Electronic Supplementary Material (ESI) for Analytical Methods.

Supporting Information for

Detection of Degraded, Adulterated, and Falsified Ceftriaxone Using Paper Analytical Devices

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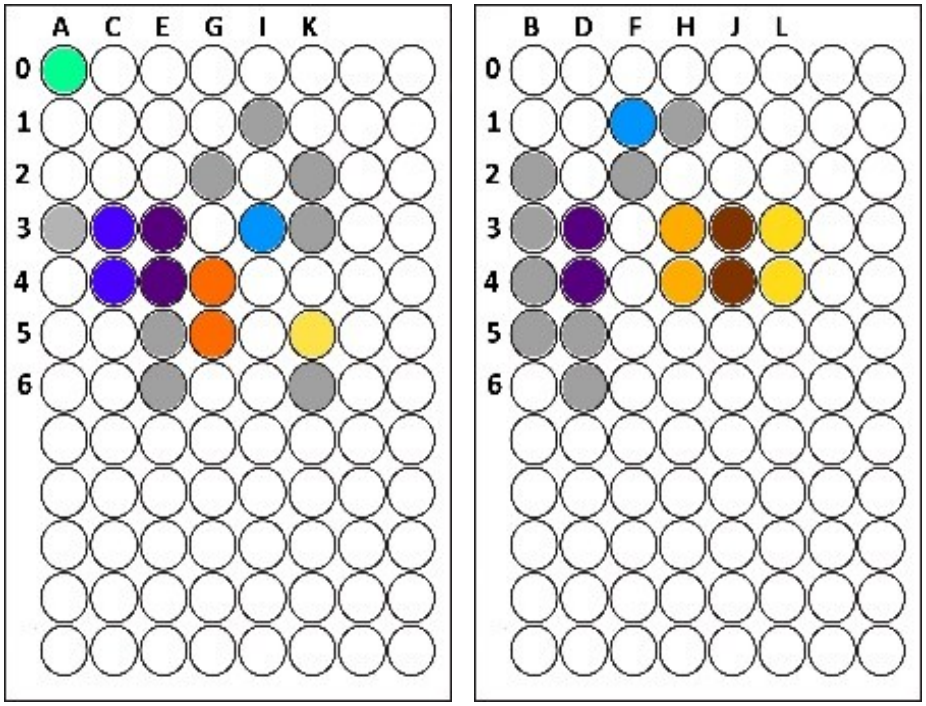
S1. PAD fabrication

The PAD is printed with front and back wax on a Xerox ColorQube 8570N printer, then is baked in a drying oven with paper towels shielding the metal racks for 7 minutes at 100°C to allow the wax to reflow. To check the sealing of the front and back wax, several lanes on each page were tested with drops of DI water to see if the water could no longer flow into adjacent lanes. The front laser features are then printed using any desired printer (an inkjet or the ColorQube can be used since the paper has already been baked). Finally, each 8.5 x 11" sheet of paper was stamped with reagents as shown below, and cut into eight PADs.



Lane	Row	Reagent
A	0	Nickel chloride
	3	Dimethylglyoxime
B	2/3	Ninhydrin
	4/5	Potassium carbonate
C	3/4	Biuret reagent
D	3/4	Cobalt thiocyanate
	5/6	Tosic acid (HOTs)
E	3/4	Cobalt thiocyanate
	5/6	Tris buffer (pH 8)
F	1	Copper sulfate
	2	Potassium carbonate
G	2	2 M Sodium hydroxide
	4/5	Sodium nitroprusside
H	1	4 M Sodium hydroxide
	3/4	Folin's reagent
I	1	2 M Sodium hydroxide
	3	Copper sulfate
J	3/4	Triiodine-povidone
K	2	2 M Sodium hydroxide
	3	Tosic acid (HOTs)
	5	P-Nitroaniline
	6	Sodium nitrite
L	3/4	Iron chloride

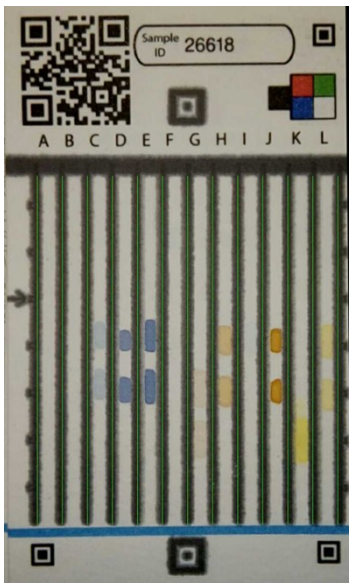


Figure S1. Reagents used to stamp PAD.

Reagents are stored in two 96-well plates. The 9 mm spacing of the spoke inoculation manifold and the 4.5 mm spacing of the lanes means that two 96-well plates and two stamping tools are necessary for high production rate. The reagents in row 2 of the 96-well plates are deposited on the swipe line of the PAD, which is indicated by the arrow printed on the sides of the PAD card.

S2: Overview of reagent chemistry in each lane

Lane A: Timer lane. Water moving up the lane by capillary action carries a chelating agent up to meet a spot of nickel(II) at the top of the lane. A pink dot visually indicates that the water has moved all the way up the lane.

Lane B uses ninhydrin to detect primary amines. Although ninhydrin usually is heated at 100°C to force the formation of Ruhrman's Purple, at room temperature and in water the reaction stops after the formation of a Schiff base, whose color can range from yellow (ampicillin) or orange (isoniazid) to green (amoxicillin).

Lane C uses the Biuret reagent, which forms a green or blue colored compounds when a copper (II) ion coordinates to multiple amide groups. (11)

In lanes D and E, cobalt thiocyanate dianion pairs with two protonated tertiary amines, forming an insoluble blue or green ion-pair; the two lanes are set up to do this reaction at either acidic or neutral pH, which allow some differentiation between tertiary amines. (12,13) Hydrophobic secondary amines also react, but tend to give more soluble ion pairs, so the blue or green color is often washed out in the center of the lane but visible near the wax boundary.

Lane F uses base and copper to detect the beta lactam functional group present in ceftriaxone and many other antibiotics. A dark green insoluble complex of unknown structure is formed.

Lane G contains sodium nitroprusside, which reacts with nucleophiles to give either addition to the electrophilic NO group, or substitution of this group.

Lane H detects free thiols and other nucleophiles via a substitution mechanism with sodium 1,2-naphthoquinone-4-sulfonate (Folin's reagent). (16)

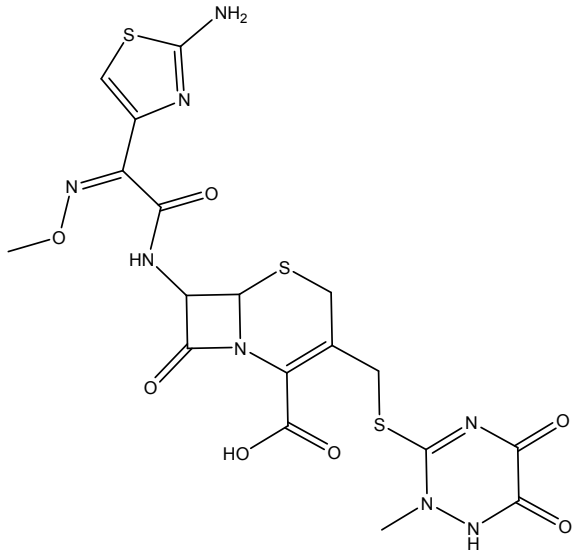
Lane I includes copper and base in locations designed to detect the TB drug ethambutol.

Lane J contains stabilized tri-iodide ion to detect starch.

Lane K typically detects phenols via a diazonium salt mechanism. Ceftriaxone lacks a phenol, but still gives a color change in this lane because the thiazole group can be diazotized under the reaction conditions.

Lane L contains iron (III) which readily coordinates to 1,3 dicarbonyl compounds such as ciprofloxacin or levofloxacin, and to polyphenols found in many natural products.

Structure of Ceftriaxone



S3. HPLC system suitability and analysis of ceftriaxone

System suitability testing was performed following USP <1225> and USP <1226>; briefly, this involves measuring analytical metrics for the HPLC chromatogram, establishing the linear range, testing accuracy and precision, and performing a matrix spike-recovery experiment to assess sample preparation and resolution of degradation products from the ceftriaxone peak.

SI Table 1. Ceftriaxone System Suitability Results.

System Suitability Test	Result
Precision	0.8%
Linearity	R2 = 0.998
Accuracy and Range	120%: 122%, 100%: 99%, 50%: 50%
Spike Recovery	100.7%

Example Chromatogram:

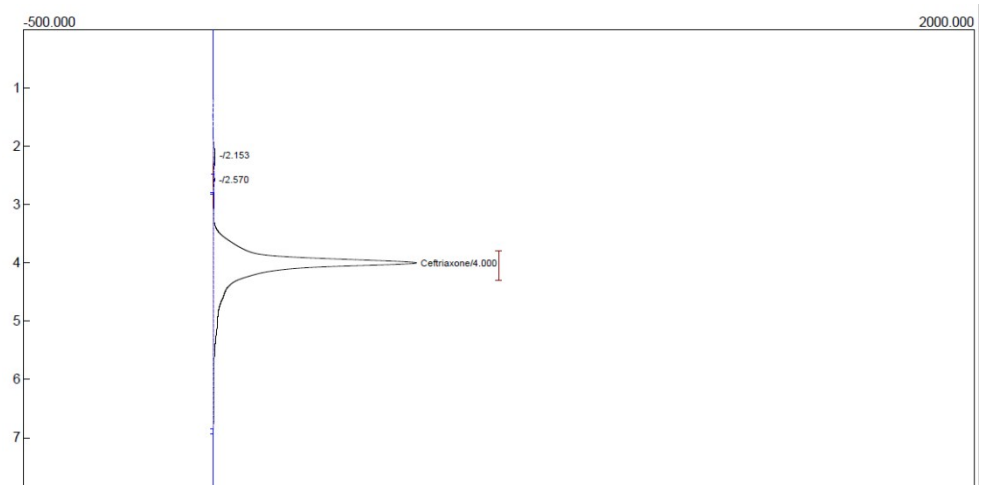


Figure S2. Typical HPLC Chromatogram for Ceftriaxone. The retention of ceftriaxone is 4.0 minutes.

S4. Thermal degradation of ceftriaxone

When a solution of 110 mg/L ceftriaxone stands at 23 °C, it undergoes hydrolysis and other degradation reactions, as shown in Figure 2.

Figure S3. HPLC Chromatogram After Thermal Degradation.

The peak area of ceftriaxone was tracked until the concentration was too low to be detected. Three degradation peaks developed throughout the course of the study.

To identify the degradation products LC-MS was completed via product catching:

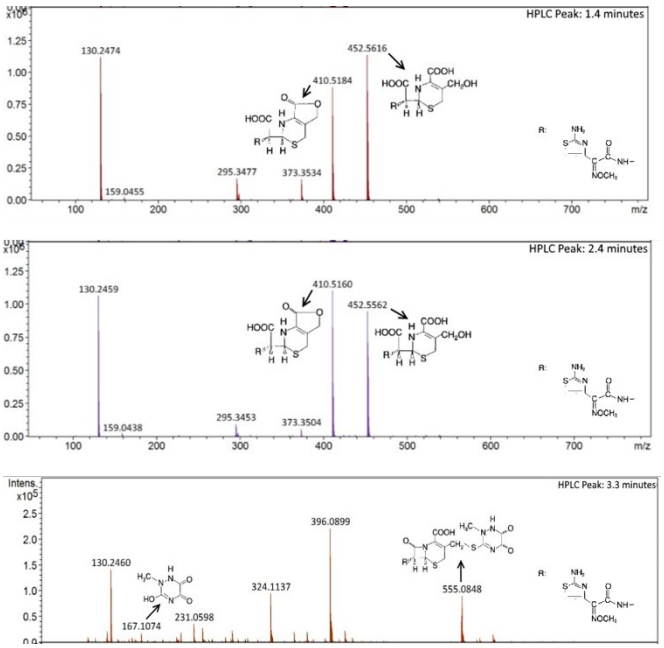


Figure S4. Mass Spectra from Degraded Ceftriaxone Standard.

The top spectrum is the HPLC peak at 1.4 minutes which is mainly the trans degradation product. The middle spectrum is the HPLC peak at 2.4 minutes which is mainly the cis degradation product. The bottom spectrum is a heterocyclic degradation product as well as some residual ceftriaxone in the HPLC peak at 3.3 minutes.

Characteristic colors for ceftriaxone are seen in lanes F, G, H, K, and L. ImageJ (NIH) software was used to quantify the red, green, and blue (RGB) intensity of color spots appearing in each lane during thermal degradation of the antibiotic. The mean intensity was determined by first, inverting the image (so large numbers correspond to high color intensity), selecting a square of 40 by 40 pixels around the strongest part of the colored spot, and then collecting the mean RGB color values. Lanes F and H showed systematic changes in R, G, or B intensity during degradation and were selected for input into the PCA analysis. Lane G, K, and L did not show strong trends during degradation, and therefore were not used in subsequent image analysis.

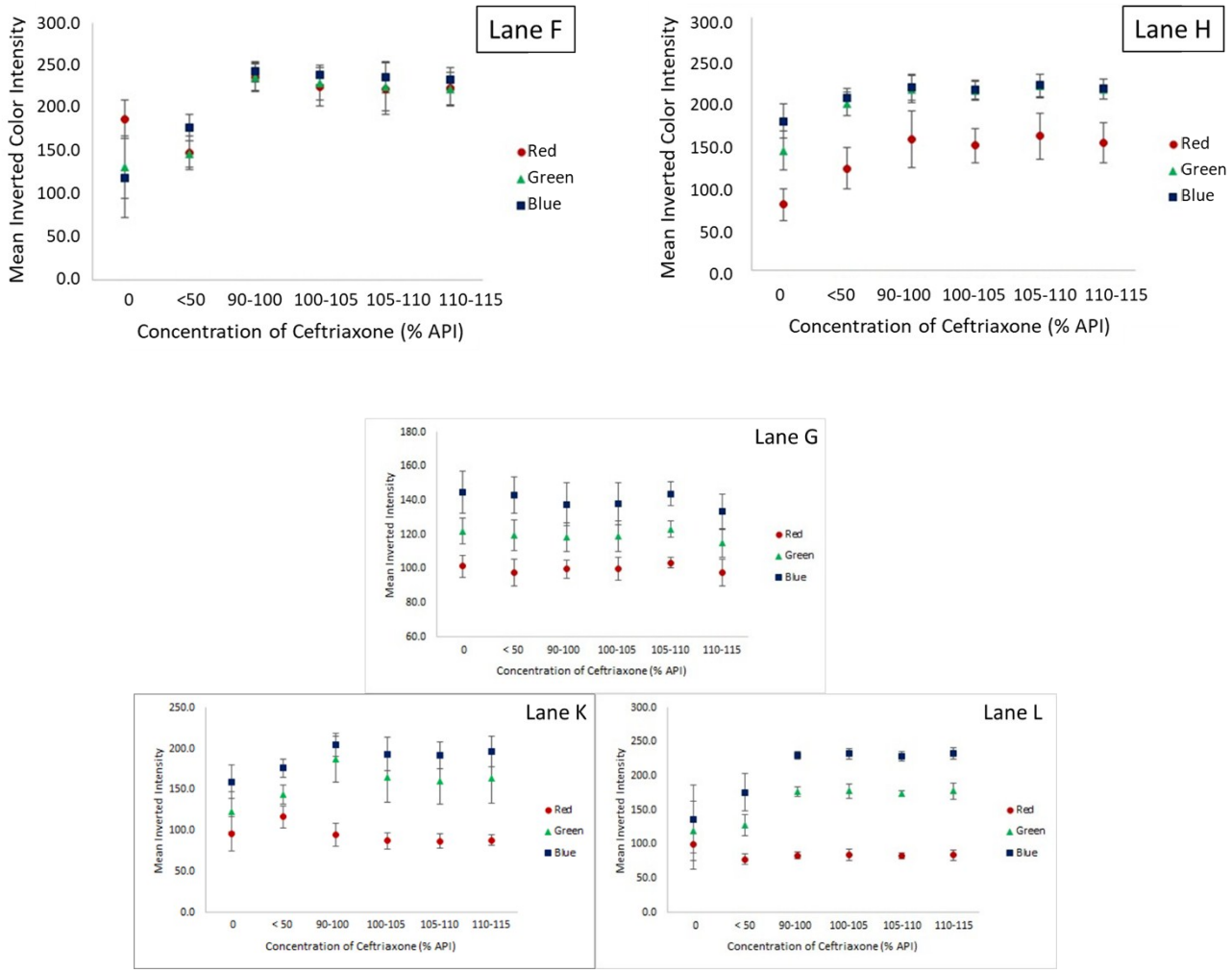


Figure S5. Image analysis for ceftriaxone during thermal degradation.