ABSTRACT: Cocaine (COC) is one of the most widely consumed illegal drugs around the world. Street COC is commonly adulterated with pharmaceutical compounds that mimic or intensify the COC’s sensory effect. Adulteration is performed to increase the profit of criminal organizations and each one has their own way of doing it. Therefore, determining the composition of seized COC samples (chemical profile) provides evidence for the police to track criminal organization networks and their activity patterns. Using filter paper as a substrate, we developed a multiple detection paper-based analytical device (PAD) that combines colorimetric and electrochemical measurements to discriminate COC samples according to adulterant’s content. A regular graphite lead modified with a gold film made from Au leaf (graphite/Au) to improve electron transfer was used as a working electrode. Silver and Ag/AgCl were used as auxiliary and reference electrodes, respectively. The colorimetric device was patterned using a laser cutter and coupled to the electrochemical device using a double-sided tape, allowing simultaneous analysis to gather more analytical information about COC samples. Graphite/Au was characterized by scanning and transmission electron microscopies and electrochemical assays. The simultaneous colorimetric and electrochemical analyses combined to principal component analysis improved the analytical characterization of COC trial samples and provided a fast discrimination based on the assembled database.

1. INTRODUCTION
Cocaine (COC) is a tropane alkaline-based compound that has stimulating properties and is used by 18 million people aged 15—64 around the world in 2017. Its production is concentrated in the Andean countries and has been growing in recent years (from 650 tons in 2013 to 1300 tons in 2017). The leading illegal destinations of production are developed countries. Individual analysis of seized drugs in 2017 indicates that 93% of the COC in the United States was of the Colombian origin, 4% from Peru, and 3% from an unknown source.

The physical (packaging and appearance) and chemical (major and minor alkaloids, purity, chemical class, and cutting agent—diluent or adulterant) analyses of the seized COC sample provide its chemical—forensic profile. Cataloged in large databases together with detailed data—criminal cases, sample mass, and date and place of seizure—such profiles provide strategic evidence to track the criminal organization networks and their activity patterns, as well as the structure and evolution in time and space in the illicit drug market. Criminal organizations often blend diluents such as sugar, starch, and carbonates8 or pharmaceutical compounds as adulterants of COC. Some of these adulterants are phenacetin and aminopyrine (AMI), which have an analgesic and antipyretic effect. At the same time, they are carcinogenic and toxic to some extent, being banned in some countries. Besides that, other chemicals such as levamisole, paracetamol, caffeine, procaine, benzocaine, boric acid, and lidocaine7—9 can be added, and each criminal organization has its own approach to adulterating or adding compounds to the drug. These substances dilute the drug raising the organization profits. Furthermore, adulterants can mimic or intensify the pharmacological effect of COC.

In a crime scene, the found substances can be examined by presumptive color tests to start an inquiry in the field. One of those tests is based on the well-known Scott reaction, where
the product between cobalt thiocyanate and COC generates blue color. However, this reaction is susceptible to produce false-positive results because of interferences of pharmaceutical compounds. In this sense, Tsumura et al. have modified the Scott test aiming for interference minimization in COC detection. Using the electrochemical method, electro-chemiluminescent-based sensors have been developed for selective detection of tropane alkaline-based compounds. In addition, the paper-based analytical devices (PAD) have drawn attention for being quickly built to improve chemical analyses and the potential to be used in-field with portable detectors such as a smartphone and a potentiostat. Thus, allowing more accurate screening analysis even in the absence of well-trained professionals mediated by chemometric pattern recognition.

The outstanding characteristics of PADs, such as low cost, simplicity, easy-to-use, and fast analysis time, attracted the researchers’ attention. The paper fibers have microfluidic properties that can be explored to separate a mixture of compounds and are an excellent platform for storing chemical (bio)reagents. Their surface can be easily modified to generate controlled flows of fluids or to confine liquid solutions to carry out spot tests. In 2007, Whitesides’ group brought up the use of paper for colorimetric analysis of glucose and protein without the need for external equipment and in 2009, the group proposed a cheaper approach using wax printing. In 2009, Henry’s group coupled the electrochemical detection to portable paper devices, providing higher selectivity and, mainly, improved sensitivity as compared to the colorimetric detection method.

In fact, both types of detection, colorimetric and electrochemical, can complement each other allowing the analysis of heavy metals for environmental application with high sensitivity for chemical species that could not be detected by a simple color change. Recently, our group demonstrated the use of electrochemistry for sample processing followed by colorimetric determination of low concentrations of COC adulterants on a microfluidic PAD (µPAD).

In this work, we present a paper-based device as an analytical platform combining colorimetric and electrochemical detections (Color-ePAD). The device was fabricated using a filter paper and hydrophobic wax barriers to delimit the wells to accommodate the electrodes. A second layer for colorimetric detection, in which the PAD was patterned using a laser cutter, was attached to the electrochemical device using a double-sided tape. A common three-electrode cell configuration was used. A regular graphite lead modified with a gold film (graphite/Au) prepared from Au leaf (usually employed in decorative crafts and wedding candies) was used as a working electrode. Silver and silver/silver-chloride were used as auxiliary and reference electrodes, respectively. The developed device was used for proof-of-concept analysis of the COC samples and for a fast-qualitative discrimination of the samples according to their composition, which was carried out through principal component analysis (PCA). To the best of our knowledge, this is the first time that both detection strategies are performed simultaneously to obtain distinct and complementary information about the sample.

2. MATERIALS AND METHODS

2.1. Reagents. All solutions were prepared using analytical grade reagents and deionized water with a resistivity of 18.2 MΩ cm (25 °C) (Direct-Q SUV, Merck Millipore, Massachusetts, USA). The reagent 4-dimethyl-aminoantipyrine (AMI) was purchased from Alfa Aesar (Massachusetts, USA). Orthophosphoric acid (85%) and cobalt(II) thiocyanate (96%) were acquired from Merck (Darmstadt, Germany). Anhydrous monomosophosphate was purchased from Synth (Diadema, Brazil). Sulfuric and hydrochloric acids were purchased from J. T. Baker (Phillipsburg, USA).

The gold leaf 23 kt (79 × 79 mm) was purchased from Nazionale Battitura Metalli srl (Milan, Italy). A qualitative filter paper (grammage 80 g m−2; thickness: 0.16 mm; particle retention: 4–12 μm; filtration speed: 20–25 s; from Unifil, Rio Grande do Sul, Brazil) of size 60 × 60 cm was cut into A4 size (210 × 297 mm) to fit into the wax printer. Whatman Grade 1 qualitative cellulose filter paper was purchased from GE Healthcare (Chicago, USA). The 0.9 mm thick graphite leads, 2B (from CIS, São Paulo, Brazil), were purchased from a local market. Ag ink was acquired from Joint Metal LTDA (Diadema, Brazil). The seized COC samples were granted by the Criminalistics Institute of the State of São Paulo (Brazil) via a license with the Institute of Chemistry. The seized COC purity (92%) and composition was determined by gas chromatography with flame ionization detection. The samples for use with the Color-ePAD were ground in a mortar and pestle and then dissolved in the phosphate buffer solution (PBS) before analysis.

2.2. Preparation of Au Film for Graphite Modification. The precursor reagent, AuCl4−, was obtained by dissolving 12 mg of Au leaf with a purity grade of 88% wt/wt in 100 μL of orange color reverse aqua regia (HNO3/HCl; 3:1 vol/vol). The resulting yellow solution was diluted to 44 mL with 0.5 mol L−1 H2SO4 aqueous solution, obtaining a 1.2 mmol L−1 AuCl4− solution.

Prior to the electrodeposition of the Au film, the graphite lead was prepared following the steps shown in Figure S1. First, the graphite surface was polished with a piece of paper (not shown in the Figure S1). Then, (step 1) ~2 mm of one end of the graphite lead was protected with a 2 × 10 mm piece of a blue adhesive tape (3M) cut with a CO2 laser engraver (Work Special Laser). The laser wavelength was 10.6 μm and the pulse duration was ∼14 μs. The remaining exposed area on the tip of the graphite was protected with an extra piece of the nonprotected area of the graphite was covered by painting with a waterproof spray (Spray Water Proof, Global) and left to dry, 15 cm apart from an infrared lamp (PAR38E 150 W, Philips) for a few minutes. Finally, (step 4) the tape was removed defining the area for electrodeposition of the Au film.

The electrodeposition of the Au film was carried out by chronoamperometry (CAMP) in the AuCl4− acidic solution, under vigorous stirring with a magnetic bar, at room temperature. The Au film deposition was evaluated at −1, −2, −3, and −4 V versus a conventional Ag/AgCl (KCl 3 mol L−1) reference electrode during 120 s. The most uniform film was obtained when −4 V was applied for 120 s using a Pt auxiliary electrode. Potentials beyond −4 V could be evaluated because potentiotstat-current range was limited up to 1 A. Throughout the electrodeposition, utmost care was taken to position the three electrodes as close as together. A potentiotstat PGSTAT 128N (Metrohm, Barendrecht, Nederland) was used for all electrochemical experiments.

2.3. Fabrication of the Color-ePAD. The ePAD device was prepared using a filter paper patterned with a wax barrier using a wax printer (Xerox ColorQube 8570) (Figure 1A). The
printed paper was then exposed to a hot plate (Hobby Line MetaInox) at 80 °C long enough to allow the melted wax to penetrate the three-dimensional structure of the paper, forming hydrophobic barriers. The template was drawn using Power Point software (Microsoft). It consisted of a 9 × 33 mm rectangular frame around three aligned circular-hydrophilic zones 6 mm apart from each other; two of them with 5 mm diameter and another one with 6 mm (right end of Figure 1A). They were interconnected by 1.5 mm wide microfluidic channels. Silver tracks were painted with silver ink onto the hydrophilic zones at both ends of the PAD to create the auxiliary and the reference electrodes. The Ag track in the 6 mm-diameter zone was oxidized to AgCl in a 0.1 mol L⁻¹ HCl solution by cyclic voltammetry (CV). The CV procedure was performed at scan rate of 100 mV s⁻¹ for five cycles, varying from −0.1 to 0.15 V (vs conventional Ag/AgCl (KCl 3 mol L⁻¹) reference electrode) with end potential at 0.14 V. Figure S2 shows an Ag/AgCl electrode aged for a month indoors. While the color of AgCl was preserved, suggesting low susceptibility to the action of ambient air, Ag darkened indicating a sulfidation of the surface mainly because of the action of traces of H₂S. Silver tracks were painted with silver ink onto the hydrophilic zones at both ends of the PAD to create the auxiliary and the reference electrodes. The Ag track in the 6 mm-diameter zone was oxidized to AgCl in a 0.1 mol L⁻¹ HCl solution by cyclic voltammetry (CV). The CV procedure was performed at scan rate of 100 mV s⁻¹ for five cycles, varying from −0.1 to 0.15 V (vs conventional Ag/AgCl (KCl 3 mol L⁻¹) reference electrode) with end potential at 0.14 V. Figure S2 shows an Ag/AgCl electrode aged for a month indoors. While the color of AgCl was preserved, suggesting low susceptibility to the action of ambient air, Ag darkened indicating a sulfidation of the surface mainly because of the action of traces of H₂S. The graphite/Au working electrode was assembled parallel to the auxiliary electrode in the sampling zone.

Shortly after the submersion of the electrodes in liquid by addition of a drop of 3 mol L⁻¹ KCl solution to the reference electrode area and a drop of sample to the sampling area, these solutions traveled by capillary action until the hydrophilic area in the center where the electrolytic contact between the semicells is established. This salt bridge action resembling the porous junction of a conventional reference electrode can be visually illustrated by using colored solutions (Figure S3). In this work, the conventional Ag/AgCl (KCl 3 mol L⁻¹) reference electrode was denominated Ag/AgCl whereas the reference electrode Ag/AgCl (KCl 3 mol L⁻¹) on the paper as Ag/AgCl⁺.

The ColorPAD was also fabricated on the same type of filter paper (Figure 1B). The microfluidic channel (3 × 10 mm), the 10 mm-diameter detection zone, and the support for connecting to the ePAD were cut with a CO₂ laser engraver with the template drawn in the RDworks software. After that, 3 μL of 4% wt/wt cobalt(II) thiocyanate solution prepared in PBS, pH 2, was deposited in the detection area and allowed to dry at room temperature. The ColorPAD was attached to the ePAD using double-sided tape thus integrating the electrochemical and colorimetric detection in a single device.

**2.4. Microscopic Characterization.** Transmission electron microscopy (TEM) images were obtained using a JEM-2100 equipment (JEOL) at an acceleration voltage of 200 kV. The samples were prepared by ultrasonic removal of the Au film in water/ethanol (1:1 vol/vol). Then, 5 μL of the suspension was deposited onto an ultrathin carbon film-coated
copper microscopy (SEM) images were obtained with a working distance of 3 mm, an electron beam acceleration of 2–5 kV, and a magnification of 5000–30,000 times with the JSM 7401F equipment (JEOL). The optical microscopy images were obtained with an USB digital microscope (Novadigital).

2.5. Electrochemical Measurements. The CVs were registered with Autolab PGSTAT 128N (Metrohm) using graphite/Au or nonmodified graphite, Ag/AgCl, and Pt as working, reference, and auxiliary electrodes, respectively. CVs were recorded in a beaker with a scan rate of 50 mV s\(^{-1}\) in 0.5 mol L\(^{-1}\) H\(_2\)SO\(_4\) solution to analyze the degree of coverage and the gold crystallographic planes.\(^{29−31}\) The redox signals of nonmodified graphite in aqueous H\(_2\)SO\(_4\) media decreased as a function of the deposition of the Au film indicating suitable coverage. The Au-anodic broad wave showed the polycrystalline nature of the Au deposit. In H\(_2\)SO\(_4\) yet the amount of deposited Au was monitored by the amount of charge involved in the reduction of the Au oxides formed in the anodic scan. The reproducibility was evaluated in this acidic medium by monitoring the anodic and cathodic waves of Au of 10 different graphite/Au electrodes. CVs were also recorded in the PBS solution at pH 2 to evaluate the graphite coverage degree by the Au film. In the same buffer solution, the electrochemical behaviors of AMI, ascorbic acid (AA), and COC were evaluated. CVs were also registered with a scan rate of 2 mV s\(^{-1}\) to evaluate the linearity of the electronic transfer from graphite/Au and nonmodified graphite electrodes. In this experiment, a conventional Au electrode (disk with \(\varnothing = 1.5\) mm) was used for comparison purpose. Differential pulse voltammograms (DPVs) of 10 mmol L\(^{-1}\) AMI were recorded in PBS (pH 2) using ePAD to evaluate the relative standard deviation (RSD) of the current intensity and the peak potential. DPV experiments were performed with 10 mV steps of 25 mV of amplitude, 50 ms of modulation time, and 400 ms of interval time.

The Nyquist plots of graphite/Au and nonmodified graphite were recorded with the ePAD after adding 50 \(\mu\)L of 0.1 mol L\(^{-1}\) KNO\(_3\) solution containing 2 mmol L\(^{-1}\) of \([\text{Fe(CN)}_6]^{3−/4−}\) to the sampling area and 50 \(\mu\)L of 3 mol L\(^{-1}\) KCl solution to the reference electrode area. The electrochemical impedance spectroscopy assays were performed at a half-wave potential of \(\sim 0.15\) V for both electrodes (graphite/Au and graphite), determined by open circuit potential (OCP) using Ag/AgCl and Ag as a reference and an auxiliary electrode, respectively. Alternating potential with an amplitude of 10 mV in the frequency range of 0.10 to 10\(^5\) Hz was used. All experiments were performed at room temperature. The semicircles were analyzed using the electrochemical circle fit method available in the Nova 2.1 software (Metrohm Autolab), which considers the Randles’ equivalent electrical circuit without taking into account the diffusion-controlled mass transport process (Warburg constant).

2.6. Colorimetric Analyses. Colorimetric assays of the PBS (pH = 2) with and without COC (7.5 mmol L\(^{-1}\)) or AMI (10 mmol L\(^{-1}\)) were performed by adding 50 \(\mu\)L of the solution to the ePAD sampling and then dipping the inlet channel of the ColorPAD into the drop until the solution reaches the upper edge of the ColorPAD detection zone through capillary action (circa 30 s). After that, the ColorPAD was manually detached from paper holders that connected it to the ePAD. Images were registered after 2, 5, 10, and 20 min of reaction using Redmi Note 7 smartphone (Xiaomi, Beijing, China) based on a 6.5 cm-height support with internal lighting to maintain lighting and focal length constant; a support similar to that was used in a previous work.\(^{18}\)

These images of the detection zone of the paper have been standardized to an average intensity of 60% of the red, green, and blue (RGB) channels using the Gimp software (Figure S4). By using the same software, the RGB values were evaluated in the half-moon shaped regions of the images where the cobalt(II) thiocyanate complex reacted with COC (zone 1) and AMI (zone 2) (Figure S4).

2.7. Analysis of COC Samples with Color-ePAD Device. Solutions containing 230 mg COC/AMI mixtures in different ratios were prepared in 100 \(\mu\)L of PBS (pH = 2), adequate to solubilize COC (pK\(_a\) 8.6).\(^{9}\) The concentrations and ratios of COC/AMI mixtures were: 230 mg of COC (7.5 mmol L\(^{-1}\), 100:0); 184 mg COC and 46 mg AMI (6 mmol L\(^{-1}\) COC/2 mmol L\(^{-1}\) AMI, 80:20); 115 mg COC and 115 mg AMI (4 mmol L\(^{-1}\) COC/5 mmol L\(^{-1}\) AMI, 50:50); 46 mg of COC and 184 mg of AMI (1.5 mmol L\(^{-1}\) COC/8 mmol L\(^{-1}\), 20:80); and 230 mg of AMI (10 mmol L\(^{-1}\), 0:100).

Each solution was loaded in the sampling zone of a new ePAD, into which the inlet channel of the ColorPAD was immersed for circa 30 s. After the end of the run, the device was detached. The CVs were started at 50 mV s\(^{-1}\), and three cycles were recorded. Concomitantly, photos of the ColorPAD were obtained after 2, 5, 10, and 20 min of the reaction, as described above.

2.8. Principal Component Analysis. The RGB and CV-data current raw data were analyzed by PCA. The principal components (PCs) are orthogonal eigenvectors in a reduced space, and they were calculated using Statistica 13.5 software. The currents and RGBs were used as the input data to compose the PCA matrices. Each CV generated 1024 variables, while colorimetry generated 24, three variables (RGB) in two different regions of analysis collected at a distinct time (2, 5, 10, and 20 min). The matrix columns were self-scaled according to eq 1 to give the same weight to all variables.\(^{32}\) Where \(x_j\) is the value of variable located at line \(i\) and column \(j\), \(\bar{x}_j\) is the average of the column \(j\), and \(s_j\) is the standard deviation of column \(j\).

\[
x_{ij}(a) = \frac{x_{ij} - \bar{x}_j}{s_j}
\]

2.9. Analysis of Au Sheets by Energy-Dispersive X-ray Fluorescence. The graphite surface modified with the Au film was prepared using the Au leaf as the precursor. The Au leaf (1 cm\(^2\)) was analyzed by energy-dispersive X-ray fluorescence spectrometry (EDXRF) with the EDX720 instrument from (Shimadzu, Columbia, USA) in atmospheric air. The analysis window was that of Na (11)—U (92) with an Rh X-ray tube with 50 kV and a current of 240 \(\mu\)A, an analysis time of 60 s, and a collimating lens of 10 mm.

3. RESULTS AND DISCUSSION

3.1. Color-ePAD Fabrication. We prepared a PAD on filter paper with colorimetric (ColorPAD) and electrochemical (ePAD) detections (Color-ePAD) (Figure 1 inset). Both techniques can be used simultaneously to extract information from the COC samples, increasing the amount of data and allowing the identification of that drug with the support of a chemometric method, PCA.
The paper platform of ePAD was fabricated, creating a 5.0 mm diameter sampling zone, defined in such a way that a small sample volume (50 μL) is enough for the electrochemical and colorimetric assays. A drop of 3 mol L\(^{-1}\) KCl needs also to be added to the reference electrode zone. The relatively narrow (1.5 mm) and long (2 × 6.0 mm) microfluidic channels connect those two electrode areas with a circular central merging area that also prevents the contamination of the reference electrode by the sample solution.\(^{28}\) A shift of +0.1 V in CV of AMI was observed when compared to other referenced to Ag/AgCl (Figure S5) caused by ohmic drop between the working electrode and the channel leading to the reference electrode.

For the fabrication of the working electrode, a graphite lead of low electric resistance (<5 Ω) was used. This electrode material has been used to detect heavy metals and drugs using electrochemical techniques.\(^{33}\) It is composed of fine graphite powder (~65%) mixed in a clay matrix (~30%) and binders (wax, resin, and polymers).\(^{33}\) The working electrode was prepared following the procedure previously reported,\(^{34}\) except that AuCl\(_4^-\) was obtained from the dissolution of the Au leaf with reverse aqua regia\(^{35}\) instead of using the commonly employed HAuCl\(_4\) reagent.\(^{34,36–38}\) In this work, taking advantage of the availability of the Au leaf used in decorative crafts and cooking,\(^{36}\) a fast, low-cost micro-scale preparation of AuCl\(_4^-\) was carried out by dissolving the gold with reverse aqua regia. The general reaction of Au with reverse aqua regia is shown in eq 2.\(^{35}\) The Au concentration in the leaf was 88% (wt/wt), which was determined by the elemental analysis technique EDXRF as shown in Table S1.

\[
\text{Au(s)} + 3\text{NO}_3^-(aq) + 4\text{Cl}^-(aq) + 6\text{H}^+(aq) \\
\rightarrow \text{AuCl}_4-(aq) + 3\text{NO}_2(g) + 3\text{H}_2\text{O}(l)
\] (2)

The manufacturing of the working electrode was optimized by applying different potentials using the CMP technique. These potentials ranged from \(-1\) to \(-4\) V (vs Ag/AgCl). The Au film distribution on the graphite surface was evaluated by monitoring the electrochemical signal of the substrate and by optical microscopy. In the case of an incomplete surface coverage, the characteristic signal of graphite in the H\(_2\)SO\(_4\) solution (between 0.2 and 0.7 V) could interfere with the measurements of the target analytes (Figure S6A).

More negative potentials applied during the reduction of AuCl\(_4^-\) decrease the graphite signal in the CV registered in the H\(_2\)SO\(_4\) solution (Figure S6B), until it disappears completely at potentials below \(-3\) V. It was also observed that the substrate signal decreased in PBS as higher energy potentials were used for AuCl\(_4^-\) reduction (Figure S6A). By means of optical microscopy, it was possible to correlate the signal decrease observed and the graphite surface coverage by Au (Figure S6C). Additionally, the faradic currents associated with the formation (1.2–1.5 V) and reduction (0.9 V)\(^{30}\) of Au oxides increased (Figure S6B). The progressively more negative potentials generated an augment in the number of active sites for AuCl\(_4^-\) electrodeposition\(^{31}\) increasing the amount of Au deposited and electrode electroactive area (Figure S6).

Besides the Au coating, the electrochemical response to the probe molecule AMI was monitored.\(^{5,7}\) The potential window of CVs using graphite/Au was limited to 1.15 V (vs Ag/AgCl) to avoid interference of the Au-oxidation faradaic current in the analyses.

At graphite/Au, AMI showed two well-defined anodic peaks at 0.65 and 0.95 V (vs Ag/AgCl). The peaks were 18 and three times higher than those obtained with a nonmodified graphite (Figure S7), indicating a larger reactivity of AMI on the graphite/Au surface than on nonmodified graphite. The higher current values observed current may be related to the increase in the surface area because of the Au-film deposition. Based on the oxidation mechanism of AMI on the platinum electrode,\(^{5}\) the first peak at 0.65 V (vs Ag/AgCl) can be attributed to the one electron oxidation of the tertiary amine attached to the pyrazole ring with the subsequent formation of a radical cation, which may undergo demethylation to form a secondary amine that can be oxidized to a hydroxyamine group at 0.95 V (vs Ag/AgCl). Another mechanism path can be described by the stabilization of the iminium cation, and the current peak at 0.95 V can be attributed to electrooxygenation of the methylated carbon of the pyrazole ring, according to the mechanism proposed at the glassy carbon electrode.\(^{39}\) The COC did not show electroactivity under these conditions (Figure S7).

The ColorPAD was prepared using the same type of filter paper. However, instead of delimiting the hydrophilic channels and detection zone with wax, the edges of the device were cut using a CO\(_2\) laser. The detection zone diameter was optimized by varying the size in a range of 4–10 mm (Figure S8). The larger diameter favored the solution spreading, which then evaporated faster, revealing the blue color of the Co/COC complex, insoluble in the aqueous medium.\(^{40}\) Therefore, 10 mm diameter was chosen for the proposed device. The width and length of the microfluidic channel as well as the type of cellulose filter paper influence the fluid transport regime (speed and flow rate) and have been used to promote efficient separation or mixing.\(^{41}\) In the present work, the channel is intended just to deliver the sample to the detection zone of the ColorPAD, so a channel of 3 × 10 mm was adopted.

The pinkish aqueous cobalt thiocyanate solution applied on the paper turned royal blue color after drying (Figure S9), because of the formation of the [Co(SCN)]\(_{6}\)\(^{2-}\) complex as water evaporates out. To evaluate whether the paper’s quality would influence the result, a cobalt thiocyanate solution was added to both Whatman no 1 paper and the filter paper used to manufacture devices. A blue color was observed on both papers (Figure S9), which is consistent with the literature\(^{40}\) that the paper could produce a false positive in the color test of a seized sample supposedly from COC. However, it immediately returned to the initial pinkish color when wetted again with the PBS solution. These color changes did not occur with the COC (Figure S9), or with the AMI (data not shown), after adding PBS to the blue complex, as expected for the formation of insoluble stable precipitates with these compounds.

When high concentrations (solid) of COC (Figure S9) and AMI (data not shown) were added to the wet paper with the Co thiocyanate solution, such Co/COC and Co/AMI complexes showed dark blue colors almost indistinguishable from the blue color of the papers. However, a change from dark blue to Capri blue or medium blue was observed when lower concentration solutions were added to the ColorPAD. Another factor that may have contributed to the change in color tone was the spread of the mixture promoted by the paper’s capillarity. Therefore, the blue hues of Co thiocyanate, Co/AMI, and Co/COC complexes can be differentiated on paper (Figure S9).
The reaction with Co thiocyanate is not selective for COC as demonstrated, and may generate blue color with controlled-use drug as lidocaine, dibucaine, chlorpromazine in addition to AMI. Therefore, there are risks of producing false-positive results or dubious results, for example, the appearance of purple color with milk powder. For this reason, Tsumura and collaborators suggested performing the Scott test in three stages: (i) reaction of the suspected sample with Co thiocyanate in an aqueous medium with glycerin for the appearance of the blue precipitate; (ii) resolubilization with concentrated HCl to monitor the color disappearance; and (iii) the extraction of the complex with chloroform producing a blue solution. If these three steps are contemplated, the accuracy of the results of the preliminary exams for COC will be higher, reducing the incidence of false positives.

Another factor that provides strong evidence for the COC presence in the seized sample is the fast formation of the complex. Figure S10 shows that the sample containing COC exhibited Capri blue precipitates in 2 min of reaction. It is important to note that the precipitate was formed and impregnated the ColorPAD fibers in a region close to where the thiocyanate reagent was deposited. It assumed an arc shape as a result of the drag generated by the solution flow. After 10 min of the reaction, the pinkish thiocyanate located on the upper part of the ColorPAD became darker, being more intense in the sample that contained only AMI (Figure S10). This color gradually became more intense over time while the excess of thiocyanate solution continued to be carried by the continuous flow.

After 20 min, it showed medium blue color indicating the presence of AMI. The dark blue that developed on the paper after running the PBS solution without any analyte appeared in the upper region, close to the border. This suggests that the formation kinetics of the Co thiocyanate and COC complex is faster than with AMI. These observations made it possible to identify the analytes according to the region of the device.

### 3.2. Microscopic and Electrochemical Characterization of the Graphite/Au Electrode

To assess the morphology of our working electrode, SEM images of the graphite with and without Au film modification were acquired. Images of bare graphite revealed thin and flat packaged structures (tactoids) composed of graphite aggregate sheets, oriented in different directions, as shown in Figure 2A,B. When the graphite was modified with the Au film, these structures were preserved, and Au was deposited on them (Figure 2C,D). In the micrograph with higher magnification, it is possible to observe the presence of submicrometric Au particles, which provide defects (i.e., folds and edges) on the surface.

TEM images revealed that the Au film is formed by aggregated Au particles of different sizes (Figure S11). In addition, high-resolution TEM images showed that the Au film exhibited a fringe distance of 0.23 nm, characteristic of the Au(111) crystallographic plane of the face-centered-cubic lattice of gold [JCPDS 04-0784]. Complementary analysis by selected area electron diffraction (SAED) suggested the presence of (200), (220), and (311) planes, as shown in Figure S12. The anodic peaks at 1.2 and 1.4 V (vs Ag/AgCl) observed in the CV recorded in the H2SO4 solution (Figure S6B) can be associated with low-index (100) and (111) crystalline planes, respectively. Additionally, the shoulder observed at 1.3 V can be attributed to the (110) plane, showing the crystalline facets exposed to the electrolyte solution.

The electrochemical behavior of the graphite/Au electrode was compared to that of a nonmodified graphite (Figure S13). Electrochemical impedance spectra (Nyquist plots) were acquired at the half-wave potential of the equimolar system of the [Fe(CN)6]3−/4− redox pair, measured at OCP. The applied potential was ~0.15 V (vs Ag/AgCl+) for both electrodes.

The semicircle in the high-frequency region of the impedance spectrum represents the electrochemical process limited by the charge transfer resistance (Rct) in the
The nonmodified graphite showed a semicircle with 644 Ω, whereas the deposition of the Au decreased the resistance to 3 Ω, suggesting an increase in the electron transfer rate at Au, favored also by the greater surface area highlighted in Figure 2. The diffusion region presented a nonlinear tail because of the capacitive behavior of electrodes resulting from the irregular microstructures of graphite and the presence of nanometric structures of Au.46

AA was selected as a redox probe for CV measurements. An increased height and definition of the broad-anodic wave attributed to the oxidation of ascorbate to dehydroascorbate on the gold electrode involving two electrons36 was observed on Au-coated graphite, in contrast to the bare graphite electrode. This effect was more noticeable when a more negative potential was used for the electrodeposition of Au, suggesting an improvement in the electron transfer rate (Figure S14). Also, a slight anticipation (~50 mV) of the anodic peak onset was observed on a graphite/Au electrode (prepared at −4 V (vs Ag/AgCl)) compared to that observed on a nonmodified graphite.

A shift of 300 mV (to lower overpotential) of the AA peak onset was also observed in CV recorded on graphite/Au compared to that of a conventional Au disk electrode (Figure S14). A similar behavior has been observed for an Au disk electrode modified with electrodeposited Au.36 This effect could be attributed to adsorption of molecules on the electrode surface affecting the transition state and, therefore, the activation-energy barrier, thus facilitating the chemical reaction on the surface.37 Atoms in kinks and edges interact more strongly with the analyte47 than the ones on bulk surfaces. The geometry of the material influences the crystallographic planes exposed to solution, and these in turn interact differently with the analytes, increasing or decreasing the catalytic activity.48 For example, copper oxide (Cu2O) nanocubes that have (100) orientation favor the nonenzymatic and selective oxidation of glucose.48 The Au crystallographic planes (311) have a greater catalytic effect than the monocrystalline (111) or (100) surface on glucose detection.49 The graphite/Au electrodes exhibited a mixture of different crystal facets, they had mostly (100) and (111) planes in contact with the solution, presenting a superior electrochemical performance than conventional Au or nonmodified graphite electrodes.

As mentioned, adsorption can decrease the energy barrier by activating the molecules50 and, consequently, can promote the occurrence of the reaction at lower overpotentials. Such an effect can explain better the definition of the anodic wave of AA.50 To evaluate if the marked increase in the current signal is a result of this effect or due simply to increased electroactive area or roughness, it is necessary to normalize the measured currents for the effective area of the electrode.50

The electrochemical surface area of nanostructures can be measured by different ways.7,50–52 One of the recommended ways is by monitoring the nonfaradaic capacitive current as a function of the scan rate, which should present a linear relationship. The parameter obtained for the nanostructured and flat electrodes can be used to obtain or compare the electroactive areas.50

It is worth noting that when graphite was used as an electrode (Figure S14), the mass transport approaches a steady state regime, observed when the radius of the electrode is smaller than the diffusion layer.53,54 The graphite surface with tactoids may have created microregions promoting such a similarity. In contrast, when a conventional Au disk electrode was used, transient mass transport was observed. This behavior is seen when the diffusion layer is smaller than the electrode radius.53

3.3. Reproducibility of Graphite/Au Electrode Fabrication. As previously discussed, when applying −3 and −4 V (vs Ag/AgCl), a well-distributed Au film was formed on the graphite surface. Because of the higher energy, a potential of −4 V reduced Au more deeply into the tactoids of graphite sheets as shown in Figure 2, thus being more suitable to fully cover the surface area accessible to the electroactive analytes. Consequently, a preparation method with a relatively high reproducibility rate of 83% was developed. Indeed, 10 out of 12 manufactured electrodes presented an adequate Au film coating and the peak currents at 1.26, 1.43, and 0.95 V of CVs registered in H2SO4 solution had a RSD of 7.5, 6.8, and 9.5%, respectively (Figure S15). On the other hand, when applying −3 V, the reproducibility decreased to 50% and only six out of 12 electrodes showed a suitable Au coating.

Additionally, when using −4 V for the deposition of the Au film on the graphite surface, the RSD of the peak height at 0.65 V for AMI was 8% (56 ± 4.5 μA) (Figure S16). It is noteworthy that the peak potential of AMI at 0.72 V versus Ag/AgCl electrode on paper presented a shift to an extent of 0.08 V (Figure S16). This represents an improvement in comparison with a Ag pseudo-reference (0.12 V) that we believe is because of the use of KCl solution to wet the true Ag/AgCl reference electrode and to the narrow “salt bridge” channel with a relatively large central contact area, protecting the reference electrode from the sample solution. The pseudoreference has the silver surface exposed to the electrolytic sample, so that its potential depends on the chloride concentration (or other halide or pseudohalide), being also susceptible to disturbances by adsorption of chemical compounds from the sample. It is worth noting that this observed variation can be lowered even more in a larger scale optimized device fabrication instead of the manual silver painting and anodization of the reference electrode for each PAD.

3.4. Proof-of-Concept Application of the Color-ePAD to COC Samples. The electrochemical analyses of the COC samples were accomplished by CV with three cycles that lasted 15 s and required 50 μL of the sample prepared in PBS (pH 2). As shown before in Figure S16, each electrode presented a slightly different behavior in the AMI solution. As expected, a similar behavior was observed for the analysis of the COC sample containing AMI as a cutting agent. Shifts in the AMI peak potential in a range of 0.07 V and different current intensities (28 ± 2 μA) were observed (Figure S17).

To circumvent these signal fluctuations, the sample analysis can be done using the standard addition method to eliminate the matrix effect53 but a different strategy is demonstrated in this work. We recorded the voltammetric and colorimetric responses (n = 5) of each COC/AMI solution prepared using the COC sample granted by Criminalistic Institute of the State of São Paulo (Brazil) to assemble the database for PCA, an unsupervised chemometric technique that reduces a larger set of variables into a smaller number of orthogonal eigenvectors, the PCs that carry as much variance as possible.18 Thus, by combining electrochemical and colorimetric data obtained with the Color-ePAD, we developed an analytical method that compare and group the samples according to similarities, thus allowing the determination of their composition. Therefore,
the analytes can be quantified quickly and without the need of trained personnel to perform the analysis.

The CV was chosen for providing information about the samples during both, the forward and reverse scans. The currents without subtracting background were introduced in the data matrix, and each column was self-scaled to minimize the influence of a dominant variable. In Figure 3A, the PC1 of the 25 samples of COC described 53% of the variance of the original data, discriminating along the axis as a function of AMI concentration. At higher AMI concentrations, the sample signal shifted to more negative values. Each sample’s position took into account the contribution of all 1024 variables (current vs potential) of each CV. However, closely analyzing the main variables in the loading plot of PCs (Figure 3B), one observes that the shift of PC1 to negative values in the samples with higher concentrations of AMI is because of the high currents at \( \sim 1.1 \) V in the forward scan and 0.9 and 0.8 V of the reverse scan.

Also, as the COC concentration in the sample increased, a broadening and a slight shift to the positive potential of the AMI peaks were observed because of possible COC adsorption on the electrode. Therefore, an increase in current at the onset of the first anodic peak of AMI was observed at 0.7 V in the sample 50:50 (COC/AMI mixture ratio), which shifted the sample to positive values of PC1. Another variable that contributed to the positive shift of PC1 was the anodic current at 0.07 V. However, this could go unnoticed, as there is no faradaic process involved. The samples containing high concentrations of COC showed high currents contributing to the displacement. Similarly, the current at 0.6 V caused a positive shift, so the sample 100:0 showed the most significant displacement among the 50:50, 80:20, and 100:0 samples. Despite the fact that samples 0:100 and 20:80 showed high currents at 0.6 V, they were positioned on the negative side, probably because of other variables such as those already mentioned. Additionally, the same current at 0.6 V contributed to a positive shift in PC2, placing the samples 0:100 and 20:80 in quadrant 1 and 100:0 in quadrant 2.

Nevertheless, PC2 contributed with 20% of the data variance to the discrimination of samples on the vertical axis. The currents at 0.5 and 0.25 V were positively displaced by the samples that presented intense cathodic currents attributed to the reduction of the product formed at \( \sim 0.8 \) V, by 0:100 and 20:80. Finally, the current at \(-0.1\) V of the reverse scan shifted to negative values for samples with small currents such as 50:50 and 80:20.

Although the CV alone provided good discrimination of the samples 100:0, 80:20, and 50:50 even without an electrochemical signal from COC, it was not able to discriminate the 20:80 and 0:100 samples.

In order to improve the discrimination of the COC samples, the microfluidic PAD device with colorimetric detection (ColorPAD) was coupled to provide more information about the samples. In this sense, PADS can easily integrate different detection techniques using only a suitable premolded design in a software program or coupling the devices in one PAD. This assay based on the reaction with cobalt thiocyanate can be performed in 20 min and allow the confirmation of the presence of COC and the quantification by the resulting color intensity, even in the presence of a compound with potential to interfere. The complexes formed with COC (or AMI) and the paper produced blue colors with different hues, which turn discrimination with the naked eye difficult. Fortunately, the paper interacts differently with each product formed and it is

**Figure 3.** (A) Graph of PCA scores plotted with CV currents of the COC samples. (B) Loading graph of the PCA variables, the potential applied where the current values were obtained is highlighted. The potentials of reverse scans are indicated with (*). (C) CVs of the COC samples recorded with a scan rate of 50 mV s\(^{-1}\) using Color-ePAD with graphite/Au, Ag/AgCl\(^{\text{″}}\), and Ag as working, reference, and auxiliary electrodes, respectively. (D) Enlarged view of the central region of the CVs shown in C. COC/AMI solutions were prepared in PBS (pH = 2) and evaluated in the ratios: 100:0 (red), 80:20 (green), 50:50 (black), 20:80 (purple), and 0:100 (blue).
possible to perform a chromatographic like separation under capillarity flow. Therefore, we transformed the colors observed in different regions into the RGB input data for PCA analysis. The raw data were used without discounting the background signal.

Figure 4A shows the color variations of the COC samples used as the calibration data as a function of time, where the intensity of colors was directly related to the time and concentration of the analytes. The RGB values were collected to compose the data matrix as a function of time and in two different regions. In the first one, Co thiocyanate formed a complex with COC (RGB) and in the second one with AMI (R* G* B*). Table S2 shows the RGB values for each data collection region at different times and concentrations. Note that the red colors R and R* were replaced by complementary blue color over time, enhancing the values of B and B*. It is interesting to emphasize that the B values were higher for samples containing larger concentrations of COC, whereas B* was larger in samples with higher concentrations of AMI. Each column of the PCA matrix was centered on its average value to give the same weight to all variables.32

According to the score plot reported in Figure S18, there is a tendency of sample discrimination, accentuated in 5 and 10 min, where the samples were almost grouped. However, the data were very scattered, and regions of overlap were observed. This problem was settled by gathering the RGB values collected in each time of analysis in a matrix, thus obtaining.

Figure 5. (A) PCA score graph combining CV currents and RGB data obtained at 2, 5, 10, and 20 min of analysis of COC/AMI-training samples using the Color-ePAD. COC/AMI mixtures were evaluated in the ratios: 100:0 (red), 80:20 (green), 50:50 (black), 20:80 (purple), and 0:100 (blue). (B) PCA score graph of the same data from the previous analysis added the synthetically simulated COC/AMI sample (stars).
more precise discrimination of the samples with greater confinement (Figure 4B). The PC1 and PC2 represented 41 and 18% of the variance of the 25-sample data, respectively (Figure 4B). PC1 discriminated samples that contained higher concentrations of AMI ordered in increasing concentration. In the PC2 axis, the samples were separated in the ascending COC concentration order. Interestingly, the variables collected in the region where the COC complex was formed had more significant contributions to discriminate samples containing high concentrations of AMI in the PC1 axis, as shown in the loading graph (Figure 4C). On the other hand, those collected in the region where the AMI complex was formed contributed to discriminate samples with high COC concentrations.

The discrimination of the samples was improved by combining the electrochemical and colorimetric information obtained with Color-ePAD. The self-scaled variables that exerted the highest weights (highlighted above) in previous PCA analyses were gathered in a matrix. Consequently, a score graph (Figure 5A) with discriminatory characteristics of the two PCAs was obtained with better grouping of the samples, despite the unsatisfactory separation on the PC1 axis for 20:80 and 0:100 samples because of the overlapped electrochemical data in the PCA.

Additionally, as proof-of-concept, COC samples were synthetically simulated and they could be discriminated according to their composition (Figure 5B). This dual-detection approach seems to be more powerful to analyze the COC samples containing a blend of adulterant compounds, the most common in real cases.7,9,58

This work was focused on COC samples blended with AMI. However, in the case of the presence of other adulterants such as paracetamol, phenacetin, caffeine, procaine, and benzocaine, the resulting CV would have a distinct pattern from those presented because of their electroactivities.7-9 Additionally, considering the variation of current intensity related to the analyte concentration, the samples could be discriminated according to the adulterant content and amount. Also, amine-containing molecules such as lidocaine can react with cobalt thiocyanate yielding a blue precipitate (data not shown) that would interact with paper devices differently from COC and AMI in a distinct scattering of products along the ColorPAD, thus allowing the sample discriminations by PCA.

The developed Color-ePAD could also be integrated with detection by surface-enhanced Raman spectroscopy (SERS) because the graphite/Au electrode had a rough Au surface appropriate for enhancement. This allowed obtaining the spectra of other cutting agents such as the levamisole molecule (data not shown) with the Color-ePAD. This strategy showed to be particularly valuable for species that do not exhibit electroactivity.7 Therefore, the SERS spectra can be added to the PCA data matrix, increasing the discriminatory potential of the analysis. Although this PCA-based method does not require trained personnel to determine the composition of a COC sample, it depends on an extensive database that needs to be constantly fed.

**4. CONCLUSIONS**

We report a PAD manufacturing method using filter paper and regular graphite leads modified with the Au film. The device combines electrochemical and colorimetric detection to provide more precise information about the COC samples adulterated with AMI. The precursor of the Au film, AuCl₄⁻, was synthesized on the microscale by dissolving the Au leaf in reverse aqua regia and electrodeposited on the graphite leads in an acidic medium at −4 V. We demonstrated that as increasingly negative potentials were used to deposit Au, the graphite surface coating became more effective, resulting in a lower charge transfer resistance (3 Ω). The structure with submicrometric particles showed edges and kinks and crystallographic gold (110) planes, mainly, (111) and (100), which facilitated the electrochemical oxidation of AA at least by 50 mV. The size of the Color-ePAD colorimetric detection zone was optimized to monitor the Co thiocyanate complex with COC and with AMI in distinct regions.

The Color-ePAD device was used, as proof-of-concept, to analyze the COC samples adulterated with AMI. The CV (i vs E) and colorimetric techniques were employed to generate the input data for PCA analysis that classified the samples according to the COC/AMI ratio. The method successfully classified the synthetically simulated samples of COC based on the database assembled in this work.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05077.

Steps and optimization of the fabrication of the working electrode by the Au electrodeposition; diameter optimization of the ColorPAD detection zone; comparison of AMI CVs recorded with Ag/AgCl and Ag/AgCl; comparison of AMI CVs recorded with graphite/Au and nonmodified graphite lead; comparison of AA CVs recorded with graphite/Au, nonmodified, and gold commercial electrodes; photos of the reference electrode on paper; Color-ePAD and papers (Whatman and Unifil) with Co thiocyanate solution and COC; comparison of Nyquist plots obtained with graphite/Au and nonmodified graphite lead; EDXRF table of the Au leaf; TEM images and SAED patterns of the graphite/Au; optical microscopy images of graphite/Au; reproducibility tests of the Color-ePAD; RGB variables; and PCA of RGB carried out at different times (PDF)

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Notes
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