Development and validation of a color spot test method for the presumptive detection of 25-NBOMe compounds.

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Development and validation of a colour spot test method for the presumptive detection of 25-NBOMe compounds

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Abstract

The great increase of new psychoactive substances over the last decade has substantially transformed the illicit drug industry to an ever-changing dynamic market. 25-NBOMe compounds are just one of these new substance groups that pose a public health risk in many countries around the world. These highly potent, hallucinogenic phenethylamines have previously been sold as 'legal highs' or 'synthetic LSD' and the necessity to rapidly identify their presence is crucial. While there are many laboratory-based analytical methods capable of identifying these compounds, the lack of presumptive test methods indicates the need for a specific and timely test that could be used in the field. Herein we outline the developed chemical spot test that can selectively identify the presence of 25-NBOMe compounds and related analogues through the reaction with a substituted benzoquinone reagent under basic conditions. This test method has been comprehensively validated showing a high level of selectivity, specificity and precision with only two other illicit substances producing similar positive results as 25-NBOMe and few false-negative results seen. The working limit of detection was determined to be 225 µg and there was no cross-reactivity from potential adulterants of significance. This test has also been shown to work directly with blotter papers containing 25-NBOMe compounds, indicating no interference from this common matrix and the ability to differentiate these compounds from LSD. This method shows a high potential to be translated to a field compatible test that is simple, rapid, and selective for 25-NBOMe compounds.

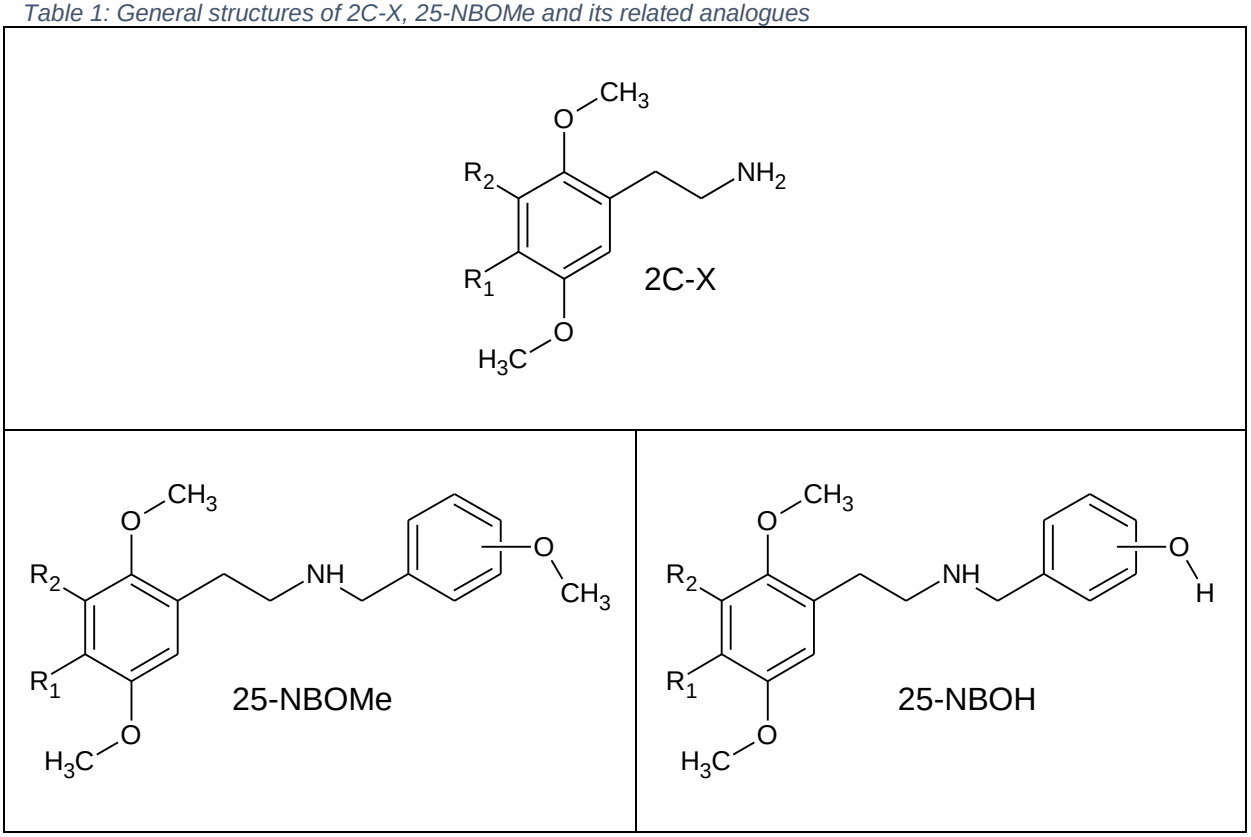
1. Introduction

The last decade has seen substantial change and development in the illicit drug industry with research and technology unveiling new substances. The occurrence of new psychoactive substances (NPS) has greatly increased since 2009 with 950 new

substances reported to the United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory up until December 2019, indicating the public health risks across many communities [1,2]. NPS encompass a wide variety of substances, with synthetic cannabinoids, cathinones, phenethylamines and opioids of significance in a highly dynamic market [3].

25-NBOMe substances are substituted hallucinogenic phenethylamines derived from the 2C-X series compounds with the addition of a 2-methoxybenzyl group on the amine functional group. They are highly potent agonists to the serotonin 5-HT_{2A} receptors even in microgram doses [4,5]. This gives them highly stimulant and hallucinogenic or psychoactive effects, similar to that of lysergic acid diethylamide (LSD). The 2-methoxybenzyl substituent, which identifies 25-NBOMe compounds, increases the potency significantly in comparison to the 2C-X substances [6-8]. The substituent commonly present at the *para*-position on the phenyl ring may also have an effect on the potency and effects of 25-NBOMe compounds [9]. Tables 1 and 2 show the structural relationship of 2C-X and 2C-X and 25-NBOMe compounds along with several 25-NBOMe related analogues.

Table 1: General structures of 2C-X, 25-NBOMe and its related analogues



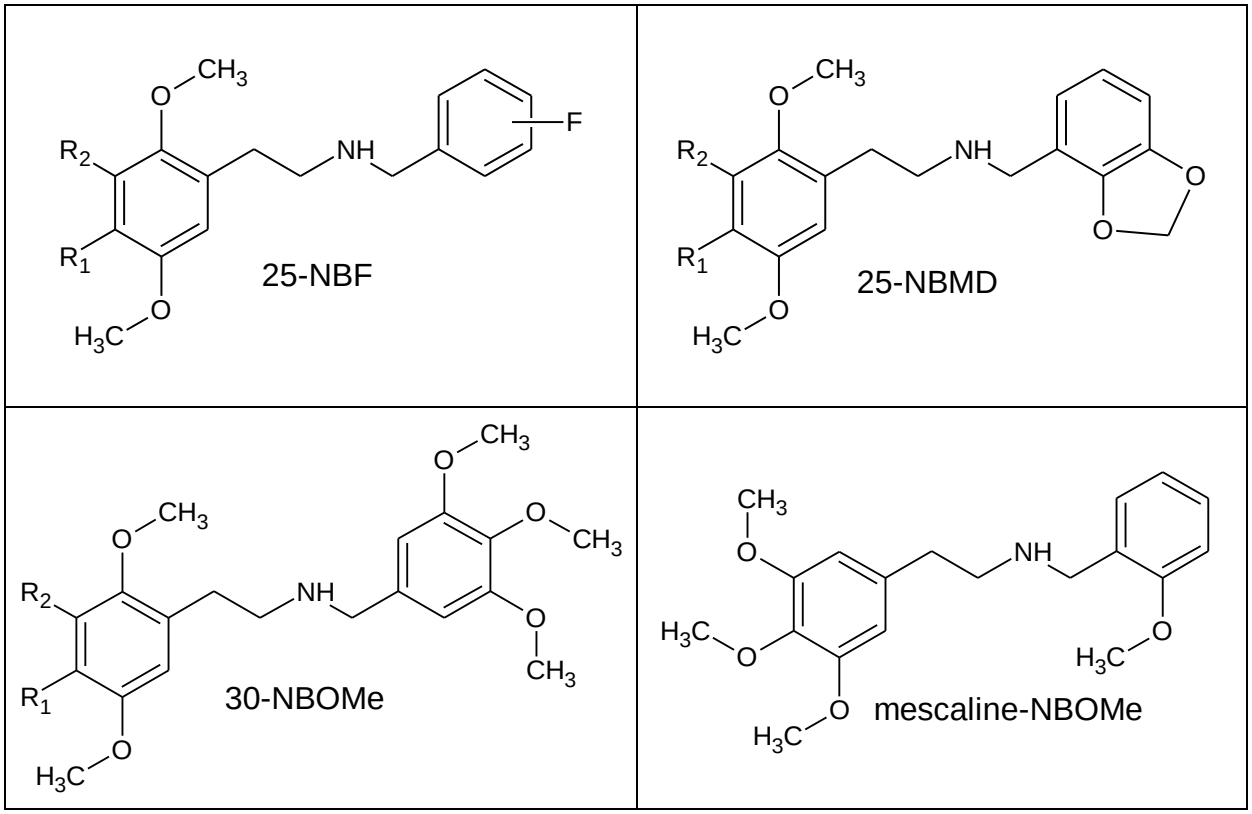


Table 2: Associated substituents for compounds outlined in Table 1

Prefix	R1 substituent	R2 substituent
25B	-Br	-H
25C	-Cl	-H
25D	-CH3	-H
25E	-CH2CH3	-H
25F	-F	-H
25G	-CH3	-CH3
25H	-H	-H
25I	-I	-H
25N	-NO2	-H
25P	-CH2CH2CH3	-H
25T	-SCH3	-H
25T2	-SCH2CH	-H
25T4	-SCHCH3CH3	-H
25T7	-SCH2CH2CH3	-H

Usage and appearance on the drug market have only been reported in recent years, initially appearing as 'legal LSD' [10]. Evidence from the literature suggests that 25-NBOMe substances are some of the more recent NPS to appear on the market [11]. Notable syntheses of 25-NBOMe compounds were first reported in 1994 [12] and more recently in 2003 [13]. It wasn't until 2011 that 25I-NBOMe first appeared on blotter papers in the designer drug market [14,15] while many countries, such as Japan, did not see 25-NBOMe substances until as late as 2013 [16]. There is data from a number of jurisdictions including Brazil, Columbia and Portugal that indicates blotter papers sold as LSD can contain 25-NBOMe compounds [17-19]. Other case reports indicate that many people present to hospital after taking what they thought to be LSD or an unknown substance and have acquired acute toxicity from an NBOMe related compound [20-22]. These cases result in severe effects on those users such as tachycardia, hypertension, hyperthermia, agitation, seizures and even kidney injury which are rarely seen after ingestion of LSD [23]. Reports of self-harm after NBOMe ingestion [21,24] along with fatalities resulting from NBOMe use [25,26] are common and provide insight into the increased risk of these drugs in comparison with LSD and other classic hallucinogens. The need to differentiate the contents of blotter papers sold as LSD is therefore apparent and the ability to do this on-site would be advantageous for police personnel, paramedics and drug checking facilities.

The analysis of 25-NBOMe compounds has been successful using gas or liquid chromatographic methods, often in conjunction with mass spectrometric techniques, and have been substantially reported in recent years [9,27-31]. The analysis of blotter papers is commonly performed using these methods and typically requires an extraction or derivatisation step before the instrumental analysis. 25-NBOMe compounds on blotter papers have also been quantified using high-performance liquid chromatography methods with a range of detection methods [5,32,33]. Other methods, including ATR-FTIR, have been reported as a potential direct analysis solution [34,35]. This said, there are few available methods for these samples to be analysed without any preparation required that can indicate the presence of 25-NBOMe compounds.

Colour tests provide analysts with preliminary information regarding the type or class of a substance. They are simple, rapid and inexpensive chemical spot tests that provide a colour change visible to the naked eye when a reagent is added to an

unknown substance [36]. The ease of use, portability, rapid results and the lack of sample preparation and expensive equipment required makes colour tests ideal for on-site identification of substances [37,38]. For these reasons, they are usually preferred by law enforcement and other forensic drug testing laboratories. They are also the most commonly used presumptive test methods with a range of commercial test kits available [39-41]. Over recent years there have been concerns regarding the selectivity of colour spot tests particularly with the appearance of many NPS. The increase in NPS availability has posed new challenges in drug detection for law enforcement, policymakers, drug testing services and scientists [42]. Currently, no presumptive test data exists for 25-NBOMe compounds [8,43].

Colour tests validated for traditional illicit drugs were studied by Cuypers et al. with a range of NPS including 25-NBOMe compounds [44]. 25-NBOMe compounds produced colour changes with some of these tests. For example, Scott's Reagent (cobalt thiocyanate), traditionally used for cocaine detection, gave a green colour change with several 25-NBOMe compounds [44]. Two main issues arise: 1) The analogues of the 25-NBOMe series produce a range of different colours, and 2) these observed colour changes cannot be distinguished from the colours seen when testing traditional illicit drugs and other NPS. Unpublished data from drug forum sites indicates that colour tests created for traditional drugs have been used in attempts to identify 25-NBOMe compounds [45]. Ehrlich's test, used to identify indoles such as LSD, and marquis reagent have been utilised to detect 25-NBOMe compounds and potentially distinguish between LSD and 25-NBOMe compounds on blotter papers. The results, however, indicate they are not reliable enough to accurately identify 25-NBOMe compounds. The high potency of 25-NBOMe compounds stresses the need for a test that can quickly and accurately identify these compounds or eliminate the possibility that one is present. A test that can identify 25-NBOMe compounds and distinguish these compounds from LSD would be highly valuable particularly directly from a blotter paper. The data indicating 25-NBOMe compounds are being sold as LSD signals the need to be able to differentiate these samples.

This study aimed to develop and validate a specific colour test method for the rapid identification of 25-NBOMe compounds, in particular, to be able to differentiate these compounds from LSD. Such a method would need to produce an obvious colour

change with 25-NBOMe compounds and have little to no cross-reactivity with other compounds that may be found in a suspected illicit drug sample.

2. Materials and methods

2.1. Reagents and chemicals

2,3,5,6-Tetrachloro-1,4-benzoquinone (TCBQ), acetaldehyde and propan-2-ol were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Ethyl acetate, 1, 4-dioxane, methanol, acetonitrile, sodium carbonate (Na_2CO_3) and sodium hydroxide (NaOH) pellets were obtained from Chem-Supply (Gillman, SA, Australia). Disodium phosphate (Na_2HPO_4) and monosodium phosphate (NaH_2PO_4) were obtained from Ajax Finechem (Taren Point, NSW, Australia). Deionised water from a laboratory supply was used throughout the methods.

2.2. Reference materials

Reference standards were purchased as hydrochloride salts including 2-(2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25H-NBOMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25C-NBOMe), 2-(4-methyl-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25D-NBOMe), 2-(4-ethyl-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25E-NBOMe), 2-(4-bromo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25B-NBOMe), 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe), 2-(3,4-dimethyl-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25G-NBOMe), 2-(4-methylthio-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25T-NBOMe), 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 4-iodo-2,5-dimethoxyphenethylamine (2C-I), 4-ethyl-2,5-dimethoxyphenethylamine (2C-E), 4-ethylthio-2,5-dimethoxyphenethylamine (2C-T-2), 2,5-dimethoxy-4-methylphenethylamine (2C-D), (±)-N-Methyl-3,4-methylenedioxyamphetamine HCl (MDMA), (+)-S-Methamphetamine HCl and d-amphetamine from the National Measurement Institute (NMI, North Ryde, NSW, Australia). Also purchased were the following NBOMe analogues 2-(4-chloro-2,5-dimethoxyphenyl)-N-[(3,4,5-

trimethoxyphenyl)methyl]ethanamine (30C-NBOMe), 2-(4-nitro-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25N-NBOMe), 2-(3,4,5-trimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (mescaline-NBOMe), 2-(4-bromo-2,5-dimethoxyphenyl)-N-[(4-methoxyphenyl)methyl]ethanamine (25B-NB4OMe), 2-(4-chloro-2,5-dimethoxyphenyl)-N-[(3-methoxyphenyl)methyl]ethanamine (25C-NB3OMe), 2-(4-Iodo-2,5-dimethoxyphenyl)-N-[(2-hydroxyphenyl)methyl]ethanamine (25I-NBOH), 2-(4-bromo-2,5-dimethoxyphenyl)-N-[(2-fluorophenyl)methyl]ethanamine (25B-NBF) and 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2,3-methylenedioxyphenyl)methyl]ethanamine (25I-NBMD) from Novachem (Heidelberg West, Victoria, Australia). LSD and ergotamine-d-tartrate were purchased as 1 mg/mL solutions and powder standards respectively from Lipomed (Arlesheim, Switzerland). Fentanyl was purchased as a powder standard from PM separations (Capalaba, Queensland, Australia) and JWH-073 was synthesised inhouse [46]. (1S,2R)-(+)-Ephedrine hydrochloride and (1S,2S)-(+)-pseudoephedrine HCl were obtained from Sigma Aldrich. Further pure drug reference standards were also obtained from NMI through the Australian Federal Police (AFP, Sydney, Australia). A complete list of these substances can be found in the results. All reference standards were obtained in powder or crystalline form.

Ibuprofen, paracetamol, 3,4-dimethoxyphenethylamine, caffeine, lidocaine, magnesium stearate, dimethyl sulfone, phenacetin, benzocaine, tetramisole hydrochloride, sorbitol, phenobarbital, salicylamide, 4-methoxyphenol (MEHQ), inositol, creatine and 4-aminophenazone were obtained from Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia). Aspirin, phenolphthalein, citric acid, benzoic acid and mannitol were obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia) and L-ascorbic acid from VWR chemicals (Campbellfield, Vic, Australia). Thiaminium dichloride (thiamine) was obtained from Merck KGA (Castle Hill, NSW, Australia) and quinine was obtained from Fluka (Castle Hill, NSW, Australia). A range of sugars were also obtained, including cellulose and starch from Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia), lactose, D(-)-fructose and D-glucose from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia), and sucrose from Mallinckrodt Pharmaceuticals (Kew, Vic, Australia). Several primary and secondary amines and amino acids were also obtained including L-threonine, L-phenyldiamine, L-valine, glycine, diphenylamine, L-

phenylalanine, methylamine HCl, methoxylamine HCl, ethylenediamine, ethylenediamine-N,N'-diacetic acid, D-alanine and piperazine hexahydrate from Sigma Aldrich Pty Ltd (Castle Hill, NSW, Australia). Diphenylamine and aniline were obtained from VWR chemicals (Campbellfield, Vic, Australia).

2.3. Preparation of solutions

A 3×10^{-3} M TCBQ solution was prepared by dissolving 0.73 g in 100 mL ethyl acetate. The phosphate buffer solution was prepared to contain sodium hydroxide (NaOH) and sodium hydrogen phosphate (Na_2HPO_4). The buffer was prepared to pH 11.4 using NaOH (0.1 M, 9.1 mL) and Na_2HPO_4 (0.05 M, 50 mL) made up to 100 mL in deionised water.

A methanolic solution of the synthesised 25H-NBOMe was prepared at a concentration of 0.3 mg/mL to be used for method validation analyses. 25H-NBOMe (0.025 mmol, 7.5 mg) was dissolved in methanol (25 mL) and when required, aliquots of this solution were added to a micro well-plate and placed in the fume hood to allow the methanol to evaporate. Testing then proceeded with the addition of 100 µL of each reagent solution.

2.4. Colour test method development

2.4.1. Preliminary testing

The initial method was based upon a published method by Walash et al. describing the spectrophotometric identification of phenylpropanolamine [47]. Several adaptations to the method were incorporated, including the removal of the extraction step and the use of the phosphate buffer solution. Initial tests were completed with 25H-NBOMe in test tubes. Approximately 1 mg of the drug was added followed by 1 mL of each reagent in the order:

1) Phosphate buffer solution (pH 11.4)
2) TCBQ in 1,4-dioxane solution (0.003 M)
3) Acetaldehyde in propanol solution (8% v/v)

Following these tests, further adaptations were made. The reagent (TCBQ) concentration was reduced from 3×10^{-2} M to 3×10^{-3} M and prepared in ethyl acetate rather than 1,4-dioxane. Acetaldehyde replaced the original 8% acetaldehyde in propanol solution after testing showed greater colour changes. A reduction in the reagent volumes was also favourable to perform this test on a micro-scale. Tests were completed on white porcelain spot plates with 3 drops of each adapted reagent on approximately 1 mg of 25H-NBOMe.

2.4.2. Reagent optimisation

Twelve solutions of TCBQ in ethyl acetate were prepared at concentrations 8×10^{-5}, 1×10^{-4}, 3×10^{-4}, 5×10^{-4}, 8×10^{-4}, 1×10^{-3}, 3×10^{-3}, 5×10^{-3}, 8×10^{-3}, 1×10^{-2}, 3×10^{-2} and 5×10^{-2} M to assess the effect on the colour change results. This testing was completed in triplicate in a micro well-plate by the addition of the previously prepared solution of 25H-NBOMe (3 mg/mL, 500 µL) to each well and the methanol allowed to evaporate before testing. A set of methanol control blanks were also prepared using 500 µL methanol in each well. 100 µL of each reagent solution was added to the blank and 25H-NBOMe containing wells and the colour changes recorded. An appropriate concentration could then be identified for future testing.

2.4.3. Buffer solution optimisation

Five buffer solutions were prepared to assess the effect of changing the pH and buffer composition on the final colour change result. Table 3 outlines the composition of each buffer and the correlating pH value. These buffers were tested on 25H-NBOMe for comparison with TCBQ in ethyl acetate (3×10^{-3} M) and acetaldehyde.

Table 3: Composition of buffer solutions prepared for analysis

pH value	Buffer composition	Final volume
8.0	NaH_2PO_4 (5.3 mL, 0.2 M) & Na_2HPO_4 (94.7 mL, 0.2 M)	200 mL
10.9	Na_2HPO_4 (100 mL, 0.05 M) & NaOH (6.6 mL, 0.1 M)	200 mL
11.0	$NaHCO_3$ (100 mL, 0.05 M) & NaOH (45.4 mL, 0.1 M)	200 mL
11.4	Na_2HPO_4 (100 mL, 0.05 M) & NaOH (18.2 mL, 0.1 M)	200 mL
12.0	Na_2HPO_4 (100 mL, 0.05 M) & NaOH (53.8 mL, 0.1 M)	200 mL

2.5. Method validation

After optimisation of the general method, validation was completed with procedures based upon guidelines outlined by the UNODC and National Institute of Justice (NIJ) [48,49]. This included assessment of specificity and selectivity, limit of detection (LOD), precision, stability and impurity testing.

2.5.1. Specificity and selectivity

A comprehensive range of drug compounds, analogues and cutting agents were tested to assess the specificity and selectivity of this test method. These included NBOMe analogues and derivatives, illicit compounds from other drug classes, illicit drug precursors, pharmaceuticals, common adulterants and diluents along with non-drug related amine-containing chemicals.

These tests were completed with no sample preparation: a pinhead-sized amount of each powder (or one drop from a Pasteur pipette if in liquid form) was added to a porcelain spot plate followed by the reagents outlined in the general procedure (see 3.2). These tests were completed as though they would be in the field with no accurate measurement of the amount of drug being tested. The colour was recorded immediately and after five minutes, and each test was completed in duplicate.

2.5.2. Limit of detection

The LOD was determined through a modified version of the method outlined by the NIJ Colour Test Standard [49]. Twelve aliquots (0, 10, 25, 50, 75, 100, 150, 200, 300, 350 and 500 µL) of methanolic 25H-NBOMe (0.3 mg/mL) solution were pipetted into a micro well-plate in triplicate. The methanol was evaporated in the fume hood before 100 µL of each reagent solution was added. The smallest sample at which the colour change was still differentiable from the reagent blank and considered a positive result was determined as the LOD. The NIJ guidelines suggest that this quantity would be multiplied by 10 to find the operational detection limit.

2.5.3. Purity testing

Twelve aliquots of 25H-NBOMe (100 µL, 1 mg/mL) were added to a micro well-plate and the solvent allowed to evaporate. To all but one of these wells, 100 µL aliquots of eleven adulterant solutions (1 mg/mL) were added and the solvent evaporated once again. These included methamphetamine, MDMA, ephedrine, paracetamol, pseudoephedrine, caffeine, ibuprofen, aspirin, lidocaine, benzocaine and creatine. 100 µL of the three reagents were then added and any colour change observed. A reagent blank was also performed with methanol for comparison to the mixtures.

2.5.4. Precision analysis

The UNODC guidelines suggest completing at least 10 replicates at concentrations between 1.25× and 2× the LOD value [48]. Using this, in a micro well-plate, twelve replicates were completed in duplicate at amounts just below 1.25× LOD and 2× the LOD value. A 1 mg/mL methanolic solution of 25H-NBOMe was used for this analysis and the methanol evaporated before 100 µL of each reagent was added to the wells in the recommended order (see 3.3). Further to this, tests were completed to assess the intra- and inter-day repeatability of the test method. Over ten days, tests with 25H-NBOMe were completed in triplicate at three separate occasions throughout each day.

The reproducibility of the test was assessed by changing the conditions in which the reaction was performed. This included testing two sets of the three reagents using reagent solutions which were prepared at different times. These tests were all carried out on porcelain spot plates using 1 mg 25H-NBOMe with 3 drops of each reagent solution from a Pasteur pipette in the order: buffer, TCBQ, acetaldehyde. A number of certified reference standards were tested in duplicate at the AFP laboratory (Sydney, Australia) with a freshly prepared reagent. Several of the NBOMe and amphetamine-type substances (ATS) reference materials (different samples) were also tested in the UTS laboratory representing an inter-laboratory investigation. These samples are identified in the selectivity results Table 4.

2.5.5. Blind tests

Blind tests were prepared in 24 wells of a 96 well plate with the addition of a selection of NBOMe, 2C-X, ATS solutions (100 µL, 1 mg/mL) and methanol blanks by a second analyst. The solvent was evaporated before 100 µL of each reagent solution was added to each well and the colour change recorded. Along with the colour changes, a determination of what the colour change identified was also recorded. After all tests had been completed and colour changes recorded, the results were compared to the compounds which had been added to each well.

2.5.6. Stability

The prepared TCBQ in ethyl acetate reagent (3×10^{-3} M) was stored in three different environments for eight weeks. One vial of the reagent was left on the laboratory bench for the study, a second wrapped in foil and stored in the laboratory cupboard and a third wrapped in foil and stored in the refrigerator (4 °C). The reagents were tested with 25H-NBOMe on porcelain spot plates over the eight-week period comparing the stored solutions each time to a freshly prepared reagent solution and colour changes observed. The coloured compound formed was also assessed for stability to determine how long the positive colour change could be seen. Photos were taken at regular time intervals over 48 hr to record the colour changes over time.

2.6. Blotter paper analysis

Two blotter paper tabs (perforated squares) were prepared with 25B-NBOMe HCl for comparative analysis. A solution of acetonitrile containing 500 µg of the compound was applied to the two blotter papers and the solvent allowed to evaporate so that each tab contained approximately 250 µg of 25B-NBOMe. One tab was placed in 500 µL of methanol and soaked for 1 h simulating an extraction process. The paper tab was removed, and the methanol evaporated. The other blotter paper tab was tested with the reagents applied directly to the paper in a microcentrifuge tube. To both samples, the buffer, TCBQ in ethyl acetate and acetaldehyde were added (100 µL each), the tubes agitated gently, and the colour change observed. A blank sample of the blotter paper was also tested as a control.

Further repetitions were completed using other NBOMe analogues and drug amounts applied. Solutions of 1 mg/mL in methanol 25D-NBOMe, 25G-NBOMe, 25I-NBOMe, 25C-NBOMe, 25T-NBOMe and 30C-NBOMe were pipetted on single blotter paper tabs from 50 – 200 µL, exact volumes tested for each drug can be found in the results (Table 7). The solvent was evaporated before the papers were placed in microcentrifuge tubes for testing. To each tube, 100 µL of each buffer, TCBQ solution and acetaldehyde were added, the tubes agitated gently, and colour changes observed.

3. Results & Discussion

3.1. Colour test method development

3.1.1. Preliminary testing

Initial testing with 25H-NBOMe produced a light green colour change. Once the outlined adaptations had been implemented, the blue colour, characteristic of this test, was seen with the 25H-NBOMe.

3.1.2. Reagent optimisation

This testing was completed to determine firstly, how much the concentration would affect the final colour change result and secondly if there was an optimal concentration which could be used to perform the test procedure. The greatest difference in colour development appeared between the concentrations of 1×10^{-3} and 3×10^{-3} M (see Figure 1). A concentration of 3×10^{-3} M was chosen (see Figure 1g) for all further testing as it was the lowest concentration that had the most distinguishable colour change from the reagent blank.

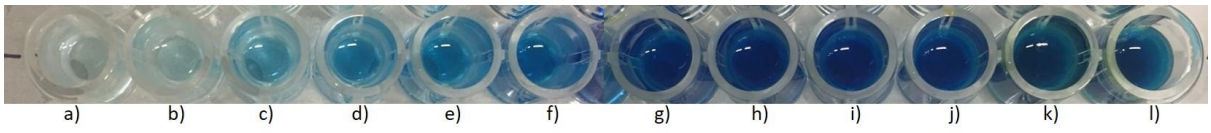


Figure 1: TCBQ reagent concentration study results. a) $8x10^{-5}$ M, b) $1x10^{-4}$ M, c) $3x10^{-4}$ M, d) $5x10^{-4}$ M, e) $8x10^{-4}$ M, f) $1x10^{-3}$ M, g) $3x10^{-3}$ M, h) $5x10^{-3}$ M, i) $8x10^{-3}$ M, j) $1x10^{-2}$ M, k) $3x10^{-2}$ M, l) $5x10^{-2}$ M TCBQ in ethyl acetate solutions.

3.1.3. Buffer solution optimisation

The tested buffer solutions showed colour change results with little variation between pH values of the phosphate-based solutions. The buffer that had the most different colour change with 25H-NBOMe was the pH 11 carbonate-based buffer solution, indicating that this may play a part in the reaction occurring even though the pH value may not be too significant. The pH 11.4 buffer solution was chosen for analysis to ensure reaction with the drug without increasing the pH unnecessarily, reducing the volume of corrosive chemicals required.

3.2. General recommended procedure

At this stage of development, a general recommended procedure was acquired and further used for all remaining testing including validation procedures, limiting variations throughout. The general procedure for this colour test method, when performed on a ceramic spot plate is:

To 1 mg of a solid sample or 1-2 drops of liquid sample add:

1. 3 drops pH 11.4 phosphate buffer solution
2. 3 drops 3×10^{-3} M TCBQ in ethyl acetate solution
3. 3 drops acetaldehyde solution
4. Observe colour change immediately and after 5 minutes

3.3. Proposed reaction mechanism

The mechanism of this type of reaction has been proposed in literature resulting in a vinylamino-substituted benzoquinone product [47]. The product of this test with NBOMe compounds has not successfully been isolated and analysed however a general proposed reaction between any primary or secondary amine and the reagents is shown in Figure 2.

3.4. Method validation

3.4.1. Specificity and selectivity

A total of 112 illicit substances and precursor chemicals along with 42 common cutting agents, sugars, amines and amino acids were tested to assess the selectivity of the method. Sixteen 25-NBOMe analogues were tested to assess the specificity of the method.

Seven 25-NBOMe analogues resulted in a blue/bright blue colour change almost immediately. Another seven analogues produced the same colour change up to five minutes after the reagents were applied. There were only two analogues, 25I-NBOH and 25I-NBMD, which resulted in a light blue and green-blue colour change respectively even after five minutes. This is most likely due to the small structural differences compared to the other NBOMe compounds (see Table 1). This indicates that these compounds may not be able to be distinguished from other secondary amine-containing compounds using this test. However, overall, the method proved to be specific for the majority of NBOMe type compounds.

Of the other illicit drugs and precursors tested, only one other compound, 4-hydroxymethcathinone (Figure 3), gave a blue colour change that would be considered a positive result (see Table 4). While cathinones are generally known to be more prevalent than the 25-NBOMe compounds, this particular analogue has not been reported in the literature for its occurrence or use in the way more common cathinones such as methylone have been [50,51]. This indicates that this cathinone would not appear to be a problematic interferant. Other secondary amine-containing compounds, particularly of the amphetamine-type substances, resulted in a light blue colour change indicating that this reagent is selective for amine-containing compounds. These results could still be differentiated from the positive colour change of the 25-NBOMe compounds at this concentration. Methamphetamine in this set of tests gave a light blue colour change indicative of the secondary amine, however, in other tests it resulted in a false positive blue colour change which could not be distinguished from the NBOMe compounds. It should also be noted that if the concentration of the NBOMe compound is below the LOD specified in Section 3.4.2, these colour changes may no

longer be able to be distinguished as an NBOMe and would simply confirm the presence of a secondary amine. Even considering this information, the ability for this test to distinguish between 25-NBOMe and LSD is apparent, as no colour change like that of the NBOMe positive result was seen with LSD.

Table 4: Resulting colour changes of illicit substances tested initially and after 5 minutes

Compound Class	**Compound**	**Initial Colour change** *	**5 min colour change** *	**Test result** **
NBOMe analogues and derivatives	25G-NBOMe HCl ‡	green-blue	blue	+
	25D-NBOMe HCl ‡	blue	NC	+
	25B-NBOMe HCl ‡	blue	NC	+
	25H-NBOMe HCl ‡	d. blue	NC	+
	25I-NBOMe HCl ‡	green-blue	blue	+
	25E NBOMe HCl ‡	blue	NC	+
	25C-NBOMe HCl ‡	green-blue	blue	+
	25B-NBF	bright blue	d. blue	+
	25I-NBOH	pale blue	l. blue	+/-
	25I-NBMD	l. green-blue	green-blue	+/-
	25T-NBOMe	l. blue	blue	+
	25N-NBOMe	l. green-blue	blue	+
	30C-NBOMe	blue	bright blue	+
	mescaline-NBOMe	l. blue	blue	+
	25C-NB3OMe	green-blue	d. blue	+
	25B-NB4OMe	blue	d. blue	+
2C-x series	2C-H HCl	l. yellow-green	yellow-green	-
	2C-T-7 HCl	l. yellow-green	yellow-green	-
	2C-I HCl ‡	NC	l. yellow-green	-
	2C-E HCl ‡	l. yellow-green	bright green	-
	2C-B HCl ‡	l. green	bright green	-
	2C-D HCl ‡	l. yellow-green	bright green	-
	2C-T-2 HCl	l. green	NC	-
Amphetamine type substances	(+)-S-Methamphetamine HCl ‡	pale blue	l. blue †	+/-
	(±)-N-Methyl-3,4-methylenedioxyamphetamine HCl (MDMA) ‡	pale blue	l. blue	+/-
	d-amphetamine	l. green	NC	-
	3-fluoroamphetamine HCl	v. pale yellow	l. yellow-green	-

	3,4-dimethoxymethamphetamine HCl	v. pale blue	l. blue	+/-
	4-hydroxyamphetamine	pale green	l. yellow	-
	2-fluoromethamphetamine HCl	pale blue	NC	-
	4-methoxymethamphetamine	l. blue	NC	+/-
	4-fluoromethamphetamine HCl	l. blue	NC	+/-
	2-methylamphetamine (oretamine) HCl	l. yellow-green	NC	-
	(+/-)-3-methylamphetamine HCl	NC	l. yellow-green	-
	(+/-)-3-methylmethamphetamine HCl	pale blue	NC	-
	(+/-)-3-methoxymethamphetamine	pale blue	colourless	-
	(+/-)-2-methylmethamphetamine HCl	NC	NC	-
	(+/-)-3-methoxyamphetamine HCl	NC	NC	-
	(+)-3-fluoromethamphetamine HCl	v. pale blue	blue-grey	-
	4-methylmethamphetamine HCl	l. blue	l. blue	+/-
	(+/-)-2-methoxyamphetamine HCl	pale green	l. green	-
	(+/-)-3,4-dimethoxyamphetamine HCl	l. yellow-green	NC	-
	(+/-)-4-methylthioamphetamine HCl	l. yellow-green	NC	-
	(+/-)-4-bromo-2,5-dimethoxyamphetamine HCl	l. yellow-green	NC	-
	(+/-)-N,N-dimethyl-3,4-methylenedioxyamphetamine HCl	NC	NC	-
	(+/-)-N,N-dimethylamphetamine HCl	NC	NC	-
	(+/-)-N-methyl-1-(3,4-methylenedioxyphenyl)-2-butylamine HCl	l. blue	NC	+/-
	2,5-dimethoxy-4-methylamphetamine HCl	l. green	NC	-
	(+/-)-2-chloroamphetamine HCl	NC	l. green	-
	(+/-)-4-chloroamphetamine HCl	NC	l. green	-
	(+/-)-2-bromoamphetamine HCl	NC	l. green	-
	(+/-)-2-bromomethamphetamine HCl	l. blue	NC	+/-
	4-isopropoxy-2,5-dimethoxyphenethylamine HCl	l. yellow	l. yellow-green	-
	(+/-)-3-bromomethamphetamine HCl	l. blue	NC	+/-
	(+/-)-3-bromoamphetamine HCl	NC	pale green	-
	(+/-)-bromo-dragonFLY HCl	pale yellow-green	NC	-
	(+/-)-4-chloro-2,5,-DMA HCl	l. green	bright green	-
	(+/-)-N-ethyl-3,4-methylenedioxyamphetamine HCl	NC	NC	-
	(+/-)-2,5-dimethoxyamphetamine HCl	l. green	bright green	-
	(+/-)-3,4,5-trimethoxyamphetamine HCl	l. green	NC	-

	N-ethylamphetamine HCl	v. pale blue	NC	-
	(+/-)-4-methoxyamphetamine HCl	l. green	NC	-
	(+/-)-4-methylamphetamine HCl	l. green	bright green	-
	3-chloromethamphetamine HCl	l. blue	NC	+/-
	(+/-)-3-chloroamphetamine HCl	v. pale green	NC	-
	(+/-)-4-bromomethamphetamine HCl	l. blue	NC	+/-
	2-chloromethamphetamine HCl	l. blue	NC	+/-
	4-chloromethamphetamine HCl	l. blue	NC	+/-
	(+/-)-4-bromoamphetamine HCl	l. green	bright green	-
	(+/-)-4-fluoroamphetamine HCl	v. pale green	NC	-
	2-fluoroamphetamine HCl	l. yellow	NC	-
Cathinones	3,4-methylenedioxy-N,N-dimethylcathinone HCl	NC	NC	-
	4-methyl-a-pyrrolidinobutiophenone HCl	NC	NC	-
	iso-ethcathinone HCl	NC	NC	-
	2,4,5-trimethylmethcathinone HCl	v. pale blue	l. blue	+/-
	3,4-dimethylmethcathinone HCl	NC	NC	-
	(+/-)-N,N-diethylcathinone HCl	NC	l. brown	-
	4-fluoromethcathinone HCl	NC	NC	-
	(+/-)-N,N-dimethylcathinone HCl	NC	NC	-
	4-methylethylcathinone HCl	NC	NC	-
	2,4-dimethylmethcathinone HCl	l. blue	NC	+/-
	2,3-dimethylmethcathinone HCl	l. blue	NC	+/-
	(+/-)-3-bromomethcathinone HCl	pale blue-green	NC	-
	4-methoxy-a-pyrrolidinopropiophenone HCl	NC	NC	-
	4-methyl-N-benzylcathinone HCl	l. green-blue	NC	-
	(+/-)-3-fluoromethcathinone HCl	NC	NC	-
	3,4-methylenedioxymethcathinone HCl	NC	NC	-
	(+/-)-4-methylmethcathinone HCl ‡	NC	NC	-
	butylone HCl	l. green-blue	NC	-
	3,4-methylenedioxypyrovalerone HCl ‡	NC	NC	-
	4-methoxymethcathinone HCl	NC	NC	-
	3-methylmethcathinone HCl	NC	NC	-
	2-methylmethcathinone HCl	l. green-blue	NC	-
	4-bromomethcathinone HCl	l. green-blue	NC	-
	(+/-)-a-pyrrolidinopentiophenone HCl	NC	NC	-
	pyrovalerone HCl	NC	NC	-
	(+)-cathinone HCl	NC	NC	-

	4-hydroxymethcathinone	l. blue	blue	+
	2-fluoromethcathinone HCl	NC	NC	-
Tryptamines	5-methoxy-N-methyl-N-isopropyltryptamine HCl	NC	NC	-
	5-methoxy-N,N-diallyltryptamine	pale purple	NC	-
	5-methoxy-N,N-dimethyltryptamine	NC	blue-grey	-
Piperazines	1-(4-chlorophenyl)-piperazine.2HCl	l. blue	NC	+/-
	Piperazine hexahydrate	green-brown	brown	-
	methylbenzylpiperazine.2HCl	NC	NC	-
	1-(4-fluorophenyl)-piperazine.2HCl	pale blue	NC	-
Other illicit substances and precursors	LSD	d. green	NC	-
	ergotamine tartarte	NC	NC	-
	fentanyl	NC	NC	-
	JWH-073	NC	NC	-
	methylamine HCl	pale green	l. green	-
	Pseudoephedrine	pale green	green-blue	+/-
	ephedrine	v. pale blue	l. green-blue	-

* l. = light, d. = dark, v. = very, NC = no change
** (+) = positive, (-) = negative, (+/-) = positive for the presence of a secondary amine
† methamphetamine resulted in some blue colour changes that could not be distinguished from NBOMe compounds
‡ compounds tested at both UTS and AFP laboratories

Of the adulterants and non-drug compounds tested, no false positives were identified and many of these tested compounds produced little or no colour change with the reagents. For ease, those compounds which did not produce a colour change have not been included in Table 5.

Table 5: Resulting colour change of common cutting agents, sugars and other amines and amino acids

Compound class	**Compound**	**Initial Colour change** *	**5 min colour change** *	**Test result** **
Common adulterants	Lidocaine	pale green	NC	-
	Paracetamol	pale yellow	v. pale orange	-
	Ibuprofen	NC	pale yellow	-
	Caffeine	pale yellow	pale yellow	-
	Tetramisole HCl	pale yellow	v. pale orange	-
	4-Methoxy phenol (MEHQ)	pale purple	purple	-
	3,4-dimethoxyphenethylamine	brown	NC	-

	Magnesium stearate	pale yellow	pale yellow	-
	Benzocaine	NC	v. pale purple	-
	Phenobarbital	brown-yellow	purple	-
	Salicylamide	pale yellow	NC	-
	Aspirin	pale yellow	NC	-
	Creatine	pale yellow-orange	NC	-
	4-aminophenazone	brown-purple	brown	-
	Quinine	pale yellow	pale grey-brown	-
Sugars	Cellulose	pale yellow	NC	-
Amines and amino acids	Aniline	d. brown	NC	-
	Glycine	pale yellow	yellow	-
	Ethylenediamine-N-N'-diacetic acid	NC	pale orange	-
	Diphenylamine	pale purple-blue	l. blue	+/-
Pharmaceuticals	Nortriptyline	black-blue	blue-black	-
	Protriptyline	black-blue	purple-black	-

* l. = light, d. = dark, v. = very, NC = no change
** (+) = positive, (-) = negative, (+/-) = positive for the presence of a secondary amine
*** Compounds which did not react are not listed in this table for ease of reading

3.4.2. Limit of detection

The limit of detection study of the TCBQ method was completed by assessing the colour change of a range of amounts of 25H-NBOMe and determining the point where the colour becomes non-differential from the developed colour.

The lowest concentration at which the colour change was differential from the reagent blank and provided what would still be classed as a positive result was 22.5 µg of 25H-NBOMe (0.075 mg/mL, in 300 µL). This correlates to Figure 4e) where 75 µL of the 0.3 mg/mL 25H-NBOMe solution was added. Below this, the colour would be described as light or pale blue rather than the bright or dark blue associated with the positive interaction and colour change result. The guidelines from NIJ's Colour Test Standard suggest a working LOD ten times this value. This working value, 225 µg, would in many cases be appropriate as a single blotter paper containing an NBOMe compound will often contain upwards of 250 µg [32]. For those analogues, notably NBOH

and NBMD, which at amounts of 100 µg only produced light blue colour changes they would not be able to be determined from this test as containing an NBOMe like structure. Particularly if the concentrations were lower, the result could only indicate the presence of a compound containing a secondary amine and further testing would be required to determine the presence of these compounds. If a compound containing a secondary amine was at a much higher concentration, there is the possibility it could be misidentified as an NBOMe. Through the completed testing, it appears only 4-hydroxymethcathinone and methamphetamine would potentially be interpreted as an NBOMe containing compound.

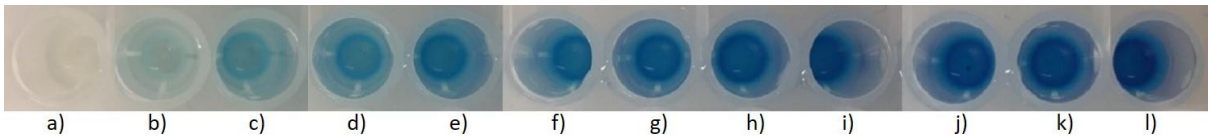


Figure 4: LOD colour change results. a) Reagent blank, b) 10, c) 25, d) 50, e) 75, f) 100, g) 150, h) 200, i) 250, j) 300, k) 350 and l) 500 µL of 0.3 mg/mL 25H-NBOMe solution

3.4.3. Purity testing

All eleven mixtures tested were differentiable from the reagent blank (not shown in Figure 5). Of these, only two mixtures showed differences from the 25H-NBOMe control (ephedrine and aspirin). Ephedrine alone gave a pale green-blue colour change with the method and has been shown to react with a similar method [52]. This may indicate why there was some interference when combined with the 25H-NBOMe. The result with aspirin, however, was not expected as it did not react to produce a colour change with the reagents on its own. It is unclear why, when mixed with the NBOMe drug, the presence would influence the final colour change result. Literature has shown that some compounds with similar functional groups (carboxylic acids and alcohols) may interfere with the reaction between the amine group of the drug and the benzoquinone [53,54]. Even with these small differences, the colour changes seen would still indicate the presence of a drug, potentially 25-NBOMe, and would require further testing.

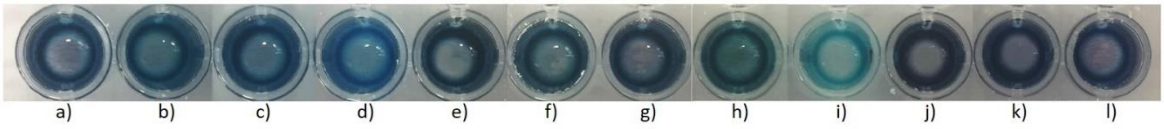


Figure 5: Results of purity tests with 25H-NBOMe and adulterants in 1:1 ratio. a) 25H-NBOMe blank, b) methamphetamine, c) MDMA, d) ephedrine, e) paracetamol, f) pseudoephedrine, g) caffeine, h) ibuprofen, i) aspirin, j) lidocaine, k) benzocaine, l) creatine

3.4.4. Precision analysis

The repeatability of this test was determined by assessing the similarity or differences in colour change across multiple 25H-NBOMe samples using the same reagents under the same laboratory conditions. The replicates at concentrations 1.25× – 2× the LOD all produced blue colour change results consistent with a positive result across the replicates. These amounts were from 28 – 45 µg of 25H-NBOMe in 300 µL of reagents (0.093 – 0.15 mg/mL). It is also noteworthy that the time of day had minimal impact on the colour change and no trends were seen for a certain result occurring at a particular time of day.

Reagent solutions that were prepared independently were tested in combinations with the other reagents. Changing the reagent solutions and the combinations had a limited effect on the final colour change result with 25H-NBOMe. Most tests with 25H-NBOMe produced a blue colour change representative of a positive result. There were some small differences between the TCBQ solutions, the older solution seeing less consistent positive results with the 25H-NBOMe in comparison to a more recently prepared reagent solution. This reagent solution was almost two months old indicating that even though some repetitions still produced a positive colour change result, the reagent may not be suitable to be used after this time.

The results seen between the compounds tested at both UTS and the AFP laboratories were also comparative. Repetitions of the colourimetric reactions of these compounds gave highly similar colour changes at both laboratories, with positive blue colour changes seen for all 25-NBOMe compounds tested at both locations.

3.4.5. Blind tests

The results of the blind tests are outlined in Table 6. Overall, these results showed a high percentage of true positives for 25-NBOMe related compounds. Some analogues such as 25I-NBOH and 25I-NBMD, previously identified to produce a light blue colour change, were only able to be identified as containing a secondary amine. Another, 25T-NBOMe, which had previously shown a light blue colour change initially, was also only able to be determined as a secondary amine-containing sample, even after several minutes. Methamphetamine was present in four of the blind samples and twice was interpreted as a 25-NBOMe compound producing a blue colour that was indistinguishable from that associated with the positive colour change. This indicates the potential for some interference of methamphetamine with this test, however, it is unlikely that methamphetamine would be found on a blotter paper. In Brazil, there have been reports of many drugs found on blotters other than LSD yet methamphetamine was not reported in any samples over a six year period [19]. All methanol blanks and 2C samples were determined as negative. Overall, the identified false negatives were classed as being positive for containing a secondary amine and would be able to be identified through further testing.

Table 6: Resulting colour change and interpretations of blind tests with corresponding compounds

Sample No.	**Colour change**	**Result interpretation ****	**Actual compound**
1	Light green	-	2C-T2
2	Dark blue	+	25I-NBOMe
3	NC	-	Methanol
4	Bright blue	+	25B-NBOMe
5	Blue	+	Mescaline-NBOMe
6	Blue	+	Methamphetamine
7	Blue	+	Methamphetamine
8	Light blue	+/-	25I-NBMD
9	Dark blue	+	25D-NBOMe
10	Light blue	+/-	Methamphetamine
11	Bright blue	+	25E-NBOMe
12	Light blue	+/-	25I-NBOH
13	NC	-	Methanol
14	Light green	-	2C-D

15	Light blue	+/-	Methamphetamine
16	Light blue	+/-	25I-NBOH
17	Blue	+	25H-NB4OMe
18	Blue	+	30C-NBOMe
19	Light green	-	2C-I
20	NC	-	Methanol
21	Light blue	+/-	25T-NBOMe
22	Blue	+	25G-NBOMe
23	Blue	+	25I-NBMD
24	Bright blue	+	25T2-NBOMe

** (+) = positive, (-) = negative, (+/-) = positive for the presence of a secondary amine

3.4.6. Stability

The stability of the reagents was tested and compared by applying the testing method using reagent solutions which were prepared and stored in different conditions. Figure 6 shows the colour change results of the three storage conditions in comparison to a freshly prepared reagent after eight weeks of storage. It can be seen that no distinct differences were observed after the storage of the reagent solution over this time nor differences between storage conditions. Based on these results and also the results of the precision analysis, it would be recommended that the TCBQ solutions are not used for this test after two months of storage.

Figure 6: Comparison of storage conditions (left to right: fresh, fridge, cupboard and laboratory bench)

The stability of the coloured compound was observed for 48 hr as after this time the colour change was not considered a positive colour. There were no significant changes in the colour for the first 24 hr. Only after this time did the blue colour become much darker than the initial test result and by 48 hr was a blue-black colour which would be too dark to be considered positive. While the regular observations stopped at this point, the vial was kept and after one week, the blue colour had completely

gone, and the solution was yellow-brown. It is appropriate that the positive blue colour remains similar to the initial change for several hours so the result can potentially be observed by another analyst or device to further reinforce the result without being there at the time of the test.

3.5. Blotter paper analysis

The blotter paper test methods were compared based on the observed colour change and the time taken for this change to occur. Both samples, extracted and direct application, showed a bright blue colour change similar to that expected from 25-NBOMe compounds in the general test method (see Section 3.2). The great similarity in the colour change of these two samples indicates that the blotter paper has no major matrix effect on the overall reaction and colour change. It is also important to note that the blotter paper used had a yellow and orange coloured print on one side and this played no effect on the colour change seen. The application of this test directly to the blotter paper is ideal for presumptive testing. While some agitation of the sample tube was required, the direct application still provided a colour change result almost immediately (see Figure 7).

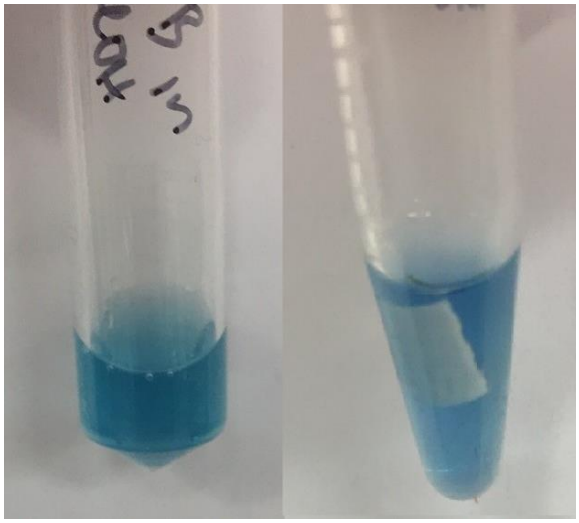


Figure 7: Comparison of blotter paper test methods. Left: colour change result of the extracted blotter paper tab; Right: colour change result of direct application to the blotter paper tab.

The repetitions of other analogues and concentrations showed similar results. Drug amounts of 150 µg and above (per blotter) all successfully showed the blue colour change expected with this test method. The 50 µg samples which were tested, produced a pale or light blue colour more indicative of a secondary amine being

present and the 100 µg samples differed between analogues (see Table 7). The values are also below the commonly found amounts of NBOMe compounds on a single blotter paper, as referred to in Section 3.4.2, upwards of 250 µg [32]. These differences indicate a concentration dependant colour change and while this may appear to be a limitation of this test, generally LSD is the only other drug commonly found on blotter papers [55].

Table 7: Results of blotter paper analysis

Compound	Amount added to blotter (µg)	Colour change result
25D-NBOMe	50	Light blue
	100	Light blue
	100	Blue
	150	Bright blue
	200	Bright blue
25C-NBOMe	100	Pale blue
	200	blue
25G-NBOMe	50	pale blue
	100	blue
	150	Bright blue
	200	Bright blue
25I-NBOMe	100	blue
	200	blue
25T-NBOMe	100	Light blue
	200	blue
30C-NBOMe	100	Light blue
	200	blue

LSD does not react with this test to produce a colour change similar to that of NBOMe compounds or other compounds containing a secondary amine. This would indicate that in most circumstances where a blotter paper was tested, and produced a blue colour change, the most likely conclusion would be the presence of an NBOMe related compound. The positive results seen with this test to blotter paper samples illustrate the real-world application of this method for the detection of NBOMe related compounds.

4. Conclusions

A simple colour spot test method was developed and sufficiently validated for the rapid and selective detection of 25-NBOMe analogues. Almost all tested 25-NBOMe analogues and derivatives produced a bright blue colour change, with the exception of those NBOH and NBMD compounds. Only two other illicit substances produced a similar result in some tests. The working limit of detection for 25H-NBOMe, of 225 µg, is sufficient for this type of test with these compounds. This method has the capacity to directly detect 25-NBOMe compounds impregnated onto blotter papers, a common matrix which they are sold, and differentiate these compounds from LSD. While no real case samples have been available for this study, this test shows the potential to be applied to these samples with no significant interferences identified.

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