who have taken the survey. The students said that their largest gains from this lab were in their ability to analyze data and other information and their skill in science writing and in learning the laboratory technique. The results suggest that the students understood how to solve problems using analytical techniques and they understand how scientific research progresses. These students had the opportunity to get a glimpse of scientific research through this experiment, which allows them to reflect on their experience to decide whether they could see themselves pursuing science.

CONCLUSION

The PADs were able to simulate a real-world analytical problem of having to identify an unknown product. The students

designed an experiment to solve their mystery pill composition in small groups with guidance from their instructor. All groups completed the goals of determining the limit of detection for the PADs and communicated this through a written scientific report at the end of the experiment. The students met the learning outcomes with their largest gains in data analysis, scientific writing, and laboratory technique.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at <https://pubs.acs.org/doi/10.1021/acs.jchemed.9b00433>.

Notes for instructors and student handout (PDF, DOCX)

PAD printing sheets (PDF)

Six lane wax outlines (PDF)

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<https://pubs.acs.org/10.1021/acs.jchemed.9b00433>

Notes

The authors declare the following competing financial interest(s): One author, M.L., holds a patent (US 009354181B2) on the paper analytical devices (PADs) used in this study. Licensing negotiations for this patent with a U.S. company are underway. The U.S. patent will not prevent others from manufacturing or selling PADs.

ACKNOWLEDGMENTS

We acknowledge the laboratory coordinator at Hamline University, Marina Gorina, for her assistance in gathering all of the required materials for the experiment.

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