TITLE:

Color Spot Test as a Presumptive Tool for the Rapid Detection of Synthetic Cathinones

AUTHORS & AFFILIATIONS:

Morgan Philp[1], Ronald Shimmon[1], Mark Tahtouh[2], Shanlin Fu[1]

[1]Centre for Forensic Science, University of Technology Sydney (UTS), Ultimo, Australia

[2]Australian Federal Police (AFP), Sydney, Australia

AUTHOR EMAILS:

Morgan Philp (morgan.philp@student.uts.edu.au)

Ronald Shimmon (ron.shimmon@uts.edu.au)

Mark Tahtouh (mark.tahtouh@afp.gov.au)

Shanlin Fu (shanlin.fu@uts.edu.au)

CORRESPONDING AUTHOR:

Shanlin Fu

Email: shanlin.fu@uts.edu.au

Tel: (+61) 2 9514 8207

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SHORT ABSTRACT:

Here we present a simple, inexpensive, and selective chemical spot test protocol for the detection of synthetic cathinones, a class of new psychoactive substances. The protocol is suitable for use in various areas of law enforcement that encounter illicit material.

LONG ABSTRACT:

Synthetic cathinones are a large class of new psychoactive substances (NPS) that are increasingly prevalent in drug seizures made by law enforcement and other border protection agencies globally. Color testing is a presumptive identification technique indicating the presence or absence of a particular drug class using rapid and uncomplicated chemical methods. Owing to their relatively recent emergence, a color test for the specific identification of synthetic cathinones is not currently available. In this study, we introduce a protocol for the presumptive identification of synthetic cathinones, employing three aqueous reagent solutions: copper(II) nitrate, 2,9-dimethyl-1,10-phenanthroline (neocuproine) and sodium acetate. Small pin-head sized amounts (approximately 0.1-0.2 mg) of the suspected drugs are added to the wells of a porcelain spot plate, and each reagent is then added dropwise sequentially before heating on a hotplate. A color change from very light blue to yellow-orange after 10 min indicates the likely presence of synthetic cathinones. The highly stable and specific test reagent has the potential for use in the presumptive screening of unknown samples for synthetic cathinones in a forensic laboratory. However, the nuisance of an added heating step for the color change result limits the test to laboratory application and decreases the likelihood of an easy translation to field testing.

INTRODUCTION:

The illicit drug market operates similarly to a traditional business by continuing to evolve and adapt to a changing marketplace. Advances in modern technology, specifically, the global proliferation of powerful communication has seen increased online purchases via the Dark Net[1] and extensive knowledge sharing among users via online forums[2]. Combined with advances in chemistry, the rapid emergence of new psychoactive substances (NPS) created a serious challenge for international and national drug control.

NPS are potentially dangerous substances of abuse that have similar effects to drugs under international control. Initially marketed as "legal" alternatives, 739 NPS were reported to the United Nations Office on Drugs and Crime (UNODC) between 2009 and 2016[3]. According to the most recent annual report, a record number of NPS were seized at the Australian border, with the majority of those analyzed, further identified as synthetic cathinones[4]. On a global scale, seizures of synthetic cathinones have been steadily increasing since first reported in 2010, and are one of the most commonly seized NPS[5].

The challenges posed by NPS have been a largely published topic of discussion[6,7]. Forensic laboratories and law enforcement personnel were left at a disadvantage without appropriate methods in place to detect and identify NPS during their rapid emergence. Extensive research into the detection of NPS, including synthetic cathinones, in seized material, has employed gas chromatography-mass spectrometry (GC-MS)[8] and liquid chromatography-high resolution mass spectrometry (LC-HRMS)[9] for confirmatory analysis. Increasing demand for minimal sample preparation has seen infrared and Raman spectroscopy[10] studies as well as ambient ionisation mass spectrometric analyses, such as direct analysis in real time mass spectrometry (DART-MS)[11,12]. The need for rapid, sensitive analysis in the field has also seen the incorporation of paper spray ionization-mass spectrometry (PSI-MS) into portable devices for use by law enforcement[13]. Many instrumental techniques offer confirmatory analysis with sensitive detection and quantitative results. However, for high-throughput analysis, they can be time-consuming due to sample preparation, run times, and instrument training and maintenance.

Presumptive color tests are designed to suggest the presence or absence of certain drug classes in a test sample[14]. The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) classifies color testing as the lowest discriminating power technique, alongside ultraviolet spectroscopy and immunoassays[15]. However, they are still widely employed by law enforcement and other security personnel as a means to provide rapid results at a significantly lower cost compared to other techniques. The main advantage offered by color spot test methods is the ability to perform them in the field using portable test kits.

The selectivity of color tests relies on individual chemical reactions occurring between the test reagent and the drug class of interest to create a color change. Current presumptive testing protocols lack a particular test for detecting synthetic cathinones only; commonly used reagents that lack specificity and contain hazardous substances are often employed. Other recommended reagents have not been screened on a large number of possible synthetic cathinone substances[16].

The aim of this work is to present a simple color test protocol that can be easily employed by interested parties for the preliminary screening of synthetic cathinones in illicit substances of unknown composition. Interested parties would include law enforcement, border protection agencies, forensic laboratories, and other relevant security personnel. The proposed methods employ a reduction-oxidation reaction occurring between the electron-accepting copper complex reagent and the electron rich synthetic cathinone drug molecules. Using these chemical methods developed, one can apply them in the form of a presumptive color test to suggest the presence of synthetic cathinones.

PROTOCOL:

1. Preparation of Color Test Reagent Solutions

1.1. Weigh 0.12 g of copper nitrate trihydrate into a dry 100 mL beaker. Add 30 mL of deionized (DI) water and carefully swirl it at room temperature to dissolve all solids. Pour this solution into a 100 mL volumetric flask and fill up to the calibrated mark with DI water. This prepared solution is reagent 1.

Note: Reagent 1 can be prepared using other copper(II) salts, *e.g.* copper(II) chloride.

1.2. Weigh 0.11 g of 2,9-dimethyl-1,10-phenanthroline (neocuproine) hemihydrate into a dry 100 mL beaker. Add 50 mL of 0.10 mol/L hydrochloric acid (HCl) and use a glass stirring rod to promote dissolution of solids at room temperature. Pour this solution into a 100 mL volumetric flask and fill up to the calibrated mark with 0.10 mol/L HCl. This prepared solution is reagent 2.

CAUTION: Neocuproine is acutely toxic can cause skin irritation and serious eye damage. Wear gloves and safety glasses while handling to minimize the risk of exposure.

Note: Neocuproine is only slightly soluble in water, therefore, dilute acid is used to prepare this reagent and ensure all solids dissolve.

1.3. Weigh 16.4 g of sodium acetate into a dry 100 mL beaker. Add 50 mL of DI water and use a glass stirring rod to promote dissolution of solids at room temperature. Pour this solution into a 100 mL volumetric flask and fill up to the calibrated mark with DI water. This prepared solution is reagent 3.

Note: The protocol can be paused here. The reagents are highly stable and can be stored for up to 12 months at room temperature.

2. Color Testing

2.1. Collect one clean porcelain spot plate, three disposable pipettes, three reagent solutions prepared in step 2.1, one clean spatula, an electric hotplate and the sample/seized material to be tested.

2.2. Using the spatula, place a small, pin-head sized amount (approximately 0.1-0.2 mg) of the unknown sample into three separate wells of a porcelain spot plate. Leave three adjacent wells empty (blank control) and another three wells with equal amounts of 4-methylmethcathinone HCl (4-MMC), a synthetic cathinone reference sample (positive control).

Note: The preferred test surface is a porcelain spot plate. If these are not available, use plastic microwell plates or semi micro test tubes.

2.3. Using a disposable pipette, add 5 drops of the copper nitrate solution (Reagent 1) to each sample well, in addition to the blank and positive control wells.

2.4. Using a second disposable pipette, add 2 drops of the neocuproine solution (Reagent 2) to each sample well, in addition to the blank and positive control wells.

2.5. Using a third disposable pipette, add 2 drops of the sodium acetate solution (Reagent 3) to each sample well, in addition to the blank and positive control wells.

Note: The solution turns light blue.

2.6. Place the porcelain spot plate directly onto an electric hotplate set at 80 °C.

Note: Do not heat plastic microwell plates directly on the hotplate. Prepare a shallow boiling water bath to set the plastic plate. Heat semi-micro test tubes in a small boiling water bath. The precise time required to observe a color change will depend on the thickness and composition of the spot plate.

CAUTION: Take care when handling spot plates to prevent burn injuries.

2.7. After heating for 10 min, observe by naked eye and note the final color change or take a photo of the final color change. Use a white background to better visualize the color changes.

REPRESENTATIVE RESULTS:

The test protocol has been validated through several studies, the results of which are described in Philp *et al.*[17]. The color test method is able to presumptively detect synthetic cathinones in an unknown sample through a color change from light blue to yellow-orange (**Figure 1**). Yellow and orange color changes occuring after the heating period are considered positive test results and any other color change, including very weak yellow or changes occurring before heatingare considered negative (**Table 1**).

The protocol has been applied to 44 synthetic cathinone analogues, 44 other illicit drugs, and 36 miscellaneous powders and cutting agents in previously published work[17]. Color changes experienced by these substances is summarized in the **Supplementary File 1**. These studies illustrate the success of the protocol in presumptively identifying the presence of synthetic

cathinones. The test protocol showed an 89% true positive test rate and a false positive rate of 10%. Representative positive test results are illustrated in **Figure 2**, and representative negative test results are provided in **Figure 3**. This test protocol can also successfully identify the presence of synthetic cathinones in mixtures containing more than one compound (**Figure 4**). This is an important result demonstrating its applicability to real-world samples.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative results from the color test protocol performed on a porcelain spot plate. (A) Color remains light blue with reagents only (blank control). **(B)** Yellow-orange color change with synthetic cathinone, 4-methylmethcathinone HCl (positive control).

Figure 2: Representative positive results from the color test protocol performed on a porcelain spot plate. The range of colors seen in a positive result is due to differences in antioxidant capacity and solubility of the compounds. **(A)** Yellow-orange color change with synthetic cathinone, N,N-dimethylcathinone HCl (true positive). **(B)** Light yellow-orange color change with synthetic cathinone, 3,4-dimethylmethcathinone HCl (true positive). **(C)** Light orange color change with a green ring around the edge with synthetic cathinone, 2,4,5-trimethylmethcathinone HCl (true positive). **(D)** Yellow color change with piperazine analog, 1-[3-(trifluoromethyl)phenyl]piperazine (TFMPP) HCl (false positive).

Figure 3: Representative negative results from the color test protocol performed on a porcelain spot plate. (A) Light green color change with synthetic cathinone, 3,4-methylenedioxypyrrolidinobutiophenone HCl (false negative). **(B)** Blue color change with miscellaneous powder, glycine (true negative). **(C)** Orange color change with drug precursor, 3,4-methylenedioxyphenyl-2-propanone (MDP2P) occurred before heating (true negative). **(D)** Color remained light blue with amphetamine sulfate (true negative).

Figure 4: Representative results of performing the color test protocol on mixtures of compounds. (A) Yellow-orange color change with a mixture of 4-methylmethcathinone HCl and ephedrine HCl. **(B)** A yellow-orange color change with a mixture of 4-methylmethcathinone HCl and 4-fluoromethcathinone (4-FMC) HCl.

Table 1: Color changes observed using the color test protocol. The proposed copper-neocuproine color test protocol was applied to 124 different substances and the color changes were recorded. Yellow and orange colors indicate a positive test result, while any other color is reported as a negative result.

Supplementary File 1. Color test results for substrates.

DISCUSSION:

This color test protocol was adapted from experimental work published by Al-Obaid *et al*.[18] in which the authors demonstrated a color change occurs in the presence of cathinone extracted from the khat plant. Modifications to the published protocol were necessary to foresee its application in presumptive illicit drug detection. The most important consideration was to reduce

the scale of the reaction. The protocol described in the present paper is designed to be applied to street samples and drug seizures.

The described protocol offers a simple presumptive indication of the presence of synthetic cathinones in a sample. Critically, the heating step of the protocol is necessary to visualize the color change of required intensity within the specified time limit. The thickness and composition of the porcelain spot plates may affect the time required for a color change to occur due to the thermal conductivity of the plate material. The 10 min heating period is designed to allow for these differences. Spot plates should also sit flat onto the hotplate so all wells experience the same amount of heat. Heating the spot plates longer than 10 min or at temperatures above 80 °C can affect the results negatively through the evaporation of the aqueous solutions. A second critical step is the addition of all three reagents, as the protocol will fail to work without all three.

Presumptive color tests are designed to be selective toward a certain drug class; provide results with rapidity, and possess a degree of portability to allow application in the field. The requirement of a heat source significantly decreases the portability of the test method. In addition, the 10 min heating period is not an ideal length of time to wait for a presumptive color test and is a limitation of this test protocol.

The basis of the color change occurring in this protocol is a non-specific reduction-oxidation reaction, which means that the synthetic cathinone molecules are not a ligand in the final colored complex. This inherent non-specific reaction means that there are likely other species that will interfere and reduce the copper(II) ions, *e.g.* ascorbic acid, and therefore lower the test specificity.

All presumptive color tests for illicit drugs are a subjective form of analysis based on the analyst's color perception. The color test protocol proposed here is particularly simple due to only one color change indicative of synthetic cathinone presence. This is unlike many general screening color tests that afford several different hues depending on the drug present.

This paper describes a useful and novel protocol for presumptively suggesting the presence of synthetic cathinones in seized material prior to confirmatory analysis. Commonly employed color test reagents are not able to afford the required specificity offered by the copper-neocuproine reagent. The most commonly used general screening color test reagent, Marquis, has been shown to afford negative results for many synthetic cathinones[19]. Although the Liebermann's reagent does react with cathinones, it also reacts with other illicit materials, including many synthetic cannabinoids[20].

The application of this protocol is ideal for forensic drug testing laboratories employing presumptive testing of seized samples. The reagent solutions are highly stable, and the protocol itself is particularly easy to follow.

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DISCLOSURES:

The authors have nothing to disclose.

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