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Research Article

# Forensic Chemistry of Alkaloids: Presumptive Color Test

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**Abstract**

Evidence collected from crime scenes often includes unknown powders, pills, and tablets, many of which are illicit drugs difficult to identify visually. Presumptive color tests help with the on-scene recognition of drug materials via rapid color changes. Most of these tests are based on qualitative chemical reactions and have since been standardized. Although simple and rapid, qualitative tests provide only preliminary analytical data. Nevertheless, these tests are still important components of crime scene investigations, and government authorities deploy them to detect illicit drugs in the field. Understanding the chemistry behind presumptive color tests makes it possible to predict reactions to known drug standards. However, in the presence of cutting agents and other chemicals, the results of presumptive color tests may not be predictable due, in part, to interference by contaminating chemicals. Most described presumptive tests are developed for 'classic' drugs, such as opiates, amphetamines, and cocaine, but new psycho-active substances and cutting agents emerge every day on the market. Among them, alkaloids (e.g., lobeline, caffeine, piperine) can be purchased easily and legally via the Internet or in local shops and are often utilized to lace or cut drugs. This study is the first to predict and document the results on 7 of the most common presumptive color tests with various alkaloid standards. We assessed these tests with mixtures of alkaloids to ascertain interference, if any, in the color results. We performed presumptive color tests on various popular cutting agents and, finally, tested several mixtures of drugs/alkaloids/cutting agents potentially similar to samples seized in the field. The results showed that color prediction worked well with pure standards, but color tests could not be predicted for mixtures in most cases. Also, alkaloid cutting agents often interfere with presumptive color test results, affecting outcomes. Better understanding of presumptive color tests, coupled with better populated databases of color results involving cutting agents, will help in reducing false positives and false negatives, thereby improving initial testing of seized evidence.

**Keywords:** Alkaloid; Forensic; Presumptive color test; Cutting products; Drugs

## Introduction

Illicit drugs represent the vast majority of chemical evidence collected from crime scenes. Drug-related crimes are common and involve large sums of money. For example, in 2012, the Federal Bureau of Investigation seized USD 1.125 billion worth of assets and drugs [1]. Investigators often encounter unknown samples (pills, capsules, stamps, tablets, powders, and liquids) at crime scenes, many of which are controlled substances, but visual identification of drugs is challenging. Two main types of tests assess whether illegal drugs are existent in samples: presumptive tests and confirmatory tests.

Presumptive tests are performed first, to help decide what to do next are less precise and indicate that illegal substances may be present. Confirmatory tests are more expensive and time-consuming, but positively identify substance(s) in question. Presumptive tests may be conducted in the field or laboratory, whereas confirmatory tests involve a battery of instrumental methods (e.g., gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry and Fourier transform infrared spectroscopy) which separate individual compounds and positively identify the chemical profiles of illegal substance(s) [2-4].

Presumptive color tests [also referred to as presumptive drug tests, chemical detection tests or spot tests] were introduced years ago to help on-scene identification of drugs via rapid color changes [2-10]. Most of these tests are based on qualitative chemical reactions to chemical functional groups, or chemical families of compounds, and have since been standardized [6,11,12]. The results of presumptive tests are indicative and non-specific: they do not categorically prove the presence or absence of drugs in test samples. However, presumptive tests allow us to confirm or invalidate the presence of functional groups or specific structures in molecules [13]. Although simple and fast, qualitative tests provide only preliminary analytical results: nevertheless, they are still important components of crime scene investigations, and government authorities deploy them to detect illicit drugs in the field.

Typically, there are two parts to presumptive drug tests: testing and interpretation. The testing portion is easier than interpretation, although electronic-based interpretation software, with red-green-blue format apps, have been developed, with database of expected results [1]. Since field samples may contain cutting agents, impurities, or substances meant to disguise the drug present, they may interfere




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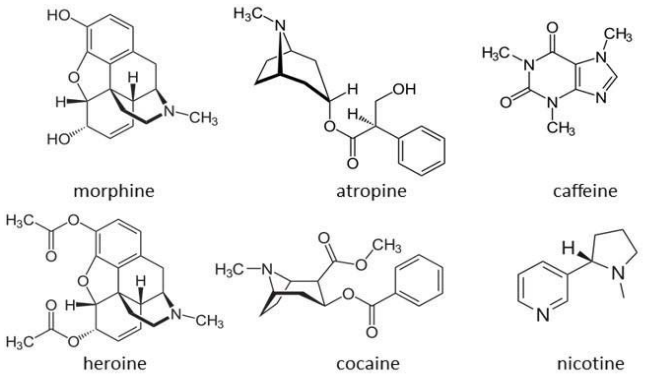



**Figure 1:** Examples of alkaloids from natural plant sources, such as morphine from opium poppy, atropine from deadly nightshade, caffeine from coffee beans, cocaine from coca, and nicotine from tobacco plants. The semi-synthetic alkaloid heroin is produced from the diacetylation of morphine, and caffeine can be synthesized in the lab with malonic acid and dimethylurea.

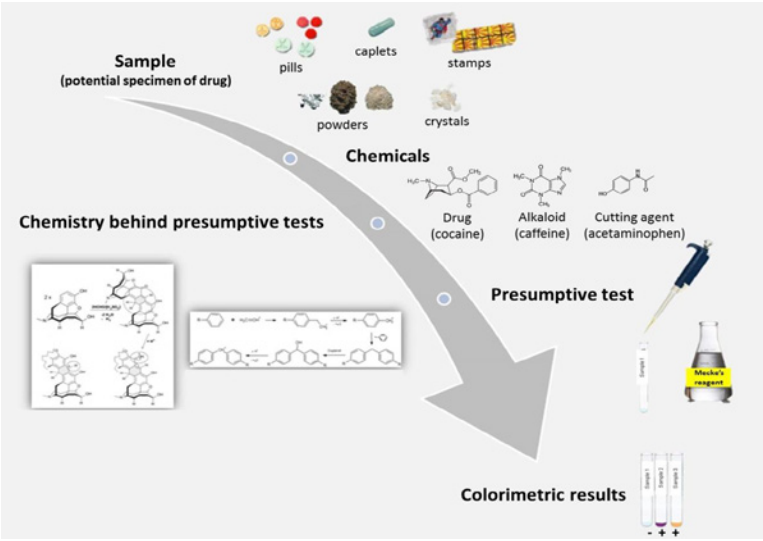



**Figure 2:** Overview of the method strategy. Samples (potential drug specimens) to be tested can come from various sources, including pills, caplets, stamps, powders and crystals. They may contain various chemicals, such as drugs of interest, but also alkaloids and other cutting agents. The chemistry behind most presumptive color tests is known, which allows for results prediction of single and pure drug samples. However, mixtures of drugs with various chemicals may interfere with presumptive test results and, therefore, need to be assayed and recorded.

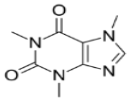
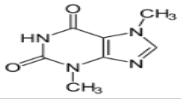
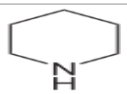
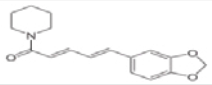
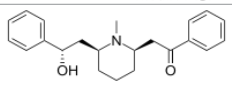
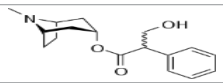
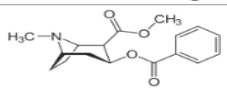
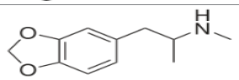
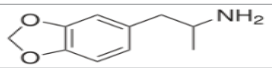
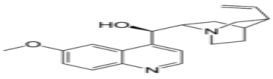
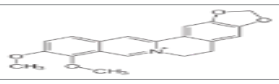
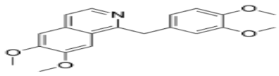
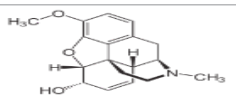
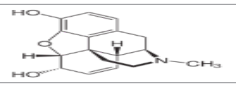
with test reactions and resulting colors. Erroneous color interpretation may elicit false-positive tests and lead to unjust warrants, arrests, and even convictions [14].

## The chemistry behind presumptive color tests

Several studies have reported the chemistry behind most presumptive color tests [6,12,15,16]. The reaction mechanisms in some tests are still hypothetical, but databanks of color reactions obtained for several known compounds are readily available. We selected 7 tests, including those of Chen, Mandelin, Marquis, Mecke, Froehde, Simon and Cobalt.

Briefly, Chen's test generally identifies narcotics since it reacts easily with functional groups present in tested chemicals [16]. Mandelin's test presumptively detects opiates and phenethylamines, with vanadate ions forming a conjugated system of electrons with different oxidation states, resulting in different colors obtained [17-20]. Marquis' reagent works with morphinan drugs and includes formaldehyde, which reacts with aromatic rings on tested chemicals and links them together [15,16,20]. Mecke's reagent contains sulfuric acid, which breaks down bonds in functional groups of tested chemicals and re-arranges them with selenious acid [15]. Froehde's presumptive color test identifies opiates and phenethylamines. Reactions of this reagent, composed of sodium molybdate, sulfuric acid and other molecules, are not clearly established. It is possible that molybdate ions link tested chemicals after bond breakage induced by sulfuric acid in a similar manner as in Froehde's test [21]. Simon's presumptive test screens chemicals containing secondary amines whereas Cobalt's test exploits cobalt thiocyanate to find protonated tertiary amines [15,16]. Knowing and understanding the chemistry behind presumptive color tests makes it possible to predict test reactions to known standards. For example, the phenethylamine MDMA reacts with all tests except Cobalt's test, since it does not possess a protonated tertiary amine. In contrast, cocaine will react with Cobalt's test, since it harbors a protonated tertiary amine in its structure. However, in the presence of cutting agents or other chemicals, the results of presumptive color tests may not be

**Table 1:** List of alkaloid standards tested in this study. Sources are natural (N), semi-synthetic (SS) or synthetic (S).

| Standard | Structure | Source | Group |
|---|---|---|---|
| Caffeine |  | N | Purine |
| Theobromine |  | N | Purine |
| Piperidine |  | N | Piperidine |
| Piperine |  | N | Piperidine |
| Lobeline |  | N | Piperidine |
| Atropine |  | N | Tropane |
| Cocaine |  | N | Tropane |
| Methamphetamine |  | S | Phenylethylamine |
| MDMA |  | S | Phenylethylamine |
| MDA |  | S | Phenylethylamine |
| Quinine |  | N | Quinoline |
| Berberine |  | N | Benzylisoquinoline (phenanthridine) |
| Papaverine |  | N | Benzylisoquinoline (simple) |
| Codeine |  | N | Benzylisoquinoline (opiate) |
| Morphine |  | N | Benzylisoquinoline (opiate) |

predictable due, in part, to interference by contaminating chemicals.

## Alkaloids

Although presumptive color tests are widely used, only a few scientific articles report their performance and validation [5,7,9]. Most described presumptive tests are developed for 'classic' drugs, such as opiates, amphetamines, cocaine, etc., but new psycho-active substances and cutting agents are emerging every day on the market. Among cutting agents with psycho-active properties, alkaloids are often utilized to lace and cut drugs.

Alkaloids are organic, nitrogenous molecules produced by specialized metabolic (aka secondary) pathways (Figure 1). They generally occur in solid crystalline form: the majority are colorless but some, especially those with many aromatic ring structures, are colored (e.g., berberine (yellow) and sanguinarine (red)) [22]. Alkaloids are basic (*alkali*) due, in part, to a free pair of electrons on the nitrogen atom. Natural alkaloids are produced in living organisms, including several plant species (e.g., opium poppy), some fungi (e.g., magic mushrooms) and, less frequently, in animals and microorganisms.

The exact functions of alkaloids remain unknown. However, owing to their biological effects, they may be involved in defence against pathogens or herbivores. The outcomes of alkaloids in human metabolism may vary, depending on their structure, their target and dose. For example, a low dose of a given alkaloid, such as morphine, can serve as a medication while a high dose can be toxic. Alkaloids act

**Table 2:** Summary of presumptive tests with authentic standards, "+" indicates positive reactions and color changes, whereas "-" represents no reaction and no color change compared to control methanol (n=3).

| Standard | Chen | Mandelin | Marquis | Mecke | Froehde | Simon | Cobalt |
|---|---|---|---|---|---|---|---|
| Caffeine | + | - | - | - | - | - | - |
| Theobromine | + | - | - | - | - | - | - |
| Papaverine | + | + | - | - | - | - | - |
| Piperidine | + | - | - | - | - | + | - |
| Piperine | + | + | + | + | + | - | - |
| Lobeline | + | - | - | - | - | - | - |
| Quinine | + | - | - | - | - | - | - |
| Atropine | + | - | - | - | - | - | - |
| Berberine | + | + | - | + | - | - | + |
| MDA | + | + | + | + | + | - | - |
| MDMA | + | + | + | + | + | + | - |
| Methamphetamine | + | - | + | - | - | + | - |
| Cocaine | - | + | - | - | - | - | + |
| Codeine | + | + | + | + | + | - | - |
| Morphine | - | - | + | + | + | - | - |

**Table 3:** Summary of presumptive tests with mixtures of 2 different alkaloids (n=3).

| Mixture of alkaloids | | Test | Reaction of single | Reaction of the mix |
|---|---|---|---|---|
| **1)** | a. Cocaine | Marquis | Colorless | Orange |
| | b. Piperine | | Brown orange | |
| **2)** | a. Morphine | Marquis | Light violet | Red orange |
| | b. Piperine | | Brown orange | |
| **3)** | a. Morphine | Mecke | Green blue | Brownish yellow |
| | b. Berberine | | Brown orange | |
| **4)** | a. Methamphetamine | Mecke | Colorless | Yellowish orange |
| | b. Berberine | | Brown orange | |
| **5)** | a. MDA | Mecke | Blackish violet | Dark brown |
| | b. Piperine | | Brownish yellow | |
| **6)** | a. Cocaine | Mecke | Colorless | Light brown |
| | b. Piperine | | Brownish yellow | |
| **7)** | a. Methamphetamine | Mandelin | No change | Red orange |
| | b. Berberine | | Dark red | |
| **8)** | a. MDMA | Mandelin | Dark blue | Blackish orange |
| | b. Berberine | | Dark red | |
| **9)** | a. Methamphetamine | Mandelin | No change | Orange |
| | b. Papaverine | | Orange | |
| **10)** | a. MDMA | Mandelin | Dark blue | Orange |
| | b. Papaverine | | Orange | |

mainly on the central nervous system and exert several physiological effects - the reason why they are frequently consumed as recreational drugs [23]. For example, morphine obtained from opium poppy is a powerful analgesic, with psychoactive properties, including sleep induction. Morphine is administered in healthcare to reduce pain, but is extremely addictive. Well-known derivatives of morphine, such as heroin, are potent drugs with extremely addictive properties [24]. Cocaine, another well-known alkaloid, is likewise seen as a recreational drug. Its rapid anesthetic and psychomimetic outcomes allow users to lose consciousness and suppress bodily sensations.

Although some alkaloids are commonplace, well-known by the public, and often abused as recreational drugs (e.g., morphine, codeine, cocaine, etc.,), other not-so-well-known substances exist: they can be purchased easily and legally via the Internet or in local

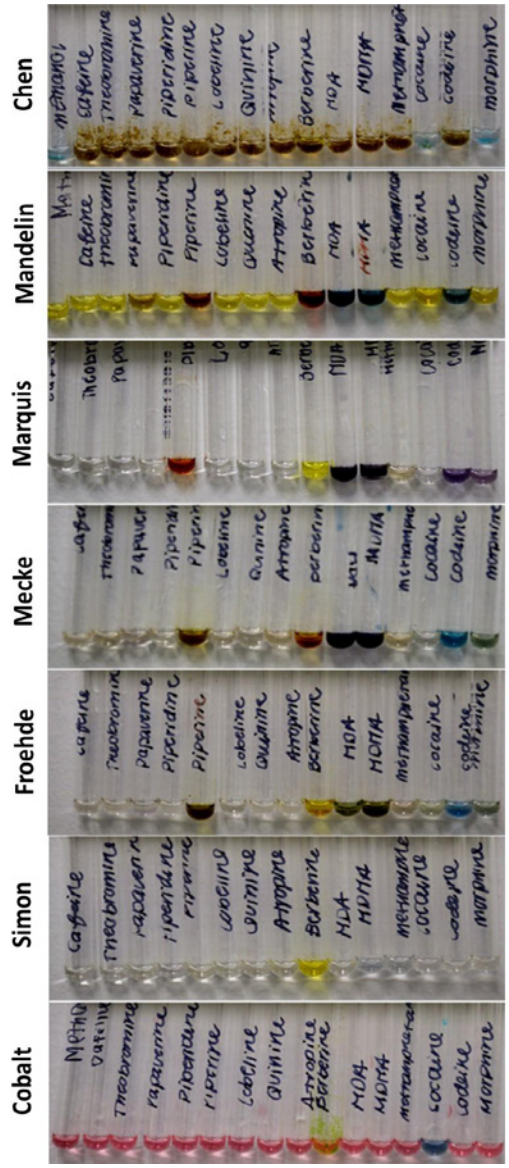



**Figure 3:** Representative images of colorimetric reactions in presumptive tests of authentic alkaloid standards.

shops in many countries (e.g., lobeline, caffeine, piperine). Many alkaloids are involved in the production of drugs as psychoactive cutting products. They possess various chemical structures, which are the bases of their classification [25] (Supplementary data 1). For example, morphine belongs to the benzylisoquinoline group, along with papaverine, whereas ephedrine, methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA; ecstasy) are phenethylamines (Supplementary data 1).

Alkaloids can be extracted from natural sources (natural) or produced synthetically in a laboratory from scratch (synthetic) or from a natural precursor (semi-synthetic). For example, cocaine is a natural alkaloid and can be extracted directly from coca leaves (Supplementary data 1). Opium poppies produce morphine and codeine (natural alkaloids), but they can be modified to form the semi-synthetic alkaloids heroin and hydrocodone, respectively (Supplementary data 1). Synthetic alkaloids, MDA and MDMA, are created in entirely legal or clandestine laboratories, via chemical synthesis (Supplementary data 1).

With the development of new synthetic drugs and different cutting products, it is increasingly challenging to detect illicit compounds in samples. Due to the structural diversity of cutting agents, especially alkaloids, we ask ourselves if presumptive color tests may be useful for identification. Databases of colorimetric presumptive tests have been established for most common drugs, but the presence of cutting agents, alkaloid or not, interfere with the color results and provide false negatives or positives.

The goal of the present study was, first, to predict and validate the results of 7 presumptive color tests of various alkaloid standards (Table 1). [Since this work was undertaken in the context of an university level course, it was not possible to test real street drug specimens. Thus, authentic standard solutions were prepared and underwent presumptive tests]. We tested alkaloid mixtures to ascertain if there was interference with the color results. Next, we performed presumptive color tests on various popular cutting agents and, finally, assessed several mixtures of drugs/alkaloids/cutting agents potentially similar to samples seized in the field. Figure 2 illustrates the overall study strategy.

## Experimental Procedure

### Chemicals, reagents and materials

Reagents, including hydrochloric acid, glacial acetic acid, nitric acid, sulfuric acid, 37% formaldehyde solution, chloroform, acetaldehyde solution, cobalt (II) thiocyanate, potassium hydroxide, sodium hydroxide, sodium carbonate, ethanol, methanol, copper (II) sulfate, cobalt (II) acetate, cobalt (II) thiocyanate, ammonium molybdate, silver nitrate, ferric chloride, ferric sulfate, anhydrous sodium sulfate, sodium nitrite, potassium iodide and ammonium vanadate, were purchased from Fisher Scientific (Pittsburgh, PA, USA). Caffeine, theobromine, papaverine, piperidine, piperine, lobeline, quinine, atropine, and berberine were sourced from Sigma-Aldrich (St. Louis, MO, USA). Controlled substances, including methamphetamine, MDA, MDMA, cocaine, codeine and morphine were procured from Cerilliant (Round Rock, TX, USA) by the UQTR Forensic Laboratory.

### Standard preparations

All standard alkaloids tested were prepared at a final concentration of 1 mg/ml in methanol. Alkaloid mixtures were produced from solution at 2 mg/ml concentration in a 1:1 ratio. Mixtures with cutting agents were formulated as indicated in Figure 4.

### Presumptive colorimetric tests

Presumptive colorimetric test protocols are available in Lajeunesse [26].

**Chen's test:** The Chen color test involves 2 solutions. The first solution is 1% (w/v) of copper sulfate in demineralized water. The second solution is sodium hydroxide at 2 M. Ten µl of the first solution is added to 10 µl of the sample to be tested. Ten µl of the second solution is added thereafter.

**Mandelin's test:** The Mandelin reagent color test comprises

| | Narcotic | Alkaloid/Cutting agent | Proportion alkaloid/cutting agent |
|---|---|---|---|
| 1.1 | MDA | Sugar + Acetaminophen | 30/35/35 |
| 1.2 | | Talc + Quinine + Caffeine | 30/23/23/23 |
| 1.3 | | Flour + Quinine + Atropine | 30/23/23/23 |
| 1.4 | | Baking soda + Sugar + Caffeine | 30/23/23/23 |
| 2.1 | MDMA | Sugar + Acetaminophen | 30/35/35 |
| 2.2 | | Talc + Quinine + Caffeine | 30/23/23/23 |
| 2.3 | | Flour+ Quinine + Atropine | 30/23/23/23 |
| 2.4 | | Baking soda + Sugar + Caffeine | 30/23/23/23 |
| 3.1 | Methamphetamine | Sugar+ Acetaminophen | 30/35/35 |
| 3.2 | | Talc + Quinine + Caffeine | 30/23/23/23 |
| 3.3 | | Flour + Quinine + Atropine | 30/23/23/23 |
| 3.4 | | Baking soda + Sucre + Caffeine | 30/23/23/23 |
| 4.1 | Cocaïne | Sugar + Acetaminophen | 30/35/35 |
| 4.2 | | Talc + Quinine + Caffeine | 30/23/23/23 |
| 4.3 | | Flour + Quinine + Atropine | 30/23/23/23 |
| 4.4 | | Baking soda + Sugar + Caffeine | 30/23/23/23 |

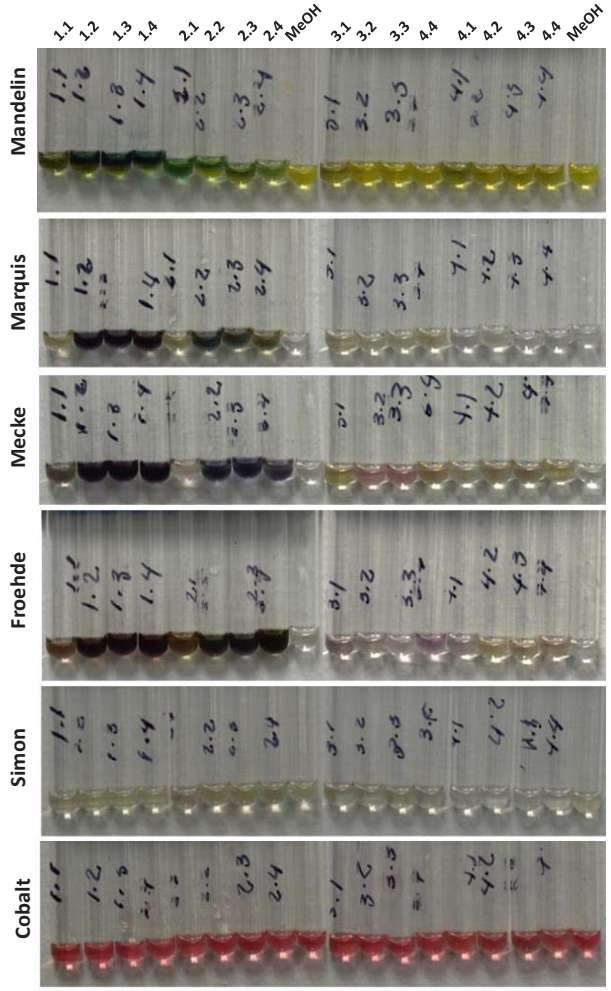



**Figure 4:** Mixtures of narcotic/alkaloid/cutting agents for presumptive tests and representative images of colorimetric reactions.

a solution of 50 mg ammonium vanadate in 5 ml of concentrated sulfuric acid. Twenty µl of the reagent is added to 10 µl of the sample to be tested.

**Marquis' test:** The Marquis reagent is a solution of 0.25 ml of formaldehyde in 5 ml of concentrated sulfuric acid. Twenty µl of the reagent was added to 10 µl of the sample to be tested.

**Mecke's test:** The Mecke color reagent is composed of 1 solution of selenious acid in concentrated sulfuric acid at 1% (w/v). Twenty µl of the reagent is added to 10 µl of sample to be tested.

**Froehde's test:** The Froehde reagent is a presumptive color test of 1 solution. This solution is sodium molybdate in concentrated sulfuric acid at 0.5 (w/v). Twenty µl is added to 10 µl of the sample to be tested.

**Simon's test:** The Simon color test comprises 2 reagents. Reagent 1 is prepared with 50 mg of sodium nitroprusside dissolved in 0.5 ml of acetaldehyde and 4.5 ml of demineralized water. Reagent 2 is 2% (w/v) sodium carbonate in demineralized water. Ten µl of reagent 1 is added to 10 µl of the test sample, followed by the addition of 10 µl of reagent 2.

**Cobalt's test:** The Cobalt reagent is composed of a cobalt thiocyanate solution in demineralized water at 2% (w/v). Twenty µl of solution is added to 10 µl of the sample to be tested.

In each experiment, which was repeated 3 times, the solution was mixed and left to stand for 2 min prior to its color detection.

## Results and Discussion

### Presumptive tests on authentic standards

We first performed 7 presumptive color tests on 15 available alkaloid standards (6 drugs and 9 potentially psycho-active cutting agents). Color reaction results were available for some, whereas, for others, predicted results were based on the chemistry behind each presumptive color test (Supplementary data 2). A representative image of each test and a table of observations were included (Figure 3; Supplementary data 3). Pictures were taken shortly after colors appeared in the different test tubes, to ensure that the color changes were due to the drug and not to side-reactions, such as photo-oxidation, after long exposure. In addition, a summary of results analysis is presented in Table 2.

Colorimetric results of Chen's test on standards revealed that all of them reacted with Chen's reagent except for the alkaloids cocaine and morphine (Figure 3; Table 2). Since Chen's test was the least specific of the presumptive color tests studied here, color changes were expected from the formation of complexes from standard functional groups and copper from the reagent. With non-reactive alkaloids, it was possible that steric hindrance prevented oxygen and nitrogen atoms from making complexes with copper, which were not accounted for in our predictions. The color results obtained for known alkaloids were not exactly similar, as expected and reported in the literature. However, the presence or absence of reactions is indicative of the presence or absence of substances [4-6,11].

Mandelin's test presumptively identifies certain phenylethylamines and opiates. Mandelin's reagent reacted with 7 standards, including the 2 phenethylamines MDA and MDMA, and the opiate codeine (Figure 3).The color changes ranged from dark yellow to dark blue, as expected from different oxidation states of vanadate ion. Indeed, Mandelin's reagent is yellow in the beginning (oxidative state +5), and, when it gets reduced to +4 or +3, it respectively becomes blue and then green [17-19].

Interestingly, standards of the phenethylamine group, such as MDA and MDMA, displayed similar dark-blue color, whereas

**Table 4:** Summary of presumptive tests with cutting agents. The first lane indicates the color of each test without agents added to the solution.

| Cutting agent | Chen | Mandelin | Marquis | Mecke | Froehde | Simon | Cobalt |
|---|---|---|---|---|---|---|---|
| | Light blue | Yellow | Colorless | Colorless | Colorless | Colorless | Pink |
| Acetaminophen | - | Fading | - | - | - | - | - |
| Baking soda | Precipitate | - | - | - | - | - | Precipitate |
| Flour | - | - | - | - | - | - | - |
| Sugar | - | Fading | Light yellow | - | Light yellow | - | - |
| Talc | - | - | - | - | - | - | - |
| Atropine | Light green-brown | - | - | - | - | - | - |
| Quinine | Light green-brown | - | - | - | - | - | - |
| Caffeine | Light green | - | - | - | - | - | - |

methamphetamine did not react. Similarly, codeine from the opiate group reacted positively whereas morphine did not. Absence of the *O*-methyl group in morphine and lack of the Methylenedioxy bridge in methamphetamine may have caused the lack of reaction with Mandelin's reagent. It suggests that these structures are important for a positive reaction with Mandelin's reagent. Indeed, the presence of the methylenedioxy bridge in piperine and berberine and of *O*-methyl groups in papaverine and cocaine supports this hypothesis (Figure 3). The orange color obtained cannot be explained by vanadate ion oxidation states: it is possible that the color resulted in side-reactions with the reagent. These findings need to be expanded with other standards, with similar chemical structures, to confirm the exact mechanism of reaction in Mandelin's test. They would help in future predictions with this test.

Given their chemical structures, it was expected that the 12 standards would react with Marquis' test (Supplementary data 2) but, surprisingly, they only half-confirmed our predictions. All phenethylamines and opiates reacted with Marquis' reagent plus piperine (Figure 3; Table 2; Supplementary data 3). These standards bear good nucleophilic structures that would allow reaction with formaldehyde in the reagent. The opiates codeine and morphine manifested a violet color when the reaction occurred (Figure 3). MDA and MDMA produced a black-violet color while piperine and methamphetamine yielded an orange color. The color results obtained for known alkaloids were not exactly similar to the color expected and reported in the literature [4-6,11]. In addition, among the alkaloids that did not react, most contained aromatic structures thought to react to Marquis' reagent. It is possible that the aromatic ring environment renders weak nucleophilic molecules unable to react with sulfuric acid to form a salt to, in turn, react with formaldehyde in the reagent. Additional standards and tests should be performed to elucidate the exact mechanism of reaction.

It was difficult to predict the results of standard alkaloids with Froehde's reagent, since this reaction is not clearly established. We proposed that most alkaloids tested had a functional group possibly able to react and yield a color with molybdate ion from Froehde's reagent (Supplementary data 2). Only 5 of the 15 alkaloids tested reacted with Froehde's presumptive test and yielded blue-green color results. The color was caused by reduction of molybdate ion [27], and the results we obtained for known alkaloids were as expected from the literature [5]. However, when we compared the chemical structures of alkaloids that reacted versus those that did not, it was challenging to understand the exact mechanism of reaction of Froehde's test. In fact, it is difficult to identify the functional group that allows reduction of molybdate ions to yield a color from Froehde's reagent. Except for berberine, the same 5 standards reacted with both Mecke's and Froehde's reagents (Figure 3; Table 2). These 2 tests assess similar components, including sulfuric acid for reaction. Thus, it is expected that the same standards react with both tests. However, berberine reacted only with Mecke's test, indicating small differences among the mechanisms of reaction of these tests. In Mecke's test, berberine and piperine both produced a brown color, whereas MDA and MDMA yielded purple-black, and the opiates codeine and morphine gave a blue color. The similarity of color reactions compared to the similarity of chemical structures suggests that Mecke's test could differentiate between specific chemical structures.

As expected, all standards with secondary amines, including piperidine, MDMA and methamphetamine, reacted with Simon's reagent, and the color obtained for MDMA and methamphetamine matched that found in the literature [5,28] (Figure 3; Table 2; Supplementary data 2 and 3). However, secondary amines from theobromine did not react. Given the position of secondary amines in structures, it was predicted that no reaction would occur (Supplementary data 2).

Berberine, with its tertiary protonated amines, reacted with Cobalt's test, as predicted (Figure 3; Table 2). Unexpectedly, cocaine reacted positively as well but not atropine. Both are tropane alkaloids with tertiary amines. However, the additional functional groups on the tropane ring present in cocaine may cause strong displacement of electrons, possibly rendering the tertiary amines ''more'' protonated and more able to react with Cobalt's reagent than atropine. The color produced by cocaine agreed with that in the literature [5,28]. Altogether, most results confirmed predicted and expected test colors. However, in tests where the mechanism of reaction was not well-defined, some results were difficult to interpret, so that additional standards and tests would be necessary to better predict the outcome of a given molecular structure. Once the reactions and results can be predicted, it may be possible to start an identification key for potentially novel drugs.

## Presumptive tests on mixtures of standards

Next, we wanted to assess if presumptive tests would react similarly with samples containing mixtures of compounds, as they are more likely to occur in the field. Thus, 10 mixtures of 2 alkaloids (1:1)

each were prepared and assayed with all 7 presumptive color tests. Observations of each alkaloid, alone and combined, are reported (Table 3). Some alkaloid mixtures displayed colors derived from the results of each alkaloid. For example, with Marquis' test, cocaine is colorless, and piperine is brown-orange. The color of Marquis' test reaction from the mixture of cocaine and piperine is orange, which is most likely derived from a mix of colorless+brown-orange (Table 3). Similarly, with Mandelin's test, methamphetamine alone showed no color change, whereas papaverine reacted and yielded orange color. The mixture of methamphetamine with papaverine resulted in orange color, most likely derived from the papaverine reaction with Mandelin's reagent. Interestingly, the last mixture tested did not react that way. Mandelin's reagent reacted with MDMA and yielded a dark blue color, whereas papaverine was orange. Unexpectedly, mixtures containing MDMA and papaverine reacted with orange color similar to papaverine alone (Table 3). In this case, it seems like the papaverine reaction took over the MDMA reaction, and the final result displayed only the color from papaverine. It is possible that in the presence of 2 alkaloids, there is competition for reaction with the reagent. In this case, papaverine succeeded over MDMA, which was not expected. Thus, multiple chemicals in a mixture may react with presumptive color tests and yield: 1) the expected color (positive result), 2) a diluted or darkened version of the expected color (mixture of 2 results (inconclusive results), or 3) an unexpected color or no color (false negative result). The latest may be problematic when we depend on presumptive color tests to decide if samples will be investigated further, by confirmatory testing. We concluded that it is not always possible to determine that more than 1 product is present in a sample, because the colors can be very different. It is possible to completely miss a drug because the reaction is hidden by another alkaloid. Thus, a thorough investigation of chemicals mixed and careful collection of the color are necessary since the results of mixed alkaloids are sometimes unpredictable.

## Presumptive tests on cutting agents

Most illicit drugs are adulterated and laced with one or more substances. Some street drugs can be as low as 10-15% of the active chemical, with the remaining (85-90%) chemicals being impurities/by-products produced during synthesis/storage or cutting agents. Cutting agents are inexpensive and easy-to-obtain chemicals that mimic the physical attributes of drugs. For example, if a drug is soluble in water, the preferred cutting agent would be water-soluble. If the drug is smoke, similar melting and boiling points are important. Common cutting agents include psycho-active drugs, such as acetaminophen, ephedrine, atropine and caffeine, and non-psychoactive chemicals, such as sugar (glucose, lactose), flour (starch), talc, cornstarch and baking soda [29-32]. It is possible that cutting agents interfere with presumptive color tests. To verify this possibility, we first performed tests with various, popular, frequently-used and easily-accessible cutting agents (Supplementary data 4). As expected, alkaloid cutting agents (atropine, quinine and caffeine) reacted positively only with Chen's test (Tables 2 and 4; Supplementary data 4). Acetaminophen and sugar did not seem to react with Mandelin's test but did cause fading of the yellow reagent (Table 4), indicating that these substances could interfere with the results of this test. Baking soda formed precipitates in the presence of Chen's and Cobalt's reagents whereas flour or talc did not react with any tests. White sugar (sucrose) is the only cutting agent that reacted with more than one presumptive color test, producing light discoloration of Mandelin's reagent and yielding yellow color with Marquis' and Froehde's tests (Table 4). Thus, some cutting agents may interfere with presumptive color test results if combined with the drugs to be assayed.

## Presumptive tests of drug, alkaloid and cutting agent mixtures

To investigate the effects of cutting agents, alkaloid or not, on the results of presumptive color tests, we prepared several drug/alkaloid/cutting agent mixtures (Figure 4). Mixtures and ratios were chosen randomly. We omitted Chen's test since it was unspecific and reacted with several chemicals (drugs, alkaloids or not).

The first set of mixtures contained MDA with various cutting agents (Figure 4). MDA alone reacted with various presumptive test reagents and generated detectable reaction products (Supplementary data 4 and Table 4). However, when MDA was mixed with sugar+acetaminophen (1.1 mix), the color reaction for 2 tests, Mandelin's and Marquis', changed significantly compared to MDA alone (Figure 4; Supplementary data 4). Similarly, 2.1 (MDMA+sugar+acetaminophen) and 3.1 (methamphetamine+sugar+acetaminophen) mixtures displayed comparable results where reaction colors with Mandelin's and Marquis' tests were significantly different from the color obtained for the corresponding drug alone (Figure 4; Supplementary data 4). This effect was not evident for 4.1 mixtures (cocaine+sugar+acetaminophen). Thus, it is possible that cutting agents sugar and/or acetaminophen alter or react with phenethylamine drugs, such as MDA, MDMA or methamphetamine, and modify their chemistry. This, in turn, may affect the response to various presumptive color tests, as reported here.

When Froehde's reagent was used on MDA and MDMA alone, the reaction produced a green color whereas no reaction was observed for methamphetamine or cocaine alone. However, when Froehde's reagent was used on mixtures of sets 1 and 2, a brown color appeared (Figure 4; Supplementary data 4). This coloration was not the one expected for MDA and MDMA, but the reaction was still positive. In contrast, mixes with methamphetamine (3.1-3.4) and cocaine (4.1-4.4) showed unexpected color reactions with Froehde's test. These results suggest that the presence of cutting agents can lead us to think that other narcotics may occur in mixes.

Simon's reagent is specific to molecules with secondary amines in the chemical structure. Thus, Simon's test is supposed to react only with MDMA and methamphetamine, producing a light blue color. Although the color was off, the mixtures containing these drugs reacted positively (Figure 4; Supplementary data 4). Simon's reagent was working, as expected, but the color differences were not expected to identify secondary amines.

Mixtures containing cocaine (4.1-4.4) showed no observable differences in Mandelin's, Marquis' and Simon's tests (Figure 4; Table 4). However, small (faded) color reactions with Mecke's and Froehde's reagents indicated false negatives for cocaine, since the drug alone did not react with these tests (Figure 4; Table 4). In addition, cocaine's blue reaction was clearly absent when mixed with cutting agents in Cobalt's test (Figure 4; Table 4). It is possible that cutting agents affected the chemical structure of cocaine's protonated

amines, yielding a negative result with Cobalt's test. Altogether, we determined that pure standards (drug/alkaloid) and mixtures with cutting agents did not always react, as expected, with different presumptive color tests.

## Conclusion

Seven presumptive color tests were evaluated to qualitatively record results in the presence or not of authentic standards, including known drugs and psycho-active cutting agents of the alkaloid family. Predictions made before experimentation were based on the chemistry behind each test. The results confirmed the predictions for pure standards but not for mixtures of standards and cutting agents. Since seized drugs are most likely to contain mixtures of compounds, it is important to better understand presumptive color tests to make better predictions. It could resolve several problems with interpretation that cannot be avoided at the moment. Furthermore, a larger database of color test results with alkaloids and cutting agents could prevent false negative or false positive outcomes.

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