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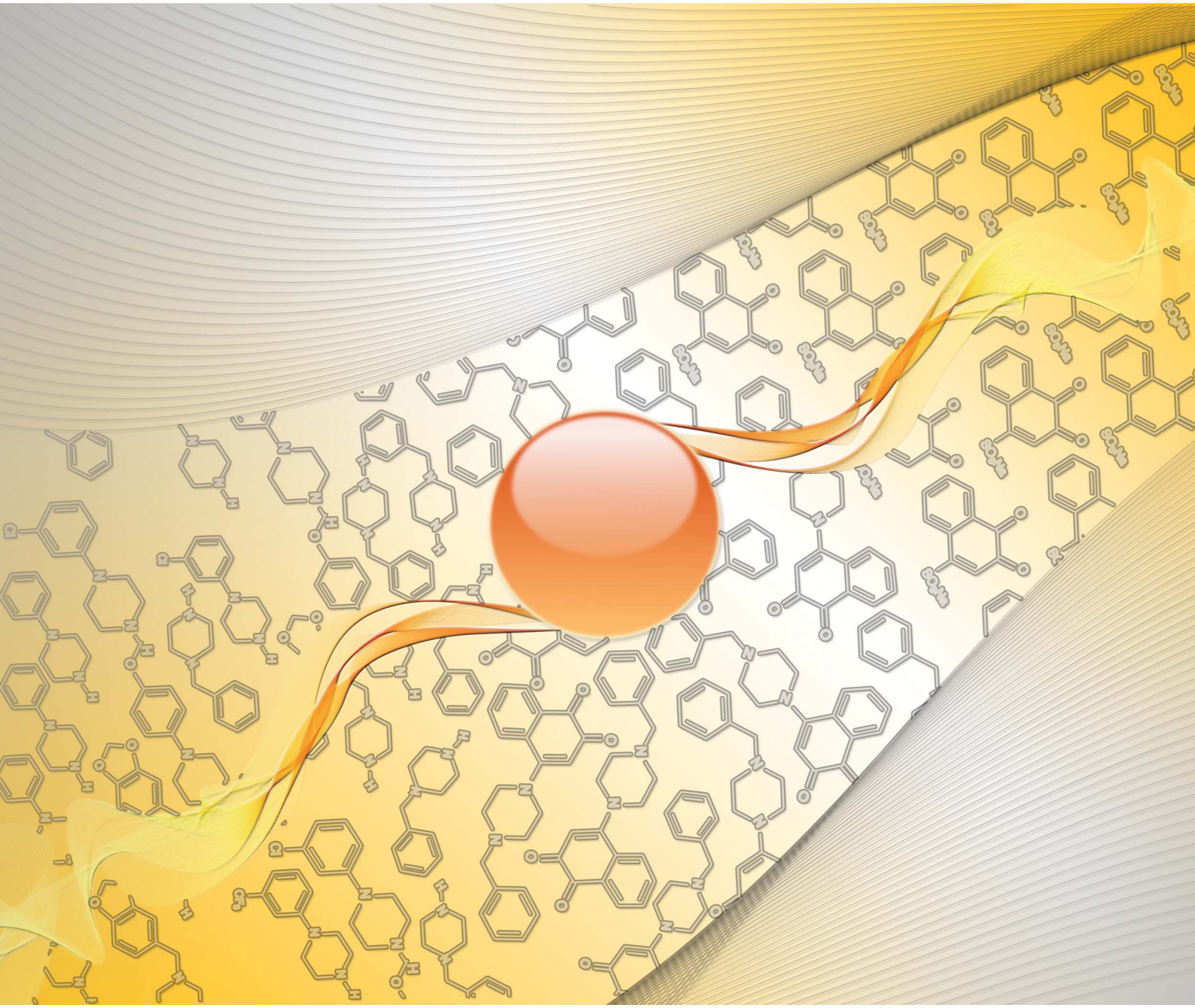

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Development and validation of a presumptive colour spot test method for the detection of piperazine analogues in seized illicit materials

Morgan Philp,[a] Ronald Shimmon,[a] Natasha Stojanovska,[a] Mark Tahtouh[b] and Shanlin Fu*[a]

The increasingly large quantities of potentially illicit samples received for confirmatory analysis highlights the importance and demand for preliminary testing procedures that are simple, rapid, selective, inexpensive and able to be used in the field. Colour testing fulfils the aforementioned requirements and is a technique frequently employed to achieve presumptive identification. Piperazine analogues (often marketed as 'legal ecstasy') are a group of psychoactive substances that have recently become established on the illicit drug market and are not effectively discriminated or identified by current colour testing methods. Herein, we report on the development and validation of a chemical spot test for piperazine analogues present in unknown seized materials using the spectrophotometric reagent, sodium 1,2-naphthoquinone-4-sulphonate (NQS). Primary testing revealed that NQS reacts almost instantly to form an intense, bright orange-red coloured complex with the representative piperazine 1-benzylpiperazine (BZP) at room temperature. The results of the test, assessed by colour development, were evaluated visually and variables affecting the coloured reaction were optimised. The colour test method was validated to meet requirements for use in preliminary screening, providing qualitative and reliable presumptive test results. Validation studies show that the characteristic colour change is unique to the piperazine class at room temperature, and is unaffected by the presence of common cutting agents, *i.e.* glucose and caffeine, in test samples of 5% purity, and other drugs such as *N*-methyl-3,4-methylenedioxyamphetamine (MDMA). The NQS reagent stability was found to be limited to storage in a refrigerated environment for no more than one week before results were affected. The operational limit of detection was found to be 40 μg.

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1 Introduction

The most recent drug seizure data from the Australian Federal Police (AFP) for the period 2010–2011 reported a 316% increase in seized illicit drugs and precursors from the previous year.[1] This statistic alone demonstrates the current prominent drug situation in Australia, while figures provided by the United Nations Office on Drugs and Crime (UNODC) highlight a similar growth pattern globally.[2]

Synthetic piperazine analogues are newly established drugs on the market that have seen a remarkable increase in abuse worldwide owing to the ease of access afforded by internet availability.[3] Piperazine analogues are central nervous system stimulants and many possess hallucinogenic properties. They are commonly used in combination with other piperazine analogues or illicit substances including MDMA, cocaine or ketamine.[4]

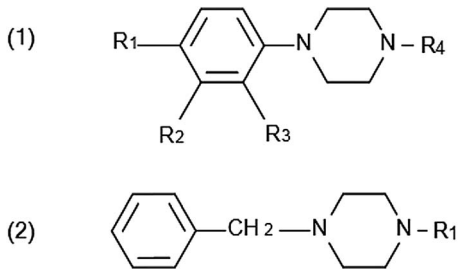
A number of piperazine analogues are currently not under international control. Many countries (including Australia) have introduced national controls to prevent the sale and distribution of 1-benzylpiperazine (BZP) in particular.[5] Following the changing legal status of piperazine analogues, the number of synthetic drugs being produced and made available on the illicit drug market predictably increased to include piperazines that were not scheduled.[6]

Piperazine is a heterocyclic compound containing four carbon atoms and two nitrogen atoms at the 1,4 position (also called 1,4-hexahydropyrazine).[7] Piperazine analogues can be divided into two classes (Scheme 1): phenylpiperazines (1) and benzylpiperazines (2). BZP is the most prevalent benzylpiperazine analogue while phenylpiperazine analogues include 1-[3-(trifluoromethyl)phenyl]piperazine (TFMPP), 1-(3-chlorophenyl)-piperazine (mCPP), and 1-(4-methoxyphenyl)piperazine (MeOPP).[8]

The confirmatory analysis of seized materials, and more specifically, piperazines, by techniques such as mass spectrometry (MS)[9–12] and nuclear magnetic resonance (NMR) spectroscopy[11,12] is well documented in the literature. However, due to the large amounts of unknown, seized materials

[a]*Centre for Forensic Science, School of Chemistry and Forensic Science, University of Technology, Sydney (UTS), Ultimo, NSW 2007, Australia. E-mail: Shanlin.Fu@uts.edu.au; Fax: +61 2 9514 1460; Tel: +61 2 9514 8207*

[b]*Australian Federal Police, 110 Goulburn St, Sydney, NSW 2000, Australia*



Scheme 1 Chemical structures of phenylpiperazine (1) and benzylpiperazine (2) analogues.

received, confirmatory testing techniques can be time-consuming and costly.

Preliminary testing of seized materials is a vital first step in the identification of potentially illicit drugs. These preliminary tests combine techniques such as Fourier Transform Infrared spectroscopy (FTIR), Raman Spectroscopy and Thin Layer Chromatography (TLC) with presumptive colour testing methods. Presumptive colour tests are rapid, cheap, effective and, in contrast to FTIR and Raman, particularly useful in the detection of target analytes within mixtures. They can also be specific, require minimal sample preparation and able to be used by untrained personnel given a sequence of steps and colour charts. These colour tests are generally performed as chemical spot tests on a small sample of the unknown material and evaluated visually in white, porcelain spotting well plates, or commercially in polyethylene cartridges containing glass ampoules.

On-site analysis is becoming one of the most important fields of modern analytical chemistry, while preliminary screening in the laboratories is also becoming necessary due to the increasing number of samples being submitted.

The requirements for colour testing have been described in detail by Zolotov *et al.*[13] These requirements include selectivity toward the analyte to be determined, high contrast and rate of colouring with the analyte, good reagent stability on storage, and a sufficiently stable analytical effect.

The need for research into novel chemical testing procedures governing piperazine analogues can be realised by looking at the unsuccessful results afforded by current preliminary testing methods. The typical colour screening tests used for on-site field testing by frontline personnel are not able to be used reliably or with any degree of specificity on piperazine analogues.[9]

The commonly encountered piperazine analogues BZP, TFMPP, mCPP, pCPP and MeOPP each possess one cyclo secondary amine functional group as part of their structure. For the purpose of developing a test that will react with each of these compounds, this secondary amine group is the likely target.

An estimation of amines using the spectrophotometric reagent NQS has previously been described in the literature in an ad-hoc manner.[14–16] Dessouky and Ismaiel[17] used the formation of a red coloured product with NQS in the detection of piperazine in pharmaceutical preparations. Supplementary to this, Cabeza *et al.*[18] studied the reaction between NQS and primary or secondary amines in the presence of a non-ionic surfactant with favourable results.

The literature contains no reports of NQS use in the detection of illicit piperazine analogues such as BZP. This work aims to investigate, modify and develop the potential use of NQS as a colour test reagent in the detection of piperazine analogues. Method validation procedures were conducted for the proposed colour test by considering the reaction with other drugs of interest, reaction of common interferents, inherent sensitivity of the method, stability of reagents and coloured products, and the method's precision. The test exhibits excellent selectivity, sensitivity and repeatability towards piperazine analogues.

2 Experimental

2.1 Reagents and reference standards

Chemicals used in the development of a novel colour test method were sodium 1,2-naphthoquinone-4-sulphonate (NQS) and triton-X-100, both purchased from Sigma-Aldrich (Castle Hill, NSW, Australia), and sodium hydrogen carbonate ($NaHCO_3$) and sodium hydroxide were supplied by Chem Supply (Gillman, SA, Australia).

Working solutions of NQS at various concentrations were freshly prepared each day and stored in the absence of light in a refrigerator kept at 7 °C while not being used. A 0.1% (v/v) triton-X-100 working solution was also prepared. A $NaHCO_3$ buffer solution was prepared by adjusting the pH of a 0.1 M $NaHCO_3$ solution to 10.8 with 5 M NaOH.

In addition to the synthesis of BZP and 4-methylmethcathinone (4-MMC) in house, a large number of certified drug reference standards were obtained from the National Measurement Institute (NMI, North Ryde, NSW, Australia) through the AFP for a selectivity study. A list of these analytes can be found in the selectivity results section.

Caffeine, ephedrine hydrochloride, codeine phosphate, starch, glucose, sucrose and an extensive range of powdered substances were obtained from Ajax Chemicals (Sydney, NSW, Australia), BDH Chemicals (Sydney, NSW, Australia), Sigma Chemical Company (Perth, WA, Australia), Glaxo Australia (Sydney, NSW, Australia), Mallinckrodt (Lane Cove, NSW, Australia), Chem-Supply and Sigma-Aldrich. Plain flour, caster sugar, protein powder and artificial sweetener were from a local supermarket. A full list of these chemicals analysed can be found in the selectivity results section.

For colour tests on drugs in solution, working standard solutions of BZP HCl and 4-MMC HCl were prepared at a concentration of 500 μg mL^{-1} in distilled water. Working standard solutions of starch, glucose, caffeine, sucrose, codeine and ephedrine HCl were prepared at a concentration of 1000 μg mL^{-1} in distilled water.

2.2 Apparatus

Polypropylene flat bottom 96-well microplates were obtained from Greiner Bio-One and porcelain spotting well plates were supplied by the AFP laboratory. A Simmerstat Plain Top 240V AC from Industrial Equipment & Control Pty Ltd was used as the hot plate during colour test procedures. All pH measurements were carried out with a pH 211 Microprocessor from HANNA

instruments. A digital CANON EFS 17-85 mm Single Lens Reflex (SLR) camera, DS126181, was employed to record all test results.

2.3 Preparation of BZP HCl and 4-MMC HCl

BZP HCl was synthesised following the method outlined by Craig and Young.[19] Equal molar piperazine hexahydrate and piperazine dihydrochloride monohydrate were dissolved (to form piperazine hydrochloride) in ethanol at 65 °C and then reacted with equal molar benzyl chloride for 25 minutes at 65 °C. The reaction solution was cooled on ice and the piperazine dihydrochloride monohydrate crystals were recovered by filtration. The filtrate was cooled on ice and treated with ethanolic HCl. BZP HCl was collected as a white precipitate. Its identity was confirmed by NMR, MS, and FTIR analysis. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.52 (1H, s), 2.41 (4H, t), 2.85 (4H, t), 3.49 (2H, s), 7.26 (5H, m) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 46.12, 54.54, 63.68, 126.94, 128.13, 129.17, 138.10 ppm. Electron impact MS: m/z 176 (M^+, 16), 134 (60), 91 (100), 85 (10), 65 (14), 56 (23). IR ν_{max} (cm^{-1}): 3241, 2993, 2759, 1631, 1557, 1444, 1063.

4-MMC HCl was synthesised in two stages following a modified method given by Camilleri *et al.*[20] The first α-bromination step involved reacting 4-methylpropiophenone with excess bromine (to form 2-bromo-4-methylpropiophenone) in the presence of glacial acetic acid at 25 °C for 1 hour. The reaction solution was poured into ice-cold water and the 2-bromo-4-methylpropiophenone was extracted with dichloromethane and concentrated under vacuum to form yellow, fluffy crystals. The final methamination step involved combining equal molar NaOH and methylamine hydrochloride solutions. This solution was then added dropwise over 1 hour to a stirred solution of 2-bromo-4-methylpropiophenone in toluene. The mixture was allowed to stir for 32 hours at 25 °C and poured into ice-cold water. The toluene layer was separated and acidified with dilute HCl solution. The acidic extracts were washed with toluene before evaporating the aqueous layer to dryness to afford the crude 4-MMC HCl product as mottled light brown/brown coloured, flaky crystals. A fine, white powder was collected following recrystallisation from isopropanol. Its identity was confirmed by NMR, MS, and FTIR analysis. ^{1}H NMR (500 MHz, D_2O): δ 1.62 (3H, d), 2.46 (3H, s), 2.82 (3H, s), 5.08 (1H, q), 7.47 (2H, d), 7.94 (2H, d) ppm. ^{13}C NMR (125 MHz, D_2O): δ 18.3, 23.8, 33.8, 62.4, 131.9, 132.6, 132.8, 150.3, 200.2 ppm. Electron impact MS: m/z 177 (M^+, 2), 119 (6), 91 (12), 65 (8), 58 (100), 56 (8). IR ν_{max} (cm^{-1}): 2912, 2740, 2453, 1686, 1605.

2.4 Colour spot test method development

2.4.1 Description of the colours. A method described by Cabeza *et al.*[18] was adapted in this study. Colour changes were described using the system adopted in Clarke's Analysis of Drugs and Poisons.[21] This system uses ten basic colours (red, orange, yellow, green, blue, violet, together with pink, brown, grey and black) with a variation in hue indicated by combining two colours (*e.g.* orange-red). The second-named colour is considered to be the dominant one.

2.4.2 Colour test in test tubes. Briefly, to a glass culture tube, in the order of addition, were added BZP HCl sample (500 μg mL^{-1}), $NaHCO_3$ buffer (pH 10.8), triton-X-100 (0.1% (v/v)) and the NQS reagent working solution (6.4×10^{-3} M) up to a total volume of 2.5 mL. The tube was then heated in a water bath kept within a temperature range of 45–50 °C for three minutes. A control reagent blank, containing a distilled water sample only, was tested simultaneously. Four preliminary experiments were performed involving different volumes of sample and reagents as specified in Table 1. Colour changes were observed and recorded before and after heating.

Table 1 Reagent volumes used in four preliminary colour test methods

Test no.	Sample 500 μg mL^{-1}	$NaHCO_3$ buffer	Triton-X-100 0.1% (v/v)	NQS 6.4×10^{-3} M
1	0.5 mL	0.5 mL	0.5 mL	1.0 mL
2	1.0 mL	0.3 mL	0.2 mL	1.0 mL
3	1.0 mL	0.4 mL	0.1 mL	1.0 mL
4	1.0 mL	0.1 mL	0.4 mL	1.0 mL

2.4.3 Reduction to a small scale. The method was then modified to be performed on a smaller scale in a plastic micro-well plate using Pasteur pipettes to add drop-sized amounts of reagents. To a plastic well were added BZP sample solution (3 drops), $NaHCO_3$ buffer (2 drops), triton-X-100 (1 drop) and NQS (4 drops). The plate was left to stand for two minutes at room temperature before being placed in a water bath set at 45–50 °C for three minutes. Colour changes were observed and recorded before and after heating.

Testing was performed on BZP HCl, 4-MMC HCl, ephedrine HCl, codeine, caffeine, glucose, sucrose and starch sample solutions alongside a control reagent blank.

2.4.4 Application to solid samples. A small pin-head sized amount of each solid sample was placed in a well of a micro-well plate. To this was added 5 drops of $NaHCO_3$ buffer, with gentle mixing for a few seconds, followed by 4 drops of NQS solution. The plate was left to stand for two minutes at room temperature and colour changes were observed and recorded.

2.5 Method validation

A series of tests were performed in order to determine the method's reliability. These tests were chosen based on the nature and purpose of the qualitative method.

2.5.1 Reproducibility. The general recommended colour test procedure was applied to solid samples of pure BZP HCl. The test was performed on five replicate samples at the same time. Further testing was completed in triplicate on the same day with the same reagents in the same laboratory.

The developed test method was also performed in triplicate on different days, using different reagents and in different laboratories and by different operators. Certified reference samples were tested in triplicate at the AFP laboratory both intra-day and inter-day.

2.5.2 Selectivity/specificity. An extensive range of analytes was collected to aid in the investigation of the selectivity and specificity of the NQS colour test. These analytes included:

- Controlled drugs in the target group, *i.e.* piperazine analogues.

• Controlled drugs from other groups.
• Mixtures of piperazine analogues and other controlled drugs.
• Common precursors to illicit drugs.
• Common diluents/excipients in the matrix of seized drugs.
• Miscellaneous powdered substances and household tablets.

The drugs and other analytes were classified as drug standards, crystals, powders or tablets. Drug standards, crystals and powders were tested without further processing, and tablets were crushed into a fine powder using a mortar and pestle.

A small pin-head sized amount of each analyte to be tested was added to the well of a porcelain spotting plate, or plastic micro-well plate. The general recommended test procedure was applied. The final colours observed after the required two minutes standing time were recorded. Each analyte was tested in triplicate.

2.5.3 Purity testing. A purity test was undertaken using caffeine and glucose as cutting agents in samples of BZP HCl, before application of the NQS test.

Methanolic solutions of BZP HCl at a concentration of 200 μg mL^{-1} and methanolic solutions of caffeine and glucose at 2000 μg mL^{-1} were prepared.

Aliquots of 100 μL of the drug standard solution were pipetted into eleven consecutive wells of a micro-well plate and the solvent was allowed to evaporate under the fume hood. To each of these wells was added, a specified aliquot of the methanolic caffeine solution: 0, 2.5, 5, 10, 15, 20, 25, 32.5, 50, 65, 100 and 200 μL (0–95% (w/w) impurity). The solvent was again left to evaporate. This was performed in triplicate. The developed NQS colour test procedure was applied to each well. The resulting colours were observed and recorded.

The process was repeated using the methanolic glucose solution in place of the caffeine solution.

2.5.4 Limit of detection. The limit of detection was determined using a modified version of that recommended in the National Institute of Justice's Colour Test Standard.[22]

A 200 μg mL^{-1} methanolic solution of BZP HCl was prepared. Five replicate aliquots of this solution of 0, 5, 10, 15, 20, 25, 30, 50, 100, 150, 200 and 250 μL were pipetted into a micro well plate and the solvent was allowed to evaporate under a fume hood. The general recommended test procedure was applied. The colours were observed and recorded. The lowest sample size producing a colour change noticeably different from that of the blank and characteristic for the target analyte for all five replicates was regarded as the limit of detection.

A small survey was undertaken asking seven volunteers to identify the sample size they considered to be the point at which a noticeable colour change was apparent. This survey was conducted over two days on two separate trials.

2.5.5 Robustness. The robustness and ruggedness of the NQS colour test were examined through deliberate minor changes made in the experimental condition, pH. Aliquots of 200 μL BZP HCl methanolic solutions were pipetted into 16 sets of triplicate wells of a micro-plate, to deposit 40 μg of material in each. NQS colour spot testing was performed on the BZP samples and an adjacent blank well using a $NaHCO_3$–NaOH buffer solution at 16 pH values ranging from 8.9 to 14.1. The developed NQS colour test procedure was carried out, employing these prepared buffer solutions in place of the first $NaHCO_3$ reagent. Colours were observed and recorded.

2.5.6 Stability. An NQS reagent solution, prepared at a concentration of 2.0×10^{-3} M, was divided into four separate containers and these were allocated a different storage environment:

• Laboratory bench top
• Laboratory cupboard, covered in aluminium foil
• Refrigerator set at 7 °C, covered in aluminium foil
• Digital water bath set at 35 °C, sealed and covered in aluminium foil

Each reagent solution was employed in the developed colour testing procedure by applying to triplicate samples of the target analyte, BZP HCl, alongside a blank reagent weekly for up to three months. The colours produced in each test were observed and recorded.

3 Results and discussion

3.1 Colour spot test method development and optimisation study

3.1.1 Colour test in test tubes. Primary testing afforded interesting results: the BZP sample produced an intense, bright orange-red colour solution with a dark red precipitate at room temperature almost immediately, notably different from the light yellow blank reagent test colour (see Fig. 1). The addition of heat to the sample tube did not affect the colour observed, and similarly, variation of the reagent and sample ratios had no effect on the colour produced.

3.1.2 Reduction to a small scale. The use of drop sized amounts of reagents in well-plates led to the colour changes observed in the large scale method being successfully replicated. The aqueous BZP HCl sample produced a bright, intense orange-red colour solution almost immediately with a slow forming red precipitate, whereas the control reagent blank solution remained yellow (see Fig. 2).

3.1.3 Modification of reagents. The results showed that variation of the triton-X-100 surfactant volume did not affect the hue of the colour produced by aqueous samples tested, or the blank reagent. Triton-X-100 is a non-ionic surfactant used in the coloured reaction as a solubilising agent. Given that the BZP

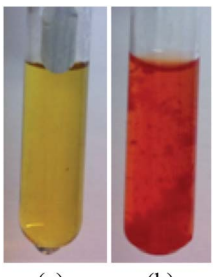


Fig. 1 Preliminary NQS colour testing performed in test tubes for reagent blank (a) and BZP HCl sample solution (b).

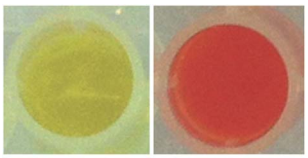


Fig. 2 Reduced scale NQS colour testing performed in plastic micro-well plates on control reagent blank (a) and BZP HCl sample solution (b) at room temperature.

reacted to form a highly coloured product with and without the triton-X-100, it was removed from further testing procedures. This would reduce the number of reagents, and associated costs of employing the surfactant.

In contrast, results show that the reaction, and hence the colour change will not take place without the addition of the $NaHCO_3$ buffer.

3.1.4 Application to solid samples. The method was demonstrated to be effective on solid samples. This testing also provided preliminary validation of the specificity of the proposed spot test method.

Seven common cutting agents, diluents and other white powdered drugs tested did not react with the NQS at room temperature, with the exception of ephedrine which reacted to produce a yellow-orange colour. This was the only sample that exhibited a possible interfering reaction; however, the colour produced was distinctly different from the orange-red product resulting with solid BZP HCl.

3.1.5 Effect of the NQS concentration. Five dilute solutions of NQS were prepared at concentrations of 3.0×10^{-3} M, 2.5×10^{-3} M, 2.0×10^{-3} M, 1.5×10^{-3} M and 1.0×10^{-3} M *via* serial dilution. The spot test method was applied to a control blank well, and pin-head sized amounts of ephedrine HCl, BZP HCl and 4-MMC HCl using each dilute NQS concentration. This study revealed that the reaction was to some extent dependent on the reagent concentration: *i.e.* an increase in intensity of the observed colour with the increasing NQS concentration.

The NQS concentration at 2.0×10^{-3} M was chosen as the optimal concentration to afford an intense orange-red coloured product with BZP HCl, and a pale, light yellow coloured product with the reagent blank.

3.2 General recommended procedure

Optimum working conditions were selected by considering the influence of reaction parameters such as reaction time, pH, NQS concentration and temperature. Reaction times were selected based on general observations in order to provide for consistent results amongst tests.

To a pin head sized amount of unknown powder sample:

1. Add *5 drops* of 0.1 M $NaHCO_3$–NaOH buffer solution, ensuring adequate mixing for a few seconds,

2. Followed by addition of *4 drops* of 2.0×10^{-3} M NQS solution, and

3. Record the colour change after 2 minutes.

3.3 Evaluation of validation data

3.3.1 Reproducibility. The repeatability of the method was determined by testing samples on the same day with the same reagents in the same laboratory. The identical colour results of these analyses demonstrate the repeatability of the proposed methods.

The reproducibility was determined by varying the conditions of testing. The test performance on different days, using different reagents and even in different laboratories with different heating apparatus and well plates showed no effect on the NQS colour test results, thus warranting the reproducibility of this method.

3.3.2 Selectivity/specificity. The UNODC recommends a minimum of 20 test samples for selectivity analyses. In this study over 90 substances were examined. The list of substances subjected to the NQS test can be found in Tables 2 and 3.

The selectivity of a colour test used in the preliminary identification of drugs is particularly important, as interferences from substances other than the analyte of interest may lead to false positives. Ideally, the NQS will only react with piperazine analogues, *i.e.* it is selective to piperazine analogues.

The application of the procedure to seven discrete aqueous samples of diluents, excipients and other drugs at room temperature showed no colour change from the reagent blank and therefore no reaction occurred within the two minute standing period. The potential of NQS as a colour test reagent for BZP specifically was realised.

The addition of heat, with a controlled water bath, to the colour testing of these particular substances had no effect on the reactions of starch, glucose, caffeine, sucrose and codeine which did not react and showed the same yellow colour as the reagent blank. Ephedrine and 4-MMC, however, reacted to produce a colour change of red-brown and brown, respectively (see Fig. 3). It should be noted that during heating, ephedrine initially produced a colour change to orange-yellow which subsequently darkened within a short time. The positive reactions with other substances allow for the potential application of the NQS reagent in their detection. It was decided to omit the heating stage and keep the reaction standing time as two minutes for testing in future in order to increase the selectivity.

The NQS reagent was found to produce an incredibly bright orange-red colour complex specific to BZP only. The five other piperazine analogues tested, TFMPP, mCPP, pCPP, MeOPP and piperazine, also produced orange-red colour. The results of BZP, TFMPP and mCPP alongside a reagent control blank can be seen in Fig. 4. Although not obvious in the image, the apparent brilliance of BZP made it distinguishable from the others upon visual examination.

The bright, intense orange-red colour exhibited by the piperazine analogues was not produced by other analytes tested. This result is highly desirable for any colour test reagent as it shows the highly selective nature of the colour test. In addition, the specificity of the NQS colour test toward the single analyte, BZP, is also apparent by the uniquely bright colour as the result.

No common cutting agent, excipient or diluent was found to react with the NQS reagent at room temperature. This is a

Table 2 Results of the NQS colour test against six common piperazines, and a range of amphetamines, common precursor chemicals and other recreational drugs

Sample analyte	Spot test colour[a]
1-Benzylpiperazine (BZP) HCl	Brilliant orange-red
1-[3-(Trifluoromethyl)phenyl]piperazine (TFMPP) HCl	Orange-red
1-(3-Chlorophenyl)piperazine (mCPP) HCl	Orange-red
1-(4-Chlorophenyl)piperazine (pCPP) HCl	Orange-red
1-(4-Methoxyphenyl)piperazine (MeOPP) HCl	Orange-red
Piperazine hexahydrate	Orange-red
5-Methoxy-*N*,*N*-diallyltryptamine	NR
p-Fluorococaine HCl	NR
(−)-Methylephedrine HCl	NR
(2*S*[a],3*R*[a])-2-Methyl-3-[3,4-(methylenedioxy)-phenyl]glycidic acid methyl ester	NR
(+)-Cathine HCl	Dark brown
Heroin HCl	NR
Cocaine HCl	NR
(+/−)-*N*-Methyl-3,4-methylenedioxyamphetamine (MDMA) HCl	Pink-orange
(+/−)-*N*-Ethyl-3,4-methylenedioxyamphetamine (MDEA) HCl	NR
(+/−)-3,4-MDA HCl	Light orange
(+/−)-*N*-Methyl-1-(3,4-methylenedioxyphenyl)-2-butylamine HCl	NR
Amphetamine sulphate	Light orange-yellow
(+/−)-Methamphetamine HCl	Light pink-orange
Gamma-hydroxy butyrate	NR
(−)-Ephedrine HCl	Light pink-orange
4-Bromo-2,5-dimethoxyphenethylamine HCl	Orange
Phencyclidine HCl	NR
Phentermine	NR
Phenylpropanolamine (racemic mixture)	Brown
3,4-Methylenedioxyphenyl-2-propanone (MDP2P)	Light brown swirls
Ketamine	NR
Dimethamphetamine (DMA)	NR
2,5-Dimethoxy-4-propylthio-phenylethylamine	Light orange-pink
2,5-Dimethoxy-4-iodophenylethylamine HCl	Orange-pink
Methylamine HCl	Light brown
4-Methylpropiophenone	Light yellow
2-Bromo-4-methylpropiophenone	Light yellow
Triethylamine	Light orange
4-Fluoromethamphetamine	NR
4-Fluoroamphetamine	Light yellow
4-Methoxymethamphetamine (PMMA)	Light orange
2,5-Dimethoxyamphetamine	Yellow-orange
3,4-Dimethoxyamphetamine	Yellow-orange
4-Methoxyamphetamine (PMA)	Orange-yellow
4-Methylmethamphetamine	Light yellow-pink
2-Fluoromethamphetamine	Light yellow-pink
2-Fluoroamphetamine	Light yellow
4-Hydroxyamphetamine	Brown-orange
4-Bromo-2,5-dimethoxyamphetamine HCl	Orange
2,5-Dimethoxy-4-methylamphetamine	Orange
4-Methylmethcathinone (4-MMC)	NR
3,4-Methylenedioxymethcathinone (3,4-MDMC)	NR
3,4-Methylenedioxypyrovalerone (3,4-MPDV)	NR
3,4-Dimethylmethcathinone (3,4-DMMC)	NR
1-(3,4-Methylenedioxyphenyl)-2-(methylamino)butan-1-one	NR
3-Fluoromethcathinone	NR
Ethylcathinone	NR
Cathinone HCl	Brown swirls
4-Methoxymethcathinone	NR
N,*N*-Diethylcathinone HCl	NR
N,*N*-Dimethylcathinone HCl	NR
Methcathinone HCl	NR
4-Methyl-α-pyrrolidinopropiophenone HCl	NR

[a] NR describes a spot test whereby no reaction occurred and therefore no change in colour was observed.

significant and important factor that can determine the practicality of a colour test when used for the purpose of identification of unknown solid sample mixtures. Two further tests involving multiple control substances were performed: a BZP and TFMPP mixed solid sample, and a BZP and MDMA solid mixture. Both of these samples turned to an orange-red colour, testing positive for the presence of piperazine and thus showing the potential for application of the test in case work.

Table 3 Results of the NQS colour test against common adulterants, excipients and other powdered substances

Sample analyte	Spot test colour[a]
Caffeine	NR
Benzoic acid	NR
Paracetamol	NR
Ephedrine HCl	Yellow-orange
O-Acetylsalicylic acid	NR
Phenolphthalein	Brilliant pink
Codeine HCl	NR
Phenethylamine	Light orange
Ascorbic acid	Colourless
Boric acid	NR
Calcium chloride	NR
Levamisole	NR
Citric acid	NR
Dimethylsulfone	NR
Glucose	NR
Glycine	Dark brown
Lactose	NR
Mannitol	NR
Magnesium sulphate	NR
Potassium carbonate	NR
Sodium carbonate	NR
Caster sugar/icing sugar/brown sugar	NR
Sodium chloride	NR
Starch/cellulose	NR
Sucrose	NR
Tartaric acid	NR
Stearic acid	NR
Maltose	NR
Artificial sweetener	NR
Protein powder	Dark yellow
Self raising flour/plain flour	NR

[a] NR describes a spot test whereby no reaction occurred and therefore no change in colour was observed.

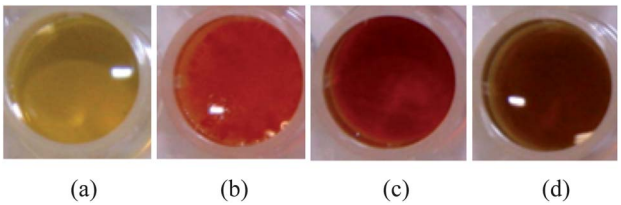


Fig. 3 Small scale NQS colour testing with addition of heat performed in plastic micro-well plates on a blank control reagent (a), BZP HCl (b), ephedrine HCl (c), and 4-MMC HCl (d) sample solutions.

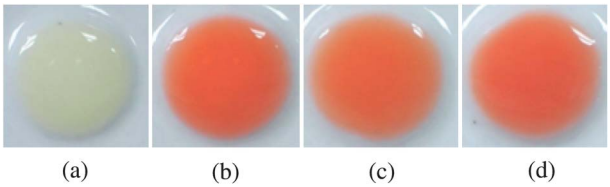


Fig. 4 Selectivity results performed in white glazed porcelain spotting plates on blank control reagent (a) and piperazine analogues: BZP HCl (b), TFMPP HCl (c) and mCPP HCl (d).

A number of non-piperazine compounds reacted with the NQS reagent at room temperature to afford a range of dull colours. This difference in colour between reacting amines could be explained by the location of the amine group in piperazine analogues being in a cyclo group.

The NQS is known to react with the amino group of target analytes. As a result, the interference of endogenous amines remains a concern.

3.3.3 Purity testing. The purity testing showed that with an impurity present in the sample at known concentrations ranging from 0% (w/w) to 95% (w/w), there is no obvious effect on the colour reaction taking place with NQS. Caffeine and glucose cut samples both appeared unaffected upon visual examination with the naked eye. The samples cut with excipients provided the requisite bright orange-red colour at all purity percentages (0–95% (w/w)) after two minutes. However, it was noticed that caffeine cut samples took longer to develop the required colour: ranging from less than 90 seconds for 0% w/w impurity to two minutes for 95% (w/w) impurity.

A given common sample matrix may contain endogenous or exogenous interfering substances, purity testing is carried out to determine the effects that these interfering substances have on the colour produced. Purity data for piperazine analogues in seized materials is not available in the Australian Crime Commission's latest Illicit Drug Data Report, nor can they be found in the UNODC's World Drug Report 2012.

3.3.4 Limit of detection. The limit of detection was determined by approaching the concentration where no change in colour could easily be determined in reference to a blank sample. Cut-off values were set at the lowest concentration tested which unmistakably resulted in a colour change as recommended by the SWGDRUG.[23] The amounts of each target compound analysed were 1, 2, 3, 4, 5, 6, 10, 20, 30, 40 and 50 μg, alongside a control blank containing 0 μg for comparison purposes. The results of testing for BZP using the NQS reagent are provided in Fig. 5.

The lowest concentration of BZP that afforded a colour change distinguishable from the background noise was determined to be 4 μg. This concentration was then multiplied by ten and recorded as the 'operational drug detection limit' at 40 μg.

It should be noted that LOD is not a particularly robust parameter and may be affected by minor changes in operational conditions. In addition, the subjective nature of the determination of the detection limit adds further room for inadequacies. In an attempt to add further validity to the proposed limits of detection, seven random participants were asked their opinion as to which concentration afforded the lowest detectable colour change. The results were in unanimous agreement with the proposed limits of detection.

For comparative purposes, the LOD of D-amphetamine HCl and D-methamphetamine HCl using the well established Marquis reagent is 20 μg and 100 μg, respectively.[22] It follows that these determined limits are well within an acceptable range for use as colour test reagents.

3.3.5 Robustness. The UNODC defines robustness as the ability of a method to remain unaffected by small but deliberate variations in the main parameters of the method. It provides reliability to a method during its normal usage for assessment.

Ruggedness is the ability to withstand small uncontrolled or unintentional changes in operating conditions and assesses the reproducibility of results obtained by analysis of the same samples under a variety of conditions.

The results of robustness testing on NQS showed that at each tested pH, with the exception of pH 14.1, the desired coloured product was observed. The time taken for development of this colour, however, varied. In the pH range of 9.0–11.0 the appropriate colour changes were developed within two minutes at room temperature, with the optimum pH level towards the end of the range. While between pH 11.1–13.3 the development time took from three minutes to six minutes. It was evident that the test method was significantly robust in regards to slight variations in pH.

3.3.6 Stability. The stability of the NQS reagent was demonstrated in this validation by comparing reagents freshly

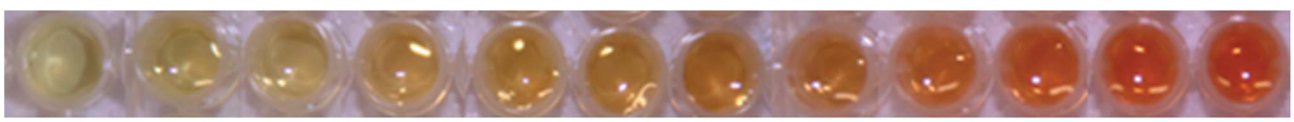
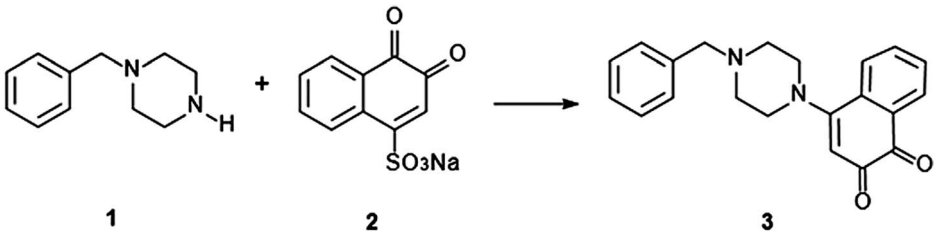


Fig. 5 Results of limit of detection testing of BZP HCl using the NQS colour test method performed in plastic micro-well plates. Amounts used are 0, 1, 2, 4, 5, 7, 9, 10, 20, 30, 40 and 50 μg, respectively.



Scheme 2 Scheme for the proposed reaction pathway of BZP (1) and NQS reagent (2) to form an orange-red product (3).

prepared with those of equal concentration that have been retained for different periods of time under various storage conditions.

The NQS reagent showed limited stability in regards to storage conditions and consequently, the storage time period. The NQS reagent has a light yellow colour when freshly prepared. Following storage in the 35 °C water bath for one week, the reagent itself turned to an intense brown colour and thus failed to produce coloured reactions with the target analytes. Similarly, the stored laboratory bench top and cupboard reagents developed dark brown precipitates after one month. After three weeks of storage in a refrigerator, the reagent had a dark orange colour and was no longer suitable for further monitoring. Colour tests were performed weekly on samples and control reagent blanks until the solution was considered unusable based on resulting colour changes.

The application of NQS as a colour test reagent would require the preparation of fresh test solutions weekly or just prior to use and be stored covered in the refrigerator in the meantime. This is not an ideal situation, however, preparation of this aqueous solution is considerably easy using distilled water only.

The stability of the BZP-NQS coloured product was demonstrated by examining the spotting well after a 24 hour standing period. The BZP-NQS complex remained slightly coloured and a red coloured precipitate crashed out of solution.

3.4 Proposed reaction mechanism

The influences of pH can be understood by investigation of the reaction mechanism involved. The buffer solution employed is alkaline to provide the means for turning the protonated amine salt ($-NH_2^+$) into an amino group (–NH). In general, a higher pH will more effectively remove this proton.

The reaction of the primary amine salt, amantadine hydrochloride, in alkaline medium with NQS was described by Mahmoud. *et al.*[15] In their proposed mechanism, the sulphonate group of the NQS is replaced by the amino group of the amine. This nucleophilic substitution reaction has also been proposed by Ali and Elbashir[16] in their validated method for determining olanzapine using NQS at pH 13.

The effect of temperature on the reaction of NQS with BZP was studied by carrying out the reaction at different temperatures (25 °C–85 °C). It was found that the reaction of BZP with NQS was not affected by the increasing temperature; no changes in the coloured product were observed upon heating. The reaction at room temperature went to completion in two minutes. Application of the test to samples other than BZP led to the increase of reaction time to eight minutes at 85 °C to provide for a wider range of coloured reactions.

The chemical structure of the coloured product formed upon reaction between NQS and BZP has been proposed in Scheme 2. Similarly, the amino groups of those substances affording a colour change with the NQS test will be the functional group involved in the reaction. The highly coloured complexes produced through reaction with NQS are the result of a high degree of conjugation afforded in the newly prepared molecule. Full structural elucidation of the product is of interest for future studies.

4 Conclusions

Preliminary investigation on the use of NQS as a colour test reagent for piperazine analogues revealed that there exists great potential for further application. A bright, orange-red product formed during the reaction of NQS with BZP is not only distinctly different from the light yellow coloured reagent blank, but selectivity tests show that this characteristic colour change and colour intensity did not match with any other substance tested.

The proposed colour test procedure fulfills laboratory screening colour test requirements, using small quantities of non-toxic reagents on limited amounts of solid samples, resulting in a characteristic colour change to orange-red within two minutes at room temperature in the presence of the piperazine analogues.

The suitability of NQS as a novel colour testing reagent for the purpose of preliminary detection of piperazine analogues was effectively evaluated through the examination of method validation parameters. The excellent selectivity of the NQS colour test toward piperazine analogues, combined with the lack of interference from the majority of analytes tested and its superior reproducibility, affords the reagent great potential for exploitation as a colour test reagent. The addition of heat provides the test with an even greater scope for identification of other reacting analytes. The results of the investigation allow for suggested additions to current preliminary testing workflows.

Future work would involve NQS reagent stability studies to investigate the means of suppressing the instability of the reagent without affecting its use as a colour test reagent. This would allow for its use in field testing kits.

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